Impact of Nitrogen Stabilizer on Nitrogen Cycling, Nitrifying Organisms, and Winter Wheat Yield and Quality in High Rainfall Zones of Northern Idaho

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Abstract

Nitrogen loss to the environment has been a constant problem for growers and timing of nitrogen (N)fertilizer application can be challenging due to seasonal precipitation in northern Idaho. Nitrogen fertilizer is especially vulnerable to loss through leaching, runoff and volatilization when applied in the fall. Prevention of nitrogen loss through the winter is crucial to supply winter wheat (*Triticum aestivum* L.) planted in the fall with sufficient nutrients. One solution to minimize nitrogen loss is the use of nitrogen stabilizers with the application of nitrogen fertilizers. The stabilizers slow down nitrate (NO_3^-) leaching by inhibiting the process of nitrification by nitrifying bacteria and archaea. However, improved crop performance after the application of nitrogen stabilizers has been variable, and little work has been done in the Pacific Northwest. Additionally, there is uncertainty in how nitrification inhibitors might influence nitrifying organisms. Therefore, experimental plots were established in Cottonwood and Cavendish, Idaho during the 2019-2020 and 2020-2021 growing seasons to test the efficacy of nitrogen stabilizers in the high rainfall zone of northern Idaho. Two wheat cultivars soft white winter wheat LCS Hulk and the hard red winter wheat LSC Jet were studied in separate trials with five urea ammonium nitrate (UAN, 32-0-0) fertilizer rates (0, 56, 112, 168, and 224 kg/ha) applied with and without the nitrification inhibitor Instinct® II (Corteva Agriscience, USA). Soil samples were taken at four dates during the growing season to monitor ammonium and nitrate soil concentrations: November after planting, March, May, and September after harvest. Agronomic measurements that were taken include yield and postharvest quality as well as monitoring populations of bacteria and archaea and soil nitrate and ammonium. Soil samples collected from both locations indicated that the nitrogen stabilizer helped to retain ammonium in the soil and decreased the concentration of nitrate in the top 15 cm and 30 cm of soil, with differences being most obvious in November and March. Most agronomic measurements were not influenced by nitrogen stabilizer application. There as not a significant impact on yield. For only the hard red winter wheat at Cavendish there was a small, but significant decrease in test weight and increase in grain protein in plots treated with Instinct® II compared to plots without Instinct® II. Nitrifying archaeal populations did not respond to nitrogen stabilizer treatment, nor were they impacted by nitrogen fertilizer rate. However, bacterial populations were significantly decreased in the Instinct® II treated soils in the spring. Populations of ammonia-oxidizing bacteria did significantly increase with

increasing nitrogen fertilizer rates. The results from this research help to further understand nitrogen stabilizer efficacy on winter wheat in rainfed areas of Idaho as well as its effect on nitrifying microorganisms' populations. This information will aid decision making for growers looking to improve their nitrogen use efficiency and crop quality while not harming their soil health.

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Dedication

To my mom and dad for always being proud of every step I take in life. Dad, thank you for placing the love of agriculture in my heart. Thank you, mom, for being there for me when me I am at my lowest. You both have been the best role models and parents. I am the luckiest daughter in the world.

To my love, my husband, who has been with me from the start. Thank you for being there at the best times and the worst. Another adventure checked off in the book of life that we share.

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Chapter 1: Literature Review

1.1 Origin and Evolution of Wheat

Scientists and historians have dated the first cultivation of wheat to about 10,000 years ago as part of the 'Neolithic Revolution' or the transition from hunting and gathering to agriculture (Weisdorf, 2005). The earlier cultivars of wheat were homozygous diploid "einkorn" with the genotype AA and homozygous tetraploid "emmer" inheriting two of the same alleles from the parents, AABB. The cultivars that are most commonly grown today are genotype hexaploid AABBDD, which was inbred to receive three of the same alleles from the parents. The process which gave rise to the tetraploid and hexaploid variants that we grow today is called amphidiploidy, which give rise to a hybrid that has a complete diploid set of chromosomes from each parent. The genetic relationships of the wild cultivars led historians to the origin of wheat in the southeastern part of Turkey (Heun et al., 1997; Nesbitt, 1998; Dubcovsky and Dvorak, 2007; Özkan et al., 2010; Pourkheirandish et al., 2018.) During the past 10,000 years, wheat evolved from landraces which were wild types of hand selected wheat by men on behalf of their superior yield and other characteristics. Two main traits of the wild-type wheat were lost due to selection which were shattering of the spike at maturity and hulled grains to free-threshing grains (Jantasuriyarat et al., 2004; Nalam et al., 2006). Einkorn and emmer wheat have been domesticated through selection from natural populations, however hexaploid wheat has evolved from the hybridization of cultivated emmer and the unrelated grass *Triticum tauschii* (Shrewry, 2009).

The spread of wheat from its site of origin across the world was described by Feldman in 2001. From its origin in the southeastern part of Turkey, wheat was domesticated in Turkey, Iraq and Iran then introduced throughout Europe, starting in 8,000 BCE. Wheat was moved

northward through the Balkans to Danube and across to Italy, France, and Spain in 7,000 BCE and finally reached the UK and Scandinavia about 5,000 BCE. About 2,000 years later, it made its route to central Asia reaching China and to Africa via Egypt. It was introduced by the Spaniards to Mexico in 1529 AD and Australia in 1788 AD (Feldman, 2001).

1.2 The Importance of Wheat

Wheat is grown in nearly every region of the world and is the main source of income and food for millions of people around the world. In 2020, China and India were the top two wheat producing countries growing over 135 million metric tons, 29% of the world's total, and 103 million metric tons, respectively (Mala, 2020). For developed countries, wheat is a large part of the diet being a major ingredient in doughs that are processed into a variety of foods such as bread, pasta and other bakery products that are valued in modern life (Peña et al., 2002).

For the United States, wheat ranks third among the field crops in planted acreage behind corn and soybeans. In the 2021, the US produced 435.5 million metric tons of winter, spring, and durum wheat on 15.1 million ha (USDA-ERS). For the 2020 growing season, winter wheat represented 70 to 80% of total US production with 32 million metric tons grown on 9.3 million hectares (USDA-NASS, 2021).

Idaho is one of the few places in the world where buyers can find several different classes of wheat in one place. The classes of wheat that are grown in Idaho are hard red winter (HRW), hard red spring (HRS), soft white (SW), durum and hard white (HW). Idaho typically ranks 7th in the nation for wheat production with an average of 486 thousand hectares grown each year (idahowheat.org). Approximately 50% of wheat grown in Idaho is sold to foreign markets such as Indonesia, Japan, Philippines, South Korea, Taiwan, and Yemen (idahowheat.org). For Idaho, wheat is the second largest crop by revenue bringing in an estimated value of 525

million dollars in 2020 with 458 thousand hectares of wheat harvested and an average yield of 4,546 kg/ha (USDA-NASS, 2020; Ellis, 2021).

For USDA reporting purposes, the state is divided into four regions: north, southwest, south central, and east Idaho. In 2019, northern Idaho planted 128,002 hectares of winter wheat and 37,190 hectares of spring wheat which yielded an average of 5,350 kg/ha with 623,700 metric tons and 166,590 metric tons total production, respectively (USDA-NASS, 2019). Southwest Idaho planted a total of 23,836 hectares of winter wheat that yielded 8,914 kg/ha with a total production of 188,865 metric tons. South central Idaho planted 34,115 hectares of winter wheat that yielded an average of 7,473 kg/ha for a total production of 237,600 metric tons. USDA did not report data for spring wheat in southwest and south-central Idaho. Finally, eastern Idaho planted 109,467 hectares of winter wheat and 124,036 hectares of spring wheat which yielded 5,826 and 6,075 kg/ha on average with a total production of 545,805 metric tons and 715,014 metric tons respectively (USDA-NASS, 2021).

Wheat contributes a substantial amount of dietary nutrients which are essential or beneficial for the humans. In particular, it contributes carbohydrates, proteins, vitamins, minerals, phytochemicals, and dietary fiber (Carbohydrates, 2015). Studies have also shown a well-established relationship between the consumption of cereals and reduced risk of cardio-vascular disease, type 2 diabetes, and many forms of cancer (Aune, 2016). A global estimation claims that cereal consumption provides 45% of protein and energy necessary for the human diet (Table 1.1) (Rosell, 2011). Essential amino acids not produced by animals such as isoleucine, leucine, phenylalanine, tyrosine, threonine, tryptophan, valine, histidine, and methionine are present in wheat in adequate amounts for adults (Shewry and Hey, 2015).

Another main source of nutrition from wheat is carbohydrates which consist of 85% of the grain and is the predominant source of carbs in the human diet (Shewry and Hey, 2015).

Table 1.1. Contribution of cereals to the daily food intake (FAO, 2007).

	Food Consumption (kg/Capita/Year)	Food Consumption (kcal/Capita/Day)	Protein Consumption (g/Capita/Day)	Fat Consumption (g/Capita/Day)
Total		2808.87	75.72	79.63
Cereals	151.07	1302.75	31.62	5.49
Wheat	67.00	518.00	15.34	2.18
Milled rice	54.21	541.92	10.07	1.28
Barley	1.13	8.04	0.23	0.03
Maize	18.54	152.72	3.66	1.22
Rye	0.98	7.42	0.20	0.03
Oats	0.52	2.94	0.12	0.05
Millet	4.05	33.26	0.89	0.35
Sorghum	3.90	32.72	0.97	0.33
Other cereals	0.74	5.73	0.16	0.02

In developing countries, wheat plays a key role in the daily food requirement and calories that are needed (Hassan et al., 2021). Because of the importance of wheat in developing countries, wheat varieties are being bred to combat zinc and iron deficiencies (Yip and Ramakrishnant, 2002; Hassan et al., 2021). One concern for wheat today is that as our world population increases, the demand for wheat also increases. According to an article written in 2009, population will climb to more than 9 billion people by 2050 which will require >70% increase in agricultural production (Bruinsma, 2009; Roser et al., 2013). Since we have no more extra land to farm, experts say that wheat is required to achievea 1.2% yield gain per year to satisfy increasing population demand (Strugnell, 2018).

1.3 Cropping Systems in the Pacific Northwest

The Pacific Northwest (PNW) is a large wheat production region with both irrigated and dryland cropping systems. In the dryland areas, which is the cropping system involved in this thesis, wheat is grown in rotation with small grains, legumes, canola, other alternative crops, and fallow. The landscape is variable starting with the glacial deposits, coulees, and scab lands in the Columbia Basin and Plateau to rolling hills with deep, fertile soil in the prairies of eastern Washington and northern Idaho. The area has a Mediterranean-like climate with

most of the precipitation falling during winter and spring months with hot, dry summers (Yorgey and Kruger, 2017). In the past, the common cultivation practice for the PNW was intensive tillage. However, many growers in the regions have adopted reduced tillage or direct seeding to reduce soil erosion and improve efficiency (Bista et al., 2017).

1.4 Use of Nitrogen Fertilizers

Nitrogen as a nutrient is essential for all living organisms from plants to humans and is in high demand. According to NASA (2018), nitrogen accounts for 78% of our atmosphere, however its lack of availability in the terrestrial ecosystem and agroecosystems causes it to be a large limiting factor (Ussiri and Lal, 2018). In fact, without the invention of reactive nitrogen fertilizers for agriculture to ensure food security for our growing population, modern society would not exist. Nitrogen is used in many different agricultural systems such as animal and plant production. In 2015, 11,802,000 metric tons of nitrogen fertilizer were applied in the United States alone (USDA-NASS, 2019) with an average price of $1.18 per kilogram of nitrogen between the years of 2008 to 2016 (Schnitkey, 2016). Growers in the United States spent an average of 13.9 billion dollars on nitrogen fertilizers annually.

Nitrogen is such an important nutrient because it is a component in many compounds and cycles needed to support life. For plants, nitrogen is a component of protein which is an essential compound for catalyzing chemical responses and transportation of electrons (Leghari et al, 2016). Nitrogen is also an important component of chlorophyll which is responsible for the process of photosynthesis (Leghari et al, 2016). Importantly, nitrogen plays a role in stimulating the uptake of other essential nutrients such as potassium and phosphorus (Bloom, 2015). For wheat, which is the focus of this thesis, nitrogen is critical to attain a high protein content which is strictly measured in hard red wheat for better quality end products. On the

other hand, if the protein content is too high in soft white wheat, the end product will be lower in quality.

1.5 Nitrogen Cycling

The nitrogen cycle is a complex series of biogeochemical processes that control the amount and form of nitrogen available for life. In agriculture, nitrogen is the major limiting factor in low-input systems such as dryland cereal farming (Dawson et al., 2008). For this reason, understanding the nitrogen cycle is important to increase crop yield and profitability. The processes in the nitrogen cycle are usually split into input/output or processes that create and reduce fixed nitrogen. The main nitrogen transformations involved in agriculture, that will be discussed in this thesis, are nitrogen fixation, nitrogen assimilation, nitrification, denitrification, and anammox.

1.5.1 *Nitrogen fixation* is carried out by biological prokaryotes called "diazotrophs," or "N_2-fixers," who reduce dinitrogen gas (N_2) to ammonia (NH_3) by the enzyme nitrogenase to fill their anabolic needs and is thought to be one of the most ancient enzyme-catalyzed reactions (Raymond et al., 2004; Zhang et al., 2020). Bioinformatics analysis of sequenced genomes has found that approximately 15% of prokaryotic species are known or could potentially fix nitrogen (Dos Santos et al., 2012). Most belong to the phylum *Proteobacteria* (Dos Santos et al., 2012) and a few are the symbiotic N_2-fixing bacteria of legumes (*Rhizobium*). This symbiotic relationship occurs under nitrogen limited conditions in which legume roots secrete a signal that is recognized by *Rhizobium* bacteria. The *Rhizobium* then use photosynthetic products made by the leguminous plant in exchange for reduced nitrogen from the atmosphere (Bergersen, 1982). For this reason, crops such as alfalfa, peas, lentils,

chickpeas, and other leguminous plants typically do not need to be amended with nitrogen fertilizer.

1.5.2 *Nitrogen assimilation*

Nitrogen assimilation is the uptake of nitrogen by plants, phytoplankton, fungi, and microbes is another process of the nitrogen cycle. Energy free ammonia assimilation into these organisms involves ammonia diffusion or transport into the cell then incorporated into amino acids by the enzymes glutamine synthetase and glutamate synthase (Xu et al., 2012). Nitrate assimilation, in contrast, requires metabolic energy because the organisms must reduce nitrate to ammonia by nitrate and nitrite reductases. Mineralization is the return of that accumulated/derived organic nitrogen back to the environment as inorganic ammonium (NH_4^+) through the means of excretion by organisms or microbial degradation of organic matter. During mineralization, particulate organic nitrogen (PON) is broken down to dissolved organic nitrogen (DON) at the C-C bonds by physical disintegration, solubilization, and breakdown (Schlesinger and Bernhardt, 2013). Deamination of protein and nucleotide macromolecules follows resulting in the release of inorganic ammonium into the environment (Zhang et al., 2020). Mineralization is very important in the nitrogen cycle to return available inorganic ammonium to the soil from unavailable nitrogen in organic materials.

1.5.3 *Nitrification*

Nitrification is the process where chemoautotropic nitrifying microbes, ammonia-oxidizing bacteria (AOB) or ammonia-oxidizing archaea (AOA), reduce ammonium or NH_3 into nitrite which is transformed by nitrite-oxidizing bacteria (NOB) into nitrate (Ward, 2018). The first and irreversible step of nitrification is the oxidation of ammonia/ammonium to hydroxylamine (NH_2OH) by the membrane bound ammonia monooxygenase (AMO) found in *Nitrosomonas* sp. and *Nitrosospira* sp (Wendeborn, 2020). Hydroxylamine oxidoreductase then catalyzes the oxidation of hydroxylamine to produce NO_2^- (Wendeborn, 2020) (Figure 1.1).

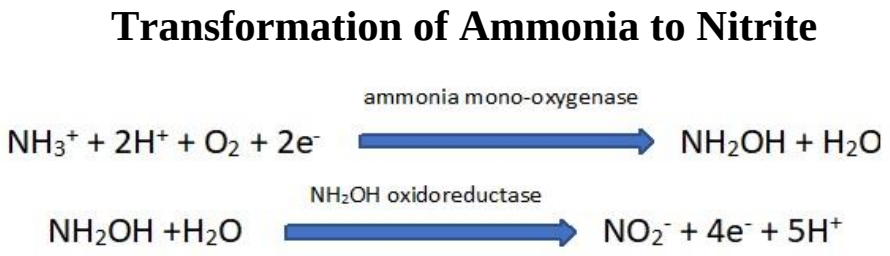

Figure 1.1. Transformation of ammonia to hydroxylamine by ammonia mono-oxygenase enzyme then to nitrite by hydroxylamine oxidoreductase.

The second step of nitrification is the oxidation of nitrite to nitrate by the NOB *Nitrobacter* sp. and *Nitrospira* sp. Like ammonia oxidation, nitrite oxidation is membrane bound and catalyzed by the enzyme nitrite oxidoreductase. Unlike ammonia oxidation, this reaction can be reversed to result in the reduction of nitrate to nitrite. Eighty percent of the energy produced by this reaction is used to fix carbon for growth (Robertson and Groffman, 2007). These nitrifiers, AOB and NOB, are not phylogenetically related and none of these organisms can oxidize both substrates (Arp and Bottomley, 2006). However, a study conducted in 2015 by Daims et al., found a bacterium (Comammox) from the genus *Nitrospira* that can perform both ammonia and nitrite oxidation. The separation of nitrification has always puzzled

microbiologists and this finding changes the picture of nitrification and key components of the nitrogen cycle (Diams et al., 2015).

1.5.4 *Denitrification*

Denitrification is a biological process by which nitrate, and nitrite are reduced to a gaseous form. This process can dominate nitrogen loss through the conversion of nitrate to N_2 which is gassed into the atmosphere (Zumft, 1997). Denitrifying organisms are mostly anaerobic, heterotrophic bacteria that use nitrate, in limited oxygen conditions, as an election acceptor during respiration. Denitrifiers that can completely reduce nitrate to N_2 use a sequence of intermediate reductions that include nitrate to nitrite, then to nitric oxide NO, nitrous oxide N_2O, which ends in N_2 (Zumft, 1997).

