Evaluating intercropping systems as a sustainable agroecosystem alternative to reduce greenhouse gas emissions

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Sole crops are commonly used in the most productive agricultural area in Argentina, the Pampas, and represent the biggest land-area in the country, and the most responsible for the release of greenhouse gas emissions to the atmosphere. For this reason, the Government has adopted sustainable practices such as intercropping systems (e.g., cereal-legume) to increase soil organic carbon and soil organic matter. An increase in soil organic matter has proven to capture greater amounts of carbon dioxide (CO_2), a threatening greenhouse gas for climate change. In addition, it has shown to decrease nitrogen losses in the form of nitrous oxide (N_2O), which is more potent than CO_2.

Agricultural soils are the main contributor to increased atmospheric nitrous oxide (N_2O) concentrations with negative impacts on global climate. In Latin America, Argentina has been enlisted as one of the highest emitter of N_2O emissions. In fact, almost half of their emissions originate from cropping systems, especially, sole crops, which tend to lose higher soil organic carbon. Cereal-legume intercrops are beneficial due to the complementary and simultaneous usage of carbon (C) and nitrogen (N) that helps to promote greater storage of C and reduce N losses. Due to the positive cycling of both elements in the intercrops this helps to store C and N more effectively and reduce greenhouse gases in the soil in the long term, making them more sustainable and beneficial with respect to climate change. Although intercropping systems have shown a vast array of benefits there is still an urge to evidence that intercrop systems can reduce both CO_2 and N_2O emissions compared to sole crop systems. The objective of this study was to determine the capacity of a cereal-legume intercrop to lower both greenhouse gases under nitrifying and denitrifying conditions compared to a cereal and legume sole crop.

Results showed that mean CO_2 emissions in the nitrification ranged from 1.77 to 2.22 mg CO_2-C g^{-1} h^{-1} in the fertilized group while in the unfertilized group ranged from 1.61 to 1.92 mg CO_2-C g^{-1} h^{-1}. Conversely, N_2O emissions in the nitrification ranged from 0.48 to 1.64 µg N_2O-N g^{-1} h^{-1}. in the fertilized group to 0.43 to

0.56 μg N_2O-N g^{-1} h^{-1} in the unfertilized group. Under nitrification conditions (60% water-filled-pore-space) in the fertilized group the maize sole crop and intercrop mean N_2O emissions were 1.64 and 0.48 μg N_2O-N g^{-1} h^{-1}, respectively. The CO_2 mean emissions favored by the nitrification in the maize sole crop, soybean sole crop, and intercrop were 2.22, 1.77, and 2.20 mg CO_2-C g^{-1} h^{-1}, respectively. Nonetheless, throughout the 48 hours of the incubation experiment, these results were not significantly different. Meanwhile, under denitrification conditions mean N_2O emissions of soybean sole crop (80% water-filled-pore-space) resulted in 12.74 μg N_2O-N g^{-1} h^{-1} and were higher than maize sole crop and intercrop (12.28 and 9.37 μg N_2O-N g^{-1} h^{-1}, respectively). Overall, the emissions followed lower increases of N_2O under the influence of intercropping systems in both nitrification and denitrification processes. In the nitrification, however, maize sole crops had a greater soil ammonium concentration (5.82 g N g_{dw}^{-1}) which seem to explain higher N_2O emissions compared to intercrop systems (5.51 g N g_{dw}^{-1}). Nitrate concentrations under nitrification were the lowest in the intercrops (1.18 g N g_{dw}^{-1}) and the highest in soybean sole crop (1.27 g N g_{dw}^{-1}), which suggests that the nitrification in the latter was occurring at faster rates than in intercrops, thus reducing emissions overall in intercrops. Greenhouse gas emissions were highly correlated to nitrifying genes indicating that the apparent source of emissions are promoted by nitrifying microbial functional genes. Although there is an evident linkage between N_2O emissions and microbial abundance, the microbial functionality needs to be further analyzed to confirm that the actual microbial source of N_2O emissions under nitrification conditions was of the nitrifying group. Moreover, isotopic measurements would elucidate the contribution of these greenhouse gases and would deliver a better understanding of the contribution of both C and N in CO_2 and N_2O emissions. In order to assess intercrop systems in terms of C storage and CO_2 reduction, long-term studies are needed (e.g., more than 10 years). Nonetheless, this study evidenced that intercropping systems are effectively reducing N_2O emissions and that have the potential to sustainably abate one of the most potent greenhouse gases that contribute to climate change.

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Dedication

This thesis goes dedicated to one of the seven indigenous communities from my country in Panama: the *Ngäbe-Bugle*. Especially to the family that welcomed me in the "Cordillera Central"; and, showed me their territories, their lifestyle, and botanical knowledge. This experience enriched me and inspired me to study a topic related to agriculture that would equip me to one day, hopefully, to serve them better. *Qui-Bononde* will be forever thankful for the wisdom, the kindness, and the generosity that you, your culture, and, your lands will forever have.

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1. Literature Review

1.1 Carbon and nitrogen dynamics in agroecosystems

The cycling of carbon (C) is important as both as an element or as a key factor for living organisms. For instance, when plants photosynthesize, these will absorb CO_2 from the atmosphere (Soussana and Lemaire, 2014). Contrarily, the release of CO_2 from plants occurs once the plant has started to be decomposed, releasing soil organic matter (SOM). The composition of SOM includes plant, and animal tissues, microbes, and carbon linked to soil minerals (Schlesinger, 1999). A soil with lower amounts of SOM is typically weak in structure (Sombrero and Benito, 2010). In agricultural soils, the relationship between soil structure and SOM has been extensively analyzed due to its linkage to C sequestration (Paustian et al., 2000). Furthermore, it has been proposed that in order to understand how C sequestration and its conservation occurs, it is required a good amount of knowledge of crop residue production and management (Sombrero and Benito, 2010). For instance, Robertson et al. (2000) found that by replacing conventional tillage with no-tillage agricultural practices net C-sequestration of soil organic carbon (SOC) was greater.

Therefore, the enhancement of SOC depends on management strategies such as residue retention (Wang et al., 2016). Subsequently, the quantity of crop residues returned to the soil influences SOC (Follett, 2001). In addition, SOC plays an important role in maintaining crop production, as well as preservation and conservation of the environment, and crop and soil quality, (Robinson et al., 1996). Therefore, quality differences can serve as an indicator of the SOC in the soil (Sombrero and Benito, 2010). Unfortunately, the transformation from forests or grasslands to agricultural production has reduced global SOC stocks (Smith et al., 2016). One approach that has been linked to SOC enhancement is the improvement of soil management practices, which has also been related to the increase of nitrogen (N) concentrations (Dikgwathlhe et al., 2014). For example, Zak et al. (1993) found that as soil C-availability decreased so did N-availability. However, they found that when C-substrate via crop-residue decomposition increased, it also increased N-availability.

This close relationship between C and N begins in the plant-soil system (Larcher, 1995). For instance, when plants incorporate CO_2 in their tissues, the N-demand increases to form amino acids and other compounds (Larcher, 1995). Once the process is over and plant decays, both C and N are decoupled to be subjected to organic breakdown (Li et al., 2005). This breakdown, however, results in the remineralization of C and N, which generates CO_2, and inorganic N-forms, ammonium (NH_4^+) and nitrate (NO_3^-) (Li et al., 2005). Researchers have found that due to the close connection between C and nitrogen (N) cycles, N turnover processes in the soil can affect gaseous exchange between the biosphere and the atmosphere as N-compound losses (Li et al., 2005). These N-losses have been extensively researched, and are typically, by products of N-forms subjected to oxidation and reductive microbial processes that lead to nitrous oxide emissions (N_2O) (Li et al., 2005). Therefore, soil C-sequestration affects the production and consumption of N_2O (Li et al., 2005).

There are various soil processes involved during C and N sequestration, for example, N competition between microbes and plants, crop residue decomposition, and mineralization (Luo et al., 2004). For this reason, it is necessary to further our understanding about how N regulates C sequestration and viceversa (Luo et al., 2004). Notwithstanding the uncertainty of this complex dynamic between C and N cycles, it becomes clear that SOC is strongly correlated to N-losses (Li et al., 2005). The decline of SOC has been a response to intense agroecosystem management practices, and have also caused an increase in greenhouse gas emissions (e.g., CO_2 and N_2O). Therefore, agricultural practices provide the opportunity to curb GHG emissions and mitigate the effect of such GHG effect (Gregorich et al., 2005). Some of the most common GHG analyzed under soil management include methane (CH_4), N_2O (Soussana and Lemaire, 2014) and CO_2 (Butterly et al., 2016), High levels of CO_2 in the atmosphere have increased as fossil fuel combustion increases (Ehlreinger et al., 2005). On the other hand, CO_2 emissions have also been ascribed to land-use changes (Gruber and Galloway, 2008). This is because land-use change alters the biogeochemical cycle of C (Forster et al., 2007; Houghton et al., 2012), leading to an increase of CO_2 into the atmosphere.

Unlike CO_2, N_2O remains in the atmosphere, namely, the stratosphere, for longer periods of time due to the photodissociation via photolysis through an electronically excited oxygen atom in the stratosphere (Prinn and Zander, 1999; Portmann et al., 2012). After N_2O becomes photodissociated it can stay around 114 years in the upper stratosphere (IPCC, 2007; EPA, 2010), which can create a powerful climate effect on Earth (Zaehle, 2013). Recent studies have shown that N_2O has a global warming potential that is 265 times higher than CO_2 in a 100-year time horizon (Myhre et al., 2013). In addition, N_2O is responsible for 89% of global annual emissions, equivalent to 16 Tg N $year^{-1}$ (Stocker et al., 2013). Agricultural soils are responsible for 60% to 80% of N_2O production (Davidson, 2009), and this is mainly due to N-fertilizers (Smith, 1997; Reay et al., 2012), but also due to agricultural management practices that impact soil organic carbon (SOC) (Dyer et al., 2012). For this reason, research in agricultural soils have been urged among other ecosystems to further understand its contribution to N_2O emissions (Stocker et al., 2013). Thus, it is imperative to understand C and N dynamics in response to different agroecosystem approaches to find sustainable agricultural production systems (Verchot et al., 2008). This expanded knowledge will produce better information tools that could potentially reduce GHGs and abate climate change.

1.1.1 The role of nitrogen in agroecosystems

A terrestrial organism such as plants or bacteria utilize N in the form of NH_4^+ or NO_3^- for growth and development. However, the availability of N in terrestrial ecosystems is not sufficient. A reduced N-availability will compromise the microorganisms' ability to decompose soil organic matter (SOM) (Plaza-Bonilla et al., 2015). One way to determine N-availability in the soil is through C:N ratios. If in the soil the C:N ratios are lower than 24:1 this will have an effect in mineralization rates within the soil; whereas ratios higher than 24:1 produces immobilization rates (USDA, 2011). Regardless of which process occurs, both will determine N-availability in the soil (Recous et al., 1999). Mineralization is the transformation from organic to inorganic forms, and the by-product consists of ammonium (NH_4^+) and nitrate (NO_3^-) (Zaehle, 2013). The process of mineralization can use either NH_4^+ or NH_3^+, which are subjected to three processes including clay fixation, nitrification, and/or denitrification. These last two transformations, in particular can

be mediated by saprophytic fungi (Philippot and Germon, 2005), or nitrifiers and denitrifiers (Snider et al., 2009). In addition, NH_4^+ or NH_3^+ can undergo immobilization, which occurs at higher ratios of C:N. Immobilization is the contrary transformation of mineralization, reversing the inorganic form to an organic form, as NH_3^+ or NH_4^+ (Myrold and Bottomley, 2008); and is usually performed by plants, microorganisms or soil organic matter (Philippot and Germon, 2005; Trehan, 1996). Both mineralization and immobilization rates proceed differently and are substantial in providing soil N and its availability to the microbial community and for plant uptake (Recous et al., 1999). If both rates are balanced, they could equally contribute maintain ideal C:N ratios in soils. However, if C:N ratios are higher and immobilization persists, either NH_3^+ or NH_4^+ will be produced in greater quantities; thus, reducing NO_3^- concentrations. This reduction means that N losses can be lessened, and decrease N_2O emissions from soils (Frimpong et al., 2012). Contrarily, if excessive mineralization takes place, the readily N availability can increase N_2O emissions.

Thus, the importance of current knowledge for both transformative processes is essential to finding sustainable alternatives that can reach this desired balance between C and N proportions, especially in agricultural soils. Some researchers found that barley (a cereal) (*Hordeum vulgare* L.) in cropping systems immobilized soil inorganic N due to the constant decomposition and microbial assimilation; whereas pea (*Pisum sativum* L.) cropping systems (a legume), showed an opposite pattern with a net mineralization (Aita et al., 1997; Evans et al., 1997; Jensen 1996b; Recous et al., 1995). Research has shown that when both crops are grown together, they produce a greater chemical (Hauggaard-Nielsen et al., 2003) and biological balance, and an increase in immobilization and mineralization rates. In addition, the integration of cereals and legumes showed an improved N-use (Dyer et al., 2012; Hauggaard-Nielsen et al., 2013; Regehr et al., 2015; Bichel et al., 2017). Typically, agroecosystems tend to be intense in its management practices, however, such alternate agricultural practices may provide a different pathway to curb GHG emissions and reduce the impact of its effect to the atmosphere (Gregorich et al., 2005). Also, with the ongoing increase of food production with an unsustainable population growth, the principle of sustainability in agroecosystems requires to be more often thought. In fact, it is estimated that the amount of food production will increase by 60% to 110% to accommodate a growing global population (FAO, WFP and IFAD, 2012). This

will result in further land-use change and an overuse of N-fertilizers in order to supply food demands world-wide (Rathmann et al., 2010). So, in order to supply food demand efficiently, while maintaining sustaina-bility at its main core, these agroecosystems besides yielding more should also be designed to reduce GHG.

The ability of agroecosystems to capture C from the atmosphere and store C in the soil makes it an attractive approach in any agricultural cropping system due to its capacity to comply with environmental standards, and mitigate climate change (Campbell et al., 2017). Thus, more research in agricultural cropping systems is required to reduce its susceptibility to climate change (Nelson et al., 2009; Campbell et al., 2017).

1.2 Agricultural cropping systems and climate change

Research suggests that fertilizers, especially N mineral fertilizers, in agricultural soils are responsible for direct top-down global N_2O emissions between 3-5% (Griffis et al., 2013) and bottom-up estimates of 1.75-1.8 (Griffs et al., 2013; Shcherbak et al., 2014). In agriculture, soils are responsible for 60% to 80% of N_2O production (Davidson, 2009). N_2O emissions have increased from an equivalent of 0.9 Tg N $year^{-1}$ (Velthof et al., 2003) to 16 Tg N $year^{-1}$ (IPCC, 2013). Therefore, it is evident that the application of N-fertilizers is building extreme pressure in agricultural systems in response to food security and crop produc-tivity for a growing global population (Campbell et al., 2017). Regehr et al. (2015) urged scientists to eval-uate N-transformations to comprehend the impact of the reliance on N-fertilizers, because this impacts GHG emissions and if it is not analyzed properly it can result in N-losses. For this reason, current agricul-tural practices that reduce GHGs while maintaining food production are essential in the political agenda to abate climate change (Raddatz, 2007).

Agricultural cropping systems have resulted impacted by the negative effect of climate change. For example, areas of northeastern Brazil increased by 40% in heavy precipitations in the first half of the 20th century. In the subtropical region this change was reported around 58% more rainfall precipitation. In Ar-gentina, annual precipitations have significantly gone from 1000 mm in the year 1945 to 2000 mm in the year 2000. Changes in annual precipitations have expectedly change the temperature, and these changes

have been detected since 1961. Such change has affected, for instance, rice cropping fields in Asian countries (Peng et al., 2004; Wassmann et al., 2009; Welch et al., 2010), which has shown to create stress to longer periods of water submersion leading to crop loss (Nelson, 2009). For this reason, there has been rising interest in the effects that weather conditions will have on harvest and sowing crop periods.

Researchers are identifying effective strategies that could aid cropping systems to adapt to this sort of climatic change. One adaptive measure consists in changing crop varieties according to climate predictions (Ghaffari et al., 2002; Alexandrov et al., 2002; Tubiello et al., 2000; Chen and McCarl, 2001; Trnka et al., 2011). Another alternative has been the inclusion of two or more crop species that belong to different sowing dates. For example, an autumn with a winter crop species (Minguez et al., 2007) or cultivating summer crops in the early spring (Olesen et al., 2007; Kaukoranta and Hakala, 2008), or both simultaneously (e.g., Dyer et al., 2012). By integrating two or more species on the same land area at the same time, this adaptive practice can lead to new ways of maximizing crop productivity and expanding production, while showing to be sustainable , which in the long-term helps to abate climate change (Tingem and Rivington, 2009; Cho et al., 2012).

1.2.1 Intercropping and sole-cropping systems

N-availability is a priority in many agricultural cropping systems. Nonetheless, N-availability without adding external N-sources remains a challenge. Therefore, low inputs of N in cropping systems with short supplies of soil inorganic N is a matter of interest in agricultural studies (Hauggaard-Nielsen et al., 2003). Legume sole crop systems, for instance, have shown lower efficient N-usage compared to cereal sole crop. This low N-efficiency has been ascribed to a a superficial root system in legume plants (Hauggaard-Nielsen et al., 2001b; Jakobsen and Nielsen, 1983; Jensen, 1985); this finding has also been reported in open fields (Hauggaard-Nielsen et al., 2003). Scientists have described this poor efficiency as the ‘spared N effect’ (e.g. Herridge et al., 1995), which prevents the uptake of inorganic N at deeper soil levels (Evans et al., 1989, 1991; Herridge et al., 1995). Legume crops, in general, generate greater N losses due to greater rates of mineralization (Aita et al., 1997; Evans et al., 1997; Jensen, 1996b; Recous et al.,

1995). For instance, these researchers have reported that pea sole crops produced net mineralization after decomposition (*Pisum sativum* L., Fabaceae Lindl.). Contrarily, the opposite transformation process has been demonstrated in barley (*Hordeum vulgare*) sole crop systems with low N availability as a result of the small inorganic fraction available after net immobilizations (Aita et al., 1997; Evans et al., 1997; Jensen, 1996b; Recous et al., 1995). Meanwhile, intercrops have the ability to balance both C and N transformational processes within the soil and efficiently use inorganic N (Hauggaard-Nielsen et al., 2003). This efficiency is rooted in that a cereal crop induces the legume to produce and fix more N (Hauggaard-Nielsen et al., 2001a; Hauggaard-Nielsen et al., 2003).

Although the mechanisms need to be further understood, this seem to be due to an enhanced nodulation produced as greater amounts of flavonoids -which play an important role in symbiotic nodulation of rhizobium and legumes (Wang et al., 2012)- are released under the presence of cereal crops (Liu et al., 2017). Once the stimulation of nodulation increases the legume's biological fixation of N_2 becomes greater too (Maj et al., 2010). In fact, cereals such as maize have shown to increase the content of quercetin and luteolin, two types of flavonoids, in a maize-faba bean intercrop, thus, enhancing faba bean nodulation (Liu et al., 2017). It is therefore not surprising that greater attention is given to intercropping systems due to the formally mentioned benefit concerning N-efficiency in intercropping systems. By definition, growing two or more crops per unit of land area enhances N and water demand simultaneously (Dyer et al., 2012; Echarte et al., 2011; Monzon et al., 2007). Nevertheless, research findings debate as to whether N_2O emissions are significant in this type of cropping systems, regardless of its natural advantage through biological fixation (Peyrard et al., 2016). For instance, Rochette and Janzen (2005) claimed that legumes can still release certain amounts of N_2O. Nonetheless, IPCC's emission factor, led to the conclusion that N_2O emissions were low enough to consider them as a global threat to the environment (Peyrard et al., 2016).

However, overall, intercrop systems have shown increased benefits compared to its counterpart, sole crops. These benefits include the biological fixation of N_2 through the inclusion of legumes, (Rochette and Janzen, 2005); a reduced effect of pests (Willey, 1979; Vandermeer, 1989; Liebman and Dyck, 1993);

control erosion (Caviglia et al., 2004); the reduction of costs for N-fertilizers that represent an environmental concern (Przednowek, 2003); a decreased dependence on external N sources (Inal et al., 2007). In addition, intercropping systems are also a sustainable agroecosystem capable of reducing GHG emission (Dyer et al., 2012), thus, enhancing N cycles regionally, and contributing to global efforts amidst climate change.

1.2.2 Intercropping systems in Argentina

Maize (*Zea mays* L.) and soybean (G*lycine max* L. [Merr.]) sole crops account for 21 Mha of land area (FAO, 2010). Argentina is one of the greatest contributors of GHG emission, releasing in the year 2000, 0.2 Gt of CO_2 eq of CO_2 (Secretaria de Ambiente y Desarrollo Sustentable, 2007). Meanwhile, Argentina's N_2O production accounted for 43% from agricultural cropping systems (Gobierno Argentino, 2007). Although this number declined in 2012, the contributions are considered as one of the largest in South America with contributions up to 28% in the agricultural sector (UNFCCC, 2015). This represents between 0.88% - 1% of the global annual N_2O emissions (Dickie and Coronel, 2016). This is important because at a regional scale all developing countries have been the most responsible for increases of N_2O (Smith et al., 2014). In fact 0.9% of N_2O has increased every year between 1990 to 2010 (Tubiello et al., 2013) representing an augmentation of 57% in the last 40 years (Blanco et al., 2014).

Therefore, even though this represents a small fraction of total global emissions, it is important when every country steps forward and becomes part of international climate commitments (UNFCCC, 2015) in order to demonstrate willingness to join international efforts to tackle climate change. Among the GHGs that are of utmost importance are CH_4, CO_2, and N_2O produced during agricultural production, thus, efforts have been mostly focused in this sector (UNFCCC, 2015). In Argentina, for instance, one of the top priorities in agriculture is improving agricultural practices and soil management strategies, as well as to create sustainable lands (IICA, 2016). Due to a massive reliance on sole crop agricultural systems for food production in Argentinian agroecosystems this practice has led to soil degradation (Posse et al., 2010). This degradation has led to SOC losses and has reduced soil organic matter (SOM) in these agroecosystems. An

increase of SOM improves carbon and nitrogen dynamics (Oelbermann and Echarte, 2011; Dyer et al., 2012). Therefore, the adoption of intercrop systems has received greater attention, especially within temperate zones such as in Argentina (Oelbermann and Echarte, 2011).

By doing so, the contribution of GHGs such as N_2O in Argentina are better understood and climate change is tackled in a more efficient way. Intercropping systems have shown to provide an arrangement of benefits compared to sole crop systems (Dyer et al., 2012). Particularly, in Argentina the enhancement of N, water demand, and the growth of two or more crops per unit of land area simultaneously has been one of the greatest contributions from intercrop systems in this region (Dyer et al., 2012; Echarte et al., 2011) has been one of the greatest contributions from intercrop systems in this region. However, more research is required to amplify other factors involved in the GHG sequestration (Pappa et al., 2011). This GHG reduction particularly applies to developing countries such as Argentina who has committed its efforts in reducing GHG emissions by 30% by 2030 (IICA, 2016).

1.3 Nitrous oxide production

1.3.1 Historic and environmental approach of N_2O

The first model that reached acceptance among scientists took place in the 1950s when Nommik (1956) described the microbial assimilation from NO_3^- pools performed by denitrifying bacteria (Zhang et al., 2015). Later on, Bremner and Blackmer (1978) supported an explanation of how soil microbes assimilate NH_4^+ pools. Both NH_4^+ and NO_3^- assimilation by soil microorganisms became the most common topic to describe N_2O production among researchers. Nevertheless, Firestone and Davidson (1989) provided further insights about both NH_4^+-N and NO_3^--N pools through the hole-in-the-pipe model; however, it has been instilled to be further investigated (Davidson and Verchot, 2000). This model is currently the most helpful to conceptually understand N_2O apportion (Davidson et al., 2000). Banerjee et al. (2016) explained that N_2O emissions originate from microsites in the soil; micro-sites are soil pockets that will each behave differently in response to variations in pH, temperature, or water content.

Hence, it is essential to understand how climatic conditions affect these microsites. One way to understand how these variables affect soil microsites is the further analysis of GHGs, such as N_2O (Banerjee et al., 2016). For example, Zhang et al. (2015) and Wunderlin et al. (2012) furthered the microbiological N cycling processes involved in the N_2O production under nitrification and denitrification. Amplifying this knowledge could help to prepare agroecosystems to be more resilient to climatic changes and sustainable in the long-term.

1.3.2 N_2O processes: Nitrification and denitrification

The nitrification (NIT) and denitrification (DNIT) process are produced by nitrifier and denitrifier microorganisms (Wrage et al., 2004); and both produce N_2O. NIT is an autotrophic oxidation sequence of NH_4 to nitrite (NO_2^-) and from NO_2^- to NO_3^- (Schmidt and Voerkelius, 1989; Hynes and Knowles, 1984; Wunderlin et al., 2012; Zhang et al., 2015); while DNIT is the reduction sequence from NO_3^- to N_2 (Payne, 1981; Zumft and Kroneck, 1990; Firestone and Davidson, 1989; Wunderlin et al., 2012; Zhang et al., 2015). Research findings suggest that 10% to 90% of N_2O in agricultural soils originate from the NIT process in agricultural soils (Skiba et al., 1993; Ambus, 1998; Bremner, 1997). The factors that regulate this process are soil temperature, NH_4-N availability, and soil pH (Mosier, 1998). Nitrifiers lead the process in the NIT process, however, there are related nitrifiers that function similarly, namely, nitrifier-denitrifier (NIT-DNIT). NIT-DNIT are capable of reducing NO_2^- to N_2O under low oxygen conditions (Ritchie and Nicholas, 1972; Poth and Focht, 1985; Voerkelius, 1990; Wrage et al., 2001). The apportion to N_2O in NIT-DNIT remains unclear (Wrage et al., 2001, 2005; Kool et al., 2007). However, it is estimated to account for 25% of the denitrification process (Dong et al., 2000).

Unlike NIT, DNIT total contribution to N_2O has not been reported to date as accurately as in the NIT process from agricultural soils; however, it is known to be the main source of N loss in agricultural systems (Philippot et al., 2007). The pathways by which denitrifier produce N_2O include soil nitrite, and abiotic decomposition of ammonium nitrate (van Cleemput and Baert, 1984; van Cleemput and Samater, 1995); chemo-denitrification of co-denitrification (Shoun et al., 1992; Spott et al., 2011); and dissimilatory

nitrate reduction to ammonia (Smith, 1982; Papen et al., 1989; Laughlin and Stevens, 2002). The factors involved in the DNIT process include water content, soil aeration, carbon and NO_3-N availability, and soil temperature (Payne, 1981; Mosier, 1998; Ruser et al., 2006). Regardless of which process takes place, NIT and DNIT are mostly governed by soil water content (Linn and Doran, 1984). Specifically, N_2O emissions are mostly related to different soil moisture levels (Pennock et al., 2010). For instance, if the soil has a water-filled-pore-space (WFPS) greater than 60% the process of DNIT dominates (Gao et al., 2016) Contrarily, the authors reported that when a soil is subjected to lesser or equal to 60% will lean towards the process of NIT.

Unfortunately, describing these two processes, and their multifaceted pathways, remains a challenge because they can both occur simultaneously (Arah, 1997; Butterbach-Bahl et al., 2013). Thus, researchers have emphasized on the description of multiple pathways by evaluating the microbial processes that are entitled to N_2O production (Ishii et al., 2011). Soil microorganisms perform different processes because of their functional genes (Xu et al., 2016). However, both functional activity and their gene abundance are efficient indicators for GHG emission, especially, N_2O (Morales et al., 2010). Gene abundance can also assist in identifying native or baseline microbial numbers following agricultural practices (Morales et al., 2010). This information can help to identify the response of the microbial population under different agricultural practices and improve it to reduce N_2O emissions. Thus, testing the response of soil microorganisms to different agricultural cropping systems will give us a clearer idea of how they respond. Agricultural cropping systems are accountable for high amounts of N_2O emissions, about 60% to 80% of N_2O production (Davidson, 2009). Investigators have reported a need to identify whether nitrifier or denitrifier are contributing to the increasing production of N_2O (Kool et al., 2011). Therefore, it becomes imperative to understand the microbial contribution to N_2O emissions under different agricultural practices.

1.4 DNA: Nitrifier and denitrifier microorganisms

1.4.1 Nitrifiers

NIT process is a sequence mediated by nitrifier microorganisms. These microorganisms convert and oxidize the NH_4^+ molecule to NO_2^- and then to NO_3^- (Theodorakopoulos et al., 2017). From an environmental perspective, the most important oxidizers, with regards to N_2O production are ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) (De Boer and Kowalchuk, 2001). AOB can produce N_2O via hydroxylamine and nitrifier-denitrification (Stein, 2011; Theodorakopoulos et al., 2017). AOB is considered to be the microorganism rate-limiting in the ammonia oxidation process in the autotrophic NIT (De Boer and Kowalchuk, 2001). They explained it is rate-limiting due to its capacity to produce hydroxylamine (NH_2OH) before it even reaches to the conversion from NH_4^+ to NO_2^- liberating N_2O as the by-product. NH_2OH is chemically produced via the catalytic reaction of ammonium monooxygenase, which corresponds to the gene *amoA* in the AOB. Although AOB is important in GHG research, it has been found that the NIT process is not limited to the bacterium domain (Venter et al., 2004). AOB shares the NIT process with ammonia-oxidizing archaea (AOA) (Theodorakopoulos et al., 2017). Although AOA belongs to a different phylum (*Thaumarchaeota*) (Francis et al., 2005; Leininger et al, 2006), it shares the *amoA* gene with AOB. It has been suggested that AOA genes have the same capacity to produce N_2O in the environment. However, as to what extent is yet to be further investigated (Erguder et al., 2009; Yao et al., 2011).

The gene *amoA* is among the most used molecular markers to identify the presence of AOA and AOB in soils (Hu et al., 2015). The reason why its more commonly analyzed its due to its significance in the global N cycle (Tang et al., 2016). This is important because more information about key genes is necessary, especially in N-limited environments (Butterly et al., 2016). The abundance and contribution of AOA and AOB (AMOs) can vary under different soil conditions, plant species, and soils' physiological response to pH (Chen et al., 2008; Prosser and Nicol, 2012). In addition it has been found to respond to

ammonia contents (Chen et al., 2008; Di et al., 2010; Prosser and Nicol, 2012). Santoro et al. (2011) suggested that AOA genes were contributing in its majority to N_2O emission. Unlike them, Carey et al. (2016) AOBs were reported higher than AOAs in agricultural soils after adding N-fertilizers to the soils.

Hence, the extent at which AOAs and AOBs dominancy and contribution are enhanced or decreased under different environmental conditions, remains unclear (Erguder et al., 2009; Yao et al., 2011). It is also unclear as to which mechanism controls the AOB and AOA groups that are responsible of the N_2O production in soils (Yao et al., 2011). This discrepancy remains particularly true under various agricultural practices (Song et al., 2007; Wang et al., 2007; Zhang et al., 2014). For instance, Huang et al. (2004) and Li et al. (2005) reported that intercropping systems were capable in reducing nitrate accumulation by keeping steady levels of ammonia in the soil; this mechanism reduces N losses in the form leaching. Nonetheless, these studies did not contribute in the knowledge as to how the microorganisms responded to the reduction of nitrate accumulation (Huang et al., 2004). To date, most findings are limited to grassland soils (Butterbach-Bahl et al., 2013) or tree-based intercrop systems (Graungaard, 2015). Hence, various researchers instill a greater inclusion in research for the *amoA* gene in AOA and AOB under different agricultural practices (Song et al., 2007; Wang et al., 2007; Zhang et al., 2014). Although intercropping systems have been studied under various scenarios, Song et al. (2007), Wang et al. (2007), and Zhang et al. (2014) informed that neither AOA nor AOB have been well-studied. Thus, *amoA* genes in both AMOs need to be better understood under different these types of cropping systems.

1.4.2 Denitrifiers

The second most important process of N_2O production, the DNIT process, is compounded by many steps with obligatory intermediates: NO_3^- to NO_2^-/NO_2^- to NO/NO to N_2O/N_2O to N_2 (Ligi et al., 2014). The two most important genes from an environmental perspective are the *nosZ* and the *nirK*. The *nosz* gene is involved in the last reductive reaction in the DNIT process (Ligi et al., 2014), and is encoded by the enzyme nitrous oxide reductase; responsible for transforming N_2O to N_2. However, the catalytical reaction performed by *nosz*, seldom occurs. Instead, the reaction is likely to end during the reduction from NO to

N_2O (Jones et al., 2008). This reduction is carried by the gene *nirK*. Therefore, the *nirK* gene is involved in the reduction step prior to the one performed by *nosz*.

The enzyme that encodes for *nirK* genes is nitrite reductase (Ligi et al., 2014). Unlike *amoA* in AMOs, this enzyme is encoded by copper containing *nirK* or cytochrome cd1 containing *nirS*; however, *nirS* and *nirK* do not share the same organism (Zumft, 1997). This gene can produce enough NO that will end up as N_2O (Shoun et al., 2012). Therefore, due to its role in gas formation this gene *nirK* has received greater attention in the DNIT process. It is imperative to determine the key factors in NO production that lead to N_2O emission, using an environmental approach. To date, there is plenty information about the factors that influence denitrifiers including competitiveness, predation, WFPS, pH, O_2, and substrate availability (Ladd et al., 1993; Franklin and Mills, 2003). For instance, one study reported greater abundance of *nirK* in treatments with a long-term N-fertilization; and lower abundance in the controls of the same experiment (Phillipot et al., 2007). Other findings explained a shift in the community structure of denitrifier after using mineral fertilizers in addition to cattle manure in agricultural soils (Zhou et al., 2011).

Hallin et al. (2009) stated that there is little agreement as to how organic fertilizers, for instance, influence denitrifier abundance in different soil types and agroecosystems. Furthermore, they explained that such discrepancy can be due to differences in the properties and the types of uses given to the soil. Ligi et al. (2014) reported that even though *nirK*-types responded to soil chemical parameters, different soil ecosystems can impact their gene expression. Assessing agroecosystems may further our understanding about gene *nirK*s. Overall, understanding the influence of crop systems over these genes will provide insights about what soil management practice can be used to lessen N_2O emissions abating climate change. In addition, it would be helpful to target genetical biomarkers as convenient indicators to develop better mitigation strategies to lessen GHG emissions (Theodorakopoulos et al., 2017).