Respiratory nitrate reduction mainly occurs via the nitrate reductase in denitrifying bacteria (Granger et al., 2008). Copper nitrite reductases carry out the reduction of NO_2^- to NO and are primarily found in denitrifying bacteria (Horrell et al., 2017). Under most anaerobic conditions, bacteria produce N_2O from nitric oxide during the process of denitrification (Takaya et al., 2003). Three NO-reducing enzymes been reported in bacteria which reduce to N_2O (Zumft, 2005; Field et al, 2008). The last reduced form of nitrogen in the sequence is nitrous oxide (N_2O) whose reduction reaction is catalyzed by the respiratory copper enzyme nitrous oxide reductase ending the denitrification pathway (Zumft and Kroneck, 2007; Zhang et al, 2020).

1.5.5 *Anammox*

Anammox is the anaerobic oxidation of ammonium in which NH_4^+ is oxidized to N_2 gas with NO_2^- as the electron acceptor. The phylum *Planctomycetes* mediates this reaction with three enzymatic reactions. The first reaction is the reduction of NO_2^- to nitric oxide (NO) then the hydrazine biosynthesis from NO and NH_4^+. The final reaction is the dehydration of hydrazine to N_2 gas (Oshiki et al., 2016).

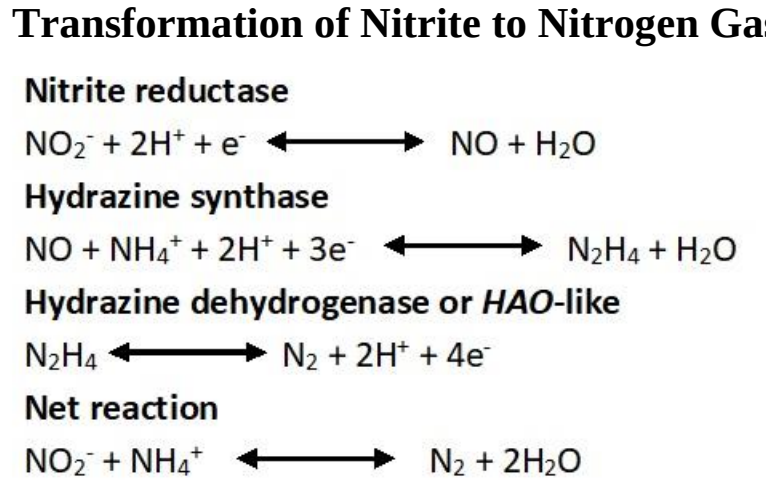

Figure 1.2. Transformation of nitrite to nitrogen gas by the enzymes nitrite reductase, hydrazine synthase, and hydrazine dehydrogenase.

Anerobic oxidation of ammonium plays a significant role in the loss of nitrogen in agricultural soils and is a fairly novel nitrogen removal pathway (Nie et al., 2019). A high input of nitrogen fertilizers has been reported to stimulate the growth of anommox bacteria resulting in greater losses of nitrogen from the soil (Humbert et al., 2010). The factors that favor anommox in agricultural soils are increased inorganic nitrogen (Hu et al., 2011), decreased dissolved oxygen concentrations (Strous et al., 1997), neutral to alkaline pH (Mulder et al., 1995; Jetten et al., 2001; Yamamoto et al., 2008), high salinity (Wang and Gu, 2013; Bai et al., 2015), and root exudates such as organic acids that provide supplementary carbon (Guven et al., 2005).

1.6 Nitrogen Pollution and Environmental Issues

Nitrogen pollution has increased dramatically after the introduction of inorganic, reactive fertilizers, and the use of combustible fossil fuels. Reactive nitrogen for fertilizer use is one of the main causes of global climate change especially in the coastal oceans of the world (Galloway et al., 2004).

Nitrate leaching is a hydrological pathway in which nitrogen is lost. It is considered to be the dominant cause of rising nitrate concentrations in groundwater, and directly responsible for ecosystem eutrophication and water quality degradation (Rivett et al., 2008; Robertson and Vitousek, 2009; Zhou et al., 2012; Sebilo et al., 2013). Under the approach of using a fertilizer-induced emission factor (EF), the Intergovernmental Panel on Climate Change (IPCC) estimated global losses of nitrate from fertilized agricultural systems (IPCC, 2006). Using this method, they found that 30% of nitrogen fertilizer is lost to leaching. A metanalysis produced in 2019 stated that soil characteristics such as soil organic carbon and total nitrogen increased nitrate leaching when these factors were high (Wang et al., 2019). Many other aspects in agriculture such as fertilizer type, application timing, crop type, and other soil characteristics can influence nitrate leaching just to name a few. The same metanalysis found that across different environments and cropping systems, nitrate leaching increases with increasing nitrogen application rate (Wang et al., 2019).

Soil acidification is another significant environmental issue resulting from nitrogen fertilizers. Nitrification of ammonium-based nitrogen fertilizers releases H^+ ions into the soil, lowering soil pH. Studies have shown that soil acidification influences crop production by decreasing wheat yields with increasing acidity. In soils with pH less than 5.5, the solubility of aluminum and manganese increases causing toxicity in winter wheat (Ernani et al., 2002; Koenig et al., 2011). Low water uptake and nutrient availability, specifically N, P, Mg, and

Mo, is also linked to low pH, negatively influencing profitability and crop production (Kemmitt et al., 2006; Kariuki et al., 2007). Additionally, soil OM decomposition rate is adversely affected by acidic soils and can impact long-term soil organic carbon and nutrient dynamics (Ghimire et al, 2017).

Nitrous oxide (N_2O), created from microbial nitrification and denitrification, is the third most important greenhouse gas and ~300 times more potent than CO_2 (Prather et al., 2001; Stocker, 2014). Nitrous oxide has a 114-year lifetime in atmosphere and has increased from ~10 Tg N/yr before the industrial era to 17.9 Tg/yr in the period from 2010 to 2015. In particular, agricultural related emissions account for 60 to 80% of nitrogen atmospheric pollution (Davidson, 2009; Coskun et al., 2017). In terms of air quality, ammonium nitrate has been found to be an important contributor to particulate matter in polluted regions. This particulate matter can cause an increase in respiratory irritation, cardiovascular disease, and premature death (Anderson et al., 2012; Kuprov et al., 2014).

Both soil microbes and terrestrial plants compromise a large portion of the genetic diversity on Earth and influence many ecosystems processes. Microbes especially have an important role to play in the cycles and processes of the environment and are important in the preservation of plant diversity. Unfortunately, a few metanalyses analyzed the effect of increased nitrogen inputs on microbes and showed a reduction in total microbial biomass (Liu and Greaver, 2010: Lu et al., 2011). Increased nitrogen addition also decreased the fungi to bacteria ratio and the abundance of arbuscular mycorrhizal fungi (Zhang et al., 2018).

In respect to terrestrial plant diversity, nitrogen deposition has certainly changed the species composition in a negative way. Nitrogen accumulation has driven competition and made conditions unfavorable for some species. Nitrogen deposition has caused adverse effects

such as direct toxicity of nitrogen gas, change in species interaction, long-term physiological damage, soil acidification, and increased susceptibility to secondary stress (Ulrich, 1983; Van Breemen et al., 1982; Pearson and Stewart, 1993; Roelofs et al., 1996; Bobbink et al., 1998; Bobbink et al., 2003; De Vries et al., 2003; Kleijn et al., 2008).

1.7 Nitrifying Bacteria and Archaea

In agricultural systems, typically only about 50% of nitrogen fertilizer applied is taken up by the crop and the rest is lost to terrestrial and atmospheric systems (Schepers et al., 2008; Cavigelli et al., 2012; Omara et al., 2019). One way that nitrogen is lost is by nitrification. As explained above is 1.5 *Nitrogen Cycling*, nitrification is process by which gram-negative autotrophic ammonia oxidizing bacteria (AOB) and archaea (AOA) reduced ammonium to nitrite then later to nitrate by nitrite oxidizing bacteria (NOB). The AOB and AOA responsible for oxidation of ammonium in autotrophic soils are *Nitrosomonas* sp. and *Nitrosospira* sp. (AOB) and *Nitrosospheara* sp. (AOA) and the bacteria responsible for nitrite oxidation are *Nitrobacter* sp. and *Nitrospira* sp. (Norton et al., 2008). The bacterial and archaeal organisms gain energy from nitrification reactions and are considered chemolithoautotrophs. These chemolithoautotrophic organisms are energetically dependent on oxidizing ammonia/ammonium and nitrite for their growth (Sedlacek et al., 2016). For this reason, the rate and extent of the nitrification process is greatly linked to the abundance and function of these microbes (Norton and Ouyang, 2016).

Not only can these organisms be found in the soil, the species *Nitrosomonas europaea*, *N. eutropha* and *N. nitrosa* and sequences of other *Nitrosomonas* lineages have been obtained from eutrophic waters, and the *Nitrosomonas marina* lineage have been detected in freshwater environments. In soil however, *Nitrosospira* lineages dominate, particularly in acid soils (Li et

al., 2018). However, because nitrification is limited by the oxidation of NH_3 or NH_4^+, NO_2^- rarely accumulates. In consequence, NO_2^- oxidizers have been studied less frequently and only a few 16S rRNA gene clone sequences are available (Bothe et al, 2007).

Less is known about the ammonia-oxidizing archaea responsible for nitrification in agricultural soils. In 2008 the new phylum Thaumarchaeota was proposed for mesophilic Crenarchaeota (Brochier-Armanet et al., 2008). The phylum Thaumarchaeota, has a ubiquitous distribution and is composed of ammonia-oxidizing archaea. A new species in the phylum, *Nitrososphaera viennensis,* was isolated from a garden soil in Vienna and was described in 2014 by Stieglmeier et al. The closest cultivated relative, based on 16S rRNA gene sequence identity, is '*Candidatus* Nitrosopumilus maritimus,' which is a marine ammonia-oxidizing archaeon (Brochier-Armanet et al., 2008). Additionally, quantitative analyses of the *amoA* gene have shown that AOAs are ubiquitous and largely outnumber AOB in diverse environments (Schuster et al., 2006; Wuchter et al. 2006; De Corte et al. 2009; Abell et al. 2010; Wankel et al. 2011; Santoro 2016).

Both AOA and AOB have an enzyme that characterizes them as ammonia oxidizing organism. This enzyme is ammonia monooxygenase (AMO) which is responsible for catalyzing the reaction of NH_3 or NH_4^+ to NO_2^-. The *amoA* gene encodes the active subunit of the AMO enzyme which is shared between the three groups of ammonia oxidizers, AOB, AOA and microorganisms that perform comammox, the complete oxidation of ammonium to nitrate (Lehtovirta-Morley, 2018). The dual process of completing both ammonium and nitrite oxidation was only demonstrated in 2015 (van Kessel et al., 2015; Daims et al., 2015; Pinto et al., 2016) with the microorganism *Nitrospira* which is the most diverse functional group of the NOB (Koch et al., 2018). Both *Nitrobacter* and *Nitrospira* contain the gene coding for NO_2^-

oxidizing enzyme which catalyzes electrons to feed into the electron transport chain and reduce O_2 to water. An H^+-electrochemical gradient across the cytoplasmic membrane is formed and NO_3^- is produced (Bothe et al, 2007).

The main factors controlling the rate of nitrification by nitrifying bacteria in soils are the availability of NH_4^+/NH_3, NO_2^- and O_2 (Grant, 1994; Bouskill et al., 2012; Nowka et al., 2015; Venterea et al., 2015; Ouyang et al., 2018), environmental conditions (Stark and Firestone, 1995; Stark, 1996; Parton et al., 2001), the population of nitrifiers and competitors (Bertagnolli et al., 2016; Han et al., 2018), and the presence of nitrification inhibitors (Subbarao et al., 2013; Coskun et al., 2017). These factors can act directly at the cell level and indirectly by affecting the soil habitat nitrifying organisms. In addition, the timescale of these factors can span from immediate change in rate of nitrification to years and decades (Norton and Ouyang, 2019). Many of these interdependent factors affecting nitrogen rates significantly interact with each other and often confound experiments making the determination of roles difficult (Stark and Firestone, 1995; Placella and Firestone, 2013).

1.8 Nitrogen Stabilizers

Nitrogen loss can be minimized using nitrogen stabilizers. Nitrogen stabilizers are products that reduce the loss of nitrogen fertilizer by inhibiting the enzymes that hydrolyze or reduce nitrogen fertilizer into forms that can be lost to the environment. The nitrogen pathways that are targeted by nitrogen stabilizers to prevent nitrogen losses are volatilization and nitrification.

Urease inhibitors prevent volatilization by inhibiting the urease enzyme which hydrolyzes urea to NH_4^+ (Soares et al., 2012). This process happens quickly in the soil environment with the majority of NH_3 loss occurring in the first week. Urease inhibitors such

as N-(nbutyl) thiophosphoric triamide (NBPT) are most effective in this short period time once the urea is applied to the surface of the soil (Watson, 2000; Trenkel, 2010). After application, urea can be incorporated into the soil either mechanically or by rain/irrigation, reducing or eliminating NH_3 losses (Christianson et al., 1993; Grant et al., 1996; Watson, 2000; Rawluk et al., 2001; Dawar et al., 2010).

The group of nitrogen stabilizers that prevents the loss of nitrogen through leaching and N_2O emission are nitrification inhibitors. Nitrification inhibitors inactivate the enzyme AMO in AOB (references). This action prevents ammonium oxidizing to nitrite and further to nitrate or nitrous oxide. Many benefits can be had from reducing nitrogen loss such as improving nitrogen efficiency and increasing crop performance (Frame and Reiter, 2013). However, these benefits are often advertised and not always achieved.

There are many examples of inconsistency with product performance on a variety of crops. Using the nitrification inhibitor dicyandiamide (DCD), many studies have found yield increase in crops such as maize, wheat, and maize-wheat as well as pastures (Sharma and Prasad, 1995; Rao 1996; Sharma and Kumar, 1998; Ball-Coelho and Roy, 1999; Rao and Popham, 1999; Di and Cameron, 2002; Smith et al., 2005). However, others have reported no yield increase and, in some cases, DCD causes adverse effects (Mason, 1987; Dachler, 1992; Macadam et al., 2003; Frye, 2005). With another nitrification inhibitor, nitrapyrin, a study conducted on rice paddy in China found an increase in yield and nitrogen use efficiency and second study found a 25 to 33% increase in profitability when nitrapyrin was added to fertilizer in a spring wheat (Gu et al., 2018; Tao et al., 2021b). Other individual studies found no effect or negative effects on crop yield (Hendrickson et al., 1978; Blackmer and Sanchez, 1988; Thapa et al., 2016; Pittelkow et al., 2017; Vetsch et al., 2019). Much of this variability is due

to management practices and environmental conditions such as the crop, type of fertilizer, time, and rate of fertilizer application, in addition to soil type, soil temperature, soil pH, soil organic matter, clay content, and rainfall (Powell and Prosser, 1991; Wolt, 2000; Subbarao, 2006).

There also have been variable results in reduced N_2O emissions with nitrapyrin. Several meta-analyses and other studies found the nitrapyrin reduced N_2O emissions 21 to 70% compared to treatment without nitrapyrin (Wolt, 2004; Singh and Verma, 2007; Akiyama et al., 2010; Thapa et al., 2016; Ruser and Schulz, 2015; Lam et al., 2017).). However, a 3-year study on N_2O emissions from spring application of UAN and nitrapyrin showed that nitrapyrin treatment had the highest emission of N_2O in the first year. In the second and third years, N_2O emission were the same for both unfertilized control and nitrapyrin treatment (Graham et al., 2018).

A side effect of nitrapyrin use that is less studied is the potential of increased ammonium volatilization due to the fact that more NH_4^+ remains in the soil following nitrapyrin application (Woodward et al, 2021). A meta-analysis done in 2016 on the volatilization of NH_4^+ in cropping systems showed that nitrification inhibitors increased the loss of ammonium by 38.0% (Pan et al., 2016). The result from this meta-analysis suggests that the volatilization of NH_4^+ might outweigh the benefit of reduced N_2O emissions (Lam et al, 2017).

1.9 Nitrapyrin Implications on Bacterial and Archaeal Populations

The nitrification inhibitor, nitrapyrin, selectively inhibits nitrification by acting as a metal chelator. Nitrapyrin is thought to bind the copper in the active site of the amoB subunit in the ammonia-monooxygenase enzyme pathway for both AOA and AOB (Subbarao et al., 2008; Subbarao et al., 2009; Ruser and Schulz, 2015). Numerous studies have shown that the nitrapyrin does in fact suppress or inhibit the activity and growth of ammonia oxidizers (Belser

and Schmidt, 1981; Zacherl and Amberger, 1990; Gu et al., 2018; Tao et al., 2021a) However, some articles claim that nitrapyrin is a “bactericide”, which by definition means that it kills the ammonia-oxidizing microbes (Espín and García-Fernández, 2014; Pérez-Castillo et al., 2021). Other research has demonstrated that nitrapyrin has an inhibitory effect on AOB, but not AOA (Cui et al., 2013; Faeflen et al., 2016; Duncan et al., 2017; Xi et al., 2017; Duan et al., 2019; Tao et al., 2021a).

1.10. Objectives

With continuing concern about acidifying soils and increasing concerns of nitrogen leaching into ground water sources, sustainable practices and products have been of interest to determine if they can reduce or slow soil acidification and be economic for farmers to practice. In this thesis, the fate of fall applied nitrogen with and without nitrogen stabilizer will be monitored through soil sampling and plant testing to determine the effectiveness and efficiency of the stabilizer to prevent nitrogen loss in winter wheat. The population of nitrifying bacteria and archaea will also be monitored after the application of nitrogen stabilizers to determine the impact of the product on the populations of these microorganisms. Additionally, the economic effectiveness of applying nitrogen stabilizers during fall seeding will be discussed and compared to alternative practices such as topdressing with additional nitrogen fertilizer in the spring in place of a fall applied stabilizer.

1.11 References

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Chapter 2: Evaluating Nitrogen Stabilizer on Soft White and Hard Red Winter Wheat in the High Rain Fall Zones of Northern Idaho

2.1 Introduction

Wheat was the 3rd most profitable crop in Idaho following potatoes and hay in 2019 with a value of nearly $501 million (USDA-NASS, 2019). In the same year, 483,600 hectares were planted to wheat with 295,421 of those hectares planted to winter wheat, and 439,000 hectares in non-irrigated land. The cropping system in northern Idaho is a rainfed, cereal-based cropping system with winter wheat comprising the majority of the farmland. The climate in northern Idaho is a Mediterranean-like climate with the majority of the precipitation falling during the winter months and annual precipitation of 58 to 66 cm (Kruger et al., 2017).

2.1.1 *Nitrogen Fertilizer Use*

Nitrogen fertilizer use around the world has increased exponentially over the past 100 years. In 1908, Fritz Harbor combined atmospheric nitrogen with hydrogen to form NH_3 (ammonia) (Haber, 1920). By 1930, farmers around the world were applying 1.3 million metric tons of nitrogen fertilizers. After World War II, use began to climb 10% every year until it reached a maximum of 80 million metric tons in 1988, almost 100 times more nitrogen fertilizers than was used in 1900 (Frink et al., 1999). In 2017, the US was using about 11.6 million metric tons of nitrogen fertilizer (FAOSTAT) which cost America farmers about $366.50 per metric ton of anhydrous ammonia. In the Pacific Norwest (PNW), nitrogen fertilization for wheat has steadily increased with rates of 39, 69 76, 83, and 84 kg/ha reported in 1964, 1974, 1981, 1996, and 2006, respectively (USDA-ERS 2012).

2.1.2 *Nitrogen Fertilizer Leaching*

Nitrogen fertilizer leaching is a significant problem in our world today and agriculture is the largest source of nitrate in groundwater (Gu et al., 2013). Leaching occurs when negatively charged molecules, in this case nitrate (NO_3^-) and nitrite (NO_2^-), move across the soil profile with soil water. Because of the negative charge, nitrate is soluble in water, repelled by negatively charged surfaces of clay minerals and soil organic matter (Gianquinto et al., 2013; Addiscott, 1996). When the molecules travel with the soil solution through the soil

profile, they travel beyond the rooting depth and are no longer plant available. At this point, they are lost vertically through ground water or horizontally during spring runoff such as through tile drains and are transported out to our waterways (Huggins et al., 2001). When the nitrogen is lost to the ground water or via horizontal movement, it is no longer available to the crops, resulting in yield and quality decline (Pratt, 1984).

There are many reasons why understanding nitrogen leaching is important. One is that nitrogen leaching in the form of nitrite and nitrate can impact environment and human health (Addiscott, 1996; Cameron et al., 2013; Watanabe et al., 2018). Perhaps the most important reason for understanding nitrogen fertilizer leaching for farmers is that it can cause a significant economic loss (Matson et al., 1998).

2.1.3 *Nitrogen Stabilizer Use and Economic Feasibility*

There are multiple ways that nitrogen loss can be managed. Common principals that every grower should know include the "4 R's": the right source, at the right time, at the right rate, in the right place (Fixen, 2020). We can use the "4 R's" with different N management strategies including the right source of nitrogen for the area and the right time to apply which includes the correct time of year due to the climate and the greatest uptake during plant growth (Thorup and Stewart, 1988). Additionally, the correct rate of nitrogen can be applied based on soil tests, soil health, crop, and yield goal along with using variable rate technology (Bruulsema, 2018). Application in the right place can involve surface application followed by incorporation, banding in furrow below the seed or foliar feeding.

Other ways that nitrogen loss can be minimized is through nitrogen stabilizers. The main types of nitrogen stabilizers include nitrification inhibitors which prevent nitrifying organisms from transforming ammonium to nitrate and urease inhibitors which prevent urea hydrolysis to ammonia. Nitrogen stabilizers are mostly used in the Midwestern United States where nitrogen contamination and leaching are common because of heavy use of fertilizer on corn and wheat crops (Denny et al., 2019; Maw et al., 2019; Woodward et al., 2019; Houser and Stuart, 2020; Houser, 2021). Nitrogen stabilizers were first evaluated in the 1960s (Swezey, 1962). However, nitrogen stabilizer testing has had its trials and tribulations with mixed effects on yield and quality. In a study conducted on corn in 2009 to 2011 at the

University of Maryland Research and Education Centers, there was no significant effect of Instinct®, a nitrification inhibitor, applied with UAN on corn yield (Watkins, 2013). In another study near West Lafayette, IN, inconsistent results were found with Instinct® when applied with UAN for corn grain yield, plant N uptake, and nitrogen use efficiency (NUE) on a year-to-year basis (Burzaco et al., 2014). On the other hand, in Nebraska corn grain yield was increased when UAN plus Instinct® was applied during a normal year for precipitation, however, in drought and excessive precipitation Instinct failed to improve UAN performance (Maharjan et al., 2017).

In the PNW, nitrogen stabilizer use is not nearly as common, especially on wheat. Due to the favorable climate, dryland farming in northern Idaho is incredibly productive. These high yields require more inputs, increasing the risk of nitrogen leaching, and this is where nitrogen stabilizers come into play for the wheat growers in the PNW. Many growers in the PNW are skeptical that nitrogen stabilizers will have a cost benefit and speculate that adding more nitrogen to their annual application would be just as or more efficient and economical.

2.1.5 *Objectives*

Little to no data is available on the effectiveness of nitrogen stabilizers to prevent nitrogen loss through leaching and improve crop performance. In this study, trials were placed in locations of high rainfall in northern Idaho, examining the impact of nitrogen stabilizers on both soft white winter wheat (SWWW) and hard red winter wheat (HRW) fields. The objectives were to test the effect of nitrogen stabilizers on yield, test weight, and protein. Throughout the growing season, soil samples were also collected to monitor the form of nitrogen present in the soil and to attempt to capture leaching of nitrate or nitrite through the soil profile.

2.2 Methods and Materials

2.2.1 *Locations*

For both the 2019-2020 and the 2020-2021 growing seasons, the plots were planted at locations near Cottonwood and Cavendish, Idaho. The 2019-2020 Cottonwood location for this trial was located 5.3 km east of Cottonwood at 46.03616 N, -116.2834 W at an elevation of 1,016 m. The plot was seeded into a Nez Perce silt loam that was summer fallow the previous

year. This trial was seeded on October 3, 2019. In 2020-2021, the trial was located 11.3 km east of Cottonwood, Idaho at 46.069143 N, -116.206522 W at an elevation of 940 m. This plot was also seeded into a Nez Perce silt loam with spring canola grown in the previous year. This trial was seeded October 2, 2020. The average annual precipitation for this location is 56.4 cm.

The 2019-2020 Cavendish, Idaho location for this trial located 1 km northwest of Cavendish at 46.567120 N, -116.442540 W at an elevation of 929 m. This plot was seeded into a Taney ashy silt loam with spring peas as the previous crop. The trial was seeded on October 1, 2019. The next growing season (2020-2021), the trial was located 4.5 km south of Cavendish at 46.519692 N, -116.433780 W at an elevation of 836 m. This plot was seeded into a Driscoll silt loam with spring peas as the previous crop. This trial was seeded on October 1, 2020. The average annual precipitation for this location is 71.1 cm.

2.2.2 *Experimental Design*

The experimental design for this trial was a randomized complete block design with four replications. Treatments consisted of five nitrogen fertilizer rates (0, 56, 112, 168, and 224 kg/ha) each with and without Instinct® II (Dow AgroSciences LLC, Indianapolis, IN) nitrogen stabilizer. A soft white winter wheat (LCS Hulk) and a hard winter wheat (LCS Jet) were tested in separate trials at each location. Prior to planting, soil samples were collected from four random sites in each trial to a depth of 60 cm at 15-cm increments. The samples were analyzed by Northwest Agricultural Consultants Soil and Plant Lab (Kennewick, WA) for nutrient content (Appendix A). Using these soils tests and the *Northern Idaho Fertilizer Guide for Winter Wheat* (Mahler, 2015), phosphorus and sulfur fertilizer rates were determined. Potassium was not required as the quantity in the soil was adequate at all locations. Phosphorus and sulfur fertilizer rates were the same for all treatments at both locations and years. Fertilizer consisted of UAN 32 (32-0-0-0, N-P-K-S), ammonium thiosulfate (12-0-0-26), and ammonium phosphate (11-37-0-0) (CHS Primeland, Lewiston, ID).

Wheat seed was treated with Albaugh Cereals F4 Premix which contains per 100 kg of seed: 326 ml difenonazole, 430 ml metalaxyl, 195.6 ml Rizolex, and 143.4 ml thiabendazole, 391.2 ml Resonate 480, and 652 ml red colorant. In the 2019-2020 growing season, ammonium phosphate and ammonium sulphate were applied to achieve 34 kg of P and 22 kg of S per hectare at Cottonwood and Cavendish. For the 2020-2021 growing season,

ammonium phosphate and ammonium sulphate were applied to achieve 67 kg of P and 22 kg of S per hectare at both locations. In all trials, Instinct® II was applied at 4.44 ml/l of fertilizer to achieve a final rate of 2.7 liters per hectare. Because the ammonium thiosulfate and the ammonium phosphate were held constant for each treatment, the 0 kg N/ha treatment in 2019 received in 18 kg N/ha and in 2020 received 29 kg N/ha.

In early October of 2019 and 2020, plots were seeded at 248 seeds per square meter using a custom Agpro Conservation (Lewiston, ID) air drill equipped with Bourgault paired-row openers on 30 cm centers. This drill allows for banding of liquid fertilizer below and between the paired seed rows. The seed was drilled at about 3.8 cm deep, and each plot measured 2.4 m wide (8 rows) by 30.5 m long.

2.2.3 *Soil Sampling and Nitrogen Analysis*

During the growing season, there were four soil samples collected. For each time point and plot, three subsamples were collected (front, middle and back of plot) from the center of the row, combined into a composite sample and homogenized. The samples were immediately placed into a cooler with ice blocks for transportation back to the laboratory and stored at -20°C until nitrogen extraction could take place. The first sample was collected in late November, about six to seven weeks after planting when the ground temperature was between 0 and 4 °C at a depth of about 10 cm. The second round of soil sampling occurred in early March once soil temperature rose above freezing and was between 0 and 4 °C. For the first two samplings, soil was collected from the upper 30 cm using a handheld corer, dividing each core into a 0 to 15 cm and 15 to 30 cm sample. The third sampling occurred in late April or early May once the field was accessible by tractor. The fourth and final soil sampling occurred immediately after harvest. For the third and fourth samples, a tractor mounted hydraulic probe (Giddings Machine Co, Inc, Windsor, CO) was used to collect a 5 cm diameter core to a depth of 120 cm. Each soil core was divided into 0 to 15 cm and 15 to 30 cm, 30 to 60 cm, 60 to 90 cm, and 90 to 120 cm increments. Before dividing the soil core into five increments, the core was split lengthwise and only half of the soil was retained for analysis.

Ammonium and nitrate concentrations were determined for all soil samples using the following procedure. The gravimetric soil moisture content of each soil sample was determined using 20 to 25 g of moist soil. Tins with the moist soil were then placed into drying

ovens at 105^{o}C and allowed to dry for a minimum of 24 hr. After drying, the tins were removed, and weight recorded. The gravimetric soil moisture content was calculated using the equation: [(Mass of wet soil + tin + lid) – (Mass dry soil+ tin + lid)] / [(Mass of dry soil + tin + lid) – (empty tin weight)]. The oven dried soil equivalent (OSDE) of the field moist sample was determined using this equation: ODSE = 4 + (4 x gravimetric soil moisture content).

Nitrogen from the samples was extracted using the procedure found in Mulvaney (1996). Extracted nitrogen samples were analyzed on a LACHAT QuiKChem QC8500 Series 2 Flow Injection Analysis ASX-410 AS with a RP-150 Pump (Lachat Instruments, Loveland, CO). Determination of inorganic ammonium and nitrate from soil samples followed the protocols provided by Lachat Instruments (Loveland, CO) (Harbridge, 2007a; Harbridge, 2007b). Standards were prepared prior to analyzing the samples. A standard stock was made with ammonium chloride and potassium nitrate to a concentration of 200 ppm (0.3819 g oven-dried ammonium chloride and 0.722 g oven-dried potassium nitrate in a total volume of 500 ml with 2 M KCl). Each day, new standards were made from the stock standard solution to calibrate the Lachat (0.1 mg/l, 0.25 mg/l, 0.5 mg/l, 1 mg/l, 2.5 mg/l, 5 mg/l, 10 mg/l, and 20 mg/l). Samples above 20 mg/l were diluted due to the Lachat's detection limit.

For nitrate, the mixture of reagents and sample were passed through a cadmium-copper column to reduce nitrate to nitrite. The nitrite concentration is determined by diazotizing with sulfanilamide and coupling with N-(1-naphthyl)-ethylenediamine dihydrochloride to form a highly colored azo dye. For the ammonium, the mixture of reagent and sample was heated, and the concentration was determined by the amount of indophenol blue produced when the ammonium reacts with alkaline phenol and sodium hypochlorite. The color is intensified by the addition of sodium nitroprusside. The color intensity that resulted from both nitrate and ammonium determined the amount of analyte present and is measured by an expanded range of detectors which converted the color intensity into a digital signal (Harbridge, 2007a; Harbridge, 2007b).

2.2.4 *Agronomic measurements*

Stand counts were collected to determine the amount of plants per square meter for the first year in the fall, but not for the second due to insufficient emergence before snowfall. The stand counts were determined by counting the number of plants in a 1 m row from three random

sites within each plot. Prior to tillering in the spring, a second stand count was collected using the same procedures described above for each site and both growing seasons. Normalized difference vegetation index (NDVI) measurements were collected biweekly for each plot and location starting the beginning of May and ending in July. These measurements were collected with a GreenSeeker Handheld Crop Sensor (Trimble, Sunnyvale, CA) by scanning a linear pass of 30 meters over a row through each plot until the plants started ripening. The NDVI measures the difference between near-infrared, in which green light is reflected, and red light absorbed. Larger index numbers indicate "greener" plants which in turn signifies a greater concentration of nitrogen in the plants.

At physiological maturity, spike counts were collected by counting the number of spikes in 1 m of row at three random sites within each plot. Plant height was also collected at this time by collecting an average height from each plot. All plots were harvested with a Wintersteiger Nurserymaster Elite^{TM} plot combine (Wintersteiger, Inc.; Salt Lake City, UT). Before plots were harvested during the same day, above ground plant biomass was collected in one square meter using a one-meter stick and rice scythes. After harvest, total grain yield was recorded, and a subsample was collected to determine the test weight, moisture and protein using a FOSS Infratec^{TM} NOVA Grain Analyzer (FOSS, Eden Prairie, MN).

An Onset HOBO USB micro station data logger (Onset Computer Corporation, Bourne, MA, USA) was placed at each location to measure rain, humidity, and temperature. The climate conditions were recorded every hour and were analyzed to determine daily averages for the seasons.

2.2.5 *Data Analysis*

Data were analyzed using SAS version 9.4 (Statistical Analysis Software (SAS) 9.4. SAS Institute, Inc., Cary, NC). Stand counts, spike counts, plant height, grain yield, test weight, protein, moisture, plant biomass yield at harvest, total nitrogen in grain at harvest were subjected to analysis of variance (ANOVA) using a generalized linear mixed model (PROC GLIMMIX). Field data from both locations and varieties were analyzed separately. Data from both years were analyzed together with no concerning year by year interaction. Soil nitrate and ammonium concentration data from both locations were analyzed separately while varieties and years were analyzed together. Crops were justified to unlikely have influence the soil

measurements. There also were no significant interactions between Instinct® II treatments and nitrogen fertilizer rates. Significant interactions between year*year were plotted and determined to have the same trends present in both data sets.

Normal random effects were assumed. Significant differences between treatments were identified using differences of least squares means with an alpha value of 0.05. Normality and homogeneity of variance assumptions were determined with PROC UNIVARIATE and PROC GLIMMIX respectively. The data were analyzed using the statistical model outlined as follows:

$$y_{ijkl} = \mu + r_i + \alpha_j + w_{ij} + \beta_k + (\alpha\beta)_{jk} + \gamma_l + (\alpha\gamma)_{jl} + (\beta\gamma)_{kl} + (\alpha\beta\gamma)_{jkl} + s_{ijkl}$$

Where y_{ijkl} is the expected response for the ith block/replicate, the jth nitrogen treatment, and the kth Instinct® II treatment; μ is the grand mean; α_j and β_k are the fixed effects of the jth nitrogen treatment and the kth Instinct® II treatment respectively; r_i is the random effect of the ith block/replicate, NID(0, σ_r^2); w_{ij} is the random whole plot error term, NID(0, σ_w^2); $(\alpha\beta)_{jk}$ is the fixed interaction between nitrogen and Instinct® II treatments; γ_l is the fixed effect of the lth year; $(\alpha\gamma)_{jl}$ is the fixed interaction between nitrogen treatment and year; $(\beta\gamma)_{kl}$ is the fixed interaction between Instinct® II treatment and year; $(\alpha\beta\gamma)_{jkl}$ is the random three-way interaction, NID(0, $\sigma_{\alpha\beta\gamma}^2$); and s_{ijk} is the random split plot error term, NID(0, σ_s^2).

A correlation analysis was performed with PROC CORR to find relationships between agronomic measurements. A simple linear regression between nitrogen rate and yield was examined to determine the yield increase per unit of nitrogen added for economic analysis. Crops and locations were analyzed separately while both growing seasons were analyzed together.

2.3 Results

2.3.1 *Precipitation, Humidity and Temperature*

Precipitation, average daily temperature, and average relative humidity for Cottonwood are show in Figure 2.1. In the winter and spring of 2020, Cottonwood experienced many heavy precipitation events such as in February in which 16 mm of precipitation occurred in a single day. May, June, and July, multiple rainfall events over 16 mm and one particular event over

24 mm occurred. During the following growing season at Cottonwood, there was only one major rainfall event in mid-October with about 20 mm. After October, the area had no precipitation over 8 mm until the end of May. For temperature, the lowest observed at Cottonwood was about -10^{o}C in both 2020 and 2021. The summer of 2020 saw temperature over 24^{o}C in the month of mid-August while the summer of 2021 saw temperatures just below 24^{o}C in the month of June. Data was not collected past the month of June due to early harvest.

Data was not recorded for much of the winter of 2019-2020 at Cavendish (Figure 2.2). Precipitation in Cavendish was similar to Cottonwood with heavy precipitation events occurring in the spring months of 2020 of up to 15 mm then declining in the summer months. Similar to Cottonwood, there were smaller precipitation events in the spring of 2021 with very little precipitation after March. The temperature for Cavendish was greater in range compared to Cottonwood with average daily temperatures below -10^{o}C in the winter and reaching 30^{o}C in the summer.