1.5 The present study

More research about GHG emission is required (Pappa et al., 2011). Moreover, various countries are accountable to climate change, therefore, quantification of N_2O emissions from agricultural practices should not be debatable. This is particularly true for Argentina, which is included among the 25 countries with higher GHG emission (Secretaría de Ambiente y Desarrollo Sustentable de la Nación, 2007). Furthermore, the country ratified its commitment in reducing GHG emissions after the Kyoto Protocol issued in 2001. Research has shown that Argentina is emitting 0.88% of the global amount of N_2O (Dickie and Coronel, 2016). The Pampa region has been one of the regions accountable for the reduction of GHG emissions from agricultural soils is. This region with an area compounded by 0.7 million hectares, distributes major agricultural crops for the country (Austin et al., 2006). Distribution includes soybean (*Glycine max* (L.) Merr.), wheat (*Triticum aestivum* L.), maize (*Zea mays* L.) and sunflower (*Helianthus annuus* L.) (Austin et al., 2006; Viglizzo et al., 2003). Videla (2014) suggested that more field investigations about N_2O emissions to contribute to the country's national inventory of GHG emitted from agricultural soils is necessary.

N dynamics are not clearly understood in temperate areas, and this is especially true for intercropping systems (Oelbermann and Echarte, 2011). Intercropping systems have been broadly studied in terms of grain quality; N-use efficiency; N-fertilizer rates; erosion, and weed control (Prasad and Brook, 2005; Waddington et al., 2007). Within the Pampa region (Figure 2.1) intercropping systems have also been found to produce optimum plant density (Echarte et al., 2011; Dyer et al., 2012). Somewhat surprising, however, is the scarce understanding of the effect of intercropping systems in the gene *amoA* in AOB and AOA (AMOs) and their close connection with the N cycle (Zhang NN et al., 2014). Furthermore, from all the genes encoding for denitrifiers, *nirK* will be the gene of more interest in this thesis due to its great contribution to gas formation. Thus, this project will expand the knowledge of intercropping systems in regards to GHG emission. The GHG analyses will proceed from maize sole crops, soybean sole crops, and intercrop systems from a rural area, situated in Balcarce, Argentina. In addition, the GHG emissions analyzed during the experiments, aim to generate insights as to which cropping system is producing greater N_2O emission and

as to whether the NIT or DNIT contributes more to the production of N_2O. Lastly, this project intends to elucidate, confirm and relate four-gene types in microorganisms present in the soil to N_2O emissions from the three previously mentioned cropping systems, by using a molecular-based analysis, quantitative qPCR.

1.6 Impact of this study

This research aims to illustrate the main contributor of CO_2 and N_2O emission between sole crops and intercrops and thus, corroborate with previous investigators about the effect of intercropping systems in reducing GHG emissions. Also, to extend the knowledge about the effect of sole crops and intercrop systems in soil microbial gene abundance in Balcarce, Argentina.

1.7 Specific research objectives

- To evaluate temporal changes of soil CO_2 and N_2O emissions from a maize sole crop, soybean sole crop, and an intercrop system
- To quantify soil ammonium and nitrate concentrations from a maize sole crop, soybean sole crop, and an intercrop system
- To assess the relationship between temporal changes of CO_2 and N_2O to nitrifier and denitrifier gene abundance from a maize sole crop, soybean sole crop, and an intercrop system

1.8 Hypothesis

- CO_2 concentrations:
  - Maize sole crop and soybean sole crop will have greater CO_2 emissions compared to the intercrop
- N_2O concentrations:
  - Maize sole crop and soybean sole crop will have greater N_2O emissions compared to the intercrop

- Ammonium and nitrate concentrations:
    - Ammonium concentrations will be lower in the intercrop, compared to the maize sole crop and the soybean sole crop
    - Nitrate concentrations will be higher in the intercrop, compared to the maize sole crop and the soybean sole crop
- Nitrifier and denitrifier abundance:
    - High nitrifier and denitrifier abundance will prevail in the intercrop
    - Lower nitrifier and denitrifier abundance will prevail in the maize sole crop and soybean sole crop
    - Higher abundance of nitrifiers and denitrifiers will prevail in the NIT process

2. Materials and Methods

2.1 Study site

The soil used in this study was collected from the *Instituto Nacional de TecnologÍa Agropecuaria* (INTA), which is located near the city of Balcarce in the city of Mar del Plata in the rolling Argentine Pampa (Dyer et al., 2012). INTA is located at 130 m.a.s.l (37°45′S, 58°18′W).

Figure 2.1 Map of location study in the city of Balcarce

https://www.google.ca/maps/place/Balcarce,+Buenos+Aires+Province,+Argentina/@-38.7141816,-62.0239632,4.25z/data=!4m5!3m4!1s0x959aaf6a677eae5b:0xe896650fcfe4517b!8m2!3d-37.8464067!4d-58.2554584

2.2 Field site condition

The county of Balcarce experiences a temperate humid climate with no dry season (Domínguez et al., 2009) according to the Köpen classification or a mesothermal subhumid-humid climate described by Thornwaite's classification (Domínguez et al., 2009; Fabrizzi et al., 2003 and Regehr et al., 2015). Precipitation

increases during the spring and summer due to the "El Niño" warm phase (Podestá et al., 1999; Iizumi et al., 2014). From 1980 to 2012 the mean annual precipitation was 860 mm yr^{-1}, evapotranspiration was 856 mm yr^{1-}, and mean annual temperature was 14.3ºC [maximum (24.2ºC); minimum (7.6ºC) (Regehr et al., 2015)] (Figure 2.2). The soils have a moderately acidic pH value of 5.77. The SOC content was 30.6 g C kg^{-1} and 1.64 g N kg^{-1}; whereas the ratio between C and N was 18.66. The P availability in the soil was low [7.87 mg P kg^{-1} (Bray-extractable P) (Videla, 2014 as cited in Oelbermann et al., 2017)].

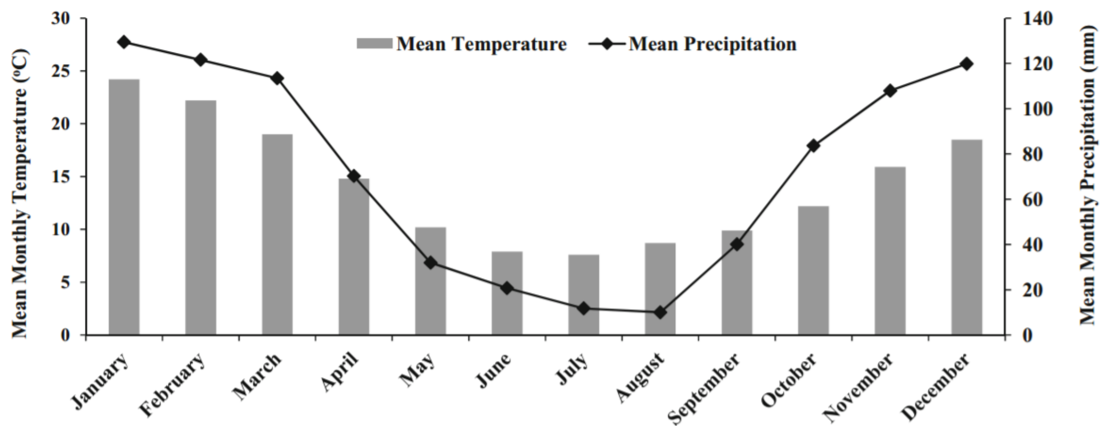


Figure 2.2 Monthly average of precipitation and temperature during 1980 to 2012 in the South region of the Pampas, Argentina alongside Balcarce (Adapted from Regehr et al., 2015).

Description of these soils is defined by the Food and Agriculture Organization (FAO) as Luvic Phaeozem (Taboada et al., 1998) or as Typic Agridudoll, according to the USDA Soil Taxonomic Key (Andrade, 1995; Regehr et al., 2015) this type of soil belongs to the *Mar del Plata* series. This type of series is characterized by a texture with 23.1% clay, 41% sand, and 36% silt (Domínguez et al., 2009) and they were formed by wind-blown silt (Domínguez et al., 2009; Nosetto et al., 2012). A second fine, mixed, illitic, and thermic Chernozemic Loam is original from the *Balcarce* series (Domínguez et al., 2009; Studdert and Echeverría, 2000) and were developed as a consequence of accumulated loess-wind-blown (Regehr et al.,

2015) that occurred in the midst of the Holocene (del Blanco and Stoops, 2007). Initially, grasslands in Balcarce were predominant but due to an intense livestock production the increase of grain production began to compete with the original landscape (Modernel et al., 2016). This land-use which occurred about 40 years ago converted natural grassland to agricultural production systems (Bichel, 2013). This shift resulted in high compacted soil leading to an increase in soil erosion, a decrease in N content, and soil organic matter losses (Hall et al., 1992; Modernel et al., 2016). As a result, the main strategy in 1990 was to improve the soils' condition by adopting conservation tillage as an alternative to conventional tillage (Álvarez et al., 2009).

To date, the strategies and sustainable alternatives have kept evolving in the Pampas region creating significant impacts over the last 20 years (Aramburu Merlos et al., 2015). In fact, the findings of the number of hectares in agricultural-land areas practicing conservation tillage increased from 25,000 to 7 million hectares (FAO, 2010). However, due to an international demand of 70% to 90% this pressure is evoking a high dependence on N-based fertilizers in crops, such as maize (*Zea mays* L.) and soybean (G*lycine max* L. [Merr.]) (Aramburu Merlos et al., 2015). Soybean crops, however, use relatively low amounts of N-fertilizer due to the inoculum of a *Rhizobium* spp. (Austin et al., 2006). Fortunately, the expansion of agricultural land areas have also resulted in the adoption of intercropping systems, which has become more noticeable in the Pampa region (Álvarez and Grigera, 2005).

2.3 Soil collection

Soil samples were collected from a randomized complete block design established in 2007. The experiment consisted of three replicates of four treatments (Figure 2.3) to measure its variation ("the power of replicates", n.d). The treatments included a maize sole crop (MSC), a soybean sole crop (SSC), an intercrop of 1:2 (one row of maize and two rows of soybean; INT), and an intercrop system of 2:3 (two rows of maize and two rows of soybean; INT). Maize was sown in October and harvested in April, while soybeans were sown in November and harvested in May. Both 1:2 and 2:3 INT systems were sown in October. Intercrop systems of 1:2 were not used, because, as reported in Regehr (2014), this system did not achieve

much, compared to intercrop systems of 2:3. Thus, in this project, intercrop systems of 1:2 were not discussed, as in previous theses documentation (e.g. Dyer, 2012; Bichel, 2013; Regehr, 2014) (Figure 2.3).

Maize in the sole crop and intercrop received N-fertilizer rates of 150 kg N ha^{-1}, whereas phosphorus (P) was added at a rate of 35 kg P ha^{-1} to all crop systems. The soybeans were inoculated with *Bradyrhizobium japonicum.* Weeds were controlled by glyphosate. Prior to the establishment of this experiment, the site was under sunflower cultivation (*Helianthus annus* L.) using minimum tillage. The experimental site was under minimum tillage (disk harrow followed by spike harrow). Each treatment plot size was 8.8 × 12 m. The maize and soybean sole crops were rotated annually. For example, treatment plots referred to as maize sole crop (MSC) were under maize production in 2008–09, 2010–11 and 2012–13. Treatment plots referred to as soybean sole crop (SSC) were under soybean production in 2008–09, 2010–11 and 2012–13. However, the intercrops were continuous (not rotated) and soybean and maize were planted in the same rows in successive years. Plant density (plants m^{-2}) was 8.0 (maize sole crop), 29 (soybean sole crop) and 4.3 (1:2 INT) with a 0.52 m distance between crop rows in all treatments.

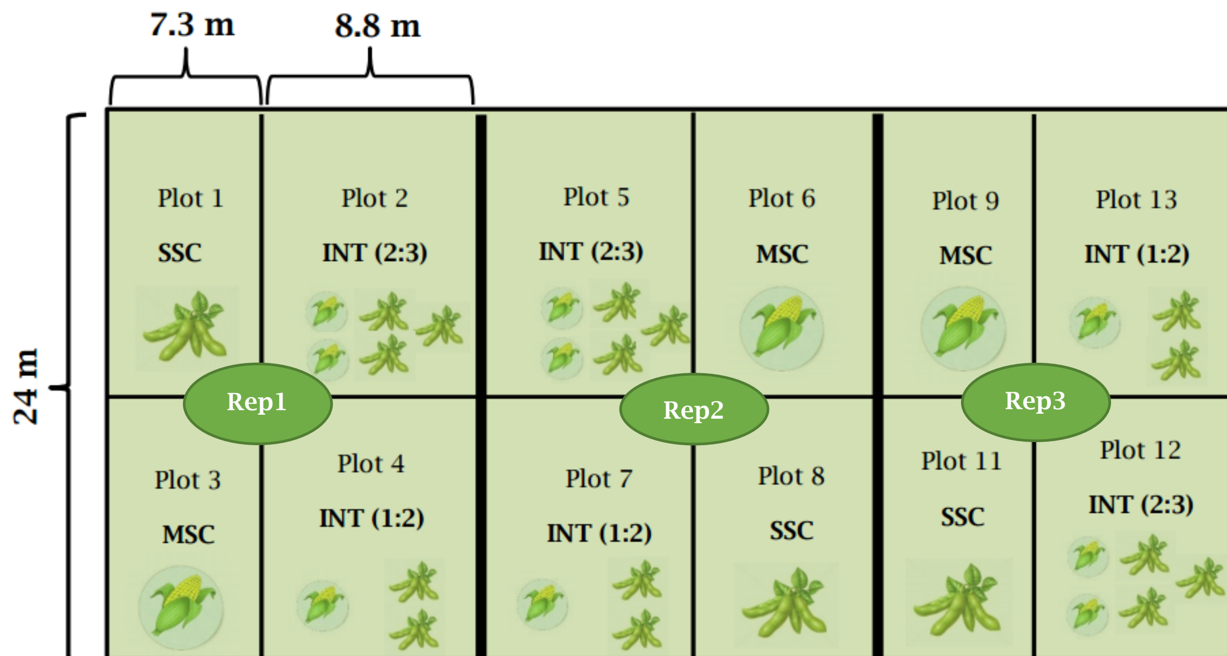


Figure 2.3 Measurements of INTA's Randomized Complete Block Design (RCBD) showing three of its replicates (rep 1, rep 2, and rep 3) from each maize sole crop (MSC), soybean sole crop (SSC), intercrop

(1:2) (not analyzed in this study), intercrop (2:3) (INT). This RCBD belongs to the cropping season 2011-2012.

Soil sampling was performed for each replicate within each treatment (Figure 2.3). Five samples were taken from the center of each treatment to avoid edge effects (Pennock et al., 2008). Soil collection was performed between 0-20 cm. In the case of the intercrop system, five samples were collected between the rows of maize, the rows of soybean, and the rows shared between maize and soybean for each replicate within each treatment. Once the sampling ended, the samples were mixed up to create homogenization since the tendency of bulk samples is to fractionate by aggregate size (Hoskins and Ross, 2003). Followed, the samples were weighed to provide greater precision in soil analysis (Hoskins and Ross, 2003) and further calculations such as bulk density. Soil samples were sent to the University of Waterloo by following Standard Operating Procedures (SOP) required by the Canadian Food Inspection Agency under Section 43 of the Plant Protection Regulation (CFIA, 2014). Thus, the soil samples for the current experiment were air dried and passed through a 2 mm mesh sieve to remove debris, gravel, and coarse crop residues.

2.4 Set up for laboratory incubation experiments

There were two set of jars with a volume of 1 L; the first set represented the nitrification (NIT) process and the second, the denitrification (DNIT) process. Both sets included a known amount of air-dried soil (60 g oven-dry equivalent). The air-dried soil samples were placed in funnels (Scienceware®) to account for the soil volume in the calculations, but also to avoid nutrient leaching from the soil samples (Sey et al., 2010). The jars and lids received sterilization in an autoclave at 121ºC for 15 minutes prior to the pre-incubation. All the jars received a secured closure in the aluminum lid using silicone septa, which make the experiment a closed system. This closure avoided any gas exchange between the headspace in the jar system and the atmospheric air in the laboratory. Silicone septas were sterilized prior to the pre-incubation; they were baked in the oven at 60 ºC for six hours. Each set of jars received three replicates corresponding to the RBCD; each set was assigned with its control group. There were a total of 18 jars per set (Figure 2.4).

Both pre-incubation and incubation were conducted at the Soil Ecosystem Dynamic's Lab (SEDL) at the University of Waterloo with the soil samples collected from INTA, Argentina.

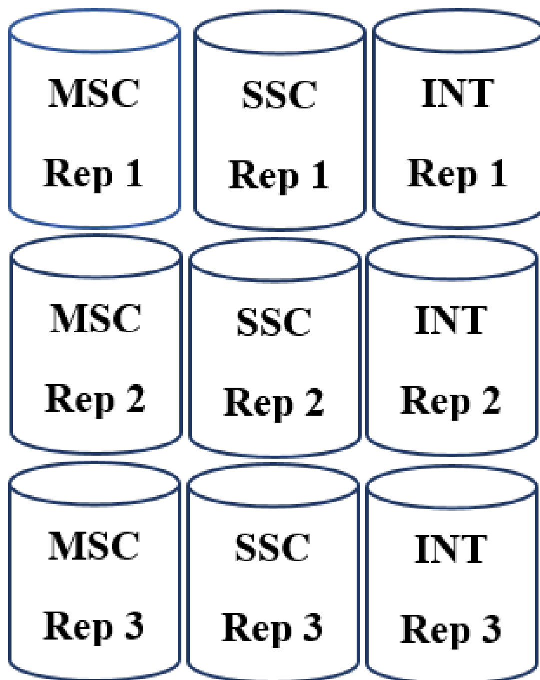
2.4.1 Pre-incubation

All soils were pre-incubated for seven days to promote microbial activity without creating a respiration pulse (stressful response) (Valentine, 2007) after rewetting the air-dried soils (Ruser, personal communication, November 22, 2016). A pulse can produce lysis-induction in the microbial community as a stressful mechanism response (the "Birch effect"; Birch, 1958). Throughout the pre-incubation period, all treatments were subjected to 50% WFPS at room temperature (Stewart al., 2009) in 1 L jars. WFPS% was determined in advanced based on prior calculations using the soil water content (Stewart et al., 2009), volume of the soil, bulk density, and pore volume (Linn and Doran, 1984; Franzluebbers, 1999; Ruser et al., 2001; R. Ruser and G. Velthof personal communication, November 22, 2016). Franzluebbers (1999) equation was used to determine WFPS%:

$$WFPS = (SWC\ x\ BD)/(1 - (\frac{BD}{PD})$$ **(Equation 1)**

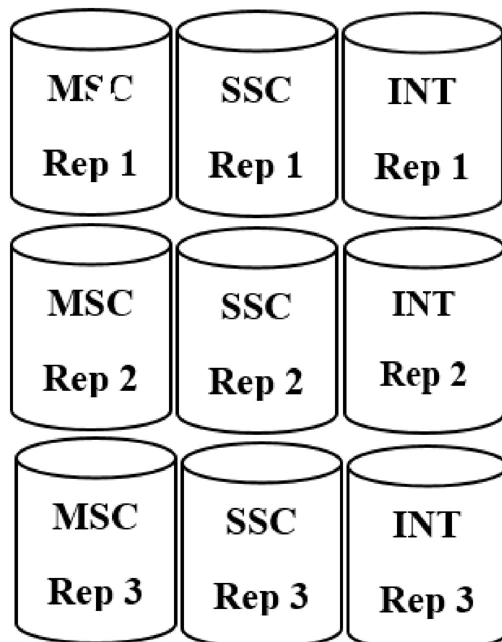
SWC = soil water content ($g\ g^{-1}$)
BD = bulk density ($Mg\ m^{-3}$)
PD = particle density (2.65 $Mg\ m^{-3}$)

During the pre-incubation, the DNIT jars received ultra-high purity (UHP) helium (He) every day for 20 minutes at 600 ml/min. The NIT jars received atmospheric air at the onset of the pre-incubation. The jars from both processes were kept in the dark during the entire pre-incubation until the end of the incubation period. Readjustment of WFPS% was applied as necessary by weighing the jars for all treatments and adding distilled water (type I) to maintain 50% WFPS before the incubation using Equation 1.



NIT process

- Control jars included the same number of jars and dry weight of soil.
- All jars contained atmospheric air inside creating an aerobic environment.
- Treatments received NH_4Cl



DNIT process

- Control jars included the same number of jars and dry weight of soil.
- All the jars contained ultra pure helium creating an anaerobic environment.
- Treatments received KNO_3^-

Figure 2.4 Illustration of the experimental set-up during the incubation period. NIT included three types of cropping systems shown in the illustration: maize sole crops (MSC), soybean sole crops (SSC), and intercrops (2:3) (INT). The amount of jars used for each replicate, the conditions at which each process was subjected to, and the type of fertilizer used for NIT and DNIT is described on the right side of the figure.

2.4.2 NIT incubation experiment

After the onset of the pre-incubation soil moisture was raised from 50% to 60% WFPS. Two different incubations were conducted with each soil: first, the control at 60% WFPS; second, the treatment at 60% WFPS with added N-mineral fertilizer in the form of NH_4Cl to promote NIT processes. The amount of fertilizer added was 0.0347 g for all treatments based on field recommendation standards in Balcarce, Argentina. The fertilizer was dissolved in the remaining 10% of the distilled water required to reach the desired WFPS%. After increasing WFPS% and adding fertilizer, both controls and treatments were kept in the dark for an hour to start with the gas collection. WFPS% was maintained by adding distilled water, if necessary (Figure 2.5). The time series throughout the entire NIT experiment consisted of 1, 6, 12, 24, 36, and 48 hours. The jars were kept in the dark throughout the experiment. Blank-jars, without soil and fertilizer, were included as well (Bichel et al., 2017). Every time a gas sample was collected from all jars, including the blank jar, the same amount of gas extracted was replaced with the atmospheric air in the laboratory to keep the process of NIT ongoing. The jars were placed in a box per group (NIT) and were enclosed with black plastic bags to simulate underground conditions.

2.4.3 DNIT incubation experiment

After the onset of the pre-incubation the soils were raised from 50% to 80% WFPS. Two different incubations were conducted with each soil: first, the control at 80% WFPS; second, the treatment at 80% WFPS with added N-mineral fertilizer in the form of KNO_3^- to promote DNIT processes. The amount of fertilizer added was 0.0680 g for all treatments based on field recommendation standards in Balcarce, Argentina. The fertilizer was dissolved in the remaining 30% of the distilled water required to reach the desired WFPS%. After increasing WFPS%, and after adding fertilizer, both controls and treatments were flushed

with ultra-high purity helium (He) for 20 minutes at 600 ml/min to form anaerobic conditions (Snider et al., 2015) (Figure 2.5). Anaerobic conditions were maintained throughout the experiment by adding distilled water, if necessary. The jars were placed in a box per group (DNIT) and were enclosed with black plastic bags to simulate underground conditions.

Figure 2.5 UHP-He flushing the jars in the DNIT process through a valve regulator attached to one needle; the second needle was used to release the oxygen out of the system.

All jars were kept in the dark throughout the experiment. The time series throughout the entire DNIT experiment consisted of 1, 6, 12, 24, 36, 48 hours. After each sampling event, the anaerobic conditions were maintained by flushing 5 minutes with UHP-He to keep the process of DNIT ongoing using the tree branch apparatus.

2.5 Measuring CO_2 and N_2O concentrations

The concentration of CO_2 and N_2O were analyzed at hour 1, 6, 12, 24, 36, 48; using a syringe to extract 10 ml of gas from the headspace, and were transposed and over-pressurized into a pre-evacuated 3 ml Exetainer vial (LabCo. Limited, High Wycombe, UK) (Parkin and Venterea, 2010). These GHG gas samples were analyzed on an Agilent 6890 (Agilent Technologies Inc., Santa Clara, CA, USA), using a

capillary column attached to a detector of thermo-conductivity detector (TCD) to measure CO_2. This column is also attached to an electron capture detector (ECD) to quantify N_2O emissions in parts per million (ppm). A linear correlation of a commercial standard was established in the gas chromatograph for minimum values of N_2O (0.98 ppm), and maximum values of CO_2 (1050 ppm) (Praxair Canada, Inc.) to calibrate the sample data before reading the gas concentrations of both GHGs. The following equation adapted from Hogg et al. (1992) was used to determine daily production rates of both CO_2 and N_2O:

$$R = (Cs - Ca) * (V * D) / {}^{M}/_{t} \quad \textbf{(Equation 2)}$$

$$R = (Ns - Na) * (V * D) / {}^{M}/_{t} \quad \textbf{(Equation 3)}$$

Where *D* is the density of CO_2 (equation 2) and N_2O (equation 3), respectively, in the jar adjusted for temperature (g/L); *V* is the volume of the jar's headspace; *t* is the time interval between samples collected (hours), *Cs* is the GHG concentration originated from the soil and *Ca* is the concentration originated from the blank jar (applicable only for nitrification); *M* is the dry weight of the soil sample expressed in grams.

2.6 Soil NH_4^+ and NO_3^- concentrations

To determine the NH_4^+ and NO_3^- concentrations, a volume of 50 ml of 2.0 M KCl was added to 10 g of soil of each crop system (McLeod, 1992; Maynard and Kalra, 1993; Smith and Li, 1993; Jones, 2001): MSC, SSC, and INT. This soil extraction occurred two times, first, at the beginning of the experiment (t=0), and second, at the end of the experiment (t=48). The soil with the KCl was shaken for 15 minutes at 180 rpm, and filter through a Whatman 42 filter paper to obtain the extracted reagent. This extraction reagent was saved after filtration, if necessary. NH_4^+ and NO_3^- assessments were made by using a Shimadzu 1800 UV-Vis spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

NH_4^+ in soil samples were quantified by preparing two reagents for concentration analysis. Reagent A was made by dissolving 0.05 g sodium nitroprusside, 13 g sodium salicylate, 10 g sodium citrate, and 10 g sodium tartrate in approximately 100 ml of water. Reagent B was done by dissolving 6 g of NaOH and 1 ml of 5% sodium hypochlorite in 100 ml water. A working stock solution was prepared for creating a standard curve with final concentrations (mg N/L). The standard curve will give us a mathematical model to replace the concentration values. For all soil samples extracted, 0.2 ml of Reagent A and B were added measuring 0.16 ml into semi-micro cuvettes. These were mixed and read at 650 nm of absorbance using the UV-Vis-spectrophotometer (Shimadzu Corporation, Kyoto, Japan) after one hour of color transformation (Forster, 1995). Similarly with NO_3^-, a working stock solution was prepared for creating a standard curve with final concentrations (mg N/L). Soil samples received 1 ml reagent into acid-resistant semi-micro cuvettes, capped and inverted to mix. Color transformation developed after 6 to 8 hours, and was stable after 1 day. Samples were left at room temperature overnight and readings were taken the next day. Absorbance was read at 540 nm (Miranda et al., 2001).

2.7 Microbial community function

2.7.1 DNA preservation and extraction

The soil samples were immediately preserved after t=48 hours (after the last extraction of the nitrification and denitrification experiment) in Power-Bead Tubes (Power Soil® DNA Isolation Kit). These tubes contain an aqueous solution of guanidine thiocyanate, which helps to disperse soil particles and, in this case, stabilize DNA prior to extraction. For each sample, 0.25 g wet soil was weighted directly into each tube in duplicate (2 DNA extraction tubes per sample) and then stored at -20°C until DNA extraction. DNA extractions were performed according to the manufacturer's protocol (MoBio Laboratories, Inc) with the following modification. Prior to bead-beating, the samples were incubated at 65°C in a water bath for 10 minutes. The centrifugation for all duplicates occurred at *10,000 x g* in an Eppendorf 5424 R (Figure 2.12). Once extractions were completed and quality was determined, duplicate DNA extractions were combined to form a single DNA sample per soil sample.

2.8 Agarose gel electrophoresis

The initial DNA quality (molecular weight and extent of shearing) from each duplicate sample was assessed on a 1% (w/v) agarose gel prepared with 1X TAE (mixture of Tris base, acetic acid and EDTA) and stained with SYBR Safe; (diluted 1: 10,000; Life Technologies, Burlington ON). A 1 kb (0.5 µg/lane) reference DNA ladder was used to assess extracted DNA size.

2.9 DNA quantification using the nanodrop and picogreen technique

The concentration, purity, and quality of the extracted DNA was assessed using spectrophotometric and fluorescent methods. Spectrophotometric measures, which provided general concentration and overall quality information, were performed using a NanoDropOne UV-Vis Spectrophotometer (ThermoScientific; Figure 2.12). The DNA samples were then pooled and final quantification was obtained using a fluorescent PicoGreen assay. The PicoGreen reagent is concentrated in DMSO solution, in 200mM Tris HCl, 20 mM EDTA, pH 7.5. The manufacturer's protocol was followed using the Quant-iT™ PicoGreen® dsDNA Reagent kit protocol. Instead of a 1:10 dilution, this protocol adjusted the volume of the DNA sample (5 µl) from a 1:5 dilution, as well as the final concentration of the standard curve to 50 cl. The C1000 Thermal Cycler coupled with a CFX-96 optical module [Bio-Rad Laboratories, Inc (Bio-Rad)] was used to read the concentration values of the extracted DNA. The PicoGreen method is not sensitive to the presence of contaminants and provides more accurate values of DNA concentration.

2.10 qPCR of nitrification and denitrification functional genes

After assessing the quality and quantity of the DNA using electrophoresis and spectrophotometry (NanoDrop One, ThermoScientific) the abundance of four different functional genes was measured, also known as reference genes, using quantitative PCR. For each functional gene, a set of forward and reverse primers that target the gene of interest was used. The descriptions of these four genes abundance are as follow (Table 2.1):

Table 2.1 These functional or reference genes were used in the quantitative PCR analyses to target the presence of nitrifier and denitrifier organisms in MSC, SSC, and INT sampling soils from INTA, Argentina, in both NIT and DNIT processes

Organism targeted	Reference gene	Gene targeted	Reference
Bacteria nitrification	*amoA1f*	5'—CCCCTCKGSAAAGCCTTCTTC—3'	Rotthauwe et al., 1997
	amoA2r	5'—GGGGTTTCTACTGGTGGT—3'	
Archaea nitrification	*amoAf*	5'—ATAGAGCCTCAAGTAG-GAAAGTTCTA—3'	Meinhardt et al., 2015
	amoAr	5'—CAAGCGGCCATCCAGCTG-TATGTCC—3'	
Bacterial denitrification	*nirK-583f*	5—TCATGGTGCTGCCGCGKGACGG—3'	Liu et al., 2003
	nirk-909r	5'—GAACTTGCCGGTKGCCCAGAC—3'	
Archaeal denitrification	*anirKa_58f*	5'—ACBYTATTCGGAAGYACATA-CACA—3'	Lund et al., 2012
	anirKa_578r	5'—GYMATTCCGTACATKCCGGA—3'	

Prior to the qPCR analyses, each reaction (occurring within each well within a q-PCR plate) contained a specific amount of Q-PCR master mix (diluted from 90 ng/ul to 10 ng/µl), primers, ultra pure grade H_2O and relevant DNA from each of the samples to make up for a final volume of 20 µl. These reactions were performed for each reference gene: *amoA*-Bacteria (AOB), *amoA*-Archaeal (AOA), *nirK*-Bacterial, (*nirK* bacteria) and *nirK*-Archaeal (*nirK* archaea). It is worth noting that the only DNA diluted from its full strength to a 1:10 dilution was the *amoA*-Archaeal. In addition, each individual reaction contained 0.5 µM from each primer, except for *nirK*-Bacteria which received 0.6 µM from each primer. These modifications were included due to low efficiency values. Also, due to negligible values of reproducibility for standard

concentrations. The first reference gene, *amoA*-Bacterial contained 1 µl from a 10µM stock primer (#25, #26); 10 µl of the q-PCR mix (Bioline Sensifast); 6 µL of ultra pure grade H_2O. After these reagents were added and mixed, then 2 µl of relevant DNA full strength was inserted into each well in the q-PCR plate. The second reference-gene, *amoA*-Archaeal received the same volume of 10 µM stock primers (#33,34) as well as the same volume for the q-PCR master mix (BioRad Sso); however, this reaction contained 5 µl of ultra pure grade H_2O. Also, each reaction contained 3 µl from the relevant DNA and was diluted from full strength to 1:10 dilution. The third reference-gene, *nirK*-Bacterial received equal volume from each reagent as specified for the *amoA*-Bacterial reaction. In addition, the q-PCR master mix was the same (BioRad Sso) and the stock primer added were #50 and #51. The fourth reference-gene, *nirK*-Archaeal received 0.5 µL from each primer (#52, #53); 10 µl from q-PCR master mix (Bioline Sensifast); 5 µl of ultra pure grade H_2O. After all reagents were well mixed, 3 µl of the relevant DNA was added. As mentioned, these volumes were per reaction; there were a total of 100 reactions per plasmid. For this reason, 2000 µl for each q-PCR tray were prepared, and duplicates of each samples were included. There was a triplicate for standards and non-template control.

The standards were diluted from 10^{-1} to 10^{-7} (Figure 2.10). Only the dilution of standards was done in separate tubes and not directly into the tray (they were then transferred from the tubes to the tray after being diluted and mixed) to avoid cross-contamination. Reagents were well-mixed and diluted prior to the addition of the DNA samples into the qPCR tray. The qPCR was set up for three phases: the first phase of denaturation for 3 minutes at 95°C; the second phase -annealing- with 35 to 40 cycles for 5 s at 95°C; the third phase, an extension from 20 to 30 s at 60°C; and lastly, the final melt curve from 65°C to 95°C using the Bio-Rad CFX 384 Real-Time PCR Detection system [Bio-Rad Laboratories, Inc (Bio-Rad)] (Figure 2.6). The standard curves were made of the following sequence identity of clone plasmids (Table 2.2):

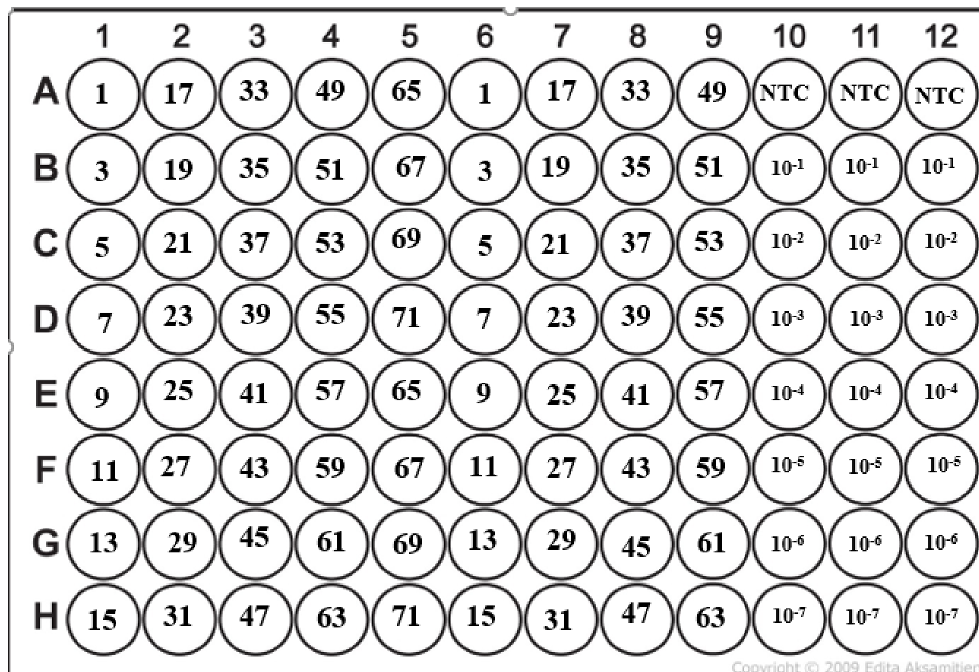


Figure 2.6 Each well represents a reaction (1-71) and every reaction had a duplicate (1 and 6; 2 and 7; 3 and 8; 4 and 9). Non-template controls (NTC) does not represent either a standard nor a plasmid. Dilutions from 10^{-1} to 10^{-7} were representative of a tenfold standard dilution and were made in replicates of three (from A to H). Every reaction contained 20 µl in the final volume.

Table 2.2 Description for clone plasmids used in MSC, SSC, and INT for both NIT and DNIT processes at Agriculture and Agri-Food Canada at Harrow's Research Station

Clone plasmid description	Accession Number
Bacterial *amoA*: *Nitrospira* sp. Nsp2; ammonia monooxygenase (*amoA*) gene.	GenBank Accession Number AY123822.1
Archaeal *amoA*; *Candidatus Nitroscosmicus* sp. G61; ammonia monooxygenase (*amoA*) gene	GenBank Accession number CP017922.1
Bacterial *nirK*: *Bradyrhizobium* sp. D209a; *nirK* gene for copper-containing nitrite reductase, partial cds;	GenBank Accession number AB480457.1
Archaeal *nirK: Candidatus Nitrosotenuis* cloacae strain SAT1; nitrite reductase	GenBank Accession number CP011097.2

2.11 Statistical analyses

All data was tested for homogeneity of variance and normality, and was evaluated for normal distribution ($p>0.05$; Shapiro-Wilk) and for equal variances ($p>0.05$; Levene's test) (Steel et al., 1997). The differences between crop treatments, and the interaction effects between crop treatment and time series, as well as the overall means (averaged over the 48 hours of incubation) were analyzed using a two-way ANOVA (univariate analysis). Sampling hour, crop treatment and presence/absence of fertilization were used as fixed factors, under "between subject factors". When ANOVA showed significant main effects, a Tukey's post-hoc multiple comparison test with a Least Significant Difference (LSD) correction was used to identify "simple effects" differences. Correlations were determined among greenhouse gas (CO_2 and N_2O), soil concentrations, and gene abundance since all are random variables (Zou et al., 2003). For all statistical analyses the threshold probability level was $p<0.05$. Each correlation measured hereby included interval levels (i.e., they were continuous), and were independent of each other; the significance of the slopes was assessed (Ligi et al., 2014); each pair of variables was bivariately normally distributed; and all sample of this data was random in the population; no outliers were detected (Kent State University Libraries, 2017). Correlations were performed using bivariate analysis. All data analyses was performed in IBM SPSS Statistics (version 24, 2017).

3. Results

3.1 Greenhouse gas emissions in the nitrification process

3.1.1 CO_2-C emissions

The interaction effect between 1) hours and crop type [F(10, 72)=0.405 p>0.05 = 0.940]; 2) crop type and treatments (fertilized and unfertilized) [F (2, 72)=0.833, p>0.05 = 0.439]; and, 3) hours-by-treatment-by-crop [F (10, 72)=0.286, p>0.05=0.982] was not significant for CO_2-C emissions. An analysis of the main effect for crop type revealed that it was statistically significant, F(2,72)=4.39, p<0.05=0.016. All pairwise comparisons were run and p-values were adjusted. The marginal means of CO_2-C emissions for MSC, SSC, and INT fertilized and unfertilized soils were 1.97±0.092 mg CO_2-C g^{-1} h^{-1}, 1.69±0.092 mg CO_2-C g^{-1} h^{-1} and 2.06±0.092 mg CO_2-C g^{-1} h^{-1}, respectively. Mean in CO_2-C emissions were significantly different [F (5,72)=78.5, p<0.0001) (Figure 3.1 and Appendix A). In the fertilized treatments soil CO_2 emissions from MSC and INT were significantly higher than SSC, especially at hour 36. The highest emission peak for MSC occurred from hour 1 to hour 24. MSC had significant differences between fertilized and unfertilized treatments (p<0.05=0.009).

CO_2-C emissions were significantly different between MSC and SSC crops (p<0.05=0.034) and INT was different to SSC (p<0.05=0.006) (Figure 3.1 and Appendix A).; however, MSC and INT were not significantly different in CO_2-C emissions. SSC and INT did not have a significant differences in CO_2-C emissions between fertilized and unfertilized treatments. Nevertheless, the results showed that SSC treatments remained the lowest from hour 24 to 48. INT treatments emitted more CO_2 compared to MSC and SSC during all 48 incubation hours in the unfertilized treatments. Meanwhile, in the fertilized treatments INTs only emitted significantly higher than sole crops at hour 36. CO_2 emissions represent the rate of organic carbon decomposed (Munoz et al., 2010) . Excessive C emitted from microbial respiration has been more often related to N-limitation (Manzoni et al., 2012). Some soils have shown to emit excessive amounts of C in the form of CO_2 as a way to compensate such limitation (Manzoni and Porporato, 2009). In addition, under N-limitation microbial communities have shown to accumulate N in the presence of high C:N ratios

(Lomstein et al, 1998). Thus, a higher respiration in unfertilized treatments seemed to be promoted to offset N-limitations in INT systems, resulting in lower N_2O emissions overall.

3.1.2 N_2O-N emissions

The interaction effect between crop type and treatment (fertilized vs. unfertilized) was significantly different compared to the other two interaction effects (crop-type-by-hours and crop-type-by-fertilization-by-hours) [$F(2, 72)=7.64$, $p<0.05=0.001$] (Table 3.2). This interaction effect was only significant in the MSC soils [$F(1,72)=26.9$, $p<0.001=0.000002$). Mean N_2O-N emissions (µg N_2O-N g^{-1} h^{-1}) were significantly different throughout the 48 hours [$F(5,72)=7.98$, $p=0.000005$]. Mean N_2O-N emissions ranged from 0.48 to 1.64 µg N_2O-N g^{-1} h^{-1} in fertilized (Figure 3.3; and Appendix A)and from 0.43 to 0.56 µg N_2O-N g^{-1} h^{-1} in unfertilized crop-type soils. Mean N_2O-N emissions in MSC soils were significantly different to SSC ($p=0.016$) and to INT soils ($p=0.001$), however, INT soils were not significantly different to SSC soils; only different to MSC soils ($p=0.001$). INT were higher in N_2O emissions in comparison to MSC before hour 24 and lower after this hour, however, these soils were the lowest overall (Figure 3.3 and Figure 3.4).

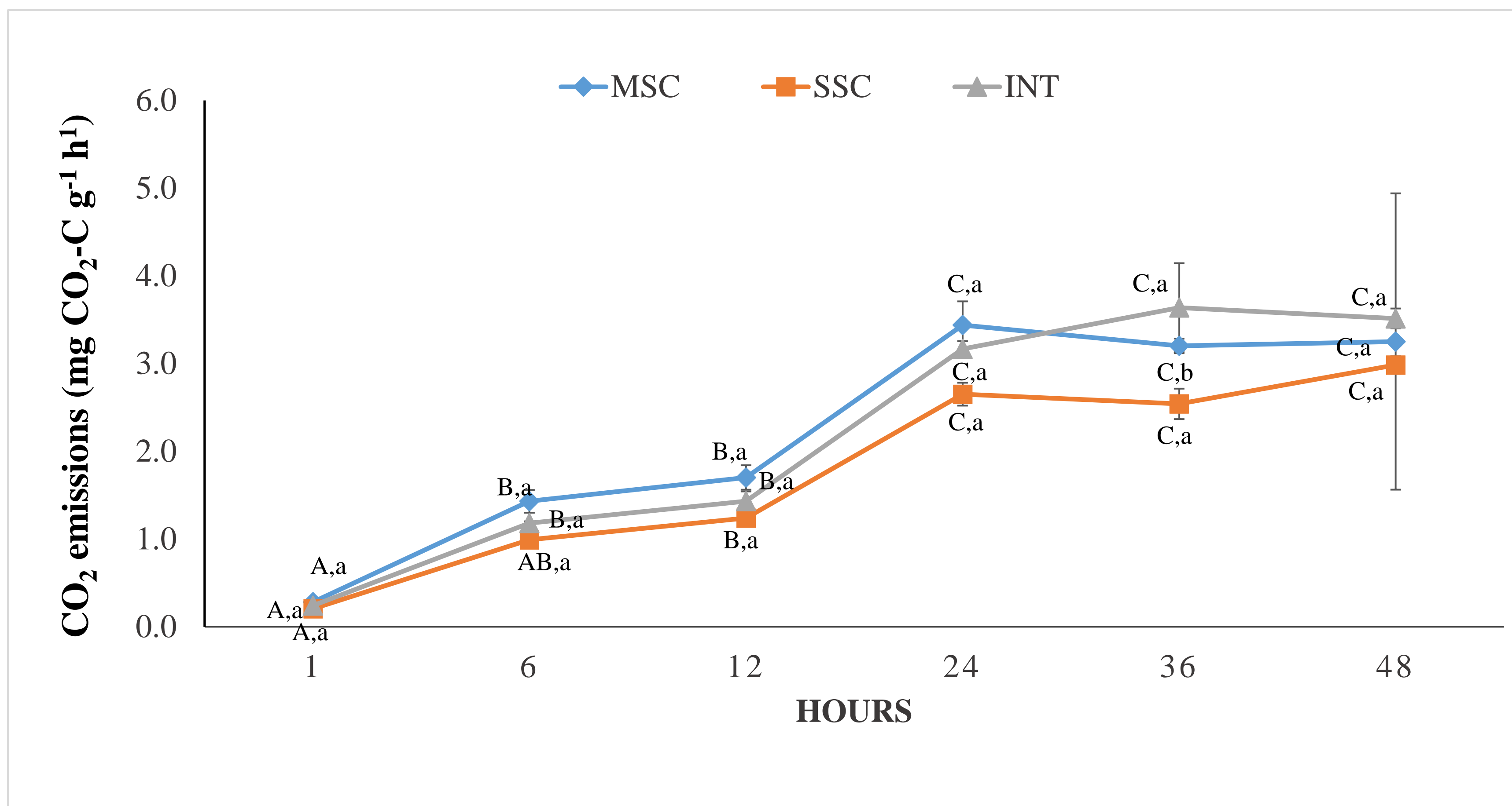


Figure 3.1 Mean CO_2 emissions (mg CO_2-C g^{-1} h^{-1}) from maize sole crops (MSC), soybean sole crops (SSC) and intercrops (INT) that received fertilized treatment with NH_4Cl and were incubated during 48 hours under 60% WFPS from Balcarce Research Station in Argentina from the period 2011-2012. Vertical bars represent standard errors. Values followed by different lower-case letters are significantly different ($p<0.05$) within time series between crop treatments. Values followed by different upper-case letters are significantly different between time series within crop treatment. Statistical tests executed in SPSS using Two-Way ANOVA and Tukey's with a Bonferroni correction if ANOVA was significant.

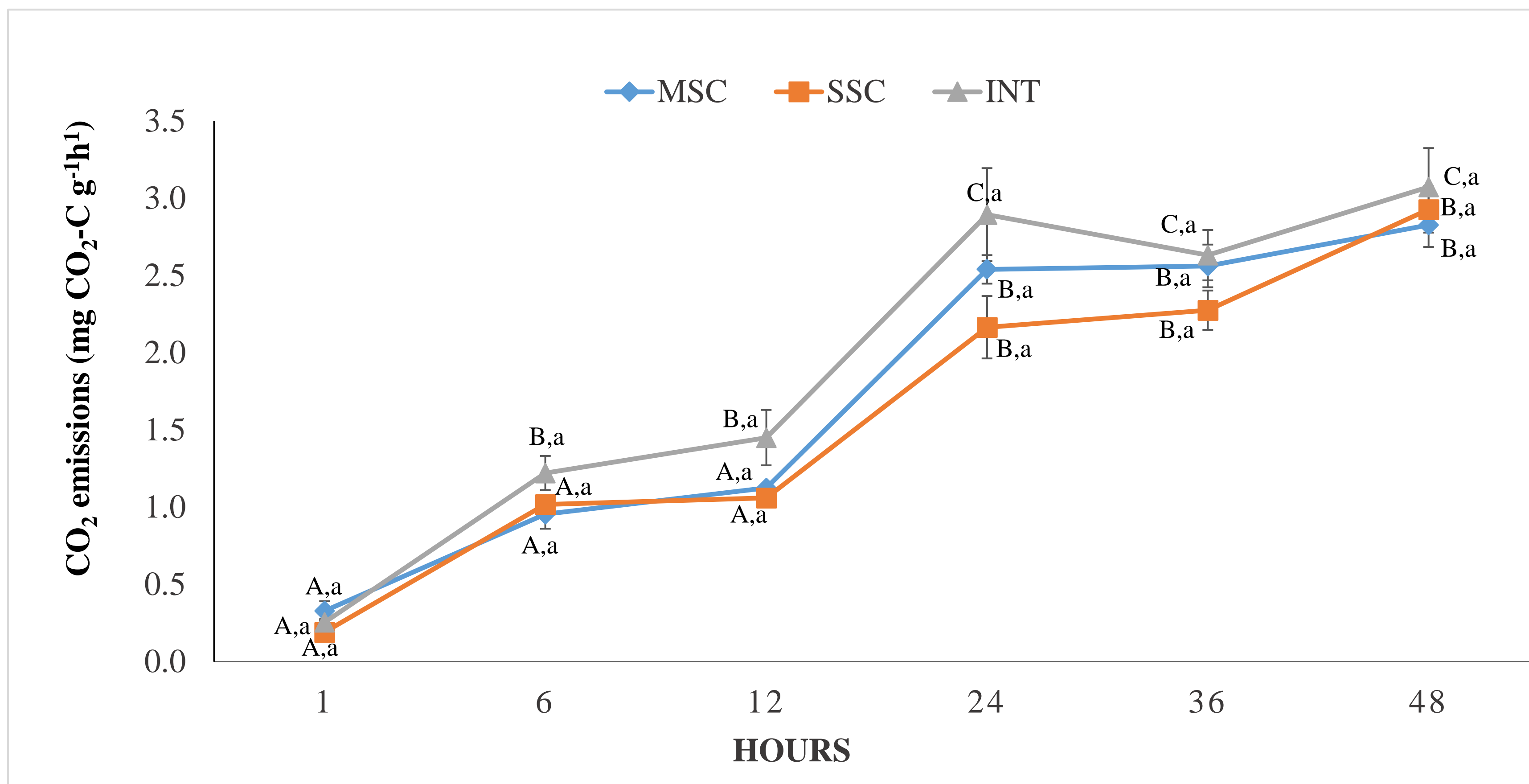


Figure 3.2 Mean CO_2 emissions (mg CO_2-C g^{-1} h^{-1}) from maize sole crops (MSC), soybean sole crops (SSC) and intercrops (INT) soils from Balcarce Research Station in Argentina that did not receive fertilized treatment with NH_4Cl and were only incubated under 60% of WFPS for 48 hours. All soils belong to the period 2011-2012. Vertical bars represent standard errors. Values followed by different lower-case letters are significantly different ($p<0.05$) within time series between crop treatments. Values followed by different upper-case letters are significantly different between time series within crop treatment. Statistical tests executed in SPSS using Two-Way ANOVA and Tukey's with a Bonferroni correction if ANOVA was significant.

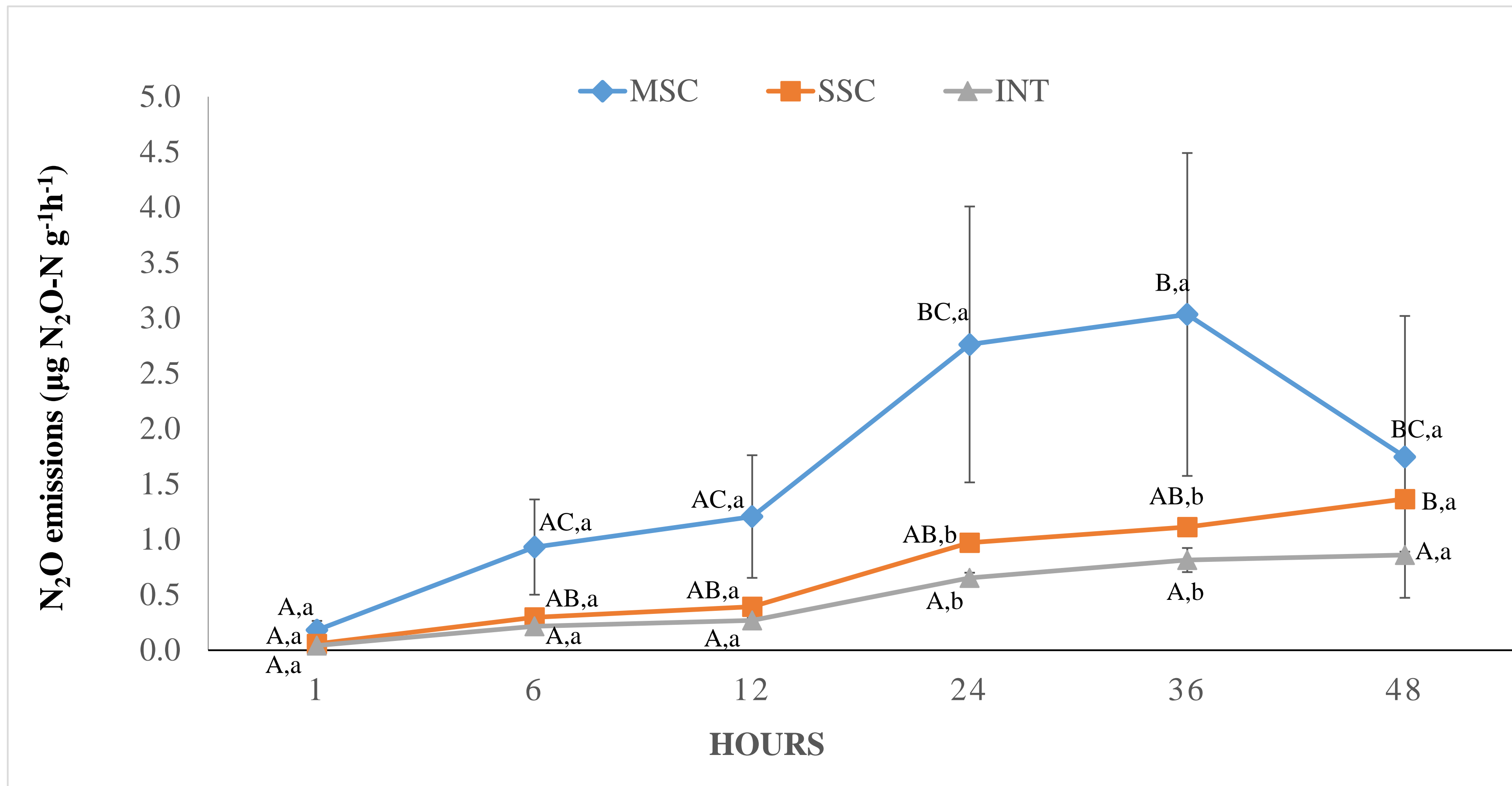


Figure 3.3 Mean N_2O emissions (μg N_2O-N g^{-1} h^{-1}) from maize sole crops (MSC), soybean sole crops (SSC) and intercrops (INT) that received fertilized treatment with NH_4Cl and were incubated for 48 hours under 60% of WFPS from Balcarce Research Station in Argentina from the period 2011-2012. Vertical bars represent standard errors. Values followed by different lower-case letters are significantly different ($p<0.05$) within time series between crop treatments. Values followed by different upper-case letters are significantly different between time series within crop treatment. Statistical tests executed in SPSS using Two-Way ANOVA and Tukey's with a Bonferroni correction if ANOVA was significant.

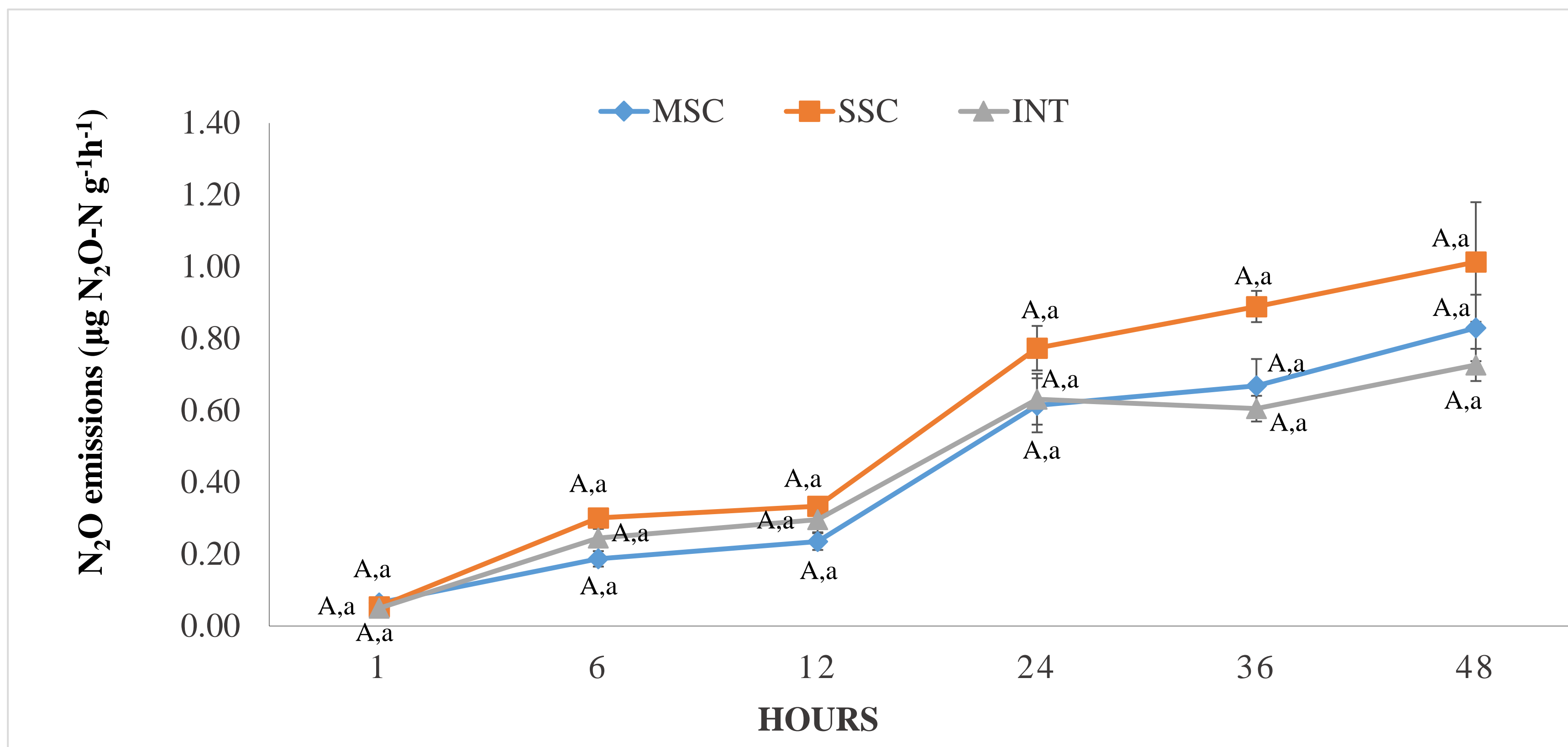


Figure 3.4 Mean N_2O emissions (µg N_2O-N g^{-1} h^{-1}) from maize sole crops (MSC), soybean sole crops (SSC) and intercrops (INT) soils from Balcarce Research Station in Argentina that did not receive fertilized treatment with NH_4Cl and were only incubated under 60% of WFPS for 48 hours. All soils belong to the period 2011-2012. Vertical bars represent standard errors. Values followed by different lower-case letters are significantly different ($p<0.05$) within time series between crop treatments. Values followed by different upper-case letters are significantly different between time series within crop treatment. Statistical tests executed in SPSS using Two-Way ANOVA and Tukey's with a Bonferroni correction if ANOVA was significant.

3.2 Greenhouse gas emissions in the denitrification process

3.2.1 CO_2-C emissions

The interaction effect between 1) hours and crop type [F (10, 72)=0.441 p>0.05 = 0.921]; 2) crop type and treatments (fertilized and unfertilized) [F (2, 72)=3.03, p>0.05 =0.054]; and, 3) hours-by-treatment-by-crop [F (10, 72)=0.980, p>0.05=0.468] was not significant for CO_2-C emissions. Moreover, an analysis of the main effect for crop type was performed, which indicated that the main effect was not statistically significant F(2,72)=2.80, p<0.05=0.067. All pairwise comparisons were run and p-values were adjusted. The marginal means of CO_2-C emissions for MSC, SSC, and INT fertilized and unfertilized soils were 2.22±0.39 mg CO_2-C g^{-1} h^{-1}, 1.77±0.092 mg CO_2-C g^{-1} h^{-1} and 2.20±0.14 mg CO_2-C g^{-1} h^{-1}, respectively.

CO_2-C emissions were significantly different between MSC and SSC crops (p<0.05=0.042) and INT was different to SSC (p<0.05=0.046) (Appendix B); however, MSC and INT were not significantly different. All soils from the three different crop types were not significantly different between fertilized and unfertilized treatments (p<0.05=0.374). Mean CO_2-C emissions were significantly different throughout the 48 hours [F (5,72)=71.1, p<0.0001). CO_2-C emissions were significantly different within crop types between different hours (Appendix B).

3.2.2 N_2O-N emissions

The interaction effect between 1) hours and crop type [F (10, 72) = 0.195 p>0.05 = 0.996]; 2) crop type and treatments (fertilized and unfertilized) [F (2, 72) = 0.668, p>0.05 = 0.516]; and, 3) hours-by-treatment-by-crop [F (10, 72) =0.118, p>0.05=1.00] was found non-significant for N_2O-N emissions. Mean N_2O-N emissions ranged from 9.37 to 12.74 µg N_2O-N g^{-1} h^{-1} in fertilized (Appendix B) and from -0.01 to 1.25 µg N_2O-N g^{-1} h^{-1} in unfertilized crop-type soils (Appendix B). Mean N_2O-N emissions in MSC, SSC, and INT soils had no significant difference between one another. Also, no significant difference was found between fertilized and unfertilized treatments. Mean N_2O-N emissions (µg N_2O-N g^{-1} h^{-1}) were significantly different throughout the 48 hours [F (5,72) =13.58, p=0.0001] (Appendix B). Emissions in the DNIT process were overall higher than the NIT process mainly due to a lower soil aeration present in these soils. The greater

the water-filled-pore-space is (80%) the smaller amount of air these pores will contain, thus, increasing N_2O production (Signor and Cerri, 2013). The main objective of the DNIT is to produce N_2 so the cycle can start all over again and be available to living organisms such as plants and microorganisms. However, under little oxygen present in the soil pores, the pathway shifted expressing genes capable of producing more N_2O instead of N_2 (Snyder et al., 2009). Therefore, caution during 60% WFPS-conditions should be taken for both MSC and SSC, since their emissions were the highest in both fertilized and unfertilized groups. In terms of cropping systems, INTs seemed to resist high water conditions compared to both MSC and SSC. Thus, they are ideally the best agricultural management approach that can be used to reduce N_2O emissions.

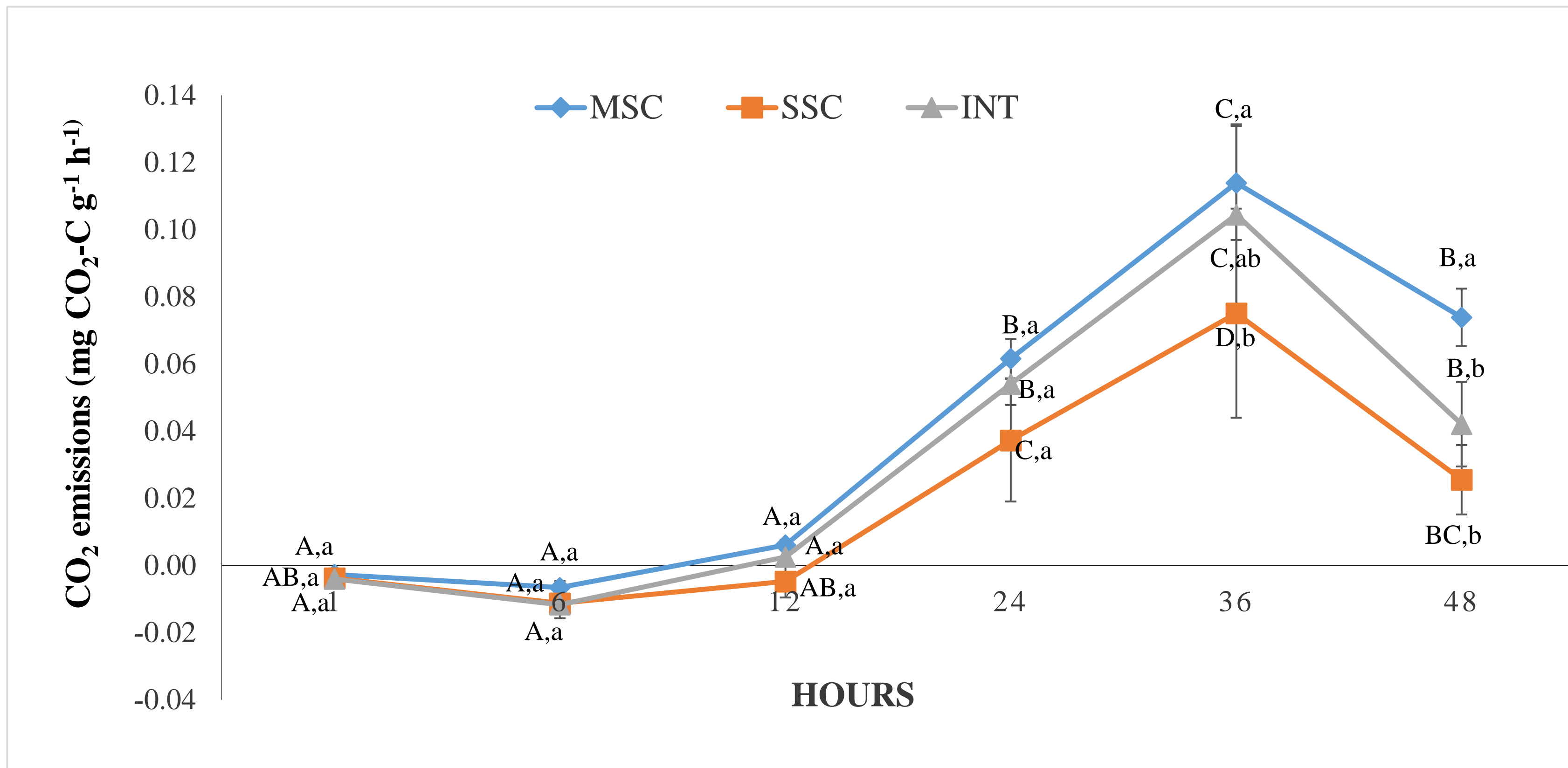


Figure 3.5 Mean CO_2 emissions (mg CO_2-C g^{-1} h^{-1}) from maize sole crops (MSC), soybean sole crops (SSC) and intercrops (INT) that received fertilized treatment with KNO_3^- and were incubated for 48 hours at 80% of WFPS. from Balcarce Research Station in Argentina from the period 2011-2012. Vertical bars represent standard errors. Values followed by different lower-case letters are significantly different ($p<0.05$) within time series between crop treatments. Values followed by different upper-case letters are significantly different between time series within crop treatment. Statistical tests executed in SPSS using Two-Way ANOVA and Tukey's with a Bonferroni correction if ANOVA was significant.