According to weather stations at the research sites, between the month of March and June 2021, Cottonwood received 135.8 mm less precipitation and had 10% lower RH than March through June 2020. The long-term (20 year) average precipitation between the months of March and June for Cottonwood is 291 mm (NOAA). At Cavendish, there was 144.2 mm less precipitation and 20% lower RH in 2021 compared to 2020 between those same months and about 200 mm less precipitation than the long-term average (NOAA). Temperature was similar in both years, however during late June and early July in 2021 there was unseasonably hot temperatures coinciding with grain fill.

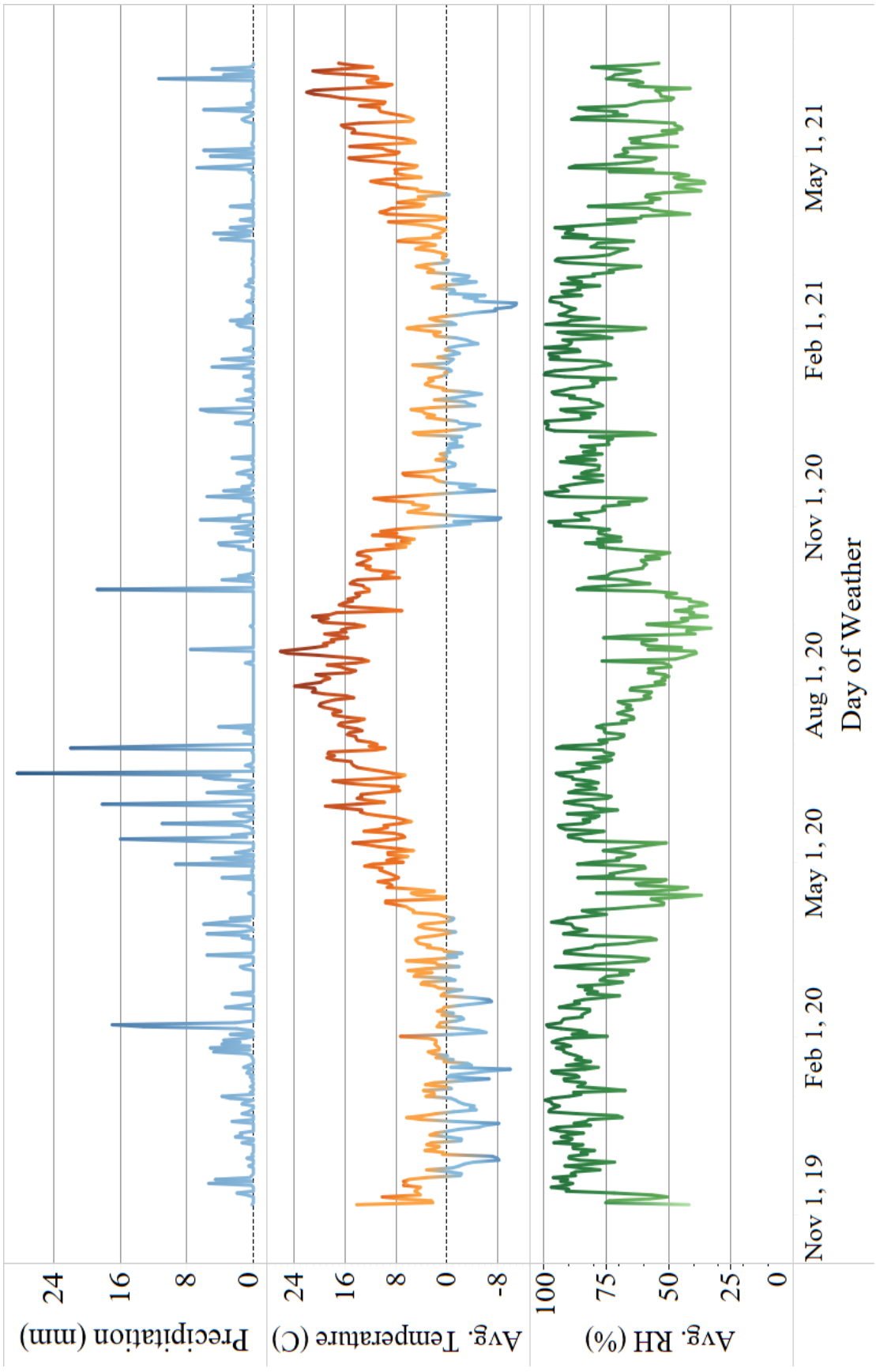

Figure 2.1. Precipitation (mm), daily average temperature (°C), and daily average relative humidity (RH) (%) for November 2019 to June 2021 for Cottonwood, ID.

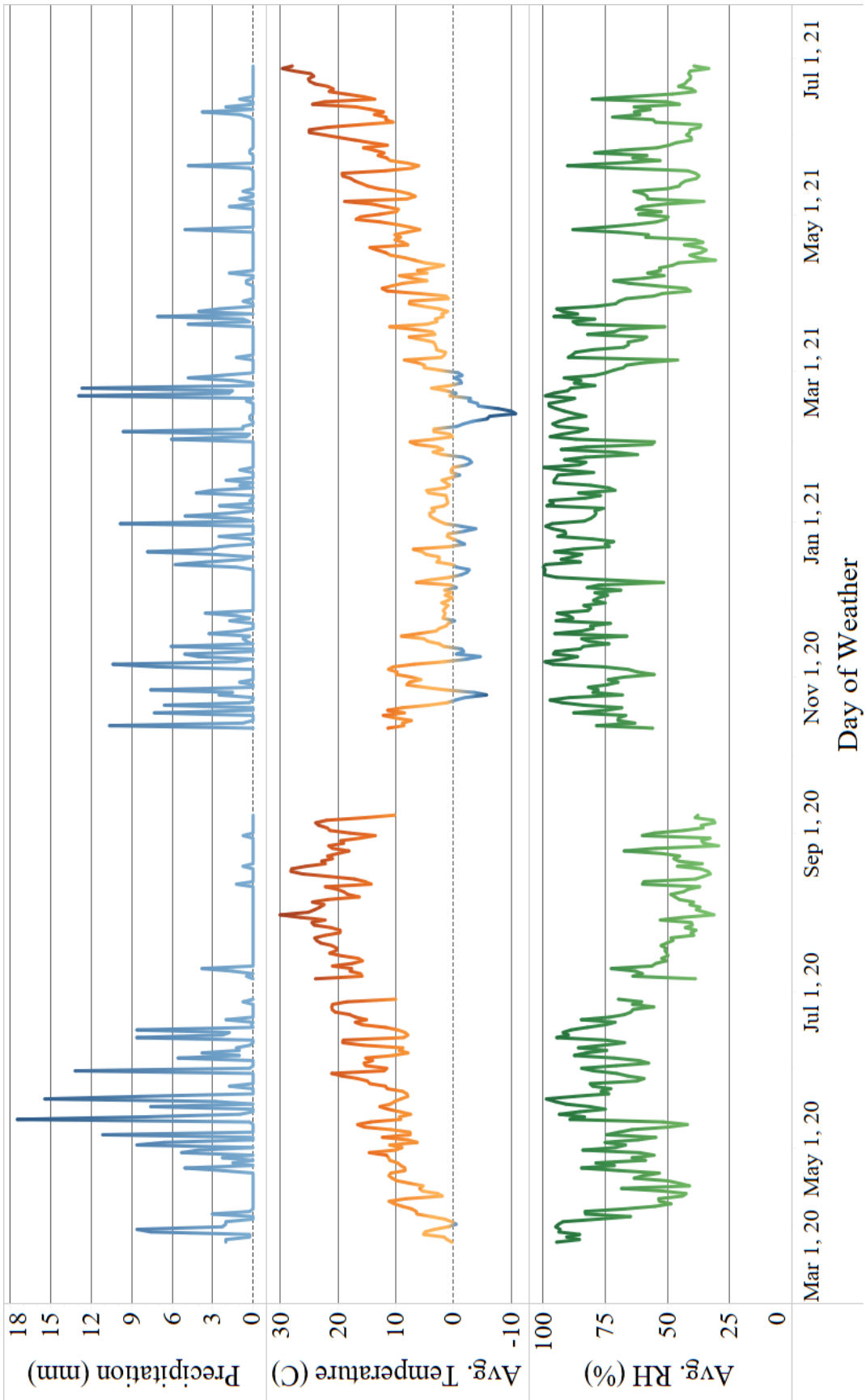

Figure 2.2. Precipitation (mm), daily average temperature (°C), and daily average relative humidity (RH) (%) for March 2020 to June 2021 for Cavendish, ID.

2.3.2 *Impact of nitrogen stabilizers on ammonium and nitrate soil concentrations*

Soil ammonium and nitrate concentrations were measured at Cavendish (Figure 2.3) and Cottonwood (Figure 2.4) during the 2019-2020 and 2020-2021 growing seasons. Four different sampling times occurred (November; March; May; September). The highest nitrate and ammonium concentrations were observed in the November and March samples at a depth of 0 to 15 cm. Nitrogen concentrations were markedly lower after harvest. The range of soil ammonia and nitrate concentrations detected in November at the depth 0 to 30 cm for Cottonwood were 4.0 to 104.9 mg of ammonium/kg of soil and 13.2 to 37.7 mg of nitrate/kg of soil, while concentration at Cavendish ranged from 4.5 to 148.8 mg of ammonium/kg of soil and 12.6 to 15.2 mg of nitrate/kg of soil. At Cavendish, soil nitrate concentrations were significantly lower with the nitrogen stabilizer treatment for the depth of 0 to 15 cm in November and March. In May, nitrate was significantly lower for the 15 to 30 cm depth (*p*-value of 0.0611). Soil ammonium concentrations were significantly higher with nitrogen stabilizer treatment for the 0 to 15 cm depth throughout all sampling occasions in Cottonwood (Figure 2.4). In March, the ammonium concentration was significantly higher at the 15 to 30 cm depth (*p*-values of 0.0797). Soil nitrate concentrations were significantly lower at the depth of 0 to 15 cm in March and May. There were no significant differences between the samples treated with and without nitrogen stabilizer for most samples taken below the depth of 15 cm as well as for postharvest soil samples.

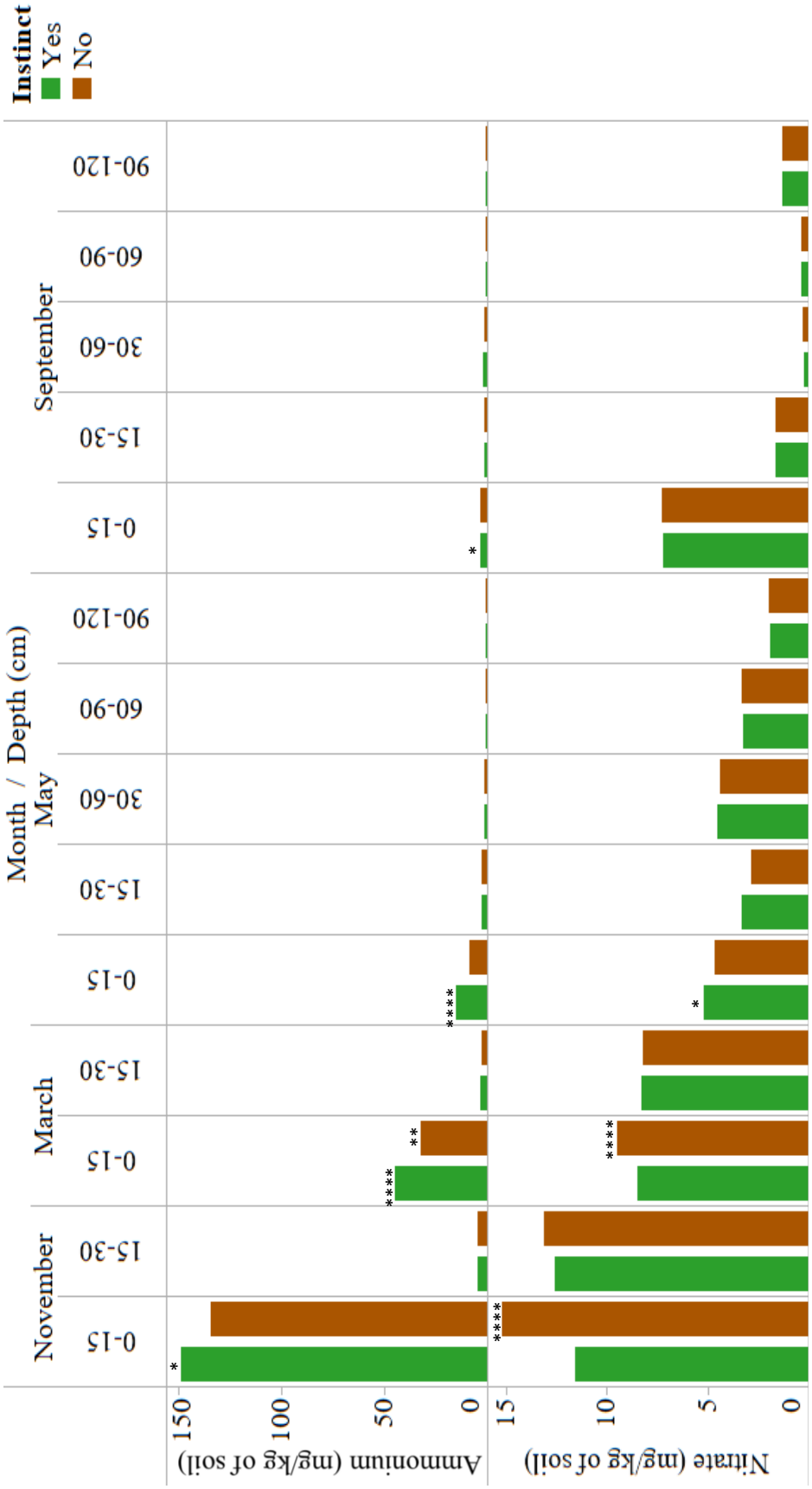

Figure 2.3. Soil ammonium and nitrate concentrations at Cavendish, Idaho during the 2019-2020 and 2020-2021 growing seasons for both soft white winter wheat and hard red winter wheat trials.
* 0.1 > p > 0.05
** 0.05 > p > 0.01
*** 0.01 > p > 0.001
**** p < 0.001

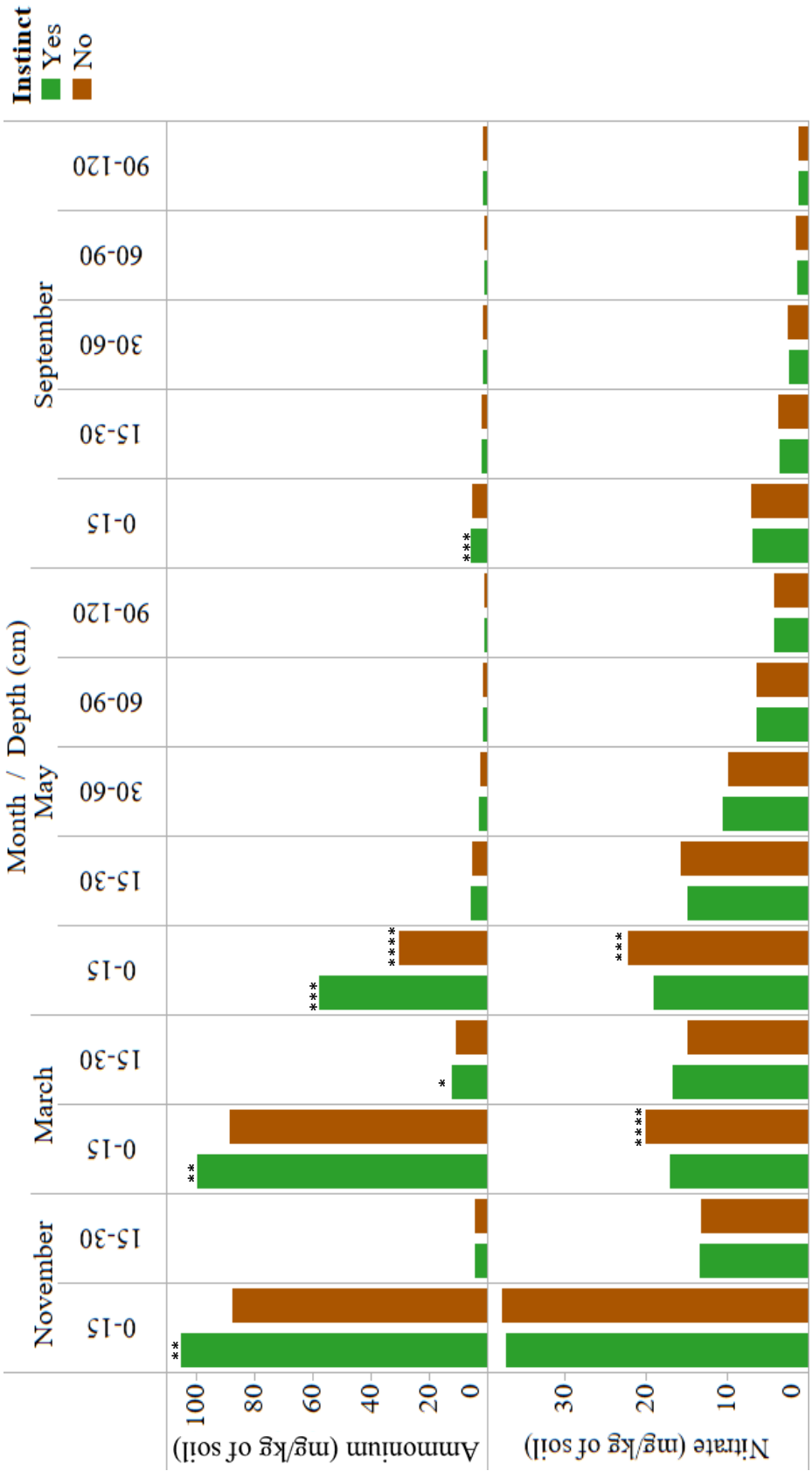

Figure 2.4. Soil ammonium and nitrate concentrations at Cottonwood, Idaho during the 2019-2020 and 2020-2021 growing seasons for both soft white winter wheat and hard red winter wheat trials.