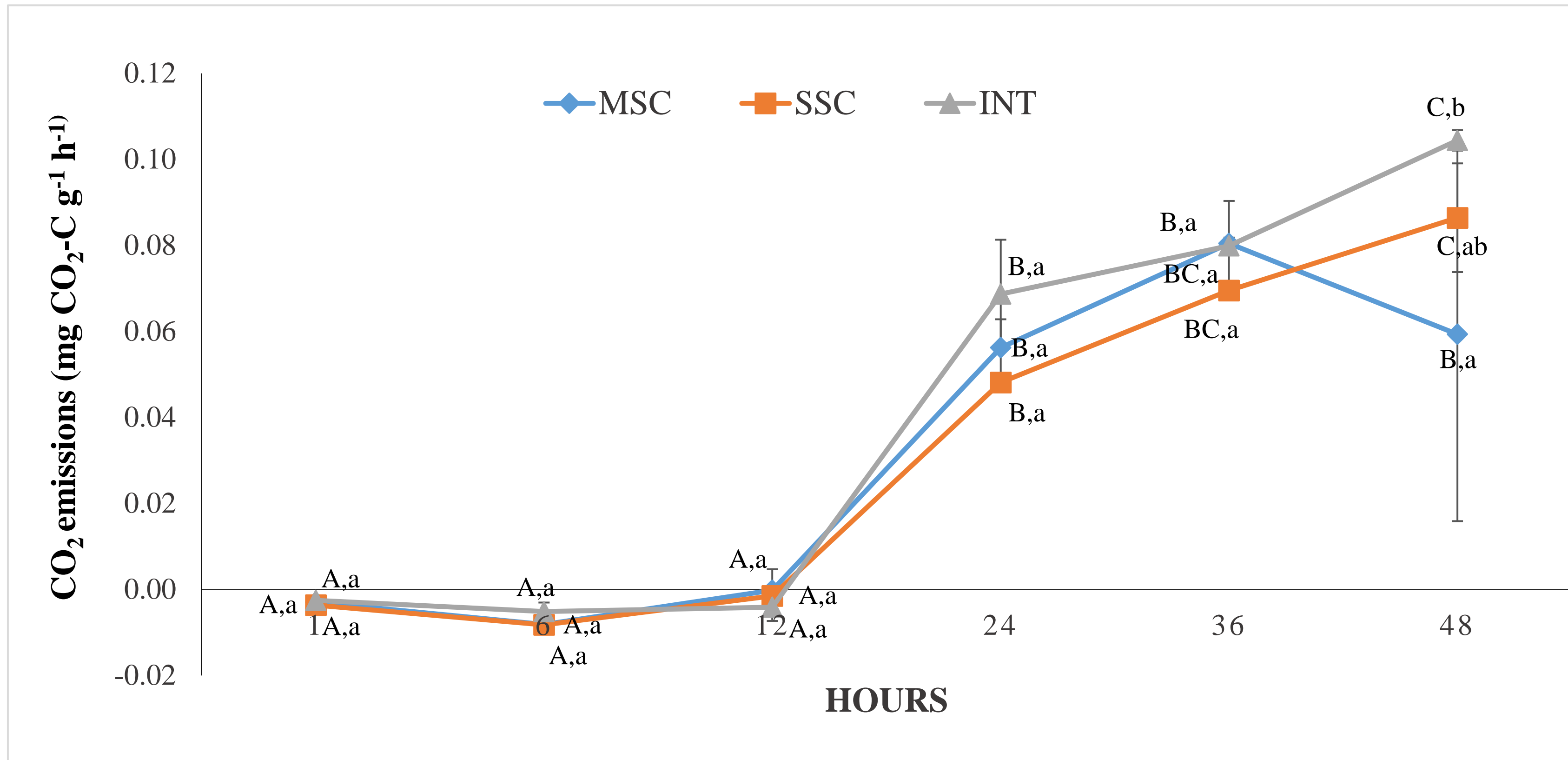


Figure 3.6 Mean CO_2 emissions (mg CO_2-C g^{-1} h^{-1}) from maize sole crops (MSC), soybean sole crops (SSC) and intercrops (INT) soils from Balcarce Research Station in Argentina that did not receive fertilized treatment with KNO_3^- and were only incubated at 80% of WFPS for 48 hours. All soils belong to the period 2011-2012. Vertical bars represent standard errors. Values followed by different lower-case letters are significantly different ($p<0.05$) within time series between crop treatments. Values followed by different upper-case letters are significantly different between time series within crop treatment. Statistical tests executed in SPSS using Two-Way ANOVA and Tukey's with a Bonferroni correction if ANOVA was significant.

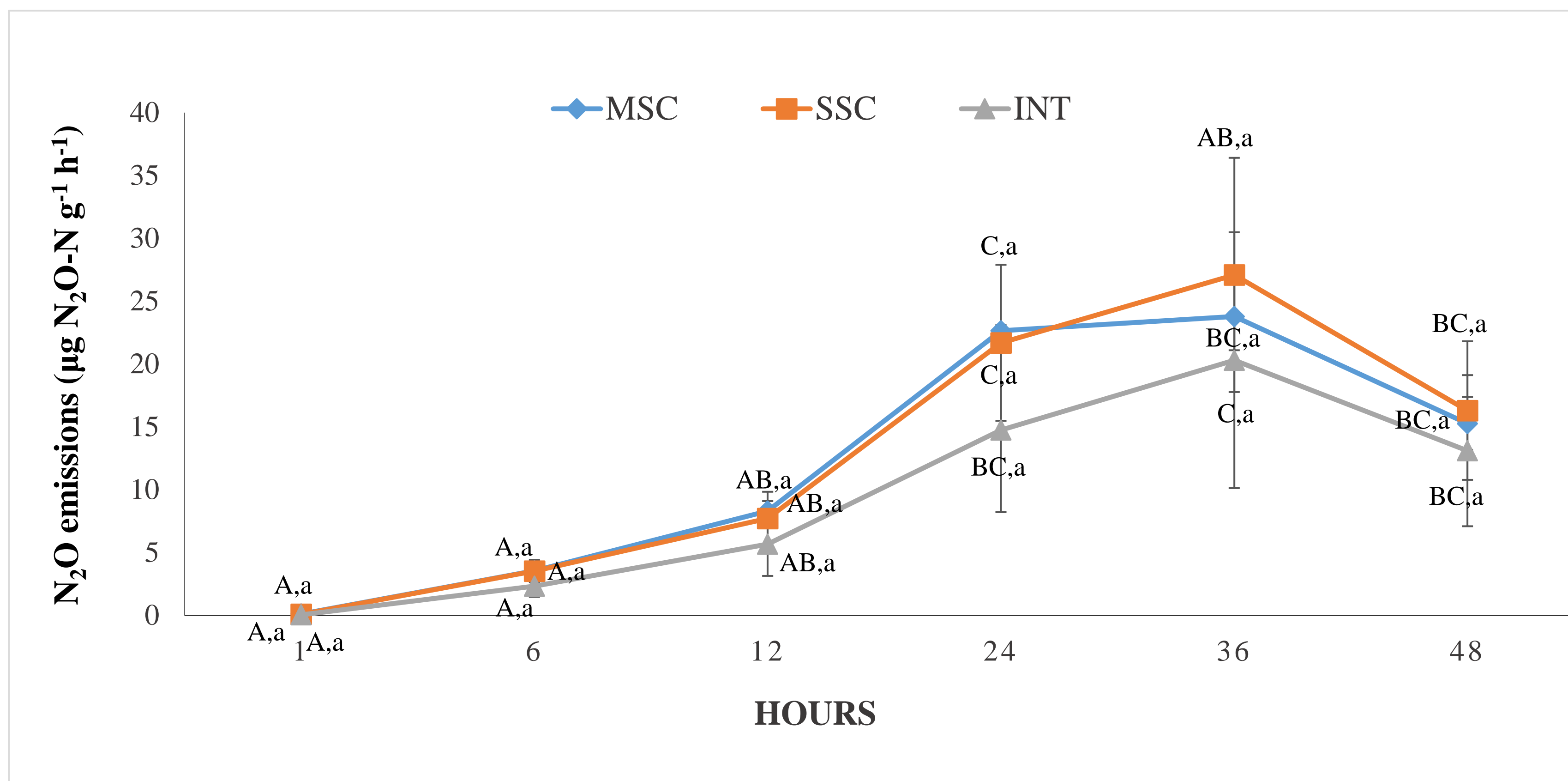


Figure 3.7 Mean N_2O emissions (µg N_2O-N g^{-1} h^{-1}) from maize sole crops (MSC), soybean sole crops (SSC) and intercrops (INT) that received fertilized treatment with KNO_3^- and were incubated for 48 hours at 80% of WFPS from Balcarce Research Station in Argentina from 2011-2012. Vertical bars represent standard errors. Values followed by different lower-case letters are significantly different ($p<0.05$) within time series between crop treatments. Values followed by different upper-case letters are significantly different between time series within crop treatment. Statistical tests executed in SPSS using Two-Way ANOVA and Tukey's with a Bonferroni correction if ANOVA was significant.

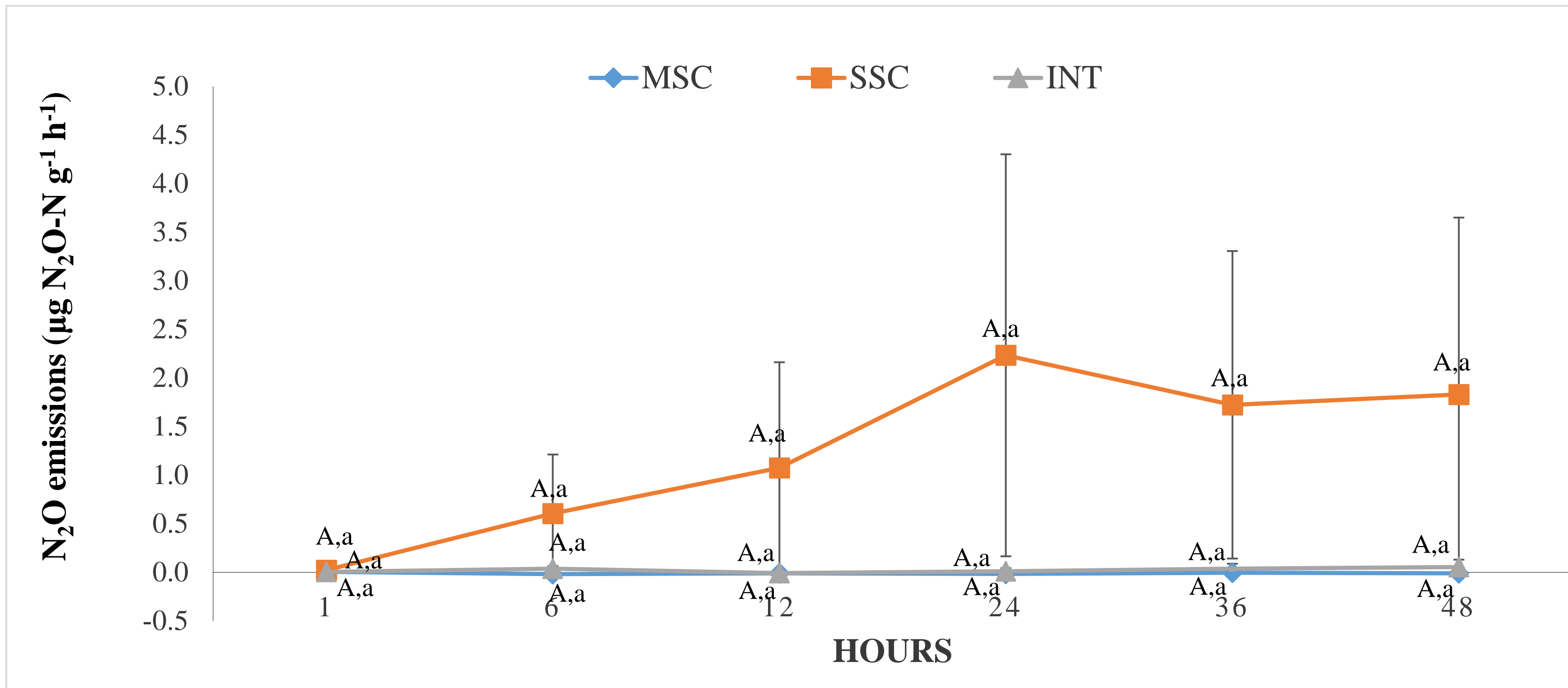


Figure 3.8 Mean N_2O emissions (µg N_2O-N g^{-1} h^{-1}) from maize sole crops (MSC), soybean sole crops (SSC) and intercrops (INT) soils from Balcarce Research Station in Argentina that did not receive fertilized treatment with KNO_3^- and were only incubated at 80% of WFPS for 48 hours. All soils belong to the period 2011-2012. Vertical bars represent standard errors. Values followed by different lower-case letters are significantly different ($p<0.05$) within time series between crop treatments. Values followed by different upper-case letters are significantly different between time series within crop treatment. Statistical tests executed in SPSS using Two-Way ANOVA and Tukey's with a Bonferroni correction if ANOVA was significant.

3.3 Soil inorganic concentrations

3.3.1 Nitrification process (Ammonium and Nitrate)

The interaction of crop-type-by-treatments (fertilized and unfertilized) was not significant. Contrast of simple effects between treatments was significantly different within crop types [F (1, 18) = 207, p<0.0001=0.00001], but not between crop types (Figure 3.9). Ammonium concentrations in INT treatments were not significantly different. Ammonium concentrations were significantly different between MSC and SSC treatments (p<0.05 = 0.025). On the other hand, in the results of nitrate concentrations, the interaction between crop-type-by-treatments (fertilized and unfertilized) was significantly different [F (2, 18) = 12.17, p<0.05=0.001]. MSC was significantly different (p<0.05 = 0.019), and INT was also significantly different (p<0.01 = 0.002). Significant differences between fertilized and unfertilized SSC crop types, however, were not observed (Figure 3.10).

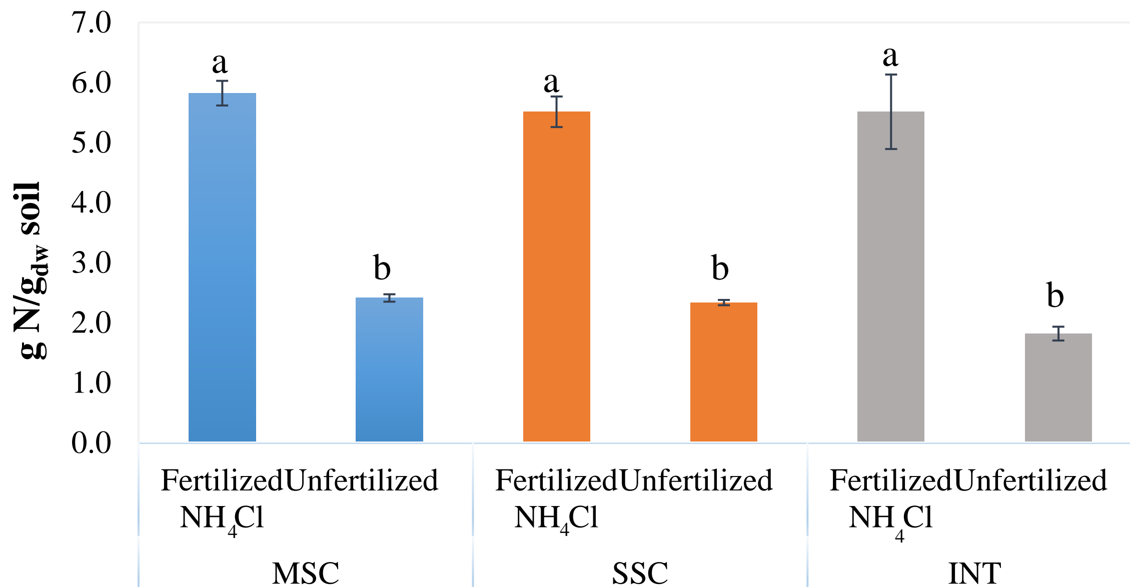


Figure 3.9 Mean NH_4^+ concentrations (g N/g$_{dw}$ soil) from maize sole crops (MSC), soybean sole crops (SSC) and intercrops (INT) soils from Balcarce Research Station in Argentina that receive fertilization and those who did not, and were incubated for 48 hours at 60% of WFPS. Measurement occurred only at hour 48. All soils belong to the period 2011-2012. Vertical bars stand for standard errors. Values followed by

different lower-case letters are significantly different ($p<0.05$) between fertilized treatments (a) and between control treatments (b). Statistical tests executed in SPSS using Two-Way ANOVA and Tukey's with a Bonferroni correction if ANOVA was significant.

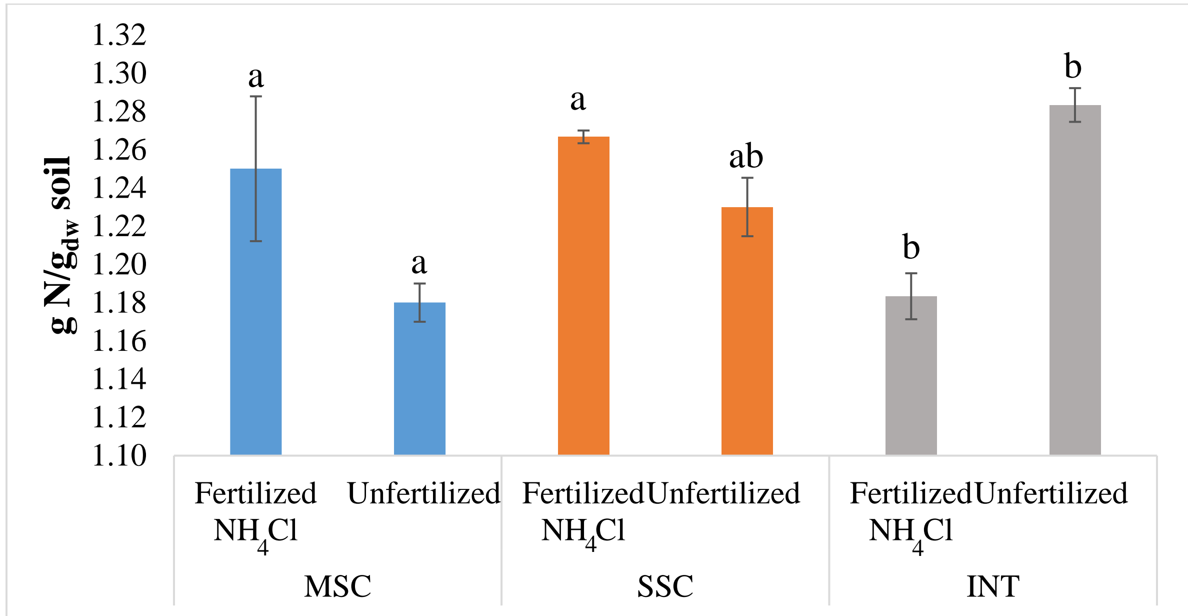


Figure 3.10 Mean NO_3^- concentrations (g N/g_{dw} soil) from maize sole crops (MSC), soybean sole crops (SSC) and intercrops (INT) soils from Balcarce Research Station in Argentina that receive fertilization and those who did not, and were incubated for 48 hours at 60% of WFPS (nitrification). Measurement occurred only at time 48. All soils belong to the period 2011-2012. Vertical bars stand for standard errors. Values followed by different lower-case letters are significantly different ($p<0.05$) between fertilized treatments (a) and between control treatments (b). Statistical tests executed in SPSS using Two-Way ANOVA and Tukey's with a Bonferroni correction if ANOVA was significant.

3.3.2 Denitrification process (Ammonium and Nitrate)

The interaction of crop-type-by-treatments (fertilized and unfertilized) was not significantly different. Contrast of simple effects between treatments, however, was significantly different [$F(1, 18) = 0.8.15$, $p<0.05=0.014$]. The difference between fertilized and unfertilized was significantly different in MSC soils only ($p<0.05 = 0.033$) (Figure 3.11). Ammonium concentrations were significantly different between MSC and SSC treatments ($p<0.05 = 0.025$). On the other hand, in the nitrate concentrations the interaction of crop-type-by-treatments (fertilized and unfertilized) did not show a significant difference [$F(2, 18) = 0.870$,

p>0.444]. Contrast of simple effects between treatments, however, were significantly different within crop types [F (1, 18) = 103, p<0.001 = 0.0001].

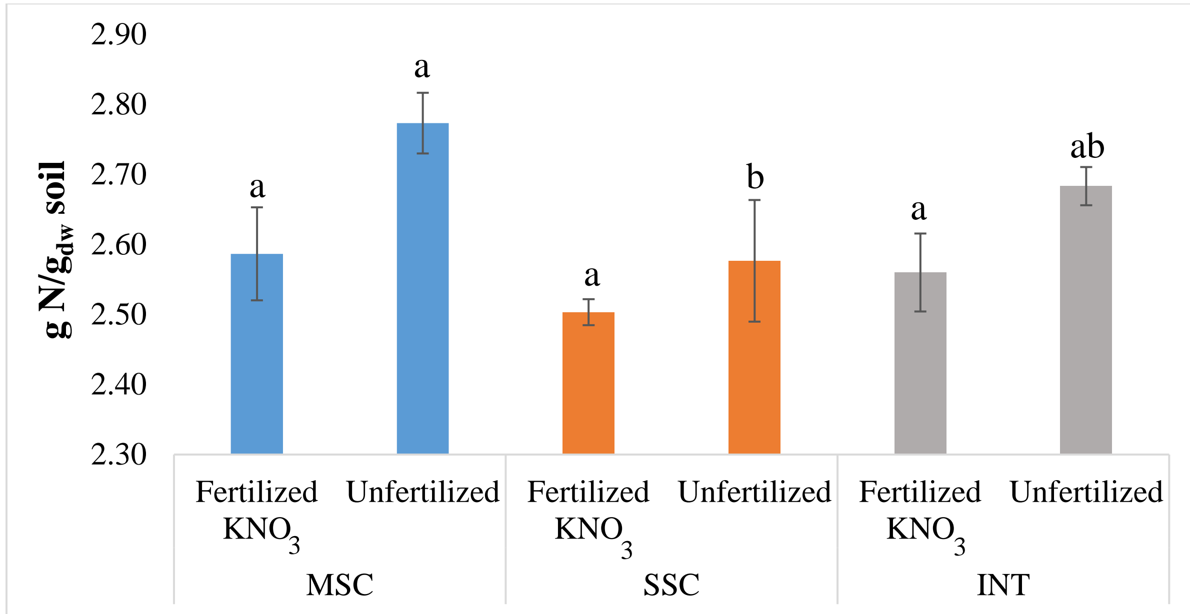


Figure 3.11 Mean NH_4^+ concentrations (g N/g_{dw} soil) from maize sole crops (MSC), soybean sole crops (SSC) and intercrops (INT) soils from Balcarce Research Station in Argentina that receive fertilization and those who did not, and were incubated for 48 hours at 80% of WFPS. Measurement occurred only at time 48. All soils belong to the period 2011-2012. Vertical bars represent standard errors. Values followed by different lower-case letters are significantly different (p<0.05) between fertilized treatments (a) and between control treatments (b). Statistical tests executed in SPSS using Two-Way ANOVA and Tukey's with a Bonferroni correction if ANOVA was significant.

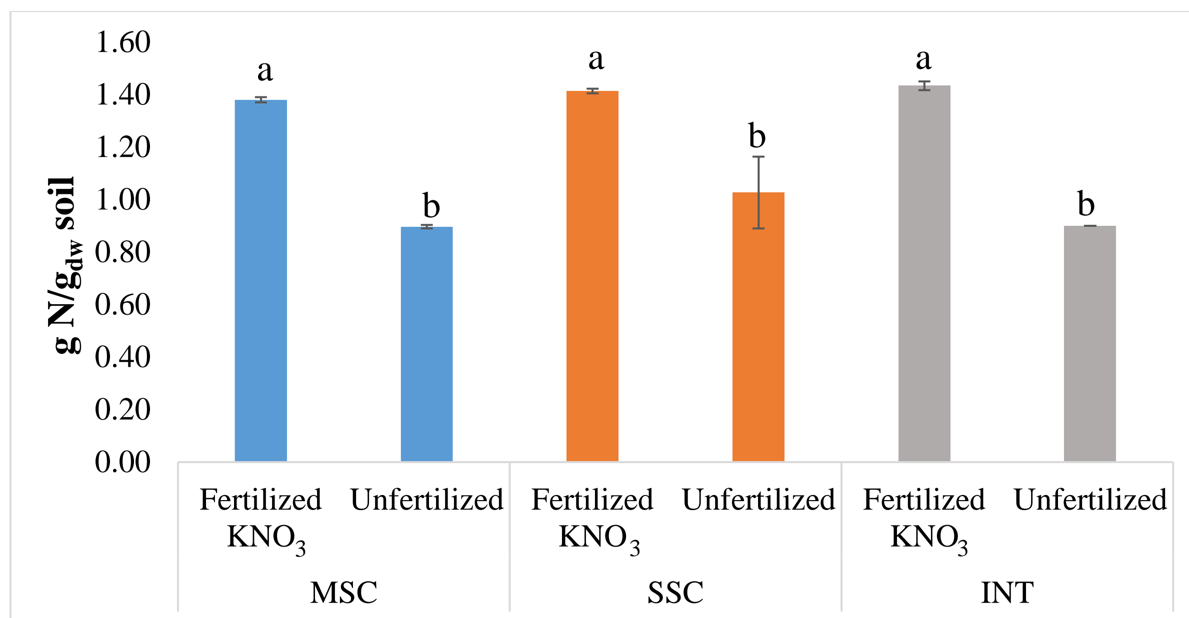


Figure 3.12 Mean NO_3^- concentrations (g N/g_{dw} soil) from maize sole crops (MSC), soybean sole crops (SSC) and intercrops (INT) soils from Balcarce Research Station in Argentina that receive fertilization and those who did not, and were incubated during 48 hours at 80% of WFPS. Measurement occurred only at time 48. All soils belong to the period 2011-2012. Vertical bars stand for standard errors. Values followed by different lower-case letters are significantly different ($p<0.05$) between fertilized treatments (a) and between control treatments (b). Statistical tests executed in SPSS using Two-Way ANOVA and Tukey's with a Bonferroni correction if ANOVA was significant.

3.4 Determining DNA concentrations: relative and absolute values

An absolute value for the standards was evaluated prior to the measurement of the absolute DNA concentration of the samples (Figure 3.13). Results from the nano-spectrophotometer allowed for an evaluation of the relative values of DNA concentration (Appendix C). The mathematical equation in Figure 3.13 was used to replace the values obtained from the samples after analyzing the absolute val-ues of all the samples.

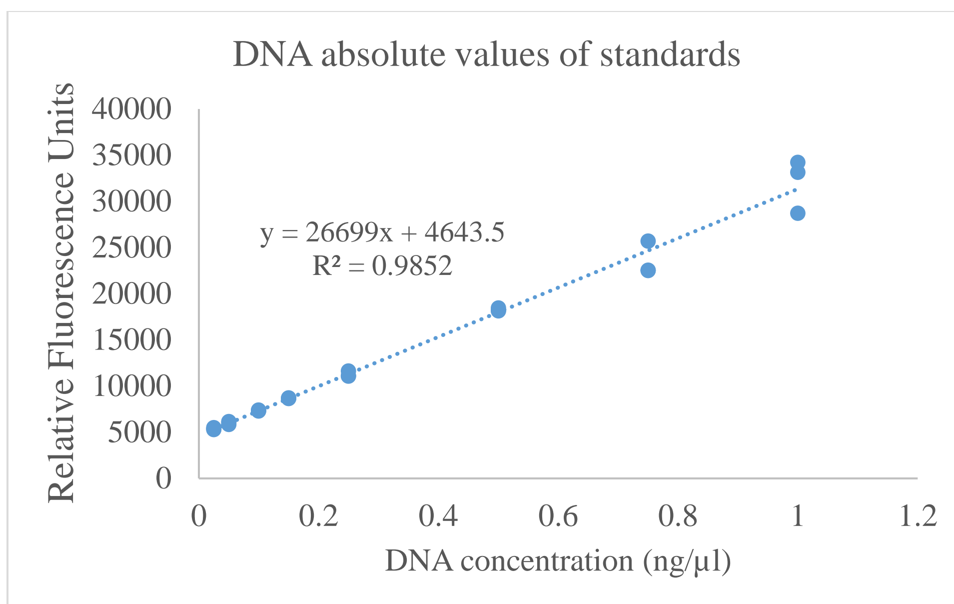


Figure 3.13 The coefficient value and precision (r^2=0.9852) for determining an accurate absolute value of the DNA concentration from all the samples from the nitrification and denitrification process.

3.5 Quantitative Polymerase Chain Reaction

Before quantifying gene abundances, the targeted genes were evaluated prior to the detection of the number of copies per ng of DNA in the soil. Below are the results of previous measurements to make sure the efficiency values followed the MIQE guidelines (Minimum Information for Publication Quantitative Real-Time PCR Experiments) (Bustin et al., 2009). These guidelines give consistency in the information provided by qPCR experiments (Bustin et al., 2009). The coefficient value, the efficiency, and the slope revealed the repeatability and reproducibility of the assays performed on the samples.

3.5.1 Electrophoresis Gel

The electrophoresis gel (1%) resulted in greater DNA concentrations in the nitrification samples (Figure 3.14) after adding 10 µl DNA plus 3 ml of dye (10 ml + 3.5 µl SYBR Safe) and running the gel for 30 minutes at 80 V, compared to the denitrification samples. The results generated from standard amplifications (reference-genes) and from the samples (targeted-genes) were higher in the nitrification process

than those of the denitrification process. The qPCR assessments contributed to similar findings (please refer to Appendix D).

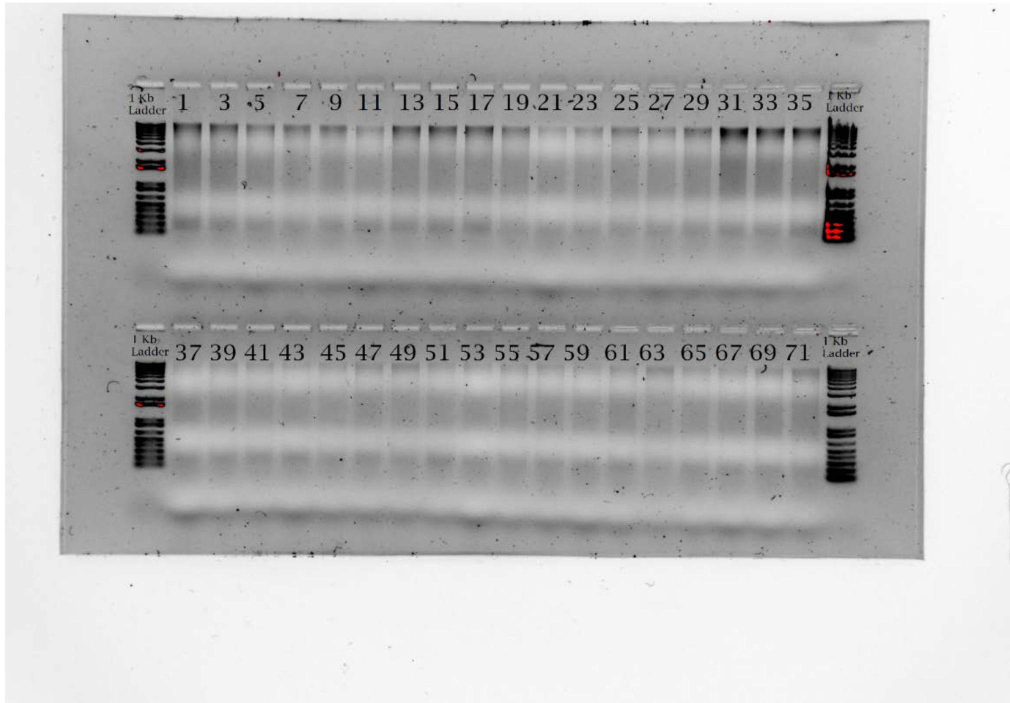


Figure 3.14 From left to right (top tray), the standard, 1 Kb ladder, followed by the nitrification samples: 1) fertilized (1-17) and 2) unfertilized (19-35). At the bottom tray followed by the standard (1 Kb Ladder) the denitrification samples: 1) fertilized treatment (37-53) and then the unfertilized treatment (55-71).

3.6 Gene abundances

Given the high efficiency and reproducibility (Appendix D) and repeatability of this assay, the following copy numbers revealed an estimated average of gene copy numbers per nanogram (ng) per each gram of dried soil (ng/DNA).

3.6.1 Gene abundance in the nitrification process

3.6.1.1 AOB gene abundance

The interaction effect between crop type and treatments (fertilized and unfertilized) was not significant. Contrast of simple effects were analyzed and were also non-significant. However, MSC soils had

higher AOB copies/ng DNA than SSC and INT. Fertilized and unfertilized INT soils resulted in higher AOB copies/ng DNA than SSC. SSC was the lowest in AOB copies/ng DNA (Figure 3.15).

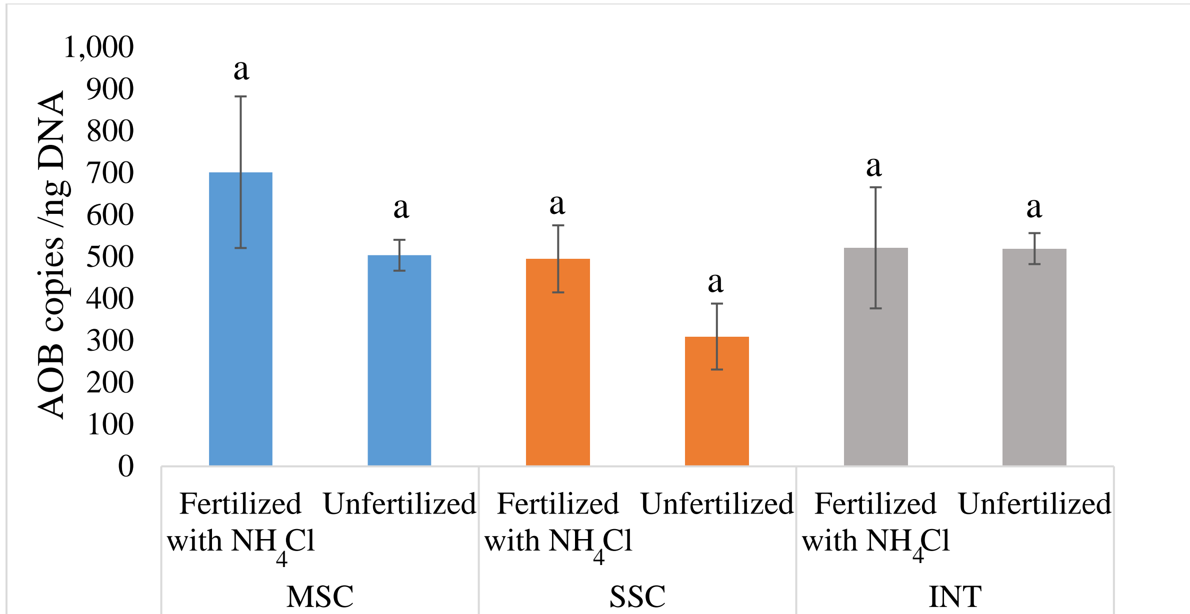


Figure 3.15 Mean AOB gene abundance (copies/ng DNA) from maize sole crops (MSC), soybean sole crops (SSC) and intercrops (INT) soils from Balcarce Research Station in Argentina that receive fertilization and those who did not, and were incubated during 48 hours under NIT conditions at 60% of WFPS. Measurement occurred only at hour 48. All soils belong to the period between 2011-2012. Vertical bars represent standard errors. Values followed by different lower-case letters are significantly different ($p<0.05$) between fertilized treatments (a) and between control treatments (b). Statistical tests executed in SPSS using Two-Way ANOVA and Tukey's with a Bonferroni correction if ANOVA was significant.

3.6.1.2 AOA gene abundance

AOA abundance was dominant in the MSC (1184.81) followed by SSC (991.36) and INT (345.57) treatments. The interaction effect between crop type and treatments (fertilized and unfertilized) was not significant. Contrast of simple effects were analyzed and all crop types were found significantly different [$F(2, 18) = 4.63$, $p<0.05 = 0.032$]. MSC soils were significantly different in AOA gene abundance compared to INT ($p<0.05 = 0.039$) (Table 3.1). In the fertilized treatment, MSC resulted with a significantly different abundance (AOA copies/ng DNA) of $p<0.05 = 0.007$ (Table 3.1). SSC soils were significantly different to INT ($p<0.05 = 0.014$) (Table 3.1). Among the fertilized treatments, INT soils were significantly

different than SSC soils (p<0.05 = 0.027) (Table 3.1). SSC, however, was not different from MSC. Unfertilized soils were all non-significantly different between one another. Nevertheless, SSC unfertilized soils showed greater AOA gene abundance compared to MSC and SSC (Figure 3.16).

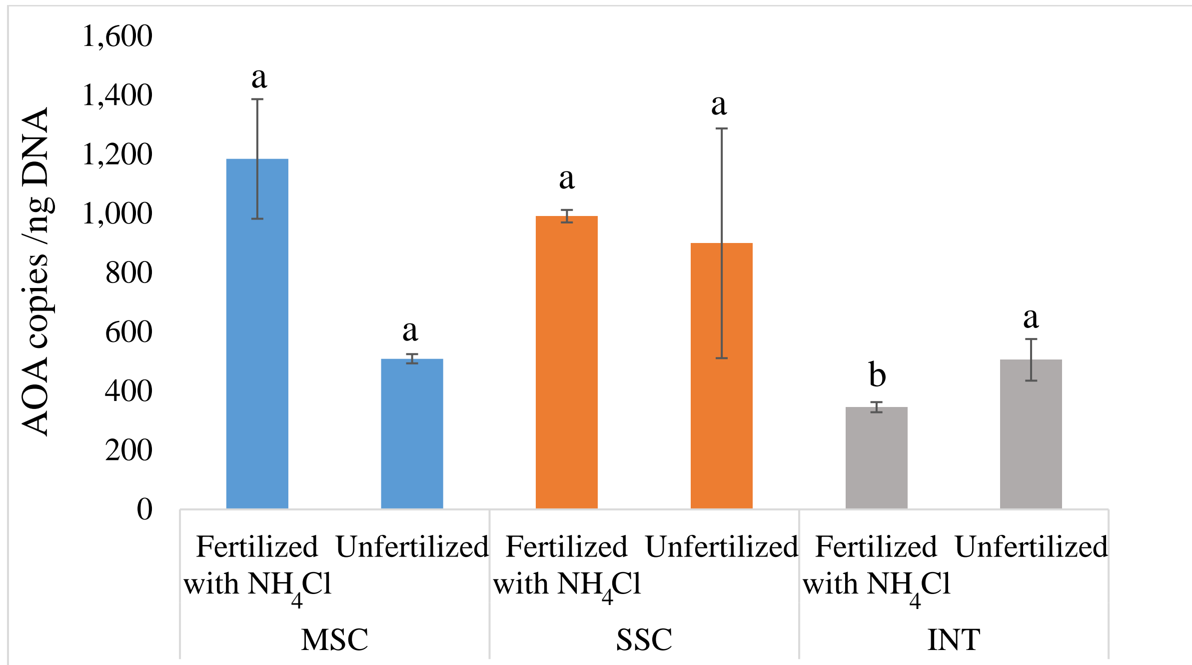


Figure 3.16 Mean AOA gene abundance (copies/ng DNA) from maize sole crops (MSC), soybean sole crops (SSC) and intercrops (INT) soils from Balcarce Research Station in Argentina that receive fertilization and those who did not, and were incubated during 48 hours under NIT conditions at 60% of WFPS. Measurement occurred only at hour 48. All soils belong to the period between 2011-2012. Vertical bars represent standard errors. Values followed by different lower-case letters are significantly different (p<0.05) between fertilized treatments (a) and between control treatments (b). Statistical tests executed in SPSS using Two-Way ANOVA and Tukey's with a Bonferroni correction if ANOVA was significant.

3.6.1.3 *nirK* bacteria gene abundance

The interaction effect between crop type and treatments (fertilized and unfertilized) was significant [F (2, 18) = 8.20, p<0.05 = 0.006). The difference between fertilized and unfertilized treatments was also significant (p<0.05 = 0.015). MSC soils were significantly different to SSC soils (p<0.05 = 0.001) (Table

3.1); also, MSC fertilized soils were greater than SSC fertilized soils (Figure 3.17). However, this significant difference occurred only for the fertilized treatments. MSC was significantly different to INT (Table 3.1). INT was not significantly different to SSC.

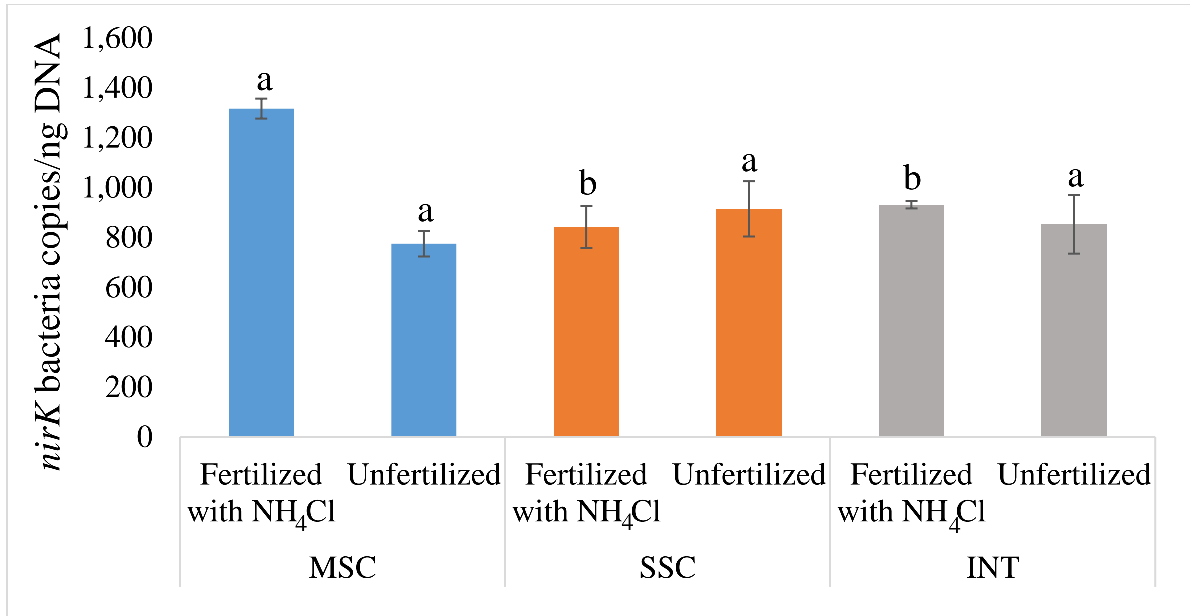


Figure 3.17 Mean *nirK* bacteria gene abundance (copies/ng DNA) from maize sole crops (MSC), soybean sole crops (SSC) and intercrops (INT) soils from Balcarce Research Station in Argentina that receive fertilization and those who did not, and were incubated during 48 hours under NIT conditions at 60% of WFPS. Measurement occurred only at hour 48. All soils belong to the period between 2011-2012. Vertical bars represent standard errors. Values followed by different lower-case letters are significantly different ($p<0.05$) between fertilized treatments (a) and between control treatments (b). Statistical tests executed in SPSS using Two-Way ANOVA and Tukey's with a Bonferroni correction if ANOVA was significant.

3.6.1.4 *nirk* archaea gene abundance

The interaction effect between crop type and treatments (fertilized and unfertilized) was not significant [$F (2,18) = 2.97$, $p = 0.089$). After performing a simple effect analysis, crop types were significantly different ($p = 0.032$). MSC ($p<0.05 = 0.029$) and SSC were significantly different to INT ($p<0.05 = 0.017$) (Table 3.1); MSC and SSC, however, were not significantly different. Although treatments (fertilized and unfertilized) almost achieved significance ($p = 0.065$) it was not significant. Nonetheless, within the fertilized treatment, crop types showed to be significantly different [$F (2, 12) = 6.75$, $p<0.05 = 0.011$). MSC

was significantly different from INT ($p<0.05 = 0.005$) (Table 3.1). SSC was likewise significantly different to INT ($p<0.05 = 0.017$) (Table 3.1). Although unfertilized treatments were not significantly different, SSC soils were greater in *nirK* archaea (copies/ng DNA) than MSC and INT soils (Figure 3.18).

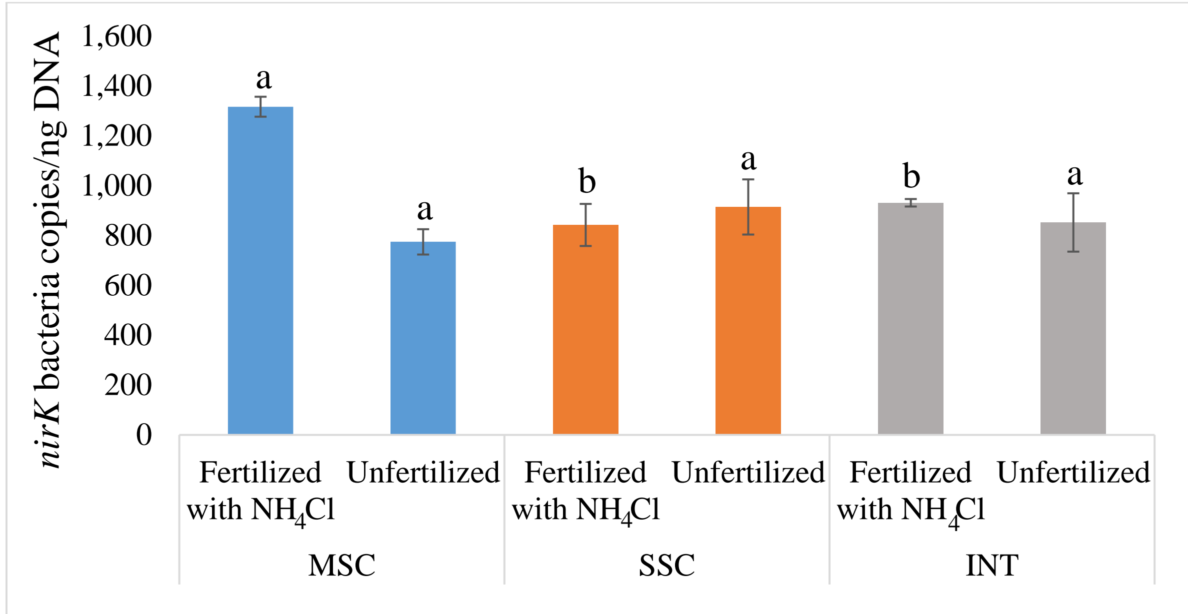


Figure 3.18 Mean *nirK* archaea gene abundance (copies/ng DNA) from maize sole crops (MSC), soybean sole crops (SSC) and intercrops (INT) soils from Balcarce Research Station in Argentina that receive fertilization and those who did not, and were incubated during 48 hours under NIT conditions at 60% of WFPS. Measurement occurred only at hour 48. All soils belong to the period between 2011-2012. Vertical bars represent standard errors. Values followed by different lower-case letters are significantly different ($p<0.05$) between fertilized treatments (a) and between control treatments (b). Statistical tests executed in SPSS using Two-Way ANOVA and Tukey's with a Bonferroni correction if ANOVA was significant.

Table 3.1 Mean gene abundance (copies/ng DNA) from soil extractions collected after 48 hours of incubation under 60% WFPS favoring NIT processes using the top 20 cm of fertilized (NH_4Cl added) and unfertilized soils from Balcarce, Argentina

	MSC		**SSC**		**INT**	
	Fertilized	Unfertilized	Fertilized	Unfertilized	Fertilized	Unfertilized
Gene abundance						
AOB	701 (181)[a]	504 (37)[a]	495 (80)[a]	310 (79)[a]	521 (145)[a]	519 (37)[a]
AOA	1185(202)[a]	509 (16)[a]	991 (21)[a]	900(388)[a]	346 (17)[b]	506 (70)[a]
nirK bacteria	1317(40)[a]	774 (51)[a]	842 (84)[b]	915 (111)[a]	931 (15) [b]	852 (117)[a]
nirk archaea	0.48(0.08)[a]	0.21 (0.04)[a]	0.41(0.05)[a]	0.31 (0.09)[a]	0.16(0.02)[b]	0.20 (0.07)[a]

[a] Means followed by a different lower case letter are significantly different ($p<0.05$) within gene abundance and treatment (fertilized and unfertilized).

3.6.2 Gene abundance in the denitrification process

3.6.2.1 AOB gene abundance

The interaction effect between crop type and treatments (fertilized and unfertilized) was significantly different [$F(2, 18) = 7.49$, $p<0.05 = 0.008$]. Fertilized versus unfertilized treatments were significantly different ($p<0.001 = 0.001$) (Table 3.2). MSC unfertilized treatments were significantly different to SSC and INT ($p<0.0001$) (Table 3.2); however, SSC was not significantly different from INT. In the fertilized treatments, MSC was different to SSC ($p<0.05 = 0.004$) (Table 3.2) and SSC was significantly different to INT ($p<0.05 = 0.003$). MSC was not significantly different to INT soils (Figure 3.19).

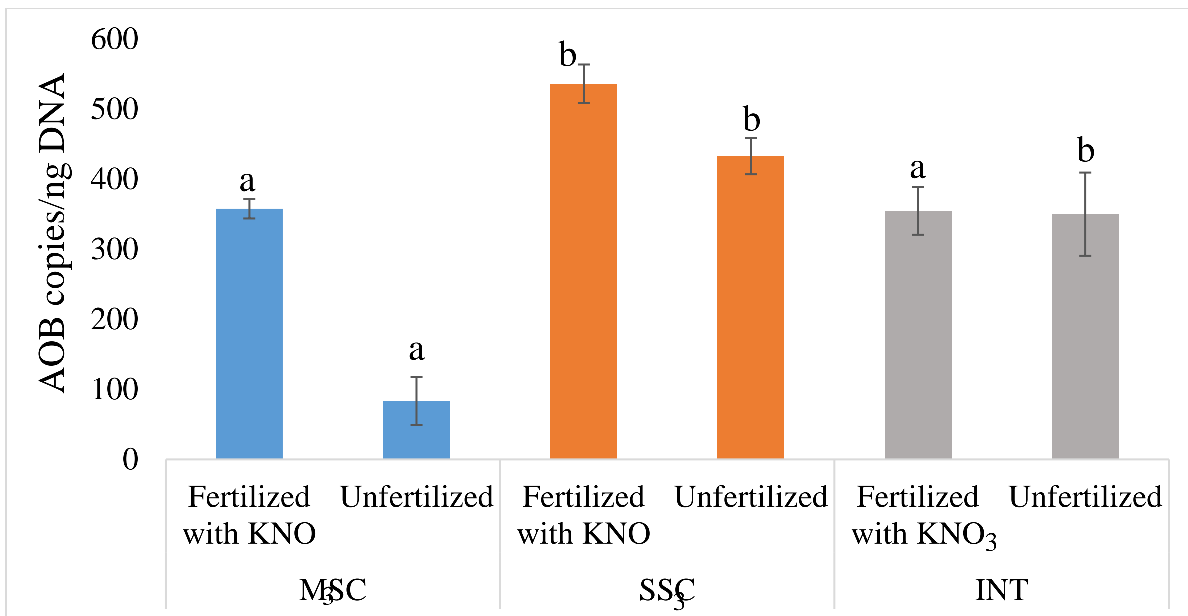


Figure 3.19 Mean AOB gene abundance (copies/ng DNA) from maize sole crops (MSC), soybean sole crops (SSC) and intercrops (INT) soils from Balcarce Research Station in Argentina that receive fertilization and those who did not, and were incubated during 48 hours under DNIT conditions at 80% of WFPS. Measurement occurred only at time 48. All soils belong to the period between 2011-2012. Vertical bars represent standard errors. Values followed by different lower-case letters are significantly different ($p<0.05$) between fertilized treatments (a) and between control treatments (b). Statistical tests executed in SPSS using Two-Way ANOVA and Tukey's with a Bonferroni correction if ANOVA was significant.

3.6.2.2 AOA gene abundance

The interaction effect between crop type and treatments (fertilized and unfertilized) was not significant. Contrast of simple effects were analyzed and were also non-significant (Table 3.2). However, AOA gene abundance (AOA copies/ng DNA) in MSC and SSC showed higher abundance than INT in both, fertilized and unfertilized treatments (Figure 3.20).

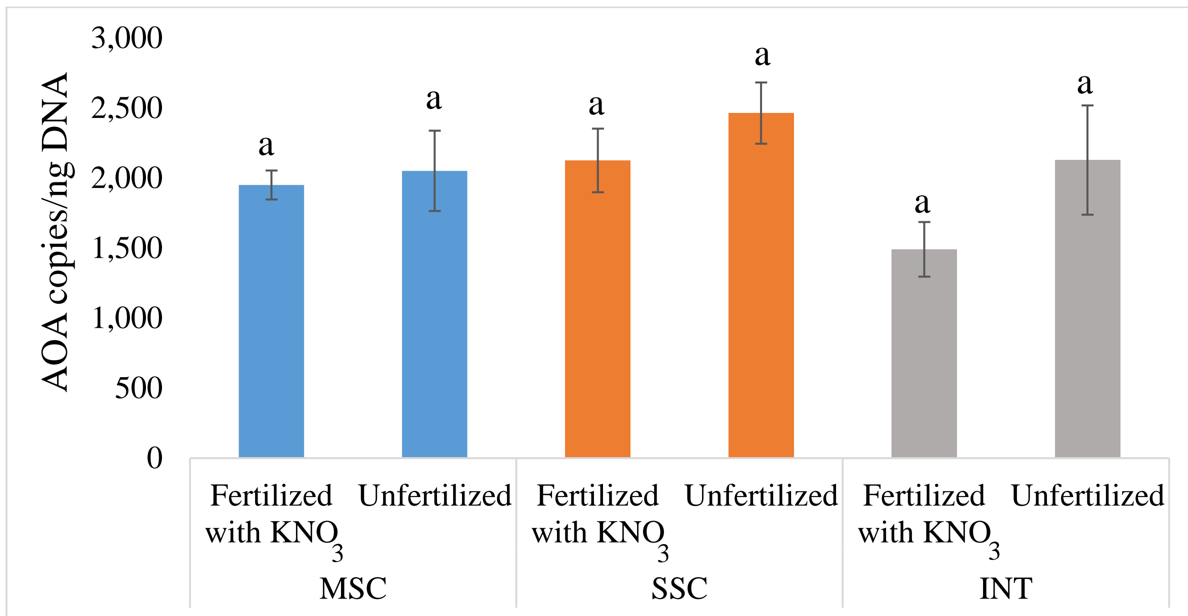


Figure 3.20 Mean AOA gene abundance (copies/ng DNA) from maize sole crops (MSC), soybean sole crops (SSC) and intercrops (INT) soils from Balcarce Research Station in Argentina that receive fertilization and those who did not, and were incubated during 48 hours under DNIT conditions at 80% of WFPS. Measurement occurred only at time 48. All soils belong to the period between 2011-2012. Vertical bars represent standard errors. Values followed by different lower-case letters are significantly different ($p<0.05$) between fertilized treatments (a) and between control treatments (b). Statistical tests executed in SPSS using Two-Way ANOVA and Tukey's with a Bonferroni correction if ANOVA was significant.

3.6.2.3 *nirK* bacteria gene abundance

The interaction effect between crop type and treatments (fertilized and unfertilized) was not significant. Contrast of simple effects were analyzed and were also non-significant (Table 3.2). Furthermore, INT fertilized soils had the greatest copies per ng DNA, although were not significantly different to the other

crop types. Even though MSC was neither significantly different, the fertilized soils of this crop type was lower in this gene compared to the other SSC and INT crop types (Figure 3.21).

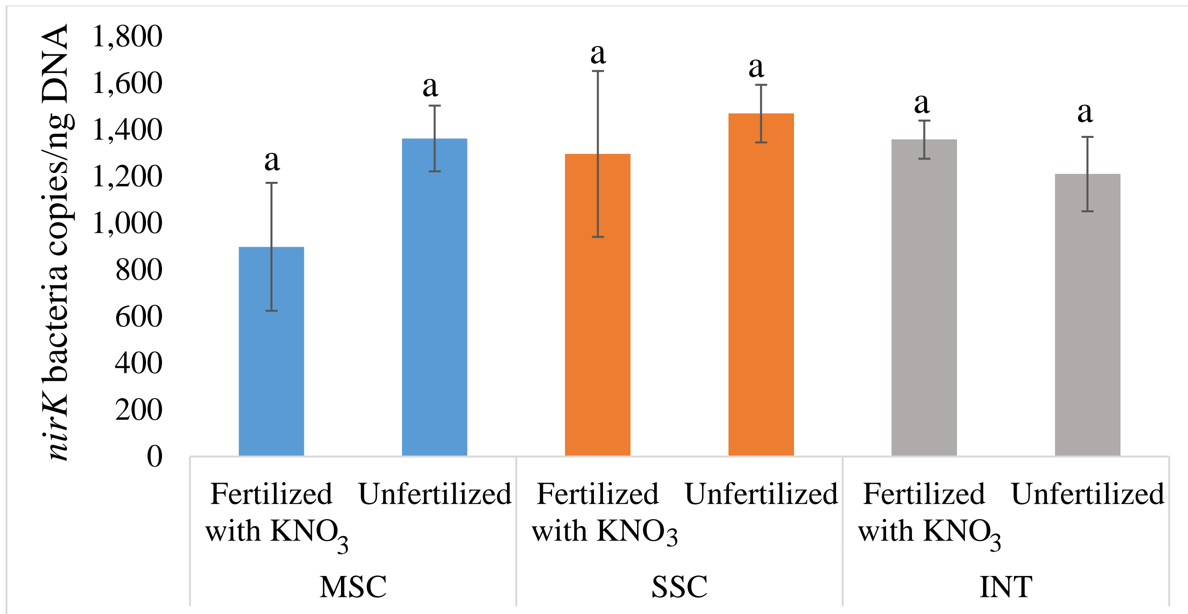


Figure 3.21 Mean *nirK* bacteria gene abundance (copies/ng DNA) from maize sole crops (MSC), soybean sole crops (SSC) and intercrops (INT) soils from Balcarce Research Station in Argentina that receive fertilization and those who did not, and were incubated during 48 hours under DNIT conditions at 80% of WFPS. Measurement occurred only at time 48. All soils belong to the period between 2011-2012. Vertical bars represent standard errors. Values followed by different lower-case letters are significantly different ($p<0.05$) between fertilized treatments (a) and between control treatments (b). Statistical tests executed in SPSS using Two-Way ANOVA and Tukey's with a Bonferroni correction if ANOVA was significant.

3.6.2.4 *nirk* archaea gene abundance

The interaction effect between crop type and treatments (fertilized and unfertilized) was not significant. Contrast of simple effects were analyzed; fertilized treatments showed greater gene abundance and the p-value approached the criterion of significance but did not reach it (Table 3.2). MSC soils resulted in the lowest amount of copies per ng DNA of this gene (Figure 3.22).

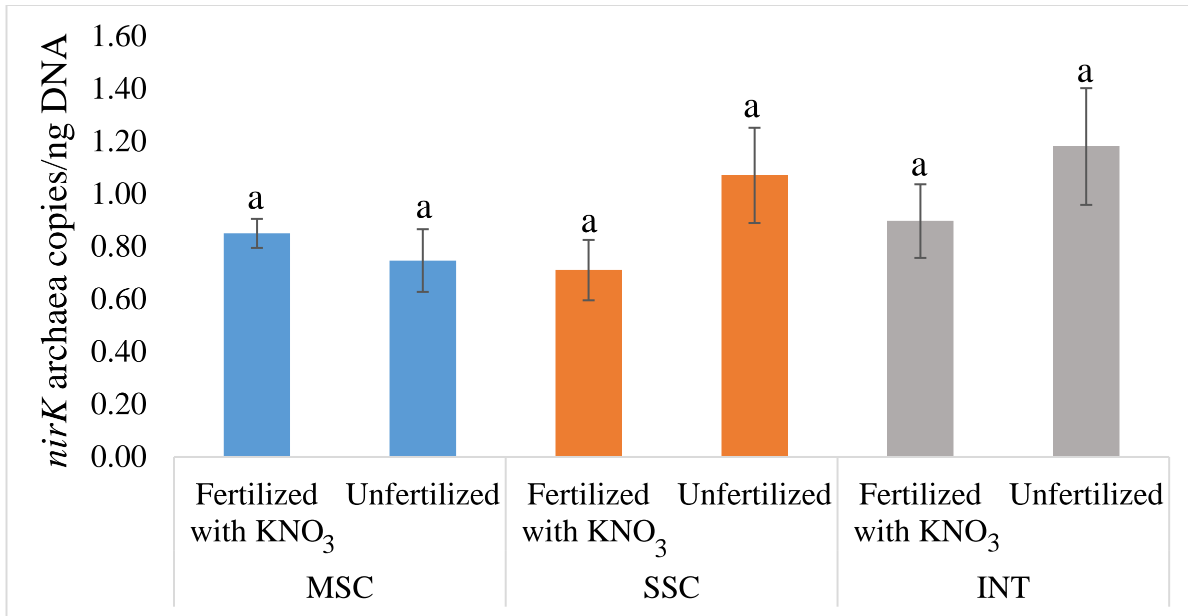


Figure 3.22 Mean *nirK* archaea gene abundance (copies/ng DNA) from maize sole crops (MSC), soybean sole crops (SSC) and intercrops (INT) soils from Balcarce Research Station in Argentina that receive fertilization and those who did not, and were incubated during 48 hours under DNIT conditions at 80% of WFPS. Measurement occurred only at time 48. All soils belong to the period between 2011-2012. Vertical bars represent standard errors. Values followed by different lower-case letters are significantly different ($p<0.05$) between fertilized treatments (a) and between control treatments (b). Statistical tests executed in SPSS using Two-Way ANOVA and Tukey's with a Bonferroni correction if ANOVA was significant.

Table 3.2 Mean gene abundance (copies/ng DNA) from soil extractions that were collected after 48 hours of incubation under 80% WFPS favoring denitrification processes using the top 20 cm of unfertilized soil (no KNO_3^- added) from Balcarce, Argentina.

	MSC		**SSC**		**INT**	
	Fertilized	Unfertilized	Fertilized	Unfertilized	Fertilized	Unfertilized
Gene abundance						
AOB	358 (14)[a]	83 (34)[a]	536 (27)[b]	433 (26)[b]	355 (34)[a]	350 (59)[b]
AOA	1950 (103)[a]	2052 (287)[a]	2126 (228)[a]	2464 (219)[a]	1491 (195)[a]	2129 (390)[a]
nirK bacteria	899 (274)[a]	1363 (141)[a]	1296(355)[a]	1469 (123)[a]	1358 (82)[a]	1210 (159)[a]
nirk archaea	0.85 (0.06)[a]	0.75 (0.12)[a]	0.71(0.12)[a]	1.07 (0.18)[a]	0.90 (0.14)[a]	1.18 (0.22)[a]

[a] Means followed by a different lower case letter are significantly different ($p<0.05$) within gene abundance and treatment (fertilized and unfertilized).

3.7 Greenhouse gas, soil concentrations, and gene abundance: correlation analysis

3.7.1 Ammonium and nitrate concentrations correlates to CO_2 and N_2O emission in the nitrification and denitrification process

A Pearson's product-moment correlation was performed to evaluate the relationship between CO_2 and N_2O emissions with the ammonium (NH_4^+) and nitrate (NO_3^-) concentrations in both, NIT, and DNIT processes. Fertilized and unfertilized treatments were not considered because the sample population was very low to compare to one another (n=6). Only hour 48 was included in these correlations because the soil concentration analysis was done as of this specific time series during the experiment. These analyses were tested in each crop type: MSC, SSC and INT.

3.7.1.1 Maize Sole crop

The correlation between CO_2 emissions and NH_4^+ concentrations from both NIT and DNIT resulted not significant. Likewise, the relationship between CO_2 emissions and NO_3^- resulted not significant in both NIT and DNIT processes. NH_4^+ concentrations were not significantly correlated to N_2O emissions in the NIT nor the DNIT process. The NO_3^- correlation with N_2O emissions was not significant in the NIT process, whereas in the DNIT process it was significant ($r= 0.966$, $n=6$, $p<0.01=0.002$) (Table 3.3).

3.7.1.2 Soybean Sole crop

Although CO_2 emissions' correlations to NH_4^+ concentrations in the NIT process resulted in relatively high values (Table 3.3), these values were still not significant. CO_2 correlations to NH_4^+ concentrations in the DNIT were not significant. In the DNIT process CO_2 emissions were negative and significantly correlated to NO_3^- concentrations ($r=-0.866$, $n=6$, $p=0.026$) (Table 3.3). Conversely, CO_2 emissions and NO_3^- concentrations in the NIT process were negative and not significant. N_2O emissions and NH_4^+ concentrations were high in values but not significant in the NIT process; whereas in the DNIT process the

values were negative, lower, and not significant. The correlation between N_2O emissions and NO_3^- correlations in the NIT process was significant (r=0.816, n=6, p=0.048) (Table 3.3). N_2O emissions were not significantly correlated to NO_3^- in the DNIT process.

3.7.1.3 Intercrop

The correlation between CO_2 emissions and NH_4^+ concentrations was not significantly correlated in the NIT process nor in the DNIT process. Correlation between CO_2 emissions and NO_3^- concentrations in the NIT process was negative and not significant. The correlation between CO_2 emissions and NO_3^- concentrations were significantly correlated at hour 48 (r=-0.901, n=6, p= 0.014) (Table 3.3) in the DNIT process. The correlation between N_2O emissions and NH_4^+ concentrations was not significant in the NIT process; in the DNIT process this correlation was likewise, not significant. The correlation between N_2O emissions and NO_3^- concentrations in the NIT process was negative and not significant; in the DNIT this value was also not significant.

Table 3.3 Pearson product-moment correlation R-values for CO_2 and N_2O emissions, and soil concentrations at hour 48 for MSC, SSC, and INT crop systems from INTA, Argentina, during the 2011-2012 crop season.

Soil concentrations		NH_4^+		NO_3^-	
Crop Type	GHG	NIT (60% WFPS)	DNIT (80% WFPS)	NIT (60% WFPS)	DNIT (80% WFPS)
MSC	CO_2-C	0.085	-0.358	-0.590	0.174
	N_2O-N	0.426	-0.690	0.171	0.966**
SSC	CO_2-C	0.096	0.203	-0.062	-0.866*
	N_2O-N	0.734	-0.336	0.816*	0.746
INT	CO_2-C	0.734	0.612	-0.732	-0.901*
	N_2O-N	0.743	-0.124	-0.751	0.737

- R-values followed by a * or ** are significant at p<0.05 and <0.01, respectively.

3.7.2 Soil concentrations' (NH_4^+ and NO_3^-) correlation to soil gene expression in the nitrification and denitrification process

3.7.2.1 Maize Sole crop

The correlation between NO_3^- concentrations and AOB gene abundance was significant under NIT processes (r=0.933, n= 6, p= 0.007). AOA gene abundance was significantly correlated to both NH_4^+ (r=0.893, n= 18, p= 0.017) and NO_3^- (r=0.941, n= 6, p= 0.005) in the soil. The abundance between *nirK* bacteria and soil concentrations was significant with NH_4^+ concentration (r= 0.959, n=18, p= 0.002) but not with NO_3- in the NIT process. *NirK* archaea was significantly correlated to NH_4^+ (r= 0.842, n= 18, p= 0.036) and NO_3^- (r=0.860, n= 6, p= 0.028) under the NIT process. In the DNIT process AOB gene abundance was highly correlated to NO_3^- concentrations (r= 0.963, n=6, p= 0.002) (Table 3.4).

3.7.2.2 Soybean sole crop

The correlation assessed between NH_4^+ concentrations and gene abundances was not significant in neither the NIT nor the DNIT process. NO_3^- concentrations were not significantly correlated to any gene in the NIT process. The results between NO_3^- concentrations and AOB gene abundance were significant in the DNIT process (r=0.861, n = 18, p= 0.028) (Table 3.4). The correlations between NO_3^- concentrations and the other gene abundances (AOA, *nirK* bacteria and *nirK* archaea) in the DNIT process were negative and not significantly different (Table 3.4).

3.7.2.3 Intercrop

The correlation between NO_3^- concentrations and AOA gene abundance was significant in the NIT process (r=0.777, n=6, p=0.0005). The correlation between NO_3^- concentrations and *nirK* bacteria in the NIT process was negative, however, this was not significant. In the DNIT process, AOB was significantly correlated to NO_3^- concentrations under the DNIT process (r=0.861, n = 18, p= 0.028) (Table 3.4). NH_4^+ concentrations were not correlated with any of the four gene abundances measured in neither the NIT, nor the DNIT process (Table 3.4).