* $0.1 > p > 0.05$

** $0.05 > p > 0.01$

*** $0.01 > p > 0.001$

**** $p < 0.001$

2.3.3 *Impact of nitrogen fertilizer rates on soil ammonium and nitrate concentrations*

At Cavendish the quantity of ammonium increased proportionally as the rate of nitrogen fertilizer applied increased at all sampling dates for the 0 to 15 cm depth (Figure 2.5). In all instances there was significantly more ammonium following the application of larger quantities of fertilizer. At the 15 to 30 cm depth, significant increases in ammonium concentrations were observed in November, March, and May, although the quantity of ammonium detected was substantially lower compared to the 0 to 15 cm depth. Below 30 cm, there were no differences in ammonium concentration. The quantity of ammonium observed in the samples declined throughout the growing season with the highest concentrations observed in November at the depth of 0 to 15 cm with a range of and 54.4 to 250.7 mg NH_4^+/kg soil. Nitrate concentrations followed a similar trend with significantly more nitrate with increasing fertilizer rates at the 0 to 15 cm depth for samples collected in November, March, and May. There also was significantly more nitrate observed with increasing fertilizer rates for the 15 to 30 cm samples collected in November and the 15 to 30, 30 to 60 and 60 to 90 cm samples collected in May. Nitrate quantities did not differ for the postharvest sample in September with the exception of the 90 to 120 cm sample. Nitrate was highest in the November samples at the depth of 0 to 15 cm at a range of 8.2 to 18.2 mg nitrate/kg soil, with much lower quantities in the March, May, and September samples.

The concentration of ammonium measured at Cottonwood followed a similar pattern as Cavendish, with significantly greater quantities of ammonium with increasing nitrogen fertilizer rates for all depths in the months of November and March (Figure 2.6). In the May and September sampling, there was a significant increase in ammonium concentration with increasing nitrogen rates at the 0 to 15 cm depth. Below 15 cm, there was no difference in ammonium concentration. As in Cavendish, the quantity of ammonium in Cottonwood declined with depth and throughout the growing season. Ammonium was the highest in November at a depth of 0 to 15 cm with a range of 37.7 to 193.1 mg ammonium/kg soil and lowest in September with a range of 4.2 to 7.3 mg NH_4^+/kg soil. Similar to soil ammonium, nitrate concentrations increased with greater nitrogen fertilizer rate. For all sampling occasions and depths, there was significantly more nitrate after a larger fertilizer application. Like ammonium, the highest concentration of nitrate occurred at a depth of 0 to 15 cm in November

with a range of 26.4 to 48.7 mg NO_3^-/kg soil and lowest in September with a range of 3.3 to 14 mg NO_3^-/kg soil. Nitrate concentrations decreased throughout the growing season and with deeper soil samples.

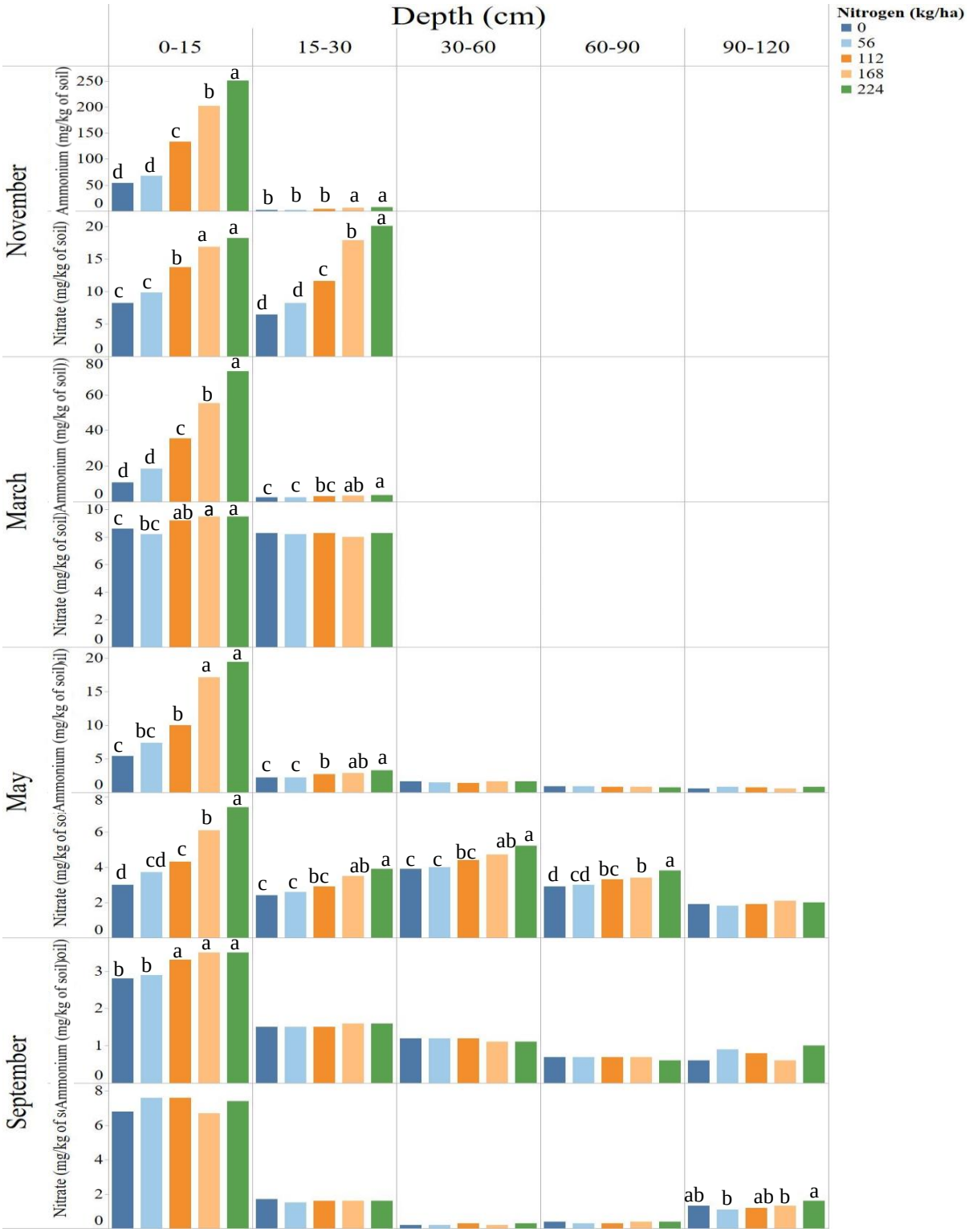

Figure 2.5. Soil ammonium and nitrate concentrations (mg/kg of soil) for hard winter wheat and soft winter wheat at Cavendish, Idaho during 2019-2020 and 2020-2021 growing seasons. For each month, nitrogen rate and depth were analyzed separately. Means presented within each subplot with same letters are not significantly different ($\alpha \leq 0.05$).

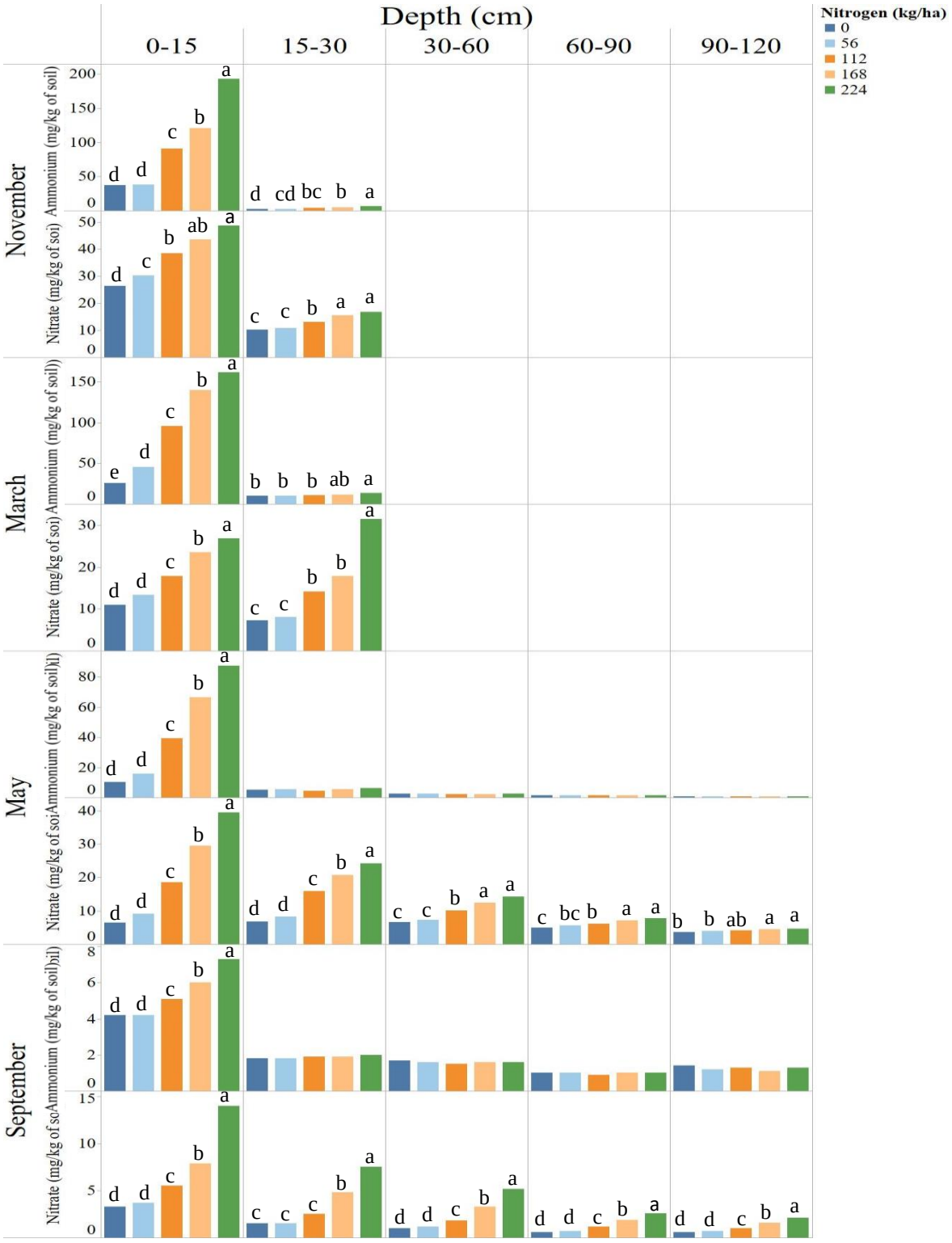

Figure 2.6. Soil ammonium and nitrate concentrations (mg/kg of soil) for hard winter wheat and soft winter wheat at Cottonwood, Idaho during 2019-2020 and 2020-2021 growing seasons. For each month, nitrogen rate and depth were analyzed separately. Means presented within each subplot with same letters are not significantly different ($\alpha \leq 0.05$).

2.3.4 *Impact of nitrogen stabilizer on agronomic performance and quality*

Stand Counts, Spike Counts, Plant Height, and NDVI. Nitrogen stabilizer treatment had no influence on spring stand counts, spike counts, or plant height for HRW or SWWW at either location (Table 2.1; Table 2.2; Table 2.3). The NDVI measurements were collected biweekly starting in early May and ending in early July for both growing seasons to quantify the vegetative greenness between treatments. The nitrogen stabilizer treatment did not impact the NDVI measurement at any timepoint throughout the growing season at Cottonwood (Figure 2.7). However, at Cavendish in the last two weeks of May, the NDVI measurement of SWWW was significantly increased by Instinct treatment (Figure 2.8). However, this increase with Instinct was not observed at any of the other sampling dates.

Table 2.1. Spring stand counts of soft white winter wheat (SWWW) and hard red winter wheat (HRW) at test sites in Cavendish and Cottonwood during the 2019-2020 and 2020-2021 growing seasons to evaluate the effectiveness of a nitrogen stabilizer.

	Cottonwood SWWW	Cottonwood HRW	Cavendish SWWW	Cavendish HRW
Treatment	---------- Stand Count per m^2 ----------			
Instinct	214 ± 4*	198 ± 4	228 ± 4	222 ± 4
No instinct	212 ± 4	194 ± 4	232 ± 2	222 ± 4

* Values are presented as mean ± standard error of mean.

Table 2.2. Average spike counts of soft white winter wheat (SWWW) and hard red winter wheat (HRW) at test sites in Cavendish and Cottonwood during the 2019-2020 and 2020-2021 growing seasons to evaluate the effectiveness of a nitrogen stabilizer.

	Cottonwood SWWW	Cottonwood HRW	Cavendish SWWW	Cavendish HRW
Treatment	---------- Spike Count per m^2 ----------			
Instinct	672 ± 12*	476 ± 4	552 ± 12	516 ± 16
No instinct	676 ± 12	480 ± 4	548 ± 12	524 ± 16

* Values are presented as mean ± standard error of mean.

Table 2.3. Plant height of soft white winter wheat (SWWW) and hard red winter wheat (HRW) at test sites in Cavendish and Cottonwood during the 2019-2020 and 2020-2021 growing seasons to evaluate the effectiveness of a nitrogen stabilizer.

	Cottonwood SWWW	Cottonwood HRW	Cavendish SWWW	Cavendish HRW
Treatment	---------- Plant Height (cm) ----------			
Instinct	76 ± 0.7*	72 ± 1.1	76 ± 0.5	71 ± 0.6
No instinct	76 ± 0.7	72 ± 1.1	75 ± 0.5	71 ± 0.6

* Values are presented as mean ± standard error of mean.

Seed yield and quality. For seed yield, there were no significant differences at either location or between crops with nitrogen stabilizer treatment (Table 2.4). There were no differences between treatments for grain test weight and protein concentration with the exception of the HRW at Cavendish (Tables 2.5 and 2.6). For the HRW at Cavendish, plots with nitrogen stabilizer treatment had significantly lower test weights while the protein concentrations were significantly higher for the same treatment. There were no significant differences between plant biomass and total nitrogen concentration in the grain.

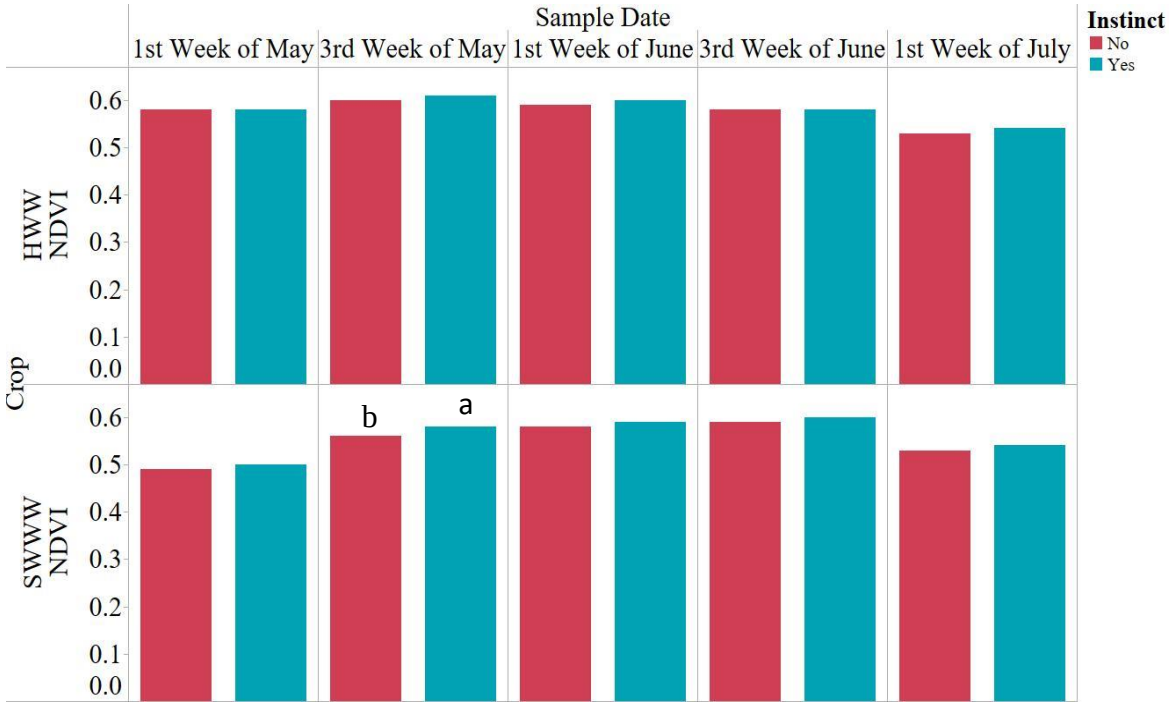

Figure 2.8. Soft white winter wheat (SWWW) and hard red winter wheat (HRW) NDVI index for nitrogen stabilizer treatment at Cavendish during 2019-2020 and 2020-2021. Means presented within each subplot with same letters are not significantly different ($p \leq 0.05$).

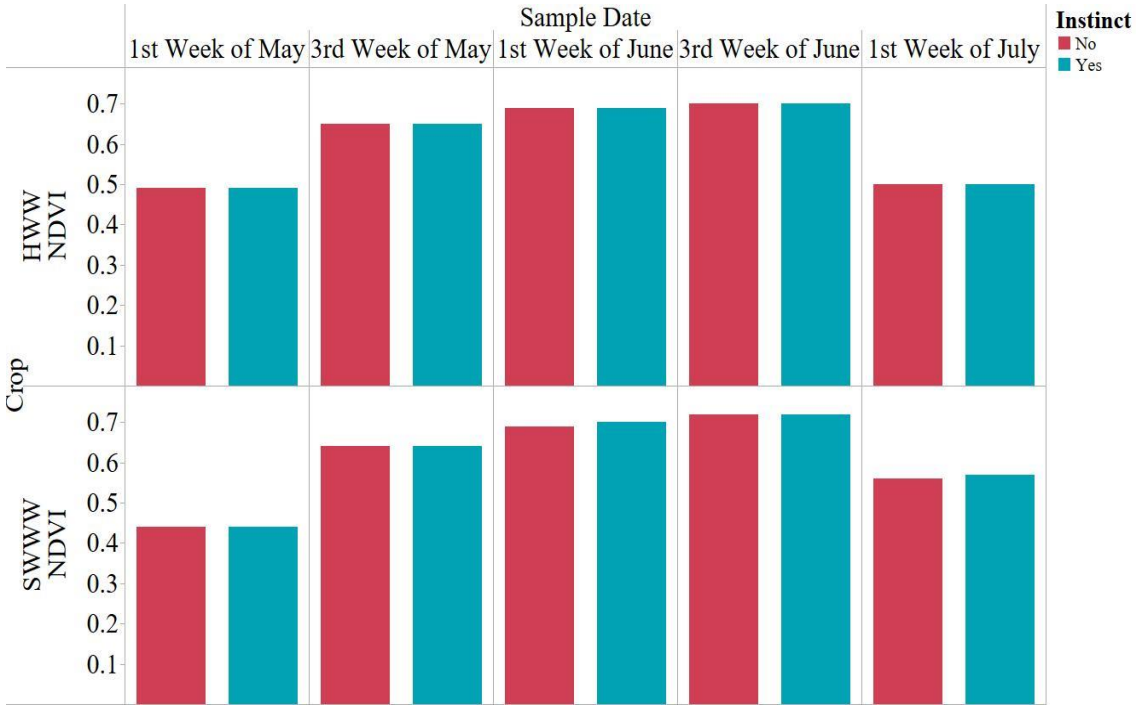

Figure 2.7. Soft white winter wheat (SWWW) and hard red winter wheat (HRW) NDVI index for nitrogen stabilizer treatment at Cottonwood during 2019-2020 and 2020-2021.

Table 2.4. Yield of soft white winter wheat (SWWW) and hard red winter wheat (HRW) at test sites in Cavendish and Cottonwood during the 2019-2020 and 2020-2021 growing seasons to evaluate the effectiveness of a nitrogen stabilizer.

	Cottonwood SWWW	Cottonwood HRW	Cavendish SWWW	Cavendish HRW
Treatment	---------- Yield (kg/ha) ----------			
Instinct	5,915 ± 206*	4,433 ± 53	4,155 ± 80	4,240 ± 157
No instinct	5,870 ± 206	4,421 ± 53	4,071 ± 80	4,252 ± 158

* Values are presented as mean ± standard error of mean.

Table 2.5. Test weight of soft white winter wheat (SWWW) and hard red winter wheat (HRW) at test sites in Cavendish and Cottonwood during the 2019-2020 and 2020-2021 growing seasons to evaluate the effectiveness of a nitrogen stabilizer.

	Cottonwood SWWW	Cottonwood HRW	Cavendish SWWW	Cavendish HRW
Treatment	---------- Test Weight (kg/hl) ----------			
Instinct	74.1 ± 0.5*	73.5 ± 0.1	74.4 ± 0.1	71.8 ± 0.2 b
No Instinct	74.0 ± 0.5	73.7 ± 0.1	75.5 ± 0.1	72.2 ± 0.2 a

* Values are presented as mean ± standard error of mean. hl:

Table 2.6. Protein of soft white winter wheat (SWWW) and hard red winter wheat (HRW) at test sites in Cavendish and Cottonwood during the 2019-2020 and 2020-2021 growing seasons to evaluate the effectiveness of a nitrogen stabilizer.

	Cottonwood SWWW	Cottonwood HRW	Cavendish SWWW	Cavendish HRW
Treatment	---------- Protein (%) ----------			
Instinct	12.2± 0.2*	12.0 ± 0.1	9.6 ± 0.2	9.8 ± 0.1 a
No Instinct	12.3 ± 0.2	12.1 ± 0.1	9.4 ± 0.2	9.5 ± 0.1 b

* Values are presented as mean ± standard error of mean. Means presented within each column with same letters are not significantly different ($p \leq 0.05$).

Table 2.7. Plant biomass yield at harvest of soft white winter wheat (SWWW) and hard red winter wheat (HRW) at test sites in Cavendish and Cottonwood during the 2019-2020 and 2020-2021 growing seasons to evaluate the effectiveness of a nitrogen stabilizer.

	Cottonwood SWWW	Cottonwood HRW	Cavendish SWWW	Cavendish HRW
Treatment	---------- Plant Biomass Yield at Harvest (kg/ha) ----------			
Instinct	519 ± 61*	333 ± 6	288 ± 9 a	270 ± 10
No Instinct	514 ± 61	325 ± 6	266 ± 9 b	272 ± 10

* Values are presented as mean ± standard error of mean. Means presented within each column with same letters are not significantly different ($p \leq 0.05$).

Table 2.8. Total nitrogen in grain at harvest of soft white winter wheat (SWWW) and hard red winter wheat (HRW) at test sites in Cavendish and Cottonwood during the 2019-2020 and 2020-2021 growing seasons to evaluate the effectiveness of a nitrogen stabilizer.

	Cottonwood SWWW	Cottonwood HRW	Cavendish SWWW	Cavendish HRW
Treatment	---------- Total Nitrogen in Grain (kg/ha) ----------			
Instinct	124 ± 3*	94 ± 1	68 ± 2 a	68 ± 2
No Instinct	123 ± 3	93 ± 1	65 ± 2 b	67 ± 2

* Values are presented as mean ± standard error of mean. Means presented within each column with same letters are not significantly different ($p \leq 0.05$).

2.3.5 *Impact of Nitrogen Fertilizer Rates on agronomic performance and quality*

Stand Counts, Spike Counts, Plant Height and NDVI. The SWWW stand counts were significantly different, decreasing with increasing nitrogen rates for Cottonwood and Cavendish, although this difference in stand was most obvious at Cottonwood (Table 2.9). The HRW stand was not influenced by nitrogen rate. Spike count and plant heights were also significantly impacted by nitrogen treatment across both sites and crops with number of spikes and plant height both increasing as the rate of applied nitrogen increased. The average increase between 0 and 224 kg N/ha for spike count was 18% and the average increase in plant height

was 9% (Table 2.10 and Table 2.11). Similar to the stand count, the greatest increase in spike count between the lowest and highest nitrogen rate was observed for the SWWW. Nitrogen rate treatment significantly impacted NDVI measurements at both sites. The range of NDVI readings was from 0.44 to 0.72 throughout the sampling season (Figure 2.9). For both SWWW and HRW at Cottonwood, the NDVI measurement increased significantly with increasing nitrogen rate for second thru fifth sampling dates. However, the 168 and 224 kg N/ha rates had similar NDVI measurements for both crops at nearly every time point. At Cavendish, there was a more pronounced impact of nitrogen rate on NDVI measurement with a significant increase in NDVI with increasing nitrogen fertilizer rate at all sampling dates (Figure 2.10). For the SWWW, every treatment was significantly different at most time points. However, for the HRW the NDVI measurement were statistically similar for the 0 and 56 kg N/ha rates as well as the 168 and 224 kg N/ha rates.

Table 2.9. Spring stand counts of soft white winter wheat (SWWW) and hard red winter wheat (HRW) at test sites in Cavendish and Cottonwood during the 2019-2020 and 2020-2021 growing seasons to evaluate the effectiveness of nitrogen rates.

	Cottonwood SWWW	Cottonwood HRW	Cavendish SWWW	Cavendish HRW
Nitrogen (kg/ha)	---------- Stand Count per m^2----------			
0	224 ± 4 a*	196 ± 6	232 ± 4 ab	224 ± 4
56	222 ± 4 ab	196 ± 6	240 ± 4 a	224 ± 4
112	210 ± 4 bc	196 ± 6	228 ± 4 b	218 ± 4
168	208 ± 4 c	192 ± 6	222 ± 4 b	224 ± 4
224	204 ± 4 c	196 ± 6	228 ± 4 b	218 ± 4

* Values are presented as mean ± standard error mean. Means presented within each column with same letters are not significantly different ($p \leq 0.05$).

Table 2.10. Average spike counts of soft white winter wheat (SWWW) and hard red winter wheat (HRW) at test sites in Cavendish and Cottonwood during the 2019-2020 and 2020-2021 growing seasons to evaluate the effectiveness of nitrogen rates.

	Cottonwood SWWW	Cottonwood HRW	Cavendish SWWW	Cavendish HRW
Nitrogen (kg/ha)	---------- Spike Count per m^2 ----------			
0	592 ± 16 d*	452 ± 8 d	474 ± 16 d	492 ± 24 bc
56	632± 16 c	474 ± 8 cd	492 ± 16 d	488 ± 24 c
112	680 ± 16 b	488 ± 8 bc	556 ± 16 c	596 ± 24 bc
168	728 ± 16 a	476 ± 8 ab	592 ± 16 b	544 ± 24 ab
224	736 ± 16 a	500 ± 8 a	644 ± 16 a	584 ± 24 a

* Values are presented as mean ± standard error mean. Means presented within each column with same letters are not significantly different ($p \leq 0.05$).

Table 2.11. Plant height of soft white winter wheat (SWWW) and hard red winter wheat (HRW) at test sites in Cavendish and Cottonwood during the 2019-2020 and 2020-2021 growing seasons to evaluate the effectiveness of nitrogen rates.

	Cottonwood SWWW	Cottonwood HRW	Cavendish SWWW	Cavendish HRW
Nitrogen (kg/ha)	---------- Plant Height (cm) ----------			
0	73 ± 1 c*	69 ± 1 b	71 ± 1 d	68 ± 1 b
56	74 ± 1 bc	71 ± 1 b	74 ± 1 c	69 ± 1 b
112	76 ± 1 ab	74 ± 1 a	76 ± 1 bc	72 ± 1 a
168	77 ± 1 a	74 ± 1 a	77 ± 1 b	73 ± 1 a
224	78 ± 1 a	75 ± 1 a	80 ± 1 a	74 ± 1 a

* Values are presented as mean ± standard error mean. Means presented within each column with same letters are not significantly different ($p \leq 0.05$).

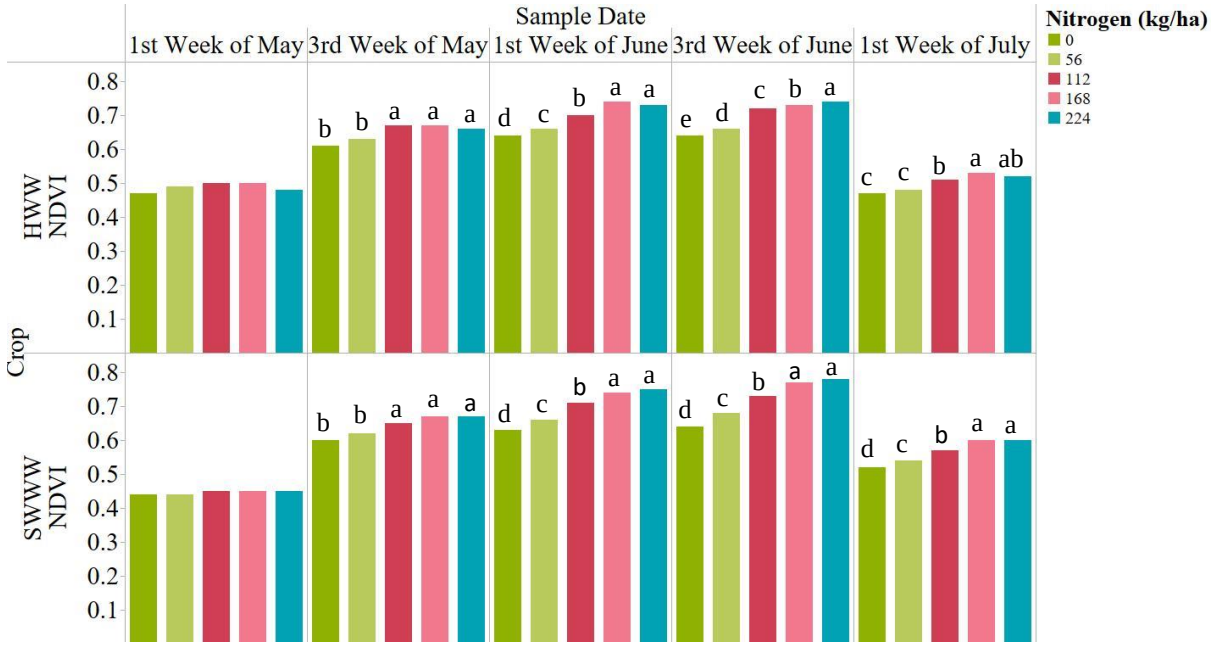

Figure 2.9. Impact of nitrogen fertilizer rate on NDVI index for soft white winter wheat (SWWW) and hard red winter wheat (HRW) at Cottonwood during the 2019-2020 and 2020-2021 growing seasons. Means presented within each column with same letters are not significantly different ($p \leq 0.05$).

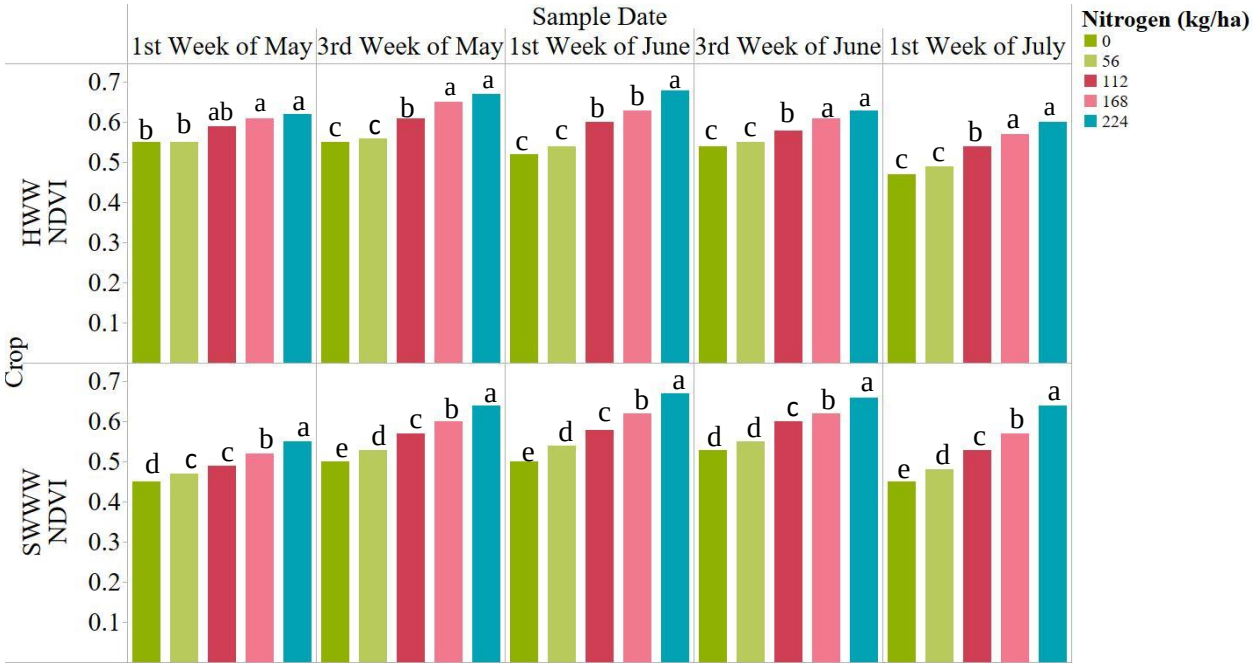

Figure 2.10. Impact of nitrogen fertilizer rate on NDVI index for soft white winter wheat (SWWW) and hard red winter wheat (HRW) at Cavendish during the 2019-2020 and 2020-2021 growing seasons. Means presented within each column with same letters are not significantly different ($p \leq 0.05$).

Yield and Quality. The yield for the nitrogen rate treatment did not vary much for the SWWW at Cottonwood (Table 2.12). Treatments with 56 to 224 kg N/ha were all significantly higher than the 0 kg N/ha control. There was more variation in yield for the HRW at Cottonwood with the 0 and 56 kg N/ha treatments resulted in significantly lower yield than the 112 and 168 kg N/ha. The yield for the 224 kg N/ha fertilizer rate was not significantly different from the other nitrogen rates. The yield for SWWW at Cavendish increased significantly with each increase in nitrogen fertilizer rate. The nitrogen rate treatment effect on HRW yield at Cavendish exhibited a similar trend to the SWWW, but the 0 and 56 kg N/ha as well as the 168 and the 224 kg N/ha were not statistically different. Although yield was higher at Cottonwood, the difference between the lowest and highest yielding treatment was 449 kg/ha compared to Cavendish which was 1,427 kg/ha.

Test weight tended to decrease with increasing nitrogen rates (Table 2.13). Results were similar for both Cottonwood SWWW and HRW with the 0 to 55 kg N/ha having a significantly higher test weight than the 112 kg N/ha, while the 168 and 224 kg N/ha had a significantly lower test weight compared to the 112 kg N/ha rate. For Cavendish SWWW, only the 224 kg N/ha rate was significantly lower while the other nitrogen rates had similar test weights. Test weight differences for the HRW were more variable with 56 and 112 kg N/ha having the highest test weights and 168 kg N/ha having the lowest test weight. Test weight values were comparable between locations and with the HRW having an arguably lower test weight than the SWWW.

Grain protein followed the same trend as yield, increasing with larger nitrogen additions (Table 2.14). Cottonwood HRW had the greatest difference between the treatments, with each subsequent nitrogen rate resulting in significantly higher grain protein concentration. The SWWW was similar however the 0 and the 56 kg N/ha were not different from each other. Cavendish SWWW and HRW had the same differences between treatments with the 0, 56, and 112 kg N/ha significantly lower in percent protein than the 168 and 224 kg N/ha rates. Between the locations, Cavendish had much lower grain protein concentration than Cottonwood for both the SWWW and HRW. It should be noted that the grain protein concentration was above the desired 10.5% for all nitrogen rates at Cottonwood.

Comparable to the grain yield results, the plant biomass yield at harvest had larger differences between the treatments at Cavendish compared to Cottonwood (Table 2.15).

However, Cottonwood had a greater plant biomass after harvest on a kg/ha basis. The plant biomass at Cavendish SWWW yielded the best at 168 and 224 kg N/ha followed the 112 kg N/ha treatment with 0 and 56 kg N/ha yielding significantly less. There were no significant differences between nitrogen rate for the SWWW biomass at Cottonwood. The plant biomass for the HRW at Cottonwood and Cavendish tended to increase with increasing nitrogen rate, but not all treatments were significantly different from each other. Total nitrogen concentration in grain (TN) followed a similar trend as the grain protein concentration with increasing content as the rate of nitrogen fertilizer increased (Table 2.16). While not all nitrogen rates were significantly different from each other, the trend was similar for both SWWW and HRW and both locations. The average TN in grain was much higher in Cottonwood (109 kg/ha) compared to Cavendish (67 kg/ha).