Table 3.4 Pearson product-moment correlation R-values for gene abundance and soil concentrations at hour 48 from MSC, SSC, and INT systems from the crop season 2011-2012.

	Gene abundance	NIT Process (60% WFPS) NH_4^+ concentration	NIT Process (60% WFPS) NO_3^- concentration	DNIT Process (80% WFPS) NH_4^+ concentration	DNIT Process (80% WFPS) NO_3^- concentration
MSC	AOB	0.549	0.933**	-0.644	0.963**
	AOA	0.893*	0.941**	0.227	-0.185
	nirK bacteria	0.959**	0.515	0.705	-0.636
	nirK archaea	0.842*	0.860*	0.180	0.339
SSC	AOB	0.641	0.645	-0.16	0.861*
	AOA	0.088	0.713	-0.365	-0.208
	nirK bacteria	-0.212	-0.145	-0.241	-0.180
	nirK archaea	0.472	0.315	-0.105	-0.165
INT	AOB	-0.231	0.146	0.031	0.861*
	AOA	-0.667	0.777*	0.563	-0.208
	nirK bacteria	0.348	-0.291	-0.062	-0.180
	nirK archaea	-0.176	0.315	0.570	-0.165

- R-values followed by a * or ** are significant at $p<0.05$ and <0.01, respectively.

3.7.3 *CO_2 and N_2O emission correlation to gene expression in the nitrification and denitrification process*

3.7.3.1 Maize sole crop

CO_2 emissions were not correlated to any other targeted gene in the NIT process. In the DNIT process *nirK* bacteria gene abundance was negatively correlated to CO_2 emissions (r=-0.900, n=6, p=0.015). No other targeted gene was correlated to neither CO_2 nor N_2O emissions during the DNIT process. The relationship between N_2O emissions and AOA gene abundances in MSC soils was significant (r=0.818, n=6, p=0.046) in the NIT process (Table 3.5). The same relationship was observed with *nirK* archaea gene abundance (r=0.852, n=6, p=0.031) in the NIT process. Conversely, *nirK* bacteria gene abundance was significantly negatively correlated to N_2O emissions under the influence of 80% WFPS (DNIT) (r= -0.927, n=6, p=0.008).

3.7.3.2 Soybean Sole crop

The correlation between CO_2 emissions and gene abundances was not significant in the NIT. In addition, although none of the values was found significant in the DNIT process, the correlation between CO_2 emissions and AOB was negative. The correlation between N_2O emissions and gene abundances in the NIT process were all not significant. Whereas in the DNIT process, AOB gene abundances were significantly correlated to N_2O emissions (r=0.875, n=6 , p=0.023) (Table 3.5). None of the other genes were correlated under the influence of the NIT nor the DNIT conditions (Table 3.5)

3.7.3.3 Intercrop

CO_2 emissions were overall negatively related to AOB, AOA, and not significant in the NIT process. In the DNIT process these emissions were mostly related to *nirK* bacteria. Nevertheless, these correlations were not significant (Table 3.5). The correlation assessed between N_2O in relation to AOB, AOA, *nirK b*acteria or *nirK* archaea was not significantly correlated in the NIT nor in the DNIT process.

Table 3.5 Pearson product moment correlation R-values between gene abundance and greenhouse gas emission of CO_2 and N_2O and at hour 48 for MSC, SSC, and INT crop systems from INTA, Argentina, from crop season 2011-2012 during the nitrification and denitrification process

		NIT process (60% WFPS)				DNIT process (80% WFPS)			
Gene abundance		AOB	AOA	*nirk* bacteria	*nirk* archaea	AOB	AOA	*nirk* bacteria	*nirk* archaea
Crop Type									
MSC	CO_2-C	0.151	0.776	0.715	0.736	-0.900*	0.054	0.515	-0.194
	N_2O-N	0.578	0.818*	0.800	0.852*	0.653	-0.210	-0.927**	0.266
SSC	CO_2-C	0.457	0.366	-0.337	-0.108	-0.653	0.616	-0.113	0.693
	N_2O-N	0.407	0.398	-0.396	0.194	0.875*	-0.582	-0.383	-0.702
INT	CO_2-C	-0.168	-0.468	0.508	0.200	0.105	0.460	-0.398	0.629
	N_2O-N	0.347	0.674	0.357	-0.089	0.100	-0.497	0.442	-0.103

- R-values followed by a * or ** are significant at $p<0.05$ and $p<0.01$, respectively.

4. Discussion

The objective of this research was to evaluate intercrops' ability to reduce greenhouse gases (CO_2-C and N_2O-N) at 60% and 80% WFPS compared to sole crops (maize and soybean). These responses were measured throughout hour 1,6,12,24,36, and 48. At the last time-measurement, both soil concentrations (ammonium and nitrate) were assessed. In addition, all treatments from intercrops and sole crops were evaluated for microbial gene abundance (AOB, AOA, *nirK* bacteria, *nirK* archeaea) to evaluate potential correlations between these four gene abundances and the greenhouse gas emissions.

4.1 Greenhouse gas emissions in the nitrification process

4.1.1 CO_2-C emissions

In the MSC fertilized treatments CO_2 emissions indicated greater C decomposition by microorganisms compared to SSC. Contrarily, the unfertilized treatment in MSC maintained a slower C decomposition, which relates to an enhancement of the soil organic carbon (SOC), and therefore, carbon sequestration (Bichel, 2013). SOC enhacenment depends on management strategies such as residue retention (Wang et al., 2016). Such retention depends upon the decomposition rate, which is governed by both residue quantity and quality (Rengel and Bowden, 2006). The quantity of crop residues returned to the soil influences SOC (Follett, 2001). However, crop residue decomposition responds to quality differences (Dikgwathlhe et al., 2014). Although lignin is another factor in the decomposition rate of crop residues, if it is low it will not influence such decomposition (Aerts and De Caluwe, 1997). Lignin concentrations in both MSC and SSC residues have been shown to be relatively low, suggesting that lignin did not exert a particular role in the decomposition rate in these crop residues (Gentile et al., 2008).

Generally, lower C:N ratios are related to a faster rate of decomposition in crop residues (Booth et al., 2005). Although soybean crops are known to have such characteristics, C-decomposition was not as fast in SSC treatments. Sole crop rotations were performed under field conditions to reduce crop disease (Oelbermann et al., 2017). Therefore, lower CO_2 emissions in SSC was likely related to the history of the

plots under sole crop rotations. A greater C input from maize sole crops from previous rotations in the soil system may have promoted greater C-immobilization processes in the SSC treatments (Matias, C., personal communication, February 2018). This process has shown to be greater in sole crops rather than in the intercrops (Regehr et al., 2015). Similarly, in a study by Sey et al (2008) CO_2 emissions in soybean were lower than in corn sole crops. They reported that prior to the year 2003, all cultivars were seeded with corn crops that resulted in similar yields and residue inputs (Whalen et al., 2007). Similar to the findings of this study Dyer et al. (2012) reported that CO_2 emissions in soybean sole crops were lower than maize sole crops in Argentinian agroecosystems. This suggests that agricultural management shifts altered C dynamics in sole crop systems. The influence of low C:N ratios in combination with crop rotations led to greater SOC enhancement (Drinkwater et al., 1998). Regehr et al. (2015) reported that after six growing seasons the organic matter from maize and soybean crop residues increased SOC accumulation and N between 2007 and 2012.

It is worth noting that under NIT conditions there is tendency to favour microbial-mediated oxidation of ammonium to nitrate (Li et al., 2005). Consequently, higher C-storage in soils may be linked to an increase in N_2O emisions (Li et al., 2005). SOC accumulation was influenced by the quantity of crop residues from previous crop rotations (Carvalho Leite et al., 2004). The quantities of crop residues for each cropping systems were reported by Vachon (2008) as follows: MSC (904 g/m^2) <INT (798 g/m^2) <SSC (502 g/m^2). Moreover, crop residues with high C:N ratios such as those containing straw have been demonstrated to lower their C:N ratio after receiving N (Pothoff et al., 2005; Chen et al., 2007). Also, Abro et al. (2011) found that a shift in the microbial community resulted in 50% of an increase in CO_2 production in a straw crop residues compared to its control due to a greater N-addition into the system capable of decomposing C more efficiently (Agren et al., 2001). The findings of this study suggest CO_2 emissions were influenced by conditions promoting NIT processes, but also due to historical rotations.

Historial SOC accumulation may have interacted with the newly added NH_4^+ in MSC treatment, resulting in greater CO_2 emissions. Conversely, this faster C decomposition can only occur up until a certain

level, at which point CO_2 production declines (Abro et al., 2011). Similarly, Studdert and Echeverria (2000) concluded that the quantity of crop residue was related to SOC, but they also suggested that the type of crop combination (e.g., crop rotation) is an important factor influencing SOC content.

On the other hand, INT did not significantly differed from that of sole crops' CO_2 emissions, namely SSC treatments, which could be ascribed to the duration of this experiment. Most research agrees that differences in SOC stocks in the Argentinian Pampas are better detected after an extended period of time (Oelbermann et al., 2017); with some suggesting 11 years or more (Studdert and Echeverria, 2000). A more active microbial community favours CO_2 reduction in intercrop soils due to shifts in its N dynamics (Bichel et al., 2017). Lower CO_2 emissions in INTs contrasted with MSCs may indicate a combination of C-degradability related-processes. Microorganisms in the INTs use new C sources to reduce C-consumption of native C; such minimal decomposition shows to increase SOC levels (Bichel et al., 2016). In addition, intercrop soils have demonstrated a more active microbial community (Redin et al., 2014). It is suggested to experiment with longer periods of time to find significant changes in SOC enhancement (Oelbermann et al., 2017). Nonetheless, the findings of INTs fertilized treatments demonstrate CO_2 response under the influence of N dynamics.

4.1.2 N_2O-N emissions

N_2O emissions from the Pampas' agricultural soils measured throughout the 48-hour incubation experiment fell within the range of values reported by authors like Tilsner et al. (2003) (0.001 to 0.03 µmol N_2O g_{dw}^{-1}). In addition, Tilsner et al. (2003) reported 3% less of N_2O production compared to that of the DNIT process, while the findings of this study reports 8% less. In addition, the values reported in this study resulted in lower N_2O emissions than in the open fields in temperate soils (Omonode et al., 2007; Ellert and Janzen, 2008; Pappa et al., 2011; Picone et al., 2014); and, lower than open field values reported in Argentinian soils (e.g., Dyer et al., 2012). Although no significant difference in N_2O emissions could be observed between fertilized and unfertilized it is well known that fertilization increases N-availability and microbial

activity; this enhances organic matter decomposition, mineralization, nitrification and denitrification, leading to increases of N_2O emissions in diverse ecosystems (Brumme and Beese, 1992; Crill et al., 2000). It is clear that by applying inorganic N fertilizer to the soil this increased mineral N content, as well as N_2O emissions under NIT conditions (Figure 3.3 and Figure 3.4) (Bowman and Focht, 1974).

A factor that has been commonly linked as a contributing to N_2O emissions is readily C-availability (Muller and Sherlock, 2004; Wu et al., 2016). Inversely, N_2O reductions has been linked to SOC enhancement (Li et al., 2005). Higher N_2O emissions in MSC and SSC treatments seemed to have responded to historical SOC accumulation. This means that in general, leguminous crops result in mineralisation processes, whereas cereals result in immobilization processes (Chen et al., 2014). Contrarily, Regehr et al. (2015) reported that mineralization processes in the Argentinian maize sole crops were greater than the Argentinian soybean sole crops. One reason maize sole crops shifted from processes was due to a higher input of N from previous legume crop residues (Chen et al., 2014). However, under such shifts (annual sole crop rotations) it was found that the amount of N inorganic content was typically higher than its N-microbial demand (Chen et al., 2014). Therefore, production of N_2O can be shifted under the effect of crop residues that have different soil inorganic N concentrations (Chen et al., 2014).

Some studies have suggested that the inclusion of low-quality crop residues do not significantly affect legume sole crops due to their N_2 fixation capacity (Hemwong et al., 2008). For example Migliorati et al. (2015) stated that crops that fix their own N leave less N in the soil for NIT or DNIT. Less N-availability have been shown to reduce the potential N loss, but agricultural shifts have altered these N dynamics (Chen et al., 2014). For instance, in the findings of this study the fertilized SSC treatments resulted in lower N_2O emissions than in the MSC treatments. Contrarily, N_2O emissions in the unfertilized treatment of SSC were higher than MSC. Thus, unlike Hemwong et al. (2008) these findings suggested that the inclusion of low-quality crop residues in SSC would have affected N dynamics. For instance, in a recent study low N inorganic concentration from maize sole crop residues showed enhancement in N-immobilization (Chen et

al., 2014). They also explained that during immobilization microorganisms absorbed N since the crop residues did not fulfil N-microbial demand (Chen et al., 2014). Thus, agricultural shifts changed the inorganic N concentration that affected the assimilation by plants or potential N loss by microbes (Sugihara et al., 2012). In the fertilized SSC treatments NIT conditions discouraged NH_4^+ conversions under the influence of N-legacy from previous sole crop rotations during NIT conditions compared to fertilized MSC treatments.

In addition, it has been suggested that when C availability is reduced in soils, microbial deaths facilitate N-sources through enzymolysis (Zelenev et al., 2006). They showed that immobilization has shown to assist in the microbial proliferation through microbial death (Chen et al., 2014). In the case of SSC, others have explained that its C-availability is low (Hart et al., 1994). Therefore, if NIT conditions are not favored in SSC treatments they seemed to promote greater N-losses through its immobilized microbial biomass, which most likely held N due to historical rotations. It is adviced that SSC receive NH_4^+-forms to avoid greater N-losses through alternate pathways. The usage of NH_4^+-based fertilizers could enhance N-storage, however, this does not guarantee lesser CO_2 emissions compared to intercrops. It is also suggested that NH_4^+-based fertilizers under 60% WFPS should be reduced or avoided in MSC to reduce N_2O emissions. Alternatively, if NH_4^+-based fertilizers are used, then nitrification inhibitors or controlled released fertilizers are advice to decrease these emissions (Shoji et al., 2007; Ward, 2013).

Initially, it was expected that both sole crops and intercrops were going to emit significantly different amounts of N_2O. It was suggested that intercrops would emit lesser amounts of N_2O than sole crops. Although this finding was not significant it clearly showed that INT is capable of reducing N2O emissions in contrast to its counterpart, sole crops. Similar findings were reported by Dyer et al. (2012), which although crop-residue biomass and N_2O emissions were greatest in maize sole crops versus intercrops, such differences were not statistically different. Such differences have been constantly discussed among researchers. For instance, Eichner (1990) suggested that the source of N determines N_2O responses, while Jarecki et al. (2008) claimed it is N-availability. According to Dyer et al. (2012) the most important factor

that had a significant effects on N_2O emissions was the type of cropping system. Lower N_2O emissions have shown to be related to a greater N-immobilization rate that reduces N losses in INTs (Accoe et al., 2004). The combination of N-rich and N-poor residues allowed microorganisms to immobilize N in more proportion as a result of higher N-availability (Redin et al., 2014). Hence, INTs do not only modulate N_2O emissions but are cropping systems that will respond better even under conditions that promote NIT. Consequently, greater increases in N-availability for successive growing crops in INTs becomes an effective approach to tackle climate change.

4.2 Greehouse gas emissions in the denitrification process

4.2.1 CO_2-C emissions

Although these emissions were relatively low when compared to those than that of the NIT process, those with higher CO_2 emissions still need to be monitored for greenhouse-gas-accountability purposes. MSC and INT were not significantly different, however, others such as Dyer et al. (2012) have reported that annual maize-soybean rotations have shown similar CO_2 emissions to intercrops; and that the similarity between different agroecosystems lies in the type of legume crop used, or in the previous crop rotation. Omonode et al. (2007) found 16% less CO_2 in a maize-soybean crop rotation compared to a maize sole crop, and that the difference relied on the crop residue quality. Sole crop rotations in the Argentinian Pampas were performed to reduce crop disease (Oelbermann et al., 2017). Previous sole crop rotations may have impact the fate of CO_2 emission rates and their residue quality. In the unfertilized treatment of INT greater CO_2 emissions may have occurred due to the absence of alternate donors such as nitrate under DNIT conditions.

Meanwhile, SSCs seemed to be influenced by previous cropping seasons (e.g., Bichel et al., 2017). Such agricultural practice may have limited N availability (Booth et al., 2005), which led to a reduced production of CO_2. Likewise, a study at an agronomic farm in McGill in Canada reported that soybean CO_2 emissions were lower than in corn plots and reported that prior in the year 2003 all cultivars were seeded with corn crops that resulted in similar yields and residue inputs (Sey et al., 2008). Similarly, Sehy et al.

(2003) found that differences in CO_2 emissions between soybean sole crops and maize sole crops were due to a greater root biomass and such difference led to higher CO_2 emission rates. Enhanced CO_2 emissions can be influenced by N dynamics, especially in soils with lower quality (e.g., Chapagain and Riseman, 2014). For instance, Staggenborg et al. (2003), for instance, reported 21 kg N ha^{-1} soil residual N in a maize crop, and explained that this was inherited from a soybean crop prior to the rotation. Similarly, Abro et al. (2011) found that 50% of an increased in CO_2-C production in a straw crop residue compared to its control occurred due to a shift in the microbial community. Such shift occurred due to a greater N-addition into the system capable of decomposing C more efficiently (Agren et al., 2001). Moreover, crop residues with high C:N ratios such as those containing straw lowered their C:N ratio after receiving N (Pothoff et al., 2005; Chen et al., 2007).

Thus, this agricultural management shift may indicate that C dynamics from previous cropping seasons decelerated decomposition of crop residues (Li et al., 2005). Crop residue decomposition responds to quality differences (Mary et al., 1996). Therefore, although the C:N ratio quantity is related to SOC, the C:N ratio quality is influenced by shifts (e.g., crop rotation) in agroecosystem practices. However, when mixing maize and soybean residues in the intercrops the outcome results in superior availability of N to the growing crops (Sakala et al., 2000). For example, a study reported that pea in a sole crop accumulated 54 kg N ha^{-1} and that this N biomass was greater compared to an intercrop. However, N decrease in the total N accumulation in the intercrop was greater in the intercrop compared to pea sole crops (Chapagain and Riseman, 2014). Although the reduction of CO_2 emissions of INT were only lower than MSC, there are researchers that have found CO_2 reduction in comparison to both sole crops (e.g., Dyer et al., 2012). In addition, the findings of this study suggest that although there is more C readily available in MSC the higher WFPS influences the level of C-decomposition. It has been suggested that under anaerobic conditions preference to N and O from sources such as NO_3^- is given instead of C, which reduces CO_2 emissions (Latati et al., 2017).

Therefore, under DNIT conditions lower oxygen levels seem to limit C-usage in MSC. This also implies that if NH_4^+ fertilizers are applied in open fields, MSC may lower CO_2 production, however, caution

should be taken due to unknown responses of N_2O emissions under those conditions. It is worth noting that C-sequestration can also lead to N_2O increases since DNIT conditions could favor NIT processes simultaneously (Azam and Farooq, 2003).

4.2.2 N_2O-N emissions

The N_2O emissions reported in this study falls into agreement with Tilsner et al. (2003) (0.01 to 0.80 µmol g_{dw}^{-1}). In other NIT incubation experiments N_2O emissions have been reported 3% lower than those incubations under DNIT (Tilsner et al., 2003). In this study, N_2O emissions represented 8% more N_2O emissions than under NIT conditions. Hence, the addition of NO_3^--fertilizer seemed to become the alternate donor under DNIT conditions. Contrastingly, the unfertilized group did not show such trend, suggesting that the DNIT may have occurred in lower proportion. Interestingly, both sole crops emitted similar amounts of N_2O (before hour 36), and were higher emitters than INT treatments, which means that the alternative hypothesis was accepted. Surprinsingly, however, MSC treatments were much lower than SSC treatments at hour 36, which suggested that crop residue differences may have encouraged such response.

Crop residue types and fertilizer application rates affected N_2O emissions (Baggs et al., 2003). Production of N_2O was shifted under the effect of crop residues that had different soil inorganic N concentrations (Chen et al., 2014). For example, low C:N ratios were related to higher N_2O emissions due to their higher quality (Baggs et al., 2000). Basically, this means that in general, leguminous crops result in mineralization processes, whereas cereals in immobilization processes (Chen et al., 2014). Contrarily, Regehr et al. (2015) reported that mineralization processes in maize sole crops were greater than soybean sole crops. The reason why maize sole crops (under annual sole crop rotation) shifted from processes was due to a higher input of N from previous legume crop residues (Chen et al., 2014). They explained that the amount of N inorganic content is typically higher than its N-microbial demand (Chen et al., 2014). These authors explained that during the seedling stage if N-microbial demand is lower than its N-input, then N-loss results inevitable (Chen et al., 2014).

Conversely, soybean sole crops received low N inorganic concentration from maize sole crops, which was held and immobilized by the microbial community (Chen et al., 2014). Some studies have suggested that the inclusion of low-quality crop residues do not significantly affect legume sole crops due to their N_2 fixation capacity (Hemwong et al., 2008). For this reason, N-fertilizers should be applied less than or equal to those required for maximum yields to emit only 1% of the applied N-fertilizer as N_2O (Halvorson et al., 2008). An excess of NO_3^- would have favored denitrifiers because NO_3^- promotes its reduction and facilitates C consumption under DNIT conditions (Li et al., 2005). This reduction is subjected however upon C-availability to promote reductive transformations of NO_3^- to N_2 (Güven, 2009). Hart et al. (1994) reported lower C availability in soybean sole crops.

Therefore, under low C availability in SSC treatments it seems that NO_3^- is loss through N_2O. Inversely, higher C-availability in MSC due to N-legacy may have favoured denitrifiers to produce N_2O emissions.Though C:N ratios are useful to understand initial processes, it cannot always be use to predict outcomes (Chen et al., 2014). Rather, C:N ratio information should be complemented by the agroecosystem history (e.g., crop rotation) as well as the climate in the region (Luce et al., 2011). If the climatic conditions allow denitrification to prevail in the soils preference for N and oxygen will mainly be dominated by C-availability, which reduces CO_2 emissions (Latati et al., 2017). This explained CO_2 reduction in SSC treatments, however, the impact in N-storage was not as effective (Figure 3.5 and Figure 3.6). Chapagain and Riseman (2014) reported 17% to 31% of difference in N-usage between intercrops and sole crops, respectively, and suggested that subsequent crops benefitted from this N-availability in intercrops. Accoe et al. (2004), for instance, reported that a greater immobilization rate in intercrops resulted from an active microbial community that was able to reduce N losses.

Such event occurred because N-soybean residues immobilize greater quantities of microbes growing in the N-poor maize residue as N-availability increases (Redin et al., 2014). Therefore, under INT agroecosystems, N-accumulation is greater due to an efficient N-mineralization and immobilization synchronization, which ultimately reduces N losses (Regehr et al., 2015). They found that intercrops not only

immobilized but also reduced N_2O under NIT, which is likewise, what these results suggested for both NIT and DNIT groups. Thus, INTs offer tangible results to abate climate change in the short- and the long-term. These results also suggest that agroecosystem management, crop residue decomposition, its quality and quantity; the quantity of residue biomass returned to the soils; will influence N dynamics (Gregorich et al., 2015). These authors suggested that the reason how these factors influence is through biochemical composition of crop residues. Such biochemical composition of crop residues ultimately affect soil processes in the soil, namely the NIT, and the DNIT process. Likewise, this study concord with Regerh et al. (2015) in that INTs are the most sustainable land management option compared to sole crops.

4.3 Soil inorganic concentrations

4.3.1 Nitrification process

The results in this experiment were not significantly different between cropping systems, and this could have happened due to one-time sampling. Measurements at one point in time may to be enough to distinguish effects of particular factors (Placella et al., 2012). It would be ideal to examine NH_4^+ concentrations throughout the entirety of the experiment to detect changes throughout time since soil tends to vary due to heterogeneity (Theodorakopoulos et al., 2017). Regehr et al. (2015), and Chapagain and Riseman (2014) found that the differences in mineralization rates between intercrops and sole crops were significant between barley intercrops and pea sole crops (*Pisum sativum* L., Fabaceae Lindl.) in British Columbia, Canada. Regehr et al. (2015) mentioned that previous cropping seasons (sole crop rotations) influenced soil N residuals in the next growing season. Hence, this outcome can be due to the influence that soil N residuals have over N dynamics in these cropping systems. The fertilized INT treatments showed higher NH_4^+ concentrations compared to the unfertilized INT treatment. Contrarily, the NO_3^- content in the fertilized INT treatment was lower than that of the NO_3^- content in the unfertilized INT treatment.

Likewise, Zhang and Li (2003) pointed out that NO_3^- levels were lower in INTs because maize crops have higher N demand compared to soybean crops, which supplies N through N_2 fixation. The decreased in NO_3^- content was not observed in the unfertilized group implying that under the influence of NIT

conditions, INTs reduced NO_3^- leaching or N losses better than when NIT conditions were absent (Figure 3.9 and Figure 3.10). The difference in soil N residuals has shown to influence NH_4^+ concentration, especially in intercrops versus sole crops (Regehr et al., 2015). For instance, Chapagain and Riseman (2014) found that in open fields maize crops used the NH_4^+ content present in soybean crops within the intercrop, which resulted in lower soil N residuals throughout the growing season. Furthermore, a wheat-maize-faba intercropping in China subjected to NIT processes was reported to mineralize NH_3^+ very fast to NH_4^+ and that the latter went through a rapid transformation to NO_3^- in the rhizosphere of maize and wheat within the intercrop compared to wheat sole crop (Song et al., 2007). In addition, they reported lower N-losses compared to the wheat sole crop. The addition of NH_4Cl which encouraged the NIT process did not affect N losses or N_2O emissions, which is in agreement with other intercropping findings (Song et al., 2007, Chapagain and Riseman, 2014; Regehr et al., 2015; Bichel et al., 2017). Song et al. (2007) found that NH_4^+ and not NO_3^- concentrations reflected NIT rates in wheat-maize-faba intercropping in China. In addition, they found that mineralization rates correlated with NIT rates. Since the findings of this study did not analyze mineralization rates it is not possible to correlate them to NIT rates.

Nonetheless, Regehr et al. (2015) found that INTs mineralization rates were lower than MSC. Similarly, the NIT by-product, NO_3^- concentration, was lower in the INT treatments. Hauggaard-Nielsen and Jensen (2001) found that in intercrops the competition for N was reduced, which allowed complementary usage of N. This complementarity resulted in a lack of competence for the same N-resource, reducing competition for NH_4^+, which was the preferred N-source for microbes (Azam and Farooq, 2003). Typically, immobilizers and nitrifiers will compete for NH_4^+, resulting in greater N losses from soil-plant systems (Burger and Jackson, 2003). In INTs, however, the N-forms seemed to have been allocated more efficiently among competitors, namely nitrifiers. Furthermore, the INTs mixed C:N ratio slowed down the decomposition process (Luxhoi et al., 2006). These researchers reported that INTs decomposition rate was slower compared to SSC due to its lower substrate availability at the beginning of its crop residue decomposition. Similarly, Bichel et al. (2017) reported that a lower amount of NIT by-product (N_2O) was due to greater

rates of immobilized N, and a lower N-availability to microbes. Thus, INTs seemed to be the best sustainable agroecosystem due to crop composition, and quality, the type of crop rotation, and legacy N (Bichel et al., 2017).

4.3.2 Denitrification process

Rates of DNIT were increased under the presence of KNO_3, however, the differences between cropping systems was not significant (Appendix B.1). Like NH_4^+ content, NO_3^- content is also another source of N-availability for plants and microbes (Xu et al., 2017). Accordingly, NIT, DNIT, and respiration (CO_2 emissions), are soil processes regulating N-forms. Within these soil processes, SOM, C:N ratios, NH_4^+ and NO_3^- concentrations affect NIT and DNIT rates (Drenovsky et al., 2004). In this study, however, NO_3^- contents did not sufficiently informed about C or N dynamics. One reason could be that due to the analysis in one time throughout the 6 time series (only analyzed at hour 48), this was not sufficient to detect changes (Placella et al., 2012). In addition, NO_3^- is the main requirement for DNIT processes, thus by the time the soils were sample (hour 48) the results of NO_3^- did not reflect variations of the DNIT substrate but the soil N residual after NO_3^- was added. Although NO_3^- dictates DNIT processes, it was surprising that NH_4^+ was able to informed best as to what happened to soil irnoganic N. Regehr et al. (2015) mentioned that previous cropping seasons (sole crop rotations) influenced soil N residuals in the next growing season. Furthermore, the difference in soil N residuals showed to influence NH_4^+ concentration, especially in intercrops versus sole crops.

According to Azam and Farooq (2003) NH_4^+ availability is given from chemical fertilizers or from SOM mineralization. As NIT progressed, N-mineralization to NH_4^+ was depleted, low available oxygen was being reduced under anaerobic conditions, and NO_3^- was accumulated (Xu et al., 2017).Therefore, if SOM mineralization was taken place, this implied that both NIT and DNIT would have co-existed. Co-existence or simultaneous NIT-DNIT is not rare and has been reported by others (e.g., Snider et al., 2009; Xu et al., 2017). In addition, these findings suggested that during DNIT conditions potential mineralization

from SOM could have simultaneously occurred, however this remains uncertain. Contrary to the unfertilized MSC treatments NH_4^+ concentrations were 1.0 times greater than its counterpart, the fertilized treatments (Figure 3.11), suggesting that a reduced NH_4^+ content in the fertilized treatment could be linked to potential nitrifier inhibition under DNIT conditions. Vervaet et al. (2004) explained that maize sole crops ability to mineralize more than intercrops negatively impacts its ability to minimize NH_4^+ pools. Unlike Frimpong et al. (2012) the potential to minimize NH_4^+ content in MSCs under DNIT conditions indicates that N-availability is reduced under such environmental conditions. Conversely, however, this study agreed with Frimpong et al. (2012) in that N-losses in maize sole crops are still greater than intercrops (Figure 3.11).

Although there were clear differences in NH_4^+ concentration between MSC and INT fertilized and unfertilized treatments, the values were not significant, but were 1.1 times higher than INT treatments. Studies have shown that maize sole crops mineralize greater amounts of NH_4^+ compared to intercrops (e.g., Regehr et al., 2015). Conversely, Regehr et al. (2015) found that SSCs generated lower mineralization rates than MSCs. The findings of this study found that the NH_4^+ concentrations in the unfertilized MSC treatments were 1.1 times significantly greater than the unfertilized SSC treatments. Although similar results were found between the fertilized MSC and SSC treatments, these values were not significant. Nonetheless, this subtle difference impacted CO_2 emissions in the fertilized SSC treatments, which were significantly different to that of the fertilized MSC treatments. Although N_2O emissions were not significantly different between MSC and SSC, hour 36 was the only time where this production was higher than SSC. Similarly, a study by Wang et al. (1993) showed that although both legume and cereal sole crop emitted more N_2O than intercropping systems, legume sole crops emitted significantly higher amounts of N_2O.

Many researchers have found that DNIT rates are consistently dependent with respiration rates since such process produces CO_2 emissions, becoming a source of C for microbial processes (Signor and Cerri, 2013). However, C-assimilation will depend on its soil N-status (Gao et al., 2015). In the case of SSC it received low N inorganic concentration from MSC, which was held and immobilized by the microbial community (Chen et al., 2015). Some studies have suggested that the inclusion of low-quality crop residue

has not affected legume sole crops due to their N_2 fixation capacity (Hemwong et al., 2008). The increase of NO_3^- in soils subjected to DNIT processes has shown to favour denitrifiers, enhancing reductive processes and facilitating C consumption to obtain N_2 (Li et al., 2005; Güven, 2009). Hart et al. (1994) reported lower C availability in soybean sole crops. Therefore, under low C availability in fertilized SSC treatments NO_3^- was lost via N_2O emissions. Therefore, it would be ideal to maintain SSCs under 80% WFPS or amend these soils with organic amendments, which are richer in C sources. Contrastingly, in the unfertilized SSC treatments, N_2O emissions were the highest probably due to its lower C-availability. However, fertilized SSC treatments were 12.2 times higher than the unfertilized treatment. Research has shown that high water content (80% WFPS) increases C and N substrates (Snider et al., 2009; Blagodatsky and Smith, 2012).

Therefore, if climatic conditions affect soil moisture in SSCs soils NO_3^- -based fertilizers should be avoided until the water content reaches a value lower than 80% WFPS. It has been suggested that legume crops should receive N-fertilizers less than or equal to those required for maximum yields to emit only 1% of the applied N-fertilizer as N_2O (Halvorson et al., 2008). Lastly, athough NH_4^+ content was greater in INTs compared to SSCs (in both fertilized and unfertilized group), N losses were the lowest. Reduced N-losses in INTs are in agreement with what other researchers have reported (Dyer et al., 2012; Regehr et al., 2015; Bichel et al., 2017).

4.4 Gene abundance, greenhouse gas, and soil concentration correlations

4.4.1 Nitrification process

The application of NH_4Cl seemed to influence gene abundance in all cropping systems. Typically, ammonia oxidizers (AMO) [bacterial (AOB) or archaeal (AOA)] have shown to respond to N-fertilizers as suggested in Tatti et al. (2014). Some studies have shown that AOA do not respond to NH_4^+ addition to the soil (Di et al., 2009; Leininger et al., 2006; Zhalnina et al., 2012). Other findings indicate that NH_4^+ substrates are mostly correlated to AOB in agricultural soils; although AOA outnumbers AOB (Jia and Conrad, 2009). The findings of this study suggested that NH_4^+ concentrations were mostly related to AOA gene abundance and not with AOB. In an attempt to explain what influences N-availability and gene abundance

responses, researchers have analyzed mineralization rates and NIT rates with regards to NH_4^+ concentration. Gleeson et al. (2010), for example, found that mineralization rates were not correlated to neither AMO gene (AOA nor AOB). Petersen et al. (2012) found that AOA and AOB (AMOs*)* were both related to NIT rates.