Table 2.12. Seed yield of soft white winter wheat (SWWW) and hard red winter wheat (HRW) at test sites in Cavendish and Cottonwood during the 2019-2020 and 2020-2021 growing seasons to evaluate the effectiveness of nitrogen rates.

	Cottonwood SWWW	Cottonwood HRW	Cavendish SWWW	Cavendish HRW
Nitrogen (kg/ha)	---------- Yield (kg/ha) ----------			
0	5,629 ± 214 b*	4,320 ± 68 b	3,469 ± 93 e	3,636 ± 175 c
56	5,879 ± 214 a	4,360 ± 68 b	3,741 ± 93 d	3,844 ± 177 c
112	5,936 ± 214 a	4,532 ± 68 a	4,080 ± 93 c	4,284 ± 175 b
168	5,939 ± 213 a	4,526 ± 68 a	4,380 ± 93 b	4,625 ± 175 a
224	6,078 ± 213 a	4,396 ± 68 ab	4,896 ± 93 a	4,840 ± 175 a

*Values are presented as mean ± standard error mean. Means presented within each column with same letters are not significantly different ($p \leq 0.05$).

Table 2.13. Test weight of soft white winter wheat (SWWW) and hard red winter wheat (HRW) at test sites in Cavendish and Cottonwood during the 2019-2020 and 2020-2021 growing seasons to evaluate the effectiveness of nitrogen rates.

	Cottonwood SWWW	Cottonwood HRW	Cavendish SWWW	Cavendish HRW
Nitrogen (kg/ha)	---------- Test Weight (kg/hl) ----------			
0	76.0 ± 0.6 a*	74.4 ± 0.2 a	74.7 ± 0.2 a	71.9 ± 0.2 ab
56	75.8 ± 0.6 a	74.3 ± 0.2 a	74.7 ± 0.2 a	72.3 ± 0.2 a
112	74.0 ± 0.6 b	73.7 ± 0.2 b	74.6 ± 0.2 a	72.2 ± 0.2 a
168	72.6 ± 0.6 c	72.8 ± 0.2 c	74.4 ± 0.2 a	71.5 ± 0.2 b
224	72.0 ± 0.6 c	72.9 ± 0.2 c	73.9 ± 0.2 b	71.9 ± 0.2 ab

* Values are presented as mean ± standard error mean. Means presented within each column with same letters are not significantly different ($p \leq 0.05$).

Table 2.14. Protein of soft white winter wheat (SWWW) and hard red winter wheat (HRW) at test sites in Cavendish and Cottonwood during the 2019-2020 and 2020-2021 growing seasons to evaluate the effectiveness of nitrogen rates.

	Cottonwood SWWW	Cottonwood HRW	Cavendish SWWW	Cavendish HRW
Nitrogen (kg/ha)	---------- Protein (%) ----------			
0	10.7 ± 0.3 d*	10.7 ± 0.2 e	9.2 ± 0.2 b	9.2 ± 0.2 b
56	10.8 ± 0.3 d	11.1 ± 0.2 d	9.1 ± 0.2 b	9.2 ± 0.2 b
112	12.1 ± 0.3 c	12.2 ± 0.2 c	9.3 ± 0.2 b	9.5 ± 0.2 b
168	13.6 ± 0.3 b	13.0 ± 0.2 b	9.9 ± 0.2 a	10.1 ± 0.2 a
224	14.2 ± 0.3 a	13.4 ± 0.2 a	10.1 ± 0.2 a	10.1 ± 0.2 a

* Values are presented as mean ± standard error mean. Means presented within each column with same letters are not significantly different ($p \leq 0.05$).

Table 2.15. Plant biomass yield at harvest of soft white winter wheat (SWWW) and hard red winter wheat (HRW) at test sites in Cavendish and Cottonwood during the 2019-2020 and 2020-2021 growing seasons to evaluate the effectiveness of nitrogen rates.

	Cottonwood SWWW	Cottonwood HRW	Cavendish SWWW	Cavendish HRW
Nitrogen (kg/ha)	---------- Plant Biomass Yield at Harvest (kg/ha) ----------			
0	465 ± 68*	308 ± 9 b	226 ± 12 c	242 ± 12 b
56	498 ± 69	319 ± 9 ab	235 ± 13 c	245 ± 12 b
112	503 ± 68	335 ± 9 a	273 ± 12 b	262 ± 12 b
168	557 ± 68	339 ± 9 a	307 ± 12 a	301 ± 12 a
224	560 ± 68	343 ± 9 a	313 ± 13 a	305 ± 12 a

* Values are presented as mean ± standard error mean. Means presented within each column with same letters are not significantly different ($p \leq 0.05$).

Table 2.16. Total nitrogen in grain at harvest of soft white winter wheat (SWWW) and hard red winter wheat (HRW) at test sites in Cavendish and Cottonwood during the 2019-2020 and 2020-2021 growing seasons to evaluate the effectiveness of nitrogen rates.

	Cottonwood SWWW	Cottonwood HRW	Cavendish SWWW	Cavendish HRW
Nitrogen (kg/ha)	---------- Total Nitrogen in Grain (kg/ha) ----------			
0	109 ± 4 b*	82 ± 1 c	55 ± 2 d	57 ± 2 c
56	117 ± 4 b	86 ± 1 c	58 ± 2 d	60 ± 2 c
112	128 ± 4 a	96 ± 1 b	65 ± 2 c	67 ± 2 b
168	128 ± 4 a	101 ± 1 a	73 ± 2 b	75 ± 2 a
224	136 ± 4 a	102 ± 1 a	83 ± 2 a	78 ± 2 a

* Values are presented as mean ± standard error mean. Means presented within each column with same letters are not significantly different ($p \leq 0.05$).

2.3.6 *Agronomy Correlation Analysis*

A correlation analysis was performed to examine the relationship between agronomic measurements and NDVI measurements. Many of the measurements compared had a strong positive correlations at both sites and both wheat varieties (Table 2.17, Table 2.18, Table 2.19, and Table 2.20). For both the SWWW and HRW at Cottonwood there was a significant correlation between each of the NDVI measurements with plant height, yield, test weight, plant biomass and total grain nitrogen. In nearly every case, these correlations increased with each successive NDVI measurement with the strongest correlation between these agronomic measurements and NDVI occurring during the last reading in early July. This correlation was particularly strong for yield and NDVI5 with a positive correlation of 0.963 for the SWWW and 0.941 for the HRW. The total grain nitrogen was also highly correlated to NDVI measurements, particularly for the NDVI5 measurement with a correlation of 0.967 for SWWW and 0.948 for HRW. The correlations between agronomic measurements and NDVI was similar at Cavendish, but the trend of increasing correlation with each successive NDVI readings did not hold for both crops. For the SWWW at Cavendish, the same increase in correlation between the yield and NDVI readings was observed with the highest correlation occurring with the July NDVI reading (0.962). However, for the HRW was not a significant correlation between yield and NDVI readings for NDVI1 and the strongest correlation was observed for the third wheat of July NDVI reading (NDVI4, 0.930). Total grain nitrogen was also highly correlated with NDIV5 for SWWW at Cavendish (0.540 to 0.964), but less so for the NRW (0.314 to 0.793).

Table 2.17. Correlation analysis for agronomic measurements and NDVI in soft white winter wheat (SWWW) at Cottonwood during the 2019-2020 and 2020-2021 growing seasons.

	†SC	Plant Height	Yield	†TWT	Protein	†PBY	†TNG	†NDVI1	†NDVI2	†NDVI3	†NDVI4	†NDVI5
†STC	-0.07544 0.5060	**0.57304** **<.0001**	**0.57346** **<.0001**	**0.67483** **<.0001**	**-0.46574** **<.0001**	**0.54756** **<.0001**	**0.50622** **<.0001**	**0.25955** **0.0201**	**0.36391** **0.0009**	**0.33952** **0.0021**	**0.33758** **0.0022**	**0.50936** **<.0001**
†SC		**0.52894** **<.0001**	**0.45841** **<.0001**	**0.16996** **0.1318**	**0.47939** **<.0001**	**0.40341** **0.0002**	**0.56319** **<.0001**	**0.61880** **<.0001**	**0.74002** **<.0001**	**0.78597** **<.0001**	**0.77398** **<.0001**	**0.58576** **<.0001**
Plant Height			**0.95804** **<.0001**	**0.83306** **<.0001**	**-0.17915** **0.1118**	**0.84504** **<.0001**	**0.95870** **<.0001**	**0.60538** **<.0001**	**0.84132** **<.0001**	**0.88297** **<.0001**	**0.89080** **<.0001**	**0.95182** **<.0001**
Yield				**0.89594** **<.0001**	**-0.29873** **0.0071**	**0.84790** **<.0001**	**0.97789** **<.0001**	**0.53699** **<.0001**	**0.80220** **<.0001**	**0.83390** **<.0001**	**0.85146** **<.0001**	**0.96328** **<.0001**
†TWT					**-0.63865** **<.0001**	**0.74274** **<.0001**	**0.81188** **<.0001**	**0.37289** **0.0007**	**0.57318** **<.0001**	**0.57469** **<.0001**	**0.60171** **<.0001**	**0.82494** **<.0001**
Protein						-0.21325 0.0575	-0.13232 0.2450	0.12571 0.2665	0.11235 0.3211	0.20031 0.0748	0.17670 0.1169	-0.14332 0.2047
†PBY							**0.83851** **<.0001**	**0.49025** **<.0001**	**0.70764** **<.0001**	**0.76371** **<.0001**	**0.76997** **<.0001**	**0.84767** **<.0001**
†TNG								**0.60736** **<.0001**	**0.85385** **<.0001**	**0.89805** **<.0001**	**0.91015** **<.0001**	**0.96651** **<.0001**

†Definitions of abbreviations: STC (stand count m^2); SC (spike count m^2); TWT (test weight kg/hl); PBY (plant biomass yield at harvest kg/ha); TGN (total nitrogen in grain kg/ha); NDVI1 (1^{st} week of May); NDVI2 (3^{rd} week of May); NDVI3 (1^{st} week of June); NDVI4 (3^{rd} week of June) NDVI5 (1^{st} week of July).

Table. 2.18. Correlation analysis for agronomic measurements and NDVI in hard red winter wheat (HRW) at Cottonwood during the 2019-2020 and 2020-2021 growing seasons.

	†SC	Plant Height	Yield	†TWT	Protein	†PBY	†TNG	†NDVI1	†NDVI2	†NDVI3	†NDVI4	†NDVI5
†STC	0.20942 0.0623	0.13073 0.2477	0.17475 0.121	**0.23743** **0.034**	-0.08467 0.4552	0.17259 0.1258	0.13413 0.2386	0.07997 0.4807	0.09708 0.3916	0.10258 0.3652	0.10962 0.3331	0.18279 0.1046
†SC		**0.84899** **<.0001**	**0.86043** **<.0001**	**0.65106** **<.0001**	0.05466 0.6301	**0.79002** **<.0001**	**0.8582** **<.0001**	**0.62925** **<.0001**	**0.76828** **<.0001**	**0.85634** **<.0001**	**0.8617** **<.0001**	**0.85077** **<.0001**
Plant Height			**0.93033** **<.0001**	**0.71302** **<.0001**	**0.00564** **0.9604**	**0.89391** **<.0001**	**0.9139** **<.0001**	**0.68151** **<.0001**	**0.80667** **<.0001**	**0.91249** **<.0001**	**0.92653** **<.0001**	**0.91469** **<.0001**
Yield				**0.8118** **<.0001**	-0.11275 0.3194	**0.93057** **<.0001**	**0.94275** **<.0001**	**0.73957** **<.0001**	**0.85551** **<.0001**	**0.92038** **<.0001**	**0.92675** **<.0001**	**0.94185** **<.0001**
†TWT					**-0.52716** **<.0001**	**0.77864** **<.0001**	**0.61451** **<.0001**	**0.52339** **<.0001**	**0.56482** **<.0001**	**0.6053** **<.0001**	**0.6087** **<.0001**	**0.68952** **<.0001**
Protein						-0.09714 0.3913	**0.22233** **0.0489**	-0.03305 0.771	0.12388 0.2736	0.20667 0.0659	0.21856 0.0514	0.07887 0.4868
†PBY							**0.87784** **<.0001**	**0.70904** **<.0001**	**0.81069** **<.0001**	**0.86413** **<.0001**	**0.87797** **<.0001**	**0.88074** **<.0001**
†TNG								**0.71102** **<.0001**	**0.87972** **<.0001**	**0.96983** **<.0001**	**0.97947** **<.0001**	**0.94808** **<.0001**

†Definitions of abbreviations: STC (stand count m^2); SC (spike count m^2); TWT (test weight kg/hl); PBY (plant biomass yield at harvest kg/ha); TGN (total nitrogen in grain kg/ha); NDVI1 (1st week of May); NDVI2 (3rd week of May); NDVI3 (1st week of June); NDVI4 (3rd week of June) NDVI5 (1st week of July).

Table 2.19. Correlation analysis for agronomic measurements and NDVI in soft white winter wheat (SWWW) at Cavendish during the 2019-2020 and 2020-2021 growing seasons.

	†SC	Plant Height	Yield	†TWT	Protein	†PBY	†TNG	†NDVI1	†NDVI2	†NDVI3	†NDVI4	†NDVI5
†STC	-0.17241 0.1262	**0.48055** **<.0001**	**0.63142** **<.0001**	**0.76809** **<.0001**	**-0.73098** **<.0001**	**0.41178** **0.0001**	**0.23442** **0.0364**	-0.11717 0.3006	-0.08708 0.4424	0.08043 0.4782	**0.55272** **<.0001**	0.00969 0.9527
†SC		**0.28306** **0.0110**	**0.29316** **0.0083**	**-0.22152** **0.0483**	**0.39511** **0.0003**	**0.25988** **0.0199**	**0.67227** **<.0001**	**0.60126** **<.0001**	**0.76840** **<.0001**	**0.74840** **<.0001**	**0.38744** **0.0004**	**0.91937** **<.0001**
Plant Height			**0.88197** **<.0001**	**0.65988** **<.0001**	**-0.52662** **<.0001**	**0.80356** **<.0001**	**0.66775** **<.0001**	**0.39627** **0.0003**	**0.54907** **<.0001**	**0.64121** **<.0001**	**0.87161** **<.0001**	**0.83647** **<.0001**
Yield				**0.78025** **<.0001**	**-0.59749** **<.0001**	**0.75916** **<.0001**	**0.77356** **<.0001**	**0.36897** **0.0008**	**0.50356** **<.0001**	**0.64007** **<.0001**	**0.95238** **<.0001**	**0.96233** **<.0001**
†TWT					**-0.92463** **<.0001**	**0.54998** **<.0001**	**0.25850** **0.0206**	-0.04792 0.6729	-0.06706 0.5545	0.08626 0.4468	**0.71533** **<.0001**	0.22016 0.1722
†Protein						**-0.39422** **0.0003**	0.03934 0.7290	0.13560 0.2304	**0.23116** **0.0391**	0.11660 0.3030	**-0.49613** **<.0001**	**0.64060** **<.0001**
†PBY							**0.63038** **<.0001**	**0.37654** **0.0006**	**0.47687** **<.0001**	**0.56439** **<.0001**	**0.75179** **<.0001**	**0.68750** **<.0001**
†TNG								**0.54015** **<.0001**	**0.78085** **<.0001**	**0.87282** **<.0001**	**0.79271** **<.0001**	**0.96448** **<.0001**

†Definitions of abbreviations: STC (stand count m^2); SC (spike count m^2); TWT (test weight kg/hl); PBY (plant biomass yield at harvest kg/ha); TGN (total nitrogen in grain kg/ha); NDVI1 (1^{st} week of May); NDVI2 (3^{rd} week of May); NDVI3 (1^{st} week of June); NDVI4 (3^{rd} week of June) NDVI5 (1^{st} week of July).

Table 2.20. Correlation analysis for agronomic measurements and NDVI in hard red winter wheat (HRW) at Cavendish during the 2019-2020 and 2020-2021 growing seasons.

	†SC	Plant Height	Yield	†TWT	Protein	†PBY	†TNG	†NDVI1	†NDVI2	†NDVI3	†NDVI4	†NDVI5
†STC	-0.09582 0.3978	**0.78873** **<.0001**	**0.74513** **<.0001**	**0.82693** **<.0001**	**-0.79615** **<.0001**	**0.65871** **<.0001**	**0.44721** **<.0001**	-0.05718 0.6144	**0.28895** **0.0093**	**0.44297** **<.0001**	**0.78251** **<.0001**	0.15626 0.3356
†SC		-0.06264 0.5810	0.01598 0.8881	**-0.24915** **0.0258**	**0.28813** **0.0095**	0.03118 0.7836	**0.30620** **0.0057**	-0.02081 0.8546	**0.47969** **<.0001**	**0.35359** **0.0013**	0.08575 0.4495	**0.82238** **<.0001**
Plant Height			**0.92625** **<.0001**	**0.87955** **<.0001**	**-0.81312** **<.0001**	**0.92032** **<.0001**	**0.70496** **<.0001**	0.13419 0.2353	**0.53110** **<.0001**	**0.63630** **<.0001**	**0.94664** **<.0001**	**0.83779** **<.0001**
Yield				**0.85654** **<.0001**	**-0.79639** **<.0001**	**0.88887** **<.0001**	**0.83318** **<.0001**	0.17290 0.1251	**0.57459** **<.0001**	**0.67879** **<.0001**	**0.92998** **<.0001**	**0.75735** **<.0001**
†TWT					**-0.95346** **<.0001**	**0.79480** **<.0001**	**0.48088** **<.0001**	0.03764 0.7403	0.20443 0.0689	**0.42975** **<.0001**	**0.85505** **<.0001**	**0.53319** **0.0004**
Protein						**-0.73750** **<.0001**	**-0.33577** **0.0023**	0.02244 0.8433	-0.11066 0.3285	**-0.32020** **0.0038**	**-0.77762** **<.0001**	**0.39209** **0.0123**
†PBY							**0.72225** **<.0001**	**0.23528** **0.0357**	**0.58165** **<.0001**	**0.63838** **<.0001**	**0.89449** **<.0001**	**0.81679** **<.0001**
†TNG								**0.31386** **0.0046**	**0.79391** **<.0001**	**0.76701** **<.0001**	**0.75135** **<.0001**	**0.79327** **<.0001**

†Definitions of abbreviations: STC (stand count m^2); SC (spike count m^2); TWT (test weight kg/hl); PBY (plant biomass yield at harvest kg/ha); TGN (total nitrogen in grain kg/ha); NDVI1 (1st week of May); NDVI2 (3rd week of May); NDVI3 (1st week of June); NDVI4 (3rd week of June) NDVI5 (1st week of July).