Likewise, in this study we found that there was higher affinity between AOA and NIT rates, which occurs more so under N-limited soil environments (Di et al., 2010; Walker et al., 2010). Such findings have suggested that SOM, NH_4^+ and NO_3^- contents are factors that indirectly influence biogeochemical processes, however, they are themselves controlled by gene abundances (Petersen et al., 2012). Hence, chemical characteristics may complement but not suggest an entire explanation of the process, and this is due to their rapid conversion within the soil (Petersen et al., 2012). Therefore, it is adviced that these cropping systems are analyzed prior to the next growing season to understand how such agroecosystem management affects both AMOs (e.g, Enwall et al., 2010). If we understand the response of both AMOs to cropping systems we can be closer to understanding N_2O contributions under NIT conditions.

The findings of this study indicated the first attempt to describe which gene expression prevails in these Argentinian agroecosystems. Hence, it is challenging to compare this study to previous status of bacteria and archaeal AMO. Nevertheless, MSC showed greater number of copies of AOA gene abundance than SSC (Figure 3.16), and was strongly correlated to both soil inorganic N content. Unlike MSCs, SSCs resulted in a linkage between NO_3^- content and N_2O emissions, however, neither AMO was correlated to neither GHGs. Enwall et al. (2010) found that DNA also responds to historical rotations. Similarly, Chan et al. (2013) explained that historical rotations can influence AMOs gene expressions. The influence of historical rotations show that favoritism for AOA in MSC contributed to these emissions. However, the lack of correlation to either CO_2 or N_2O emissions with AMOs gene abundances in this study could not contribute to additional information. Unfortunately, in agricultural soils it is very common to find functional redundancy between AOA and AOB (Schauss et al., 2009). Therefore, AOA and AOB gene functionality still needs to be further analyzed to understand their real contributions to the production of N_2O (Prosser and Nicol, 2008; Jia and Conrad, 2009).

Similar to MSC, the gene abundance of AOA in INTs was significantly linked to NO_3^- content (Table 3.4). However, similar to SSC, its lack of correlation to neither GHG creates a challenge to assign a specific function to AOA in these soils. Schauss et al. (2009) reported that AOAs influence may be a back-up function providing a way to generate N-availability under poor environmental conditions (e.g., low nutrient availability). Interestingly, in the INT unfertilized treatments AOAs were 1.5 times higher than the fertilized treatments, suggesting that under NIT conditions a reduction of AOA gene abundance may be the genetic mechanism that INTs use to reduce N_2O emissions. Inversely, the fertilized MSC treatment was 2.3 times higher than its unfertilized counterpart. AOA gene expression has shown to be the limiting step process in the NIT process (Francis et al., 2007; Shen et al., 2008). For this reason, some studies have suggested that the presence of AOA is related to stressful conditions (Valentine, 2007). Meanwhile, Levy-Booth et al. (2014) proposed that AOA gene expression can also promote by-products using other pathways such as nitrification-denitrification, DNRA nitrate reduction, or anammox processes (Levy-Booth et al., 2014). Thus, it could be possible to postulate that AOA gene abundance was related to a mechanism by which INTs reduce N_2O emissions. Unlike INT, this principle may not apply to MSC fertilized treatments.

It is important noting that AOB gene abundance was also correlated to one of the N-forms in MSC soil (Table 3.4). Zhang, J. et al. (2015) explained that within NIT pathways, nitrite (NO_2^-) was the only stage where N_2O was reduced to N_2. The presence of AOB genes has been related to the oxidation of NH_4^+ to NO_2^- and of NO_2^- to NO_3^- (Francis et al., 2007; Shen et al., 2008). Thus, the correlation between AOB gene abundance and NO_3^- content may have indicated the genetical mechanism by which N_2O was loss to the atmosphere and impacted N-availability in MSC fertilized treatments (Table 3.4). Meanwhile, AOAs influence may be a back-up function providing a way to generate N-availability under poor environmental conditions (e.g., low nutrient availability) (Schauss et al., 2009). Further research about AOA and AOB is warrant to further our understanding of each gene's functionality in SSC treatments.

The gene abundance in *nirK* bacteria was higher overall in all cropping systems compared to *nirK* archaea. Further research of *nirK* archaea should be performed since the values were negligible (Figure 2.1(). Even under the right fertilization, wetting, and incubation conditions there may have been microsites

or ‘hotspots’ leading to different aimed processes (Banerjee et al., 2016). If successful, these microsites would have turned from aerobic to anaerobic favouring denitrifiers. Such development seemed to have occurred mainly in MSC treatments. Denitrifiers are well known to be favored by C-sources in soils under competent alternate electron donors such as NO_3^- (Tiedje, 1988; Phillippot et al., 2007; Su et al., 2010). This implies that denitrifiers were influenced by resource availability, but most importantly, by quantity and composition of organic compounds emitted by crop residue decomposition (Henry et al., 2008). Bichel et al. (2017) explained that C-availability is enhanced by N-legacy. Many researchers have found that DNIT rates are consistently dependent with respiration rates since such process produces CO_2 emissions, becoming a source of C for microbial processes (Signor and Cerri, 2013). This increase in C-availability may have impacted CO_2 emissions in MSC fertilized treatments (Figure 3.1 and Figure 3.3). Furthermore, since the findings of this study suggested that AOA gene abundance may have had an influence over NO_3^- it would be adviced to further our understanding of AOAs functionality over N-legacy in MSC. It seems that historical rotations may have facilitated *nirK* bacteria, as well as the amount of water filling the pores in the soil, and this outcome may have been encouraged by AOA gene abundance.

Moreover, the relationship between NH_4^+ content is most likely related to mineralization rates as MSC is the cropping system with greatest mineralization rates compared to SSC and INT (Regehr et al., 2015). This explains how both nitrifiers and denitrifiers were correlated to NH_4^+ in MSCs. The activation of all the genes at once and their relationship with both N-forms suggests that rapid conversion affected N_2O production the most in MSC. Contrarily, Hart et al. (1994) demonstrated that soybean sole crops are low in C-availability slowing down the internal N cycle. Thus, this explains how the redundancy among AMOs in SSC treatments. Studies have shown that the combination or crop interaction between N-rich and -poor residues facilitates greater N-immobilization as microbial demand increases for N-availability in intercrops (Redin et al., 2014). INTs negative correlates between CO_2 and NO_3^- may have indicated that AOA gene abundance was potentially responsible for the slowed down decomposition of substrate in INTs residues. However, since the findings of this study establishes a genetical-information baseline, this postulate should be further tested.

4.4.2 Denitrification process

The application of KNO_3 seemed to have influenced gene abundance in all three cropping systems. Large quantities of NO_3^- have shown to provide electrons to denitrifiers, thus becoming the most preferred form of N in soil systems (Firestone et al., 1980). Based on the findings of this study, it was surprising to see that AMOs were still dominant in these soils since DNIT conditions were encouraged and maintained throughout the incubation experiment. Moreover, although AOB was correlated to NO_3^- in all cropping systems, AOA still outcompeted AOB (Wessen et al., 2010). Jia and Conrad (2009) stated that although AOA gene abundance tends to prevail in agricultural soils, AOB is most likely more active than AOA. In addition, AOA gene abundance is typically related to agricultural soils under stressful conditions, namely water and nutrient availability (Verhamme et al., 2011). Thus, the dominancy of AOA gene abundance may be a reflection of the stress at which these soils are subjected to. Since NO_3^- content was correlated to N_2O emissions, and to AOB gene abundance in MSC (Table 3.3 and Table 3.5, respectively), it is AOB that was, most likely, more active in MSC treatments.

Others have demonstrated that functional redundancy between AOA or AOB is usually common in agricultural soils (Schauss et al., 2009). In addition, research has shown that *nirK* denitrifying genes has also been recognized in AOB (Casciotti and Ward 2001, 2005). Similar to *nirK* genes, AOBs have shown to reduce NO_2 to N_2O and N_2 (nitrification-denitrification pathways) using hydrogen, hydroxylamine, or organic compounds (Ritchie and Nicholas 1972; Stuven et al. 1992; Bock et al. 1995). Therefore, for future research purposes, both AMOs should be functionally identified in order to make conclusions about which one contributes more to N_2O production in MSC treatments. Phillips et al. (2015) found that microbial communities previously exposed to historic crop rotation changed under changing soil moisture. In addition, Enwall et al. (2010) explained that DNA of genes in their soils responded to historical agroecosystem management. Similarly, C and N dynamics seem to respond to such shifts in agricultural management. For instance, Chan et al. (2013) reported that previous legume-cereal rotations inherited less N in contrast to cereal-legume rotations since cereals such as maize sole crops demanded higher amounts of N.

These researchers established a maize and alfalfa crop rotation between 2008-2009 with manure amendements promoted an effect in both AOA and AOB gene abundance; however, AOA still dominated the soil, regardless of crop type (Chan et al., 2013). In the contrary, the authors reported that in a second agronomic location in Woodslee, Ontario (2008-2009), AOA abundance was higher in maize than alfalfa, however, neither maize sole crops nor alfalfa sole crops received amendements or inorganic N (Chan et al., 2013). These authors reported that previous sole crop rotations (e.g., cereal-legume) promoted AOAs affinity to NH_4^+, and its dominance in these soils. Furthermore, Wessen et al. (2010) reported that SOC and C:N ratios mostly impacted AMO selectivity in agricultural soils. Unlike a preference for NH_4^+, this study linked NO_3^- concentrations to AOB in the three cropping systems analyzed [e.g., Wagner and Jones (2006); Graungaard (2015)]. Therefore, AMOs selectivity seem to be irrespective of cropping system but instead depends on soil parameters such as SOM quality, N- and C–content, and on the type and quantity of organic residues (Wessen et al., 2010). Although correlations between NH_4^+ concentrations and gene abundances (Table 3.4) are not reported in this study, significant NH_4^+-content differences among cropping systems have suggested that NH_4^+ was a by-product of N-mineralizers.

Chan et al. (2013) explained that soils showed a tendency to nitrified NH_4^+ from native SOM through AMO genes. In this study, this outcome was more clear in the unfertilized treatments (Figure 3.11). For instance, in the unfertilized MSC treatments, the NH_4^+ concentration was significantly higher than the unfertilized treatments of SSC (Figure 3.11), suggesting mineralization from native SOM. The lack of correlation between AOA and any N-form suggested that AOB (correlated to NO_3^- content) influenced soil processes (Table 3.5). However, the high abundance of this gene (AOA) may suggest that under DNIT conditions this gene has an ecological role (Schauss et al., 2009). Walker et al. (2010) found that AOA can oxidize NH_3^+ via nitroxyl under anaerobic conditions. In addition, it has been proposed that AOA gene expression takes place via nitrification-denitrification, via DNRA nitrate reduction, or via anammox processes (Levy-Booth et al., 2014). These pathways require almost very little oxygen allowing AOAs to perform anaerobic processes (Schleper and Nicol, 2010). Thus, under waterlogged conditions this appear to be

the one possible explanation as to how mineralization from SOM occurred; in addition, it explains an ecological function under DNIT conditions. It is adviced to further our understanding about the relationship between mineralization rates along with functional gene analysis under DNIT conditions.

Moreover, denitrifiers seemed to compete with AOA gene abundance under DNIT conditions. Hallin et al. (2009) found that AOA and denitrifying gene abundance were indicative of which process rate was occurring in agricultural soils. For instance, SSC and INT were 35% and 31% higher in *nirK* bacteria gene abundance than in the NIT process, respectively. This was expected, since the conditions promoting DNIT conditions, would theoretically activate denitrying genes (Güven, 2009). Thus, it may seem that the negative correlation between CO_2 and NO_3^- in SSC and INT (Table 3.3) is a by-product of denitrifying gene activity. Denitrifiers are well known to be favored by C-sources in soils under competent alternate electron donors such as NO_3^- (Tiedje, 1988; Phillippot et al., 2007). Re-mineralization of C in the absence of oxygen levels is supplied through NO_3^- (alternative electron donors). For instance, Szukics et al. (2009) found that agricultural soils with 70% WFPS increased its organic C-content by 16% and that these soils increased *nirK* genes (Levy-Booth et al., 2014). Therefore, if C-availability is lower, although the supply of NO_3^- is enough, C-sources will become a limitation, and will generate higher N-losses (e.g., Brentrup et al., 2000; Ciampitti et al., 2008). Hart et al. (1994) demonstrated that soybean sole crops are low in C-availability slowing down the internal N cycle.

Therefore, this correlation demonstrates that CO_2 emissions were lower due to lower C-availability. Meanwhile, the same negative correlation in the INT may suggest that these soils are able to decompose substrates more slowly (Luxhoi et al., 2006). Since there is no existing correlation between none of these genes, there is still no information about which group enhanced or reduced CO_2 emissions in INT treatments. Nonetheless, AOA gene abundance was 1.4 times higher in the unfertilized treatment than the fertilized INT treatments. However, abundance on its own does not always suggest which specific group contributed the most (Wessen et al., 2010). Thus, this can only suggest that AOA gene abundance was present due to stressful conditions (Valentine, 2007). But it could also suggest a different ecological role (e.g., Schauss et al., 2009). Moreover, AOA gene abundance in the INT was not correlated to NO_3^- as in the NIT

process. Instead, the correlation was significant between NO_3^- and AOB gene abundance. The presence of AOB genes has been related to the oxidation of NH_4^+ to NO_2^- and of NO_2^- to NO_3^- (Francis et al., 2007; Shen et al., 2008). Studies have shown that under the right conditions the reduction of NO_2 to N_2O and N_2 by AOB genes can successfully occur via nitrification-denitrification pathways using hydrogen, hydroxylamine, or organic compounds (Ritchie and Nicholas 1972; Stuven et al. 1992; Bock et al. 1995). Thus, the findings of this study suggests genetical mechanisms that INT uses to reduce N_2O emissions under DNIT conditions. Hence, it would be ideal to investigate the functional relationship between AOA, AOB, and *nirK* bacteria gene abundance over soil N-contents in these cropping systems. The gene abundance in *nirK* bacteria was higher in all cropping systems compared to *nirK* archaea. Banerjee et al. (2016) examined bacterial and archaeal denitrifier, however, their primer was only capable of amplifying a fraction of bacterial denitrifier involved in N_2O emissions. Likewise, the primer may have been able to identify and amplify small binding regions in the DNA. Therefore, more research about *nirK* archaea with different primers could further our understanding about this particular gene in INT treatments.

5. Conclusions and Recommendations

Cropping systems in agriculture represent approximately 1.7 billion hectares globally (Paustian et al., 2000). The constant cultivation in these systems increases the mineralization of SOC (Reicosky, 1999).This means that cropping systems can influence CO_2 emissions and C-content, which affects climate change (Sainju et al., 2008). For this reason, C sequestration in soils has been considered as one of the main approach to mitigate climate change (Li et al., 2005). However, some researchers have reported that C-availability increases as N-availability does (Luo et al., 2004). Similarly, others have found that the enhancement of SOC in the soil leads to an increase in N_2O production (e.g., Brentrup et al., 2000; Li et al., 2005). Then, it becomes important to consider other greenhouse gases such as N_2O. Compared to CO_2, N_2O emissions represent a higher risk to the environment due to its long-life in the ozone layer (Prinn and Zander, 1999). N_2O is subjected to photodissociation via photolysis through an electronically excited oxygen atom occurring in the stratosphere (Prinn and Zander, 1999; Portmann et al., 2012).

Recent studies have shown that N_2O has a global warming potential that is 265 times higher than CO_2 in a 100-year time horizon (Myhre et al., 2013). In agriculture, soils are responsible for 60% to 80% of N_2O production (Davidson, 2009). N_2O production takes place through oxidation-reduction reactions (Li et al., 2005). These reactions use the inorganic N provided through SOM decomposition, which also encourages soil processes such as nitrification and denitrification (via anoxic microsites) (Gregorich et al., 2005). This means that SOM is the linkage between nitrification and denitrification, fulfilling the N cycle (Ward, 2013). Similarly, SOC influences microbial growth and activity, providing with organic C to denitrifiers (Cameron et al., 2013). Therefore, both the nitrification and denitrification can elucidate C and N dynamics in cropping systems to tackle climate change more effectively.

The findings of this study concluded that CO_2 emissions dependent strongly on the quality of the C:N ratio due to previous sole crop rotations. For instance, Dyer et al. (2012) reported that the intercrops emitted similar amounts of CO_2 compared to maize-soybean sole crop rotation. Such similarities between intercrops and maize-soybean rotations have been ascribed to crop-residue quality (Omonode et al., 2007).

Regehr et al. (2015) stated that both maize and soybean sole crops increased SOC and soil total N by 2012 due to greater crop residue inputs. Therefore, intercrop systems may require greater amount of time so as to perceive significant changes between these two cropping systems; some suggesting 11 years or more (Studdert and Echeverria, 2000). C sequestration did not imply N storage in soybean sole crop since N_2O emissions in soybean sole crops resulted higher than the intercrops in the nitrification and denitrification process. Contrastingly, intercrops showed a decrease of N_2O production under both nitrification and denitrification conditions suggesting that in the long-term N storage would represent greater C-sequestration. Nonetheless, CO_2 emissions in intercrops were only lower with regards to maize sole crop but not compared to soybean sole crops.

Therefore, longer-incubation experiments may facilitate more accurate predictions of CO_2 emissions in intercrops versus sole crops. An apparent increase in CO_2 emissions in unfertilized-intercrop systems suggested that NO_3^- fertilization may be adviced, if under field conditions, intercrops reach 80% WFPS. However, longer-incubation experiments may be better predictors of the behavior of CO_2 in intercrops as well as predictive SOC models (e.g., Oelbermann et al., 2017). Similarly, without NO_3^- fertilizers soybean sole crops emitted similar amounts of CO_2 as intercrops, however, N_2O emissions were affected greatly in soybean sole crops. The difference between CO_2 emissions indicated that overall, intercrops represented the most sustainable agroecosystem compared to maize sole crops but not compared to soybean sole crops in this short-term incubation study. Nevertheless, the response of N_2O emissions indicated that N-losses in intercrops was minimal compared to both sole crops, and that this agricultural practice could potentially preserve C in these agroecosystems. This demonstrated that intercrops are an effective agroecosystem approach that offers curbing agricultural emissions to reduce the impact of climate change (Dyer et al., 2012; Regehr et al., 2015; Bichel et al., 2017).

On the other hand, the hypothesis established for soil N-inorganic concentrations suggested that NH_4^+ and NO_3^- availability was dependent mostly on crop residue composition as well as to historic agroecosystem management practices. Soil concentrations, which are the indirect by-product of nitrifiers- and

denitrifiers-N-preference informed us about the chosen soil microbial pathway (nitrification or denitrification) performed under the aimed process. For instance, under nitrification conditions, the expected by-product was NO_3^-. Therefore, NO_3^- seemed to inform better about C and N dynamics in the nitrification group. The amount of NO_3^- concentrations from the intercrop treatments reflected the lack of competence for the same N-resource compared to sole crops since its concentration was significantly the lowest. Typically, immobilizers and nitrifiers will compete for NH_4^+, resulting in greater N losses from soil-plant systems (Burger and Jackson, 2003). However, the N-forms seemed to be allocated more efficiently among these competitors in the intercrops. Meanwhile, in the denitrification group the interpretation of N-forms was challenging because N_2 was not measured. Surprisingly, the NH_4^+ content informed the best under denitrification conditions so as to understand the response of these cropping systems. For instance, maize sole crop treatments without fertilizer showed significant variations in the NH_4^+ concentration. This elucidated the difference in soil N residuals and their influence over NH_4^+ during the denitrification process.

The results showed that potential mineralization from SOM may have occurred since NH_4^+ were significantly higher compared to soybean sole crop and intercrop during the denitrification process. Therefore, it was postulated that this enhanced N-availability in maize sole crops favoured higher N-losses under denitrification conditions. Unlike unfertilized-maize-sole-crop treatments, unfertilized-soybean-sole-crop treatments did not mineralize as much NH_4^+, but this outcome seemed not to explain the difference between this treatment and maize sole crop. Hart et al. (1994) explained that reduction of N-availability follows lower C availability in soybean crops. Conclusions about the correlates between soil concentrations of soybean sole crops and gene abundance described that potential nitrification-denitrification may have had occurred, namely performed by AOB genes. The linkage between nitrifiers and soil concentrations in soybean sole crops may describe the influence of quality crop-residue over gene expression and greater N-losses.

Nitrifiers and denitrifiers were not dependent on cropping system, but rather on crop composition (quality over quantity) and historic agroecosystem management practices. Likewise, other recent studies have found that rotation history influences the resilience and resistance of microbial communities to soil moisture changes (Phillips et al., 2015). Preference to nitrifier genes was clearly favoured under nitrification

conditions, which is in agreement with other authors (e.g., Nicol et al., 2008; Verhamme et al., 2011). Greater NH_4^+ availability in maize sole crops may have encouraged greater abundance of nitrifiers. Interestingly, AOA nitrifier genes seemed to be present regardless to the ongoing soil process. Gene functional analysis is advice since some recent studies have found that changes in the soil water-filled-pore-space shifts soil processes favouring N-mineral cycling processes (Phillips et al., 2015). In addition there should also be an analysis to detect gene abundances at 50% WFPS to measure the impact of these genes over N_2O production. By doing so, we can determine a more healthy gene abundance in cropping systems that can perhaps support an explanation for greater N_2O reductions in sole crops.

Denitrifier genes such as *nirK* bacteria were favoured greatly under denitrification conditions. However, they were still present under nitrification conditions. This suggested that both nitrifiers and denitrifiers co-existed (e.g., Snider et al., 2009; Xu et al., 2017). Interestingly, the results of this study showed that *nirK* bacteria was reduced under denitrification conditions in maize sole crop fertilized treatments compared to both soybean sole crop and intercrop treatments. This decrease in maize sole crops may have impacted N_2O production in hour 36. Others have reported that AOA is favoured under low NH_4^+ soil environments (Di et al., 2010). Gene functional analysis is advice since some recent studies have found that changes in the soil water-filled-pore-space shifts soil processes favouring N-mineral cycling processes (Phillips et al., 2015). There have been findings where nitrification inhibitors and controlled release fertilizers seemed to mitigate N_2O emissions (Shoji et al., 2007; Ward, 2013). Nitrification inhibitors, for instance, have shown to slow NIT and increase N-assimilation in cereals (e.g., Delgado and Mosier, 1996). Therefore, it is recommended that under denitrification and nitrification conditions, nitrification inhibitors and controlled release fertilizers are considered and analyzed over AOA gene abundance prior to its usage in maize sole crops. It is advice to perform similar studies using different soil depths to detect potential gene expression shifts along soil gradients (Zhang et al., 2015; Lori, P., personal communication, February 2017).

Furthermore, crop composition and historic agroecosystem management seemed to have influenced the response of *nirK* bacteria in the unfertilized-maize-sole-crop treatments under denitrification conditions. Therefore, *nirK* bacteria is most likely the best indicator of lower N_2O production in maize sole crops. It is

likely that soybean sole crop used alternative genes to promote nitrification-denitrification processes, namely AOB genes. AOB gene expression has shown to reduce NO_2 to N_2O and N_2 (nitrification-denitrification pathways) using hydrogen, hydroxylamine, or organic compounds (Ritchie and Nicholas 1972, Stuven et al. 1992, Bock et al. 1995). AOB is considered to be the microorganism rate-limiting in the ammonia oxidation process in the autotrophic NIT (De Boer and Kowalchuk, 2001). It is rate-limiting due to its capacity to produce hydroxylamine (NH_2OH) before it even reaches to the conversion from NH_4^+ to NO_2^- liberating N_2O as the by-product (De Boer and Kowalchuk, 2001). Nitrification-denitrification pathways account for 25% of the denitrification process (Dong et al., 2000). Therefore, further functional analysis of AOB gene should be furthered in the literature in all cropping systems.

The AOA gene abundance seemed to have outgrowth AOB in both maize and soybean sole crop. This outgrowth was related to stressful water conditions in the soil (e.g., Valentine, 2007). However, in the intercrops there was equal gene abundance of AOA and *nirK* bacteria. This similar gene abundance in intercrops may have been favoured by the type of C substrates and inherited N from previous cropping seasons (Bichel et al., 2016; 2017). Since values for *nirK* archaea were negligible and outnumbered by *nirK* bacteria, it is advice to perform more qPCR analysis, perhaps with a different p-value or with different primers. Denitrifiers tend to be extensive and heterogenous, which creates mismatches during amplification protocols (Appendix D.12). Thus, primers should be improved and expanded based on latest DNA information about denitrifiers (Penton et al., 2013).

Thus, gene abundances exemplified other mechanisms by which sole crops and intercrops respond under different soil processes. This information suggests that if AOB is functionally active during denitrification conditions in soybean sole crops, these soils could receive substrates that inhibit this particular gene. Similarly, if AOA are functionally active and promoting N_2O emissions, nitrification inhibitors could assist in their reduction to reduce GHG emissions. Thus, further research in relation to genes' functionality is encouraged. Overall, intercrop systems represented an agroecosystem approach that commits to curbing N_2O emissions in the long-term under both nitrification and denitrification processes; also, denoting sustainable cropping systems that would secure food productivity and still abate climate change.

6 References

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Appendix

Appendix A – GHG emissions from MSC, SSC and INT under nitrification conditions

Table A.1 Mean carbon dioxide emission (mg CO_2-C g^{-1} h^{-1}) in different fertilized (F) and unfertilized (UF) crop treatments during 48 hours under 60% WFPS

Crop type	1 hour	6 hours	12 hours	24 hours	36 hours	48 hours	Total mean
MSC-F	$0.28(0.02)^{A,a}$	$1.43(0.13)^{B,a}$	$1.70(0.14)^{B,a}$	$3.44(0.27)^{C,a}$	$3.20(0.08)^{C,a}$	$3.25(1.69)^{C,a}$	2.22
MSC-UF	$0.33(0.06)^{A,a}$	$0.96(0.10)^{A,a}$	$1.13(0.02)^{A,a}$	$2.54(0.09)^{B,a}$	$2.56(0.14)^{B,a}$	$2.83(0.14)^{B,a}$	1.72
SSC-F	$0.21(0.01)^{A,a}$	$0.99(0.06)^{AB,a}$	$1.24(0.08)^{B,a}$	$2.65(0.13)^{C,a}$	$2.54(0.17)^{C,a}$	$2.99(0.10)^{C,a}$	1.77
SSC-UF	$0.19(0.01)^{A,a}$	$1.02(0.01)^{A,a}$	$1.06(0.05)^{A,a}$	$2.17(0.20)^{B,a}$	$2.28(0.13)^{B,a}$	$2.93(0.15)^{B,a}$	1.61
INT-F	$0.24(0.00)^{A,a}$	$1.18(0.02)^{B,a}$	$1.43(0.11)^{B,a}$	$3.17(0.09)^{C,a}$	$3.64(0.51)^{C,b}$	$3.52(0.11)^{C,a}$	2.20
INT-UF	$0.25(0.02)^{A,a}$	$1.22(0.11)^{B,a}$	$1.45(0.18)^{B,a}$	$2.89(0.30)^{C,a}$	$2.63(0.16)^{C,a}$	$3.07(0.25)^{C,a}$	1.92

[A] Means followed by a different uppercase letter are significantly different ($p<0.05$) within crop treatment between different hours
[a] Means followed by a different lowercase letter are significantly different ($p<0.05$) between crop treatments within hour

Table A.2 Mean nitrous oxide emissions (μg N_2O-N g^{-1} h^{-1}) in different fertilized (F) and unfertilized (UF) crop treatments during 48 hours under 60% WFPS

Crop type	1 hour	6 hours	12 hours	24 hours	36 hours	48 hours	Total mean
MSC-F	0.18 (0.08) ^{A,a}	0.93(0.43)^{AC,a}	1.21(0.55)^{AC,a}	2.76(1.24)^{BC,a}	3.03(1.46)^{B,a}	1.75(1.27)^{BC,a}	1.64
MSC-UF	0.06 (0.01) ^{A,a}	0.19(0.02)^{A,a}	0.24(0.02) ^{A,a}	0.61(0.08)^{A,a}	0.67(0.07)^{A,a}	0.83 (0.09)^{A,a}	0.43
SSC-F	0.06 (0.00) ^{A,a}	0.3 (0.00) ^{AB,a}	0.39(0.01)^{AB,a}	0.97(0.08)^{AB,b}	1.11(0.04)^{AB,b}	1.37 (0.06)^{B,a}	0.70
SSC-UF	0.05(0.00) ^{A,a}	0.30 (0.00)^{A,a}	0.33 (0.01)^{A,a}	0.77(0.06) ^{A,a}	0.89 (0.04)^{A,a}	1.01 (0.17)^{A,a}	0.56
INT-F	0.04(0.002)^{A,a}	0.22 (0.01)^{A,a}	0.27 (0.02)^{A,a}	0.65(0.05) ^{A,b}	0.81 (0.11)^{A,b}	0.86 (0.03)^{A,a}	0.48
INT-UF	0.05 (0.00) ^{A,a}	0.24 (0.03)^{A,a}	0.30 (0.04)^{A,a}	0.63(0.07) ^{A,a}	0.61 (0.04)^{A,a}	0.73 (0.04)^{A,a}	0.43

[A] Means followed by a different uppercase letter are significantly different ($p<0.05$) within crop treatment between different hours
[a] Means followed by a different lowercase letter are significantly different ($p<0.05$) between crop treatments within hour

Appendix B – GHG emissions from MSC, SSC, and INT in denitrification conditions

Table B.1 Mean carbon dioxide emissions (mg CO_2-C g^{-1} h^{-1}) in different fertilized (F) and unfertilized (UF) crop treatments during 48 hours at 80% WFPS

Crop type	**1 hour**	**6 hours**	**12 hours**	**24 hours**	**36 hours**	**48 hours**	**Total mean**
MSC-F	0.00 (0.00)^{A,a}	-0.01 (0.00) ^{A,a}	0.01 (0.00) ^{A,a}	0.06(0.01)^{B,a}	0.11(0.02)^{C,a}	0.07(0.01)^{B,a}	0.04
MSC-UF	0.00 (0.00)^{A,a}	-0.01 (0.00) ^{A,a}	0.00 (0.00) ^{A,a}	0.06(0.01)^{B,a}	0.08 (0.00)^{B,a}	0.06 (0.04)^{B,a}	0.03
SSC-F	0.00 (0.00)^{AB,a}	-0.01 (0.00)^{A,a}	-0.00(0.00)^{AB,a}	0.04(0.02)^{C,a}	0.08(0.03)^{D,b}	0.03(0.01)^{BC,b}	0.02
SSC-UF	0.00 (0.00)^{A,a}	-0.01 (0.0)^{A,a}	0.00 (0.00)^{A,a}	0.05(0.00)^{B,a}	0.07(0.00)^{BC,a}	0.09(0.01)^{C,ab}	0.03
INT-F	0.00 (0.00)^{A,a}	-0.01 (0.00)^{A,a}	0.00 (0.00)^{A,a}	0.05(0.01)^{B,a}	0.10(0.03)^{C,ab}	0.04 (0.01)^{B,b}	0.03
INT-UF	0.00 (0.0)^{A,a}	-0.01 (0.0)^{A,a}	0.00 (0.0)^{A,a}	0.07(0.01)^{B,a}	0.08(0.01)^{BC,a}	0.10 (0.0)^{C,b}	0.04

[A] Means followed by a different uppercase letter are significantly different ($p<0.05$) within crop treatment between different hours
[a] Means followed by a different lowercase letter are significantly different ($p<0.05$) between crop treatments within hour

Table B.2 Mean nitrous oxide emissions (μg N_2O-N g^{-1} h^{-1}) in different fertilized crop treatments during 48 hours at 80% WFPS

Crop type	1 hour	6 hours	12 hours	24 hours	36 hours	48 hours	Total mean
MSC-F	0.12 (0.00) ^{A,a}	3.57(0.33) ^{A,a}	8.31(0.79)^{AB,a}	22.64(0.47)^{C,a}	23.78(2.69)^{BC,a}	15.28(2.10)^{BC,a}	12.28
MSC-UF	0.00(0.00)^{A,a}	-0.02(0.00)^{A,a}	-0.01(0.00)^{A,a}	-0.02 (0.01) ^{A,a}	-0.00 (0.02) ^{A,a}	-0.01 (0.00) ^{A,a}	-0.01
SSC-F	0.09(0.01) ^{A,a}	3.53(0.89) ^{A,a}	7.72(2.12)^{AB,a}	21.69(6.20)^{C,a}	27.09(9.32)^{AB,a}	16.30(5.51)^{BC,a}	12.74
SSC-UF	0.02(0.02)^{A,a}	0.61 (0.61)^{A,a}	1.07 (1.09) ^{A,a}	2.23 (2.07) ^{A,a}	1.72 (1.58) ^{A,a}	1.83 (1.82) ^{A,a}	1.25
INT-F	0.05 (0.01)^{A,a}	2.34 (0.86) ^{A,a}	5.67(2.53)^{AB,a}	14.74(6.52)^{BC,a}	20.30(10.18)^{C,a}	13.11 (6.01)^{BC,a}	9.37
INT-UF	0.00(0.00)^{A,a}	0.04 (0.03) ^{A,a}	-0.01 (0.02) ^{A,a}	0.01 (0.03) ^{A,a}	0.04 (0.05) ^{A,a}	0.05 (0.08) ^{A,a}	0.02

[A] Means followed by a different uppercase letter are significantly different ($p<0.05$) within crop treatment between different hours
[a] Means followed by a different lowercase letter are significantly different ($p<0.05$) between crop treatments within hour

Appendix C – Nanospectrophotometer and PicoGreen Method

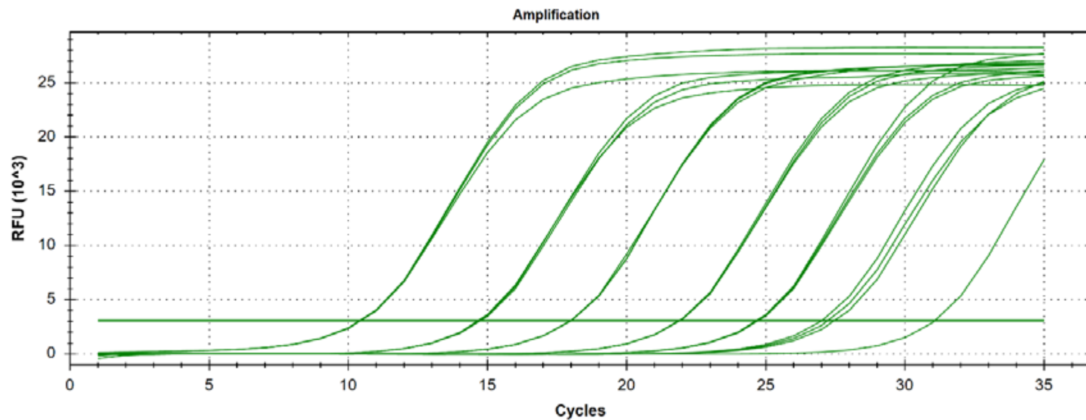
Table C.1 - Relative DNA concentrations from MSC, SSC, and INT crop treatments using the nanospectrophotomter

Sample ID	Average concentration (ng/ul) nanodrop
NITMS1	17.40
NITMS2	18.45
NITMS3	15.50
NITSS1	13.65
NITSS2	14.30
NITSS3	10.35
NIT231	21.10
NIT232	24.90
NIT233	13.95
CNITMS1	10.50
CNITMS2	11.15
CNITMS3	8.60
CNITSS1	11.00
CNITSS2	13.10
CNITSS3	10.17
CNIT231	19.45
CNIT232	19.60
CNIT233	17.20
DNITMS1	8.80
DNITMS2	4.90
DNITMS3	3.45
DNITSS1	3.60
DNITSS2	8.35
DNITSS3	8.30
DNIT231	5.15
DNIT232	4.70
DNIT233	5.40
CDNITMS1	9.20
CDNITMS2	13.60
CDNITMS3	6.90
CDNITSS1	7.50
CDNITSS2	6.00
CDNITSS3	5.60
CDNIT231	6.95
CDNIT232	7.20
CDNIT233	10.50

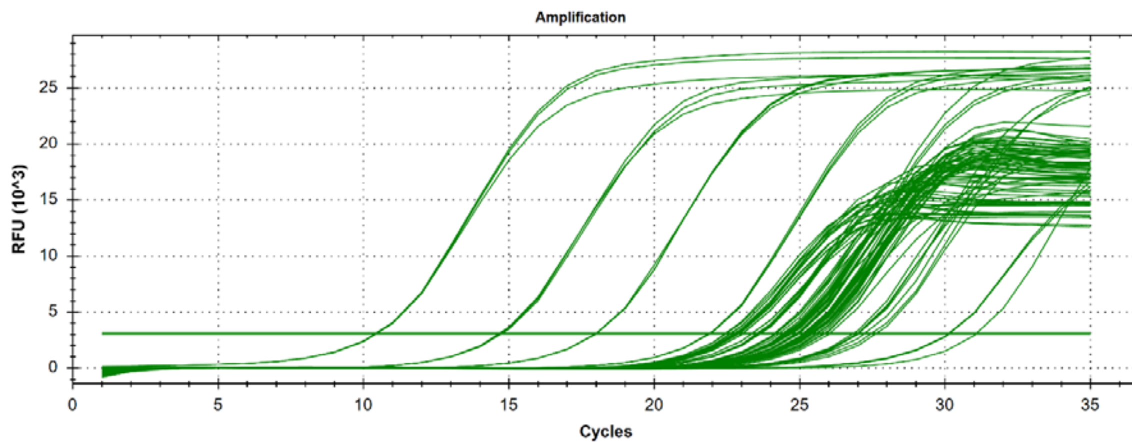
Table C.2 - Values that resulted from the PicoGreen Assay to evaluate the absolute concentration value of the DNA in MSC, SSC, and INT crop treatments

Sample ID	Average concentration (ng/ul) pico
NITMS1	14.05
NITMS2	10.18
NITMS3	8.85
NITSS1	7.31
NITSS2	8.21
NITSS3	4.32
NIT231	9.35
NIT232	8.90
NIT233	14.26
CNITMS1	7.27
CNITMS2	4.53
CNITMS3	4.41
CNITSS1	5.76
CNITSS2	5.59
CNITSS3	5.86
CNIT231	12.99
CNIT232	13.07
CNIT233	11.55
DNITMS1	4.77
DNITMS2	3.81
DNITMS3	3.03
DNITSS1	2.76
DNITSS2	2.16
DNITSS3	3.68
DNIT231	3.52
DNIT232	2.19
DNIT233	3.34
CDNITMS1	3.51
CDNITMS2	3.12
CDNITMS3	2.86
CDNITSS1	3.75
CDNITSS2	3.84
CDNITSS3	2.70
CDNIT231	3.16
CDNIT232	4.49
CDNIT233	4.26

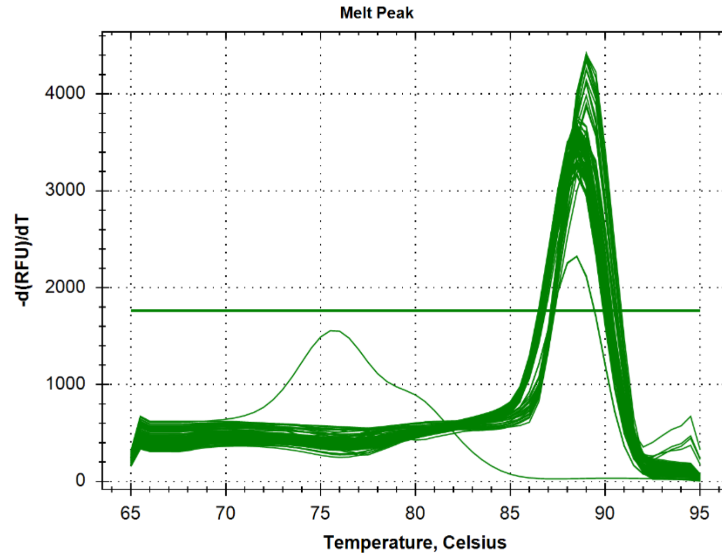
Appendix D – qPCR Assessment Graphs Prior to gene-reference copy analyses



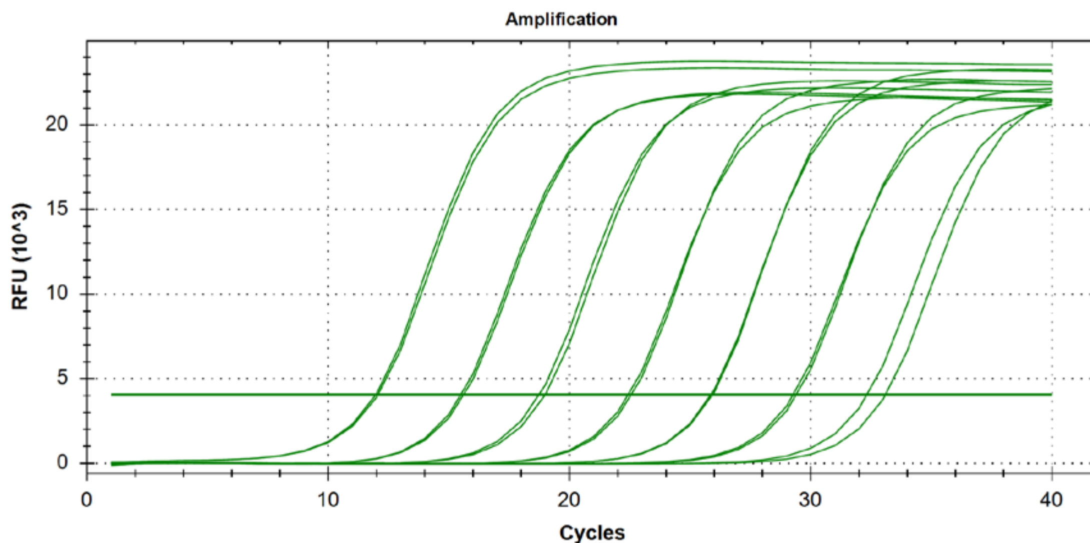
Appendix D.1 Amplification of AOB standards only. In general, the standards' height resulted all in singleplex and Cq values remained constant with the set of triplicates of the AOB gene-reference and were lower than 40 and greater than 15 cycles. Thus, the reproducibility of this analysis was high enough to accept the efficiency r value from the targeted-gene of the samples from the nitrification and denitrification process.



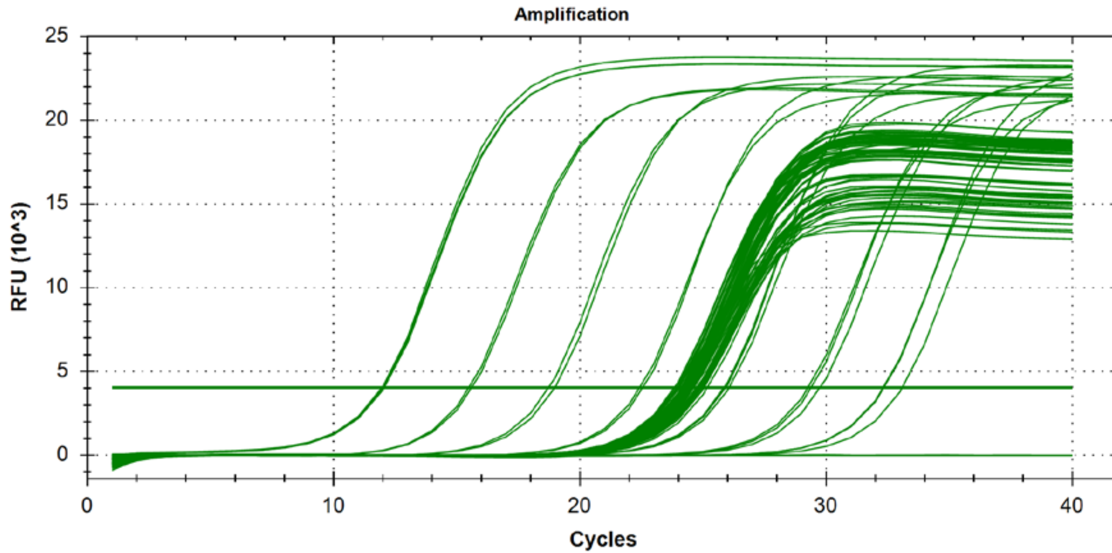
Appendix D.2 Amplification of AOB standards with targeted genes from nitrification and denitrification samples. Cq values remained constant with the set of triplicates of the AOB gene-reference previously assessed and were lower than 40, but greater than 15; all targeted genes started revealing an exponential stage at around 20 cycles around 3 x 10^{-3} RFU (relative fluorescent units). This revealed a good efficiency overall.



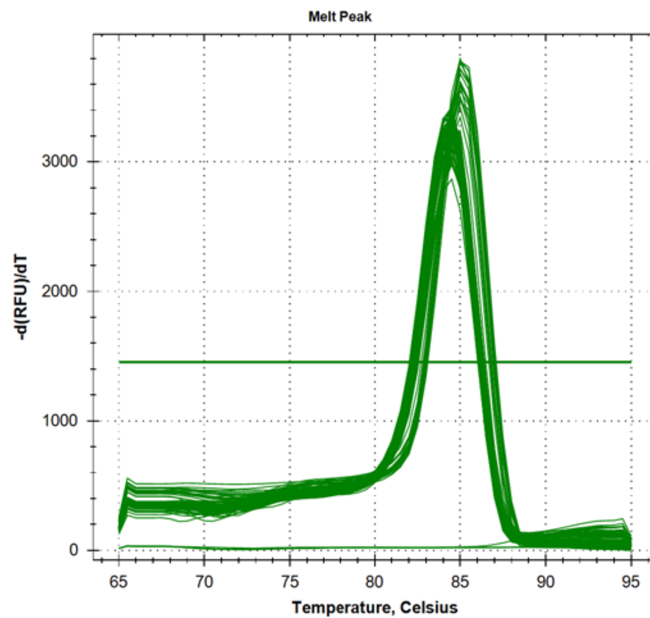
Appendix D.3 Melt peak of AOB targeted-genes and reference-genes (samples) generated a single peak for 99.9% of all samples that belong to the nitrification and denitrification processes. The second amplicon shown between 73-77 ºC belongs to a contaminant present in one of the samples, a primer dimer or non-specific amplification (Lori, P., personal communication, February 2017).



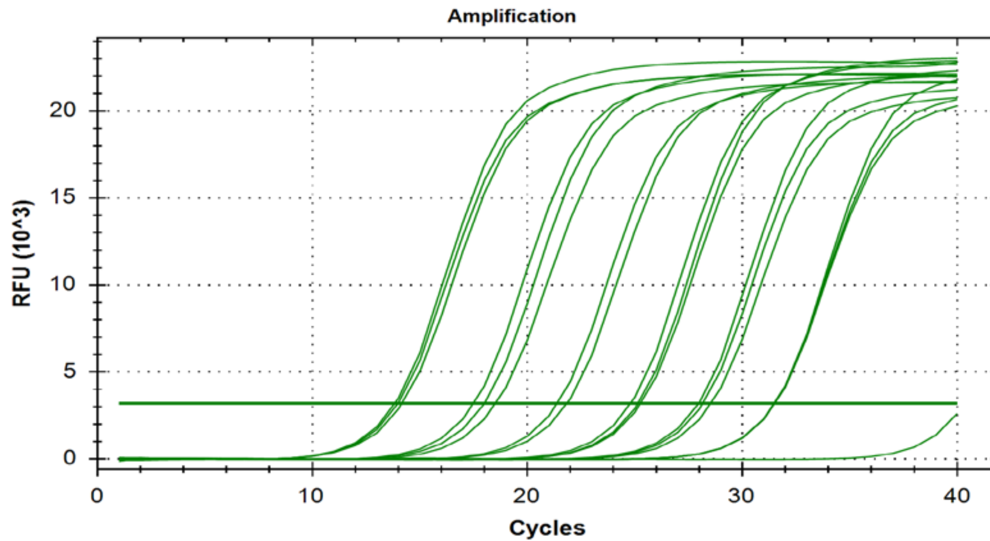
Appendix D.4 Amplification of AOA standards only. In general, the standards' height resulted all in singleplex and Cq values remained constant with the set of triplicates of the AOA gene-reference and were lower than 40 and greater than 15. Thus, the reproducibility of this analysis was high enough to accept the efficiency r value from the targeted-gene of the samples from the nitrification and denitrification process.



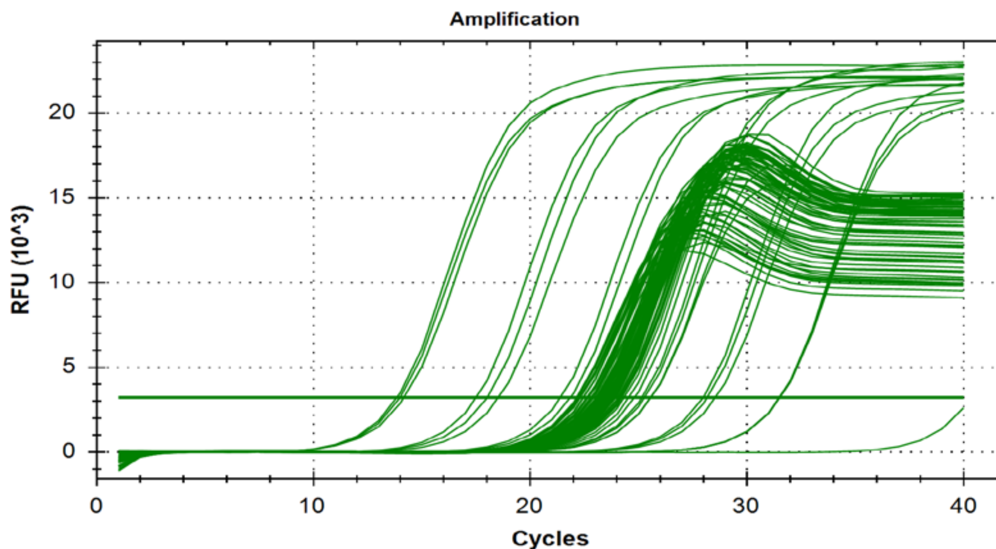
Appendix D.5 Amplification of AOA standards with targeted genes from nitrification and denitrification samples. Cq values remained constant with the set of triplicates of the AOA gene-reference previously assessed and were lower than 40, but greater than 15 cycles. all targeted genes started revealing an exponential stage at around 20 cycles around 4 x 10^{-3} RFU (relative fluorescent units). This revealed a good efficiency overall.



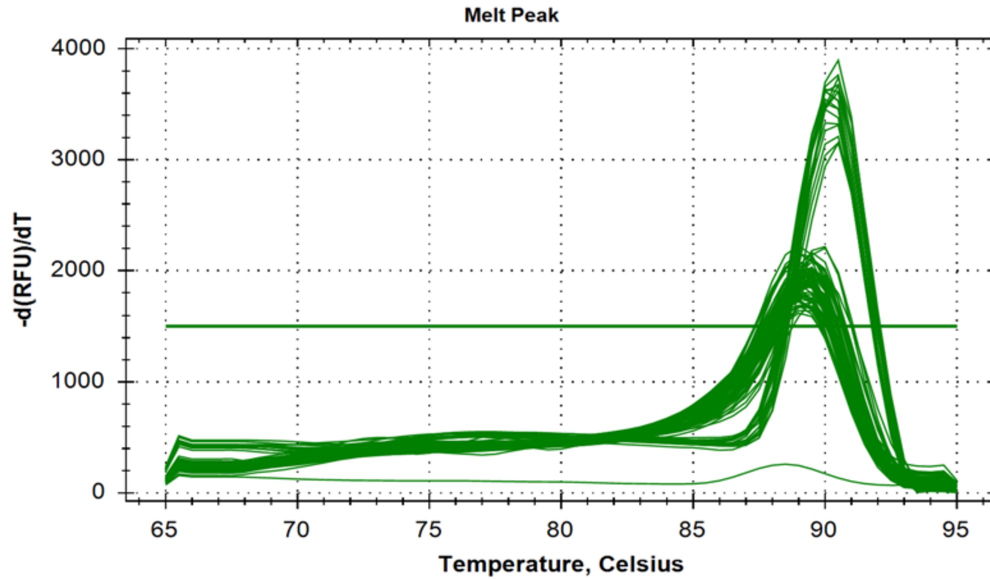
Appendix D.6 Melt peak of AOA targeted-genes and reference-genes (samples) generated a single peak for all the samples that belong to the nitrification and denitrification processes.



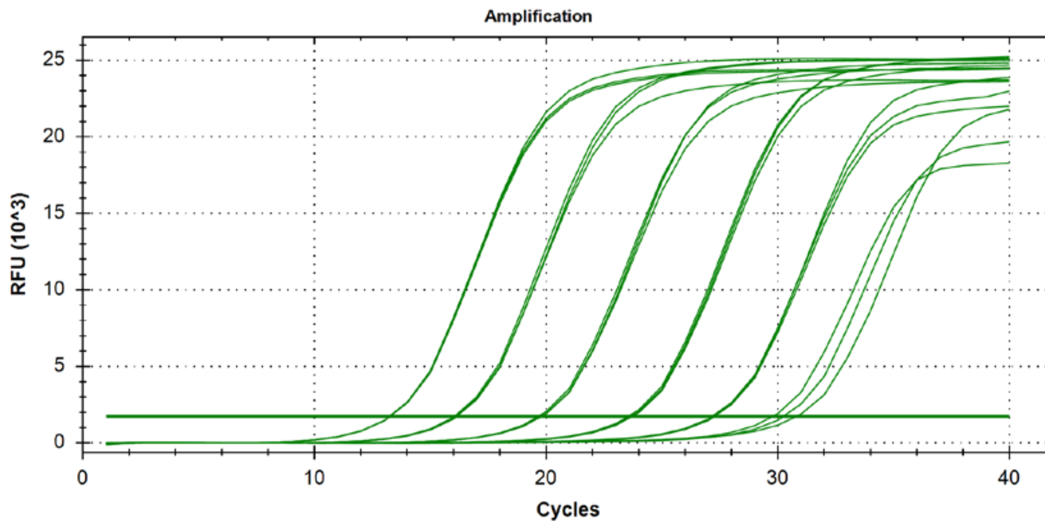
Appendix D.7 Amplification of *nirK* bacteria standards only. In general, the standards' height resulted all in singleplex and Cq values remained constant with the set of triplicates of the *nirK* bacteria gene-reference and were lower than 40 and greater than 15. Thus, the reproducibility of this analysis was high enough to accept the efficiency r value from the targeted-gene of the samples from the nitrification and denitrification process.



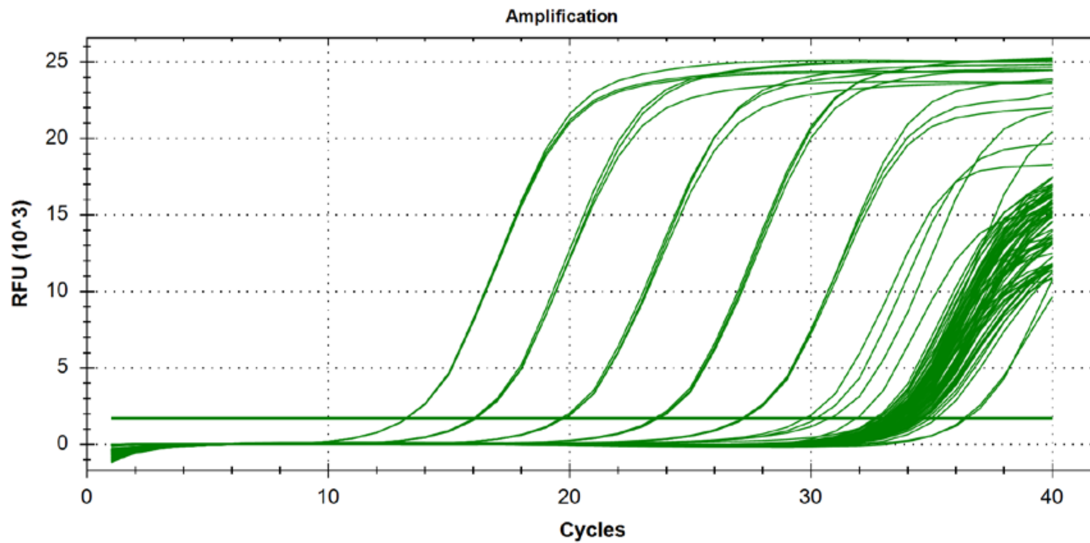
Appendix D.8 Amplification of *nirK* bacteria standards with targeted genes from nitrification and denitrification samples. Cq values remained constant with the set of triplicates of the nirK bacteria gene-reference previously assessed and were lower than 40, but greater than 15 cycles. all targeted genes started revealing an exponential stage at around 20 cycles around 3.5 x 10^{-3} RFU (relative fluorescent units). This revealed a good efficiency overall.



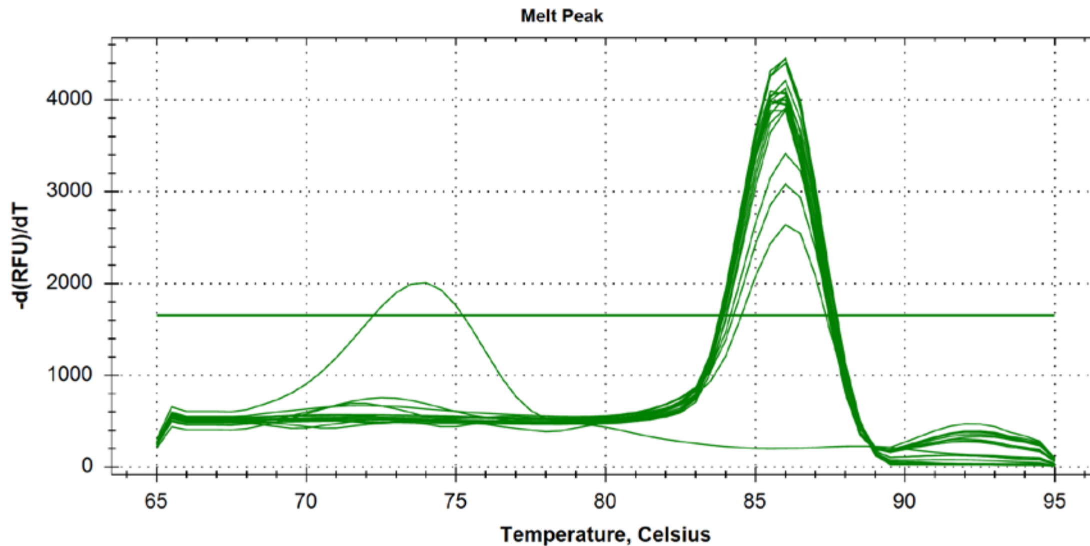
Appendix D.9 Melt peak of *nirK* bacteria targeted-genes and reference-genes (samples) generated a single peak for 99.9% of all samples that belong to the nitrification and denitrification processes. The second amplicon shown between 73-77 ºC belongs to a contaminant present in one of the samples, a primer dimer or non-specific amplification (Lori, P., personal communication, February 2017).



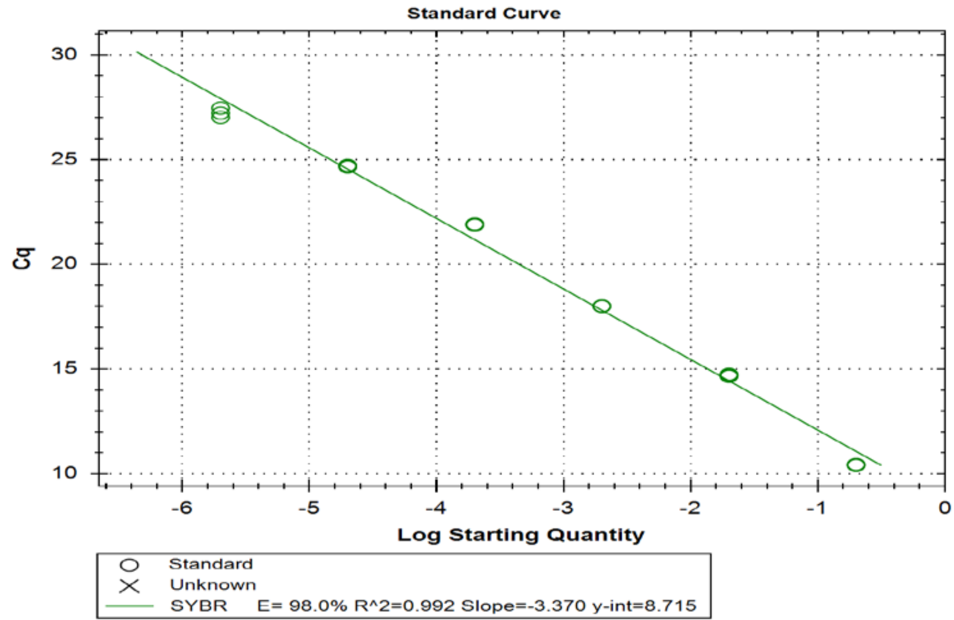
Appendix D.10 Amplification of *nirK* archaea standards only. In general, the standards' height resulted all in singleplex and Cq values remained constant with the set of triplicates of the *nirK*-archaea gene-reference and were lower than 40 and greater than 15. Thus, the reproducibility of this analysis was high enough to accept the efficiency r value from the targeted-gene of the samples from the nitrification and denitrification process.



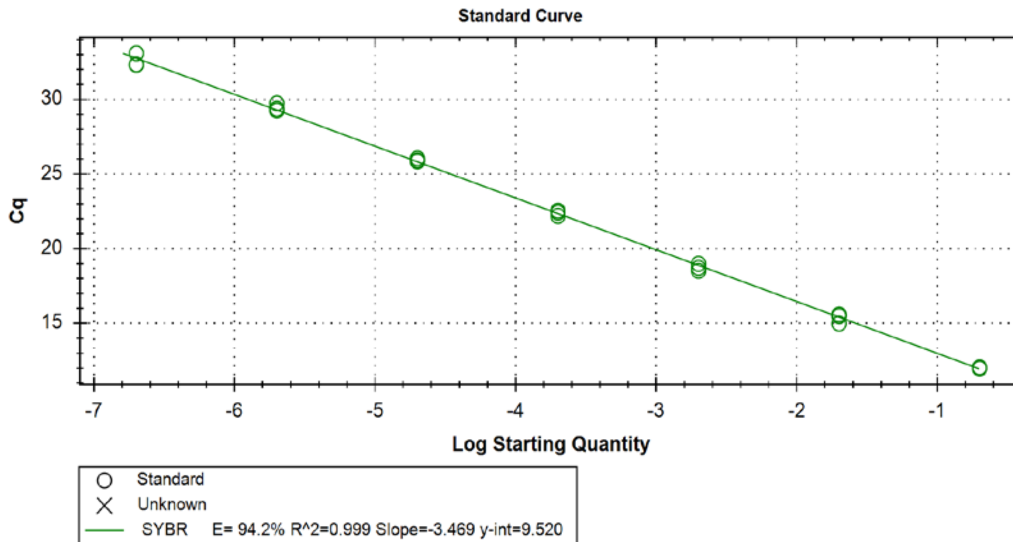
Appendix D.11 Amplification of *nirK* archaea standards with targeted genes from nitrification and denitrification samples. Cq values remained constant with the set of triplicates of the *nirK* archaea gene-reference previously assessed and were still lower than 40 and greater than 15 cycles. all targeted genes started revealing an exponential stage at around 20 cycles around 2 x 10^{-3} RFU (relative fluorescent units). This revealed a good efficiency overall.



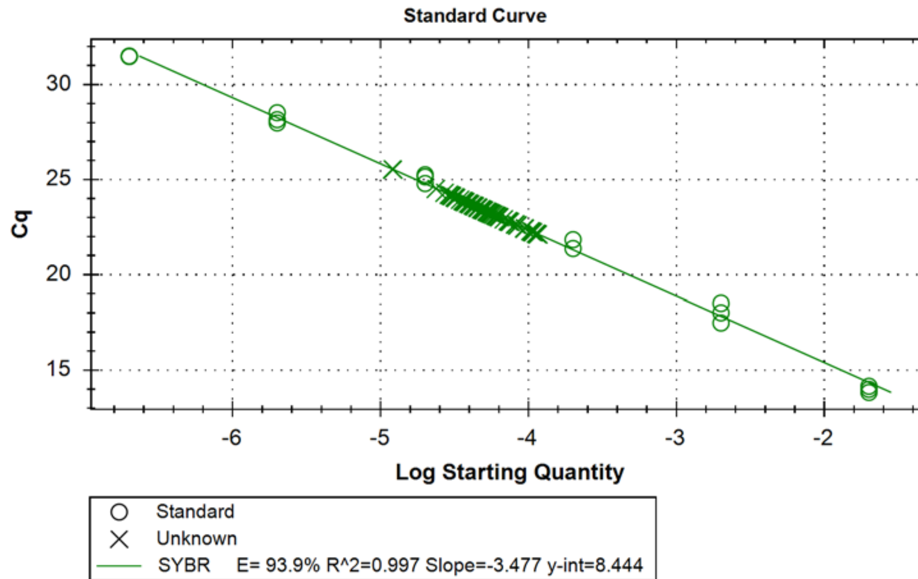
Appendix D.12 Melt peak of *nirK* archaea targeted-genes and reference-genes (samples) generated few single peaks from samples that belong to the nitrification and denitrification processes. This graph shows that the targeted-gene *nirK* archaea was not dominant in all soil samples. The second amplicon shown between 73-77 ºC belongs to a contaminant present in one of the samples, a primer dimer or non-specific amplification (Lori, P., personal communication, February 2017).



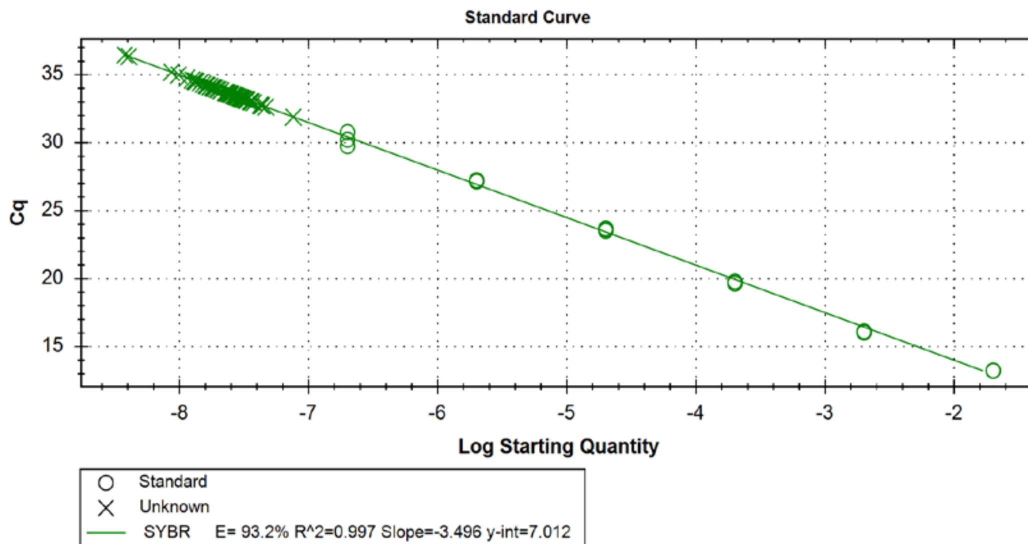
Appendix D.13 Cq values revealed a high coefficient value (r^2=0.992) with an efficient value within accepted range limits in AOB gene abundance; this slope also resulted in a negative slope (-3.37). The efficiency value tells us that 98% of the targeted genes were amplified (Svec et al., 2015).



Appendix D.14 Cq values revealed a high coefficient value (r^2=0.999) with an efficient value within accepted range limits in AOA gene abundances; this slope also resulted in a negative slope (-3.5). The efficiency value tells us that 94.2% of the targeted genes were amplified (Svec et al., 2015).



Appendix D.15 Cq values revealed a high coefficient value (r^2=0.997) with an efficient value within accepted range limits in nirK bacteria; this slope also resulted in a negative slope (-3.47). The targeted-genes resulted between 21 to 24 cycles. The efficiency value tells us that 93.9% of the targeted genes were amplified (Svec et al., 2015).



Appendix D.16 Cq values revealed a high coefficient value (r^2=0.997) with an efficient value within accepted range limits in *nirK* archaea; this slope also resulted in a negative slope (-3.49). Targeted-genes were mostly amplified between 30 to 35 cycles. The efficiency value tells us that 93.2% of the targeted genes were amplified (Svec et al., 2015).