2.4 Discussion

Nitrogen stabilizers added to nitrogen fertilizer at planting can be an effective tool to prevent nitrogen loss and improve nitrogen use efficiency. In northern Idaho, a major loss of nitrogen is through leaching due to high annual rainfall. Therefore, field trials were established to determine the potential success of retaining nitrogen in the soil by applying nitrogen stabilizers with fertilizer in the at planting of winter wheat. Soil samples taken throughout the season demonstrated that conversion of ammonium to nitrate was being inhibited by the stabilizer. In both fall and spring samples there was a greater proportion of ammonium and lower nitrate in the plots treated with Instinct compared to those that were not. However, this did not appear to translate into an improvement in crop performance as yield and postharvest quality was similar between plots. As expected, there was a significant impact of nitrogen fertilizer rates on soil ammonium and nitrate concentrations as well as agronomic measurements.

In this study, soil ammonium retention was increased to a depth of 15 cm for both locations at all four sampling occasions with nitrogen stabilizer. In March, plots with nitrogen stabilizer retained more ammonium down to 30 cm for both locations. Below 30 cm the ammonium concentrations were very low due to positive charge of ammonium ion and immobility in the soil (Kowalenko and Cameron, 1976). Similar results with increased ammonium concentrations following the application of nitrogen stabilizers at the depth of 15 cm can be found in other studies. Ten trials in cereal and oil seed growing areas of Alberta, Manitoba, Saskatchewan, and Ontario, Canada, tested nitrapyrin products applied with anhydrous or urea in the fall and found that samples taken around May 1st maintained 51 to 63% more nitrogen in the ammonium form than soils treated with non-stabilized fertilizer (Degenhardt et al., 2016). In New Zealand the effect of the nitrification inhibitor DCD was evaluated in combination with cow urine on a grazed pasture system (Zaman et al., 2009). They found that DCD significantly held soil ammonium compared to urine alone in the top 20 cm. In the same study, the ammonium ions were mostly retained in the upper 10 cm of soil and decreased with time and depth. Zaman and Nguyen (2012) conducted another experiment in New Zealand on the application timings of urease and nitrification inhibitors and observed how they effected nitrogen loss from urine patches in the pastoral system. They found that autumn

application of DCD to the urine patches compared to the spring exhibited high soil ammonium concentrations in the top 5 cm of soil, and this soil ammonium concentration was significantly higher compared to treatments of urine alone. A study conducted on winter wheat in Missouri during 1991 and 1992 discovered nitrapyrin improved soil ammonium retention during the fall and the winter (Kidwaro and Kephart, 1998). In contrast, only one research trial conducted in Missouri that evaluated the effects of nitrapyrin and pronitridine on winter wheat soil, found that both nitrification inhibitors did not affect soil ammonium concentrations at soil depths of 0 to 15 cm and 16 to 30 cm over a period of three months after application (Habibullah et al., 2018).

Similarly, nitrate concentrations were mainly impacted at the 0 to 15 depth as well. At Cavendish, nitrate was decreased by nitrogen stabilizer at the 0 to 15 cm depth during November and March while at Cottonwood, a similar reduction at the 0 to 15 cm depth was observed in March and May. These findings are supported by previous studies that found that DCD slowed down nitrification and for autumn application significantly reduced nitrate leaching by 43% (Zaman et al., 2009; Zaman and Nguyen, 2012). In other studies, the nitrification rate was slowed when urea and UAN were stabilized with nitrapyrin (Instinct® II) which significantly reduced nitrate concentrations compared to the control (Peng et al., 2015; Thapa et al., 2015). In 1999, Goos and Johnson discovered that nitrapyrin banded with aqua ammonia in the fall yielded decreased nitrate concentrations in both fall and spring soil samples with larger overall total nitrogen concentration compared to aqua ammonia alone. Only one study has been found where nitrapyrin did not affect soil nitrate concentrations and reduction of nitrification (Habibullah et al., 2018).

Soil nitrate concentrations at Cavendish were reduced with nitrogen stabilizer treatment in the 0 to 15 and 15 to 30 cm depths most dramatically in the month of November, while the difference between treatments declined throughout the year. Data in the current study shows a decrease in nitrate and ammonium after fertilization throughout time. In the months of November, March and May, soil nitrate concentrations are similar across all depths. In contrast, the month of September shows a decrease in nitrate concentrations at the depths of 15 to 90 cm when compared to May concentrations. Since this is the primary rooting zone for winter wheat plants in this region, these nutrients have likely been utilized by the plants.

There have been few studies following nitrate leaching below 30 cm with and without the application of a nitrification inhibitor. However, many studies have stated that nitrate does leach past the 30 cm depth so it would be assumed that Instinct could influence the quantity of nitrogen traveling past this depth. In this study, there were no effects of Instinct on nitrate concentration below 15 cm compared to the control. These results could be related to the lack of precipitation that occurred in the spring in the 2021 growing season, although near normal precipitation was observed in 2020 with similar results. The lack of precipitation in 2021 could have limited nitrogen loss through leaching and minimized the benefit of nitrapyrin which was also found in Degenhardt et al (2016).

Despite the nitrogen stabilizer increasing ammonium retention and reducing the conversion to nitrate over the winter and spring months, there was no gain in the yield. Averaged across Cottonwood and Cavendish SWWW and Cottonwood HRW, the nitrogen stabilizer treatment only increased wheat yield by 47 kg/ha, and this increase was not statistically significant. Previous studies have shown mixed results with either slightly increased yields or no increase in yields from nitrapyrin application. Most yield comparisons with and without the addition of Instinct or Instinct II in UAN fertilizer have been in spring corn crop. These studies have found either reduced yields or a lack of response from treatment (Fernandaz, 2010; Cook et al., 2015; Barker and Sawyer, 2017; Franzen and NCERA-103 Committee, 2017; Maharjan et al. 2017; Sassman et al., 2018). On the other hand, a few articles under the same treatments with both spring and fall applications of nitrification inhibitors have stated increase in corn yields (Randall et al., 2003; Wolt, 2004). In a trial under summer waterlogged conditions, there was an increased grain yield for corn hybrids (22 to 33%) with application of nitrapyrin with fertilizers (Ren et al., 2017).

While the majority of the work with nitrogen stabilizers has been conducted in corn, there are several similar studies that have looked at winter wheat. A winter wheat study conducted in Lansing, MI, indicated that when urea plus a nitrification inhibitor was applied with nitrogen fertilizer to soft white winter wheat at a nitrogen fertilizer rate of 134.5 kg N/ha, wheat yield decreased 510 kg/ha. In the same study for the 161 kg N/ha treatment without nitrification inhibitor, yield decreased 520 kg/ha (Quinn and Steinke, 2019). Habibullah et al.

(2018) saw no winter wheat yield difference near Novelty, MI between Instinct II treatment and control with UAN as a nitrogen fertilizer.

Use of Instinct in this study did not influence any other agronomic measurements including plant stand, plant height, spike count, grain test weight and protein concentration. The only exception was a slight, but significant decrease in test weight and increase in protein for the HRW at Cavendish. Poor crop response to nitrogen stabilizer treatment may also be a result of the nitrogen source used in the trials. UAN-32 nitrogen fertilizer is 32% total nitrogen and contains 16.50% urea, 7.75% ammonium-nitrogen, and 7.75% nitrate-nitrogen. Since 7.75% of the fertilizer is already in the nitrate form, there may have been less of a response to the nitrification inhibitor. On the other hand, there was a response of ammonium retention in soil testing to nitrogen stabilizer with UAN-32, but perhaps not a large enough response to reflect in the agronomic results.

Another factor affecting the results may perhaps be the lack of rain that occurred in the PNW during the spring and summer of 2021. According to the U.S. Drought Monitor, more than 94% of the region was in drought Aug. 17, 2021. An article from Parma, ID stated that 2021 was one of the most severe droughts for the PNW with only 1924, 1931, 1977, and 1994 being drier (Ansah and Walsh, 2021). Previous work has indicated that lower than normal precipitation may influence crop performance and negate any benefits from using a nitrogen stabilizer. A study comparing the benefits of urea treated with and without stabilizer was conducted during 2013 and 2015 at Saginaw Valley Research and Extension Center near Richville, MI (Steinke and Bauer, 2017). Fertilizer was applied in the spring during the sugar beet planting. During the study, there was 20 to 23% lower precipitation compared to the 30-yr mean. The authors reported that sugar beet yield and quality data were not influenced by nitrification inhibitors because of the absence of nitrogen loss limited the usefulness of the inhibitors.

Another factor affecting the lack of field performance with Instinct II could be related to the formulation of the microencapsulation. Instinct® II is a polymer-encapsulated form of nitrapyrin that is ineffective until the nitrapryin is released form the microcapsule (Ferrel, 2012). Because of this, greater application rates of these polymer-encapsulated formulations

may be required to enhance inhibition of nitrification than the labeled rate (Ferrel, 2012; Franzen and NCERA-103 Committee, 2017; Maharjan et al., 2017). Additionally, cool soil temperatures (less than 15^{o}C) significantly decrease nitrification rates (Shammas, 1986). In this study, Instinct II was applied in the beginning of October when air temperatures were below 15^{o}C, and soil nitrification may have been slowing down. Another study in Western Australia found that the production of nitrate ions declined in a loamy sand soil as soil temperatures were reduced from 20^{o}C to 5^{o}C (Russell et al., 2002).

As expected, both soil nitrate and ammonium concentrations responded to nitrogen fertilizer rate in both years and locations of the study. At Cavendish, soil nutrient concentrations increased significantly as nitrogen rate increased for all four sampling times at the depth of 0 to 15 cm. However, at the depth of 15 to 30 cm, ammonium concentrations substantially declined and were only impacted by nitrogen rate for the months of November, March, and May. Below the depth of 30 cm, ammonium was virtually undetectable which can also be seen in Riley et al. (2001). Results of nitrogen rate treatments at Cottonwood were similar to those observed at Cavendish. However, differences between treatments were more significant and could be found at deeper depths. Soil ammonium concentrations were impacted by nitrogen rate to the depth of 30 cm in November and March, while samples collected in May and September were only significant to a depth of 15 cm. As in Cavendish, ammonium concentrations drastically declined below 30 cm.

The trends in nitrate and ammonium concentrations relative to nitrogen fertilizer rates in this current study are similar to those in previous work. In Ottawa, ON, Canada, soil ammonium, ammonium and nitrate concentrations increased with increasing nitrogen rate in a corn field with rates ranging from 30 to 900 µg N/m^2 (Ma et al., 2010). The authors sampled multiple times during the growing season at different depths and found that mineral ammonium and nitrate decreased over time and with depth. The decrease in ammonium and nitrate concentrations through deeper depths and across time can be supported by data from a study in 2011-2012 that measured ammonium and nitrate concentrations four times throughout the growing season and at four depths at 10 cm increments in Indiana on corn (Omonode and Vyn, 2013).

As nitrogen rates increased, there was a positive response in most of the agronomic measurements collected. The NDVI values increased significantly with increasing nitrogen for both locations and both wheat cultivars. The only occasion where treatments were not significantly different from each other was at Cottonwood for the first NDVI measurement in the beginning of May for both cultivars. This may have been caused by a lack of response due to the young age of the plants and difference of precipitation between the two years. The lack of response in the early growing season was not due nitrogen deficiency since all of the nitrogen fertilizer was applied in the fall and would have been available at that time. These increased NDVI values with greater rates of nitrogen may indicate better plant health and increased yield. As indicated by the increased correlation between NDVI and yield throughout the season, with the greatest correlation occurring in early July (the last measurement date). Several studies back this claim that NDVI values can be used to predict potential yield and monitor plant health (Jarocinska and Zagajewski, 2009; Thapa et al., 2019).

Wheat quality measurements such as grain protein concentration and grain TN increased with increasing nitrogen on most occasions expect test weight which decreased with greater nitrogen rates. Wheat yield at Cavendish and Cottonwood also increased with more nitrogen fertilizer applied except Cottonwood HRW 224 kg N/ha. The Cottonwood HRW yielded 130 kg N/ ha less for the 224 kg N/ha treatments than 168 kg N/ha treatment. Similar decreased test weights with more nitrogen applied can be seen in the study conducted in northern Idaho in 2015 at Genesee and Peck (Senefsky, 2017). In addition, Senefsky's findings on spike counts, plant height, and protein were similar to the results found in this study. The relationship between nitrogen fertilizer rate and the agronomic measurements grain yield, spike count, and plant biomass yield at harvest are also comparable to results is other previous studies (Halvorson et al., 1999; Halvorson et al., 2004; Dabin et al., 2015; Khan et al., 2020). Dabin et al. (2015) points out in their study that "increasing the number of spikes is an effective way to increase wheat yields". This claim was also demonstrated in many previous studies (Garcia del Moral et al., 2003; Moragues et al., 2006) and is supported by the strong correlation between yield and spike count in this current study.

As of December of 2020, according to the University of Wisconsin-Madison Extension, the price of UAN-32 nitrogen fertilizer was $0.84/kg, and the average price of Instinct II was

$28.19/hectare at a rate of 2.7 l/ha (Fertilizer Price Survey, 2020). The argument for the growers is that if they apply 10% more fertilizer or about 11.2 kg N/ha, it would be $18.35 per hectare cheaper than applying UAN nitrogen stabilizer to the normal amount of N applied. The average yield increase across Cottonwood and Cavendish SWWW and Cottonwood HRW was only 47 kg/ha with the addition of nitrogen stabilizer. With the cost of nitrogen stabilizer at $28.19/hectare the yield increase of winter wheat would need to be 136 kg/ha to break even. Based on the results from this study, the yield gain would not be sufficient to offset the costs. To attain the same yield gain with adding more nitrogen fertilizer compared to adding nitrogen stabilizer, a grower would need to add about 11 kg/ha more nitrogen. This would only cost $8.92 more with UAN 32 at the price of $0.84/kg. According to this model, a grower would save $19.27/ha just adding more nitrogen fertilizer rather than a nitrogen stabilizer (Figure 2.11).

The use of nitrogen stabilizer can be beneficial to retain ammonium in the soil for a longer period of time. However, the amount of ammonium retained may not be enough to reflect in the winter wheat yields of northern Idaho. On the other hand, protein in the HRW of Cavendish did benefit from Instinct application and would have provided the grower profit. Growers in the region may use this information to take the risk of increasing grain protein concentration in their wheat. However, the costs may outweigh yield the benefits. Future work needs to be conducted to study the use of multiple types of fertilizers and multiple types of nitrogen stabilizers to see which combination works best for the typical fertilizer application timing and various weathers of northern Idaho.

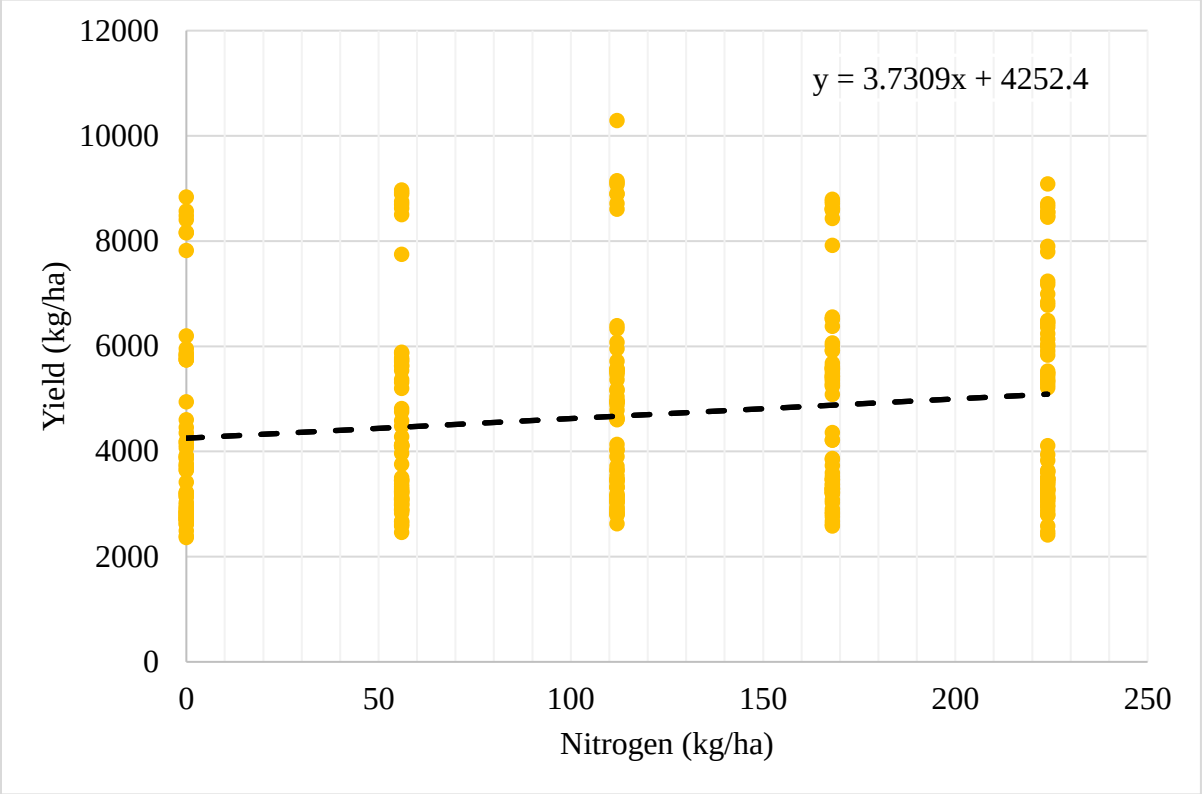

Figure 2.11. Simple linear regression of yield to nitrogen rate for the soft white winter wheat and the hard red winter wheat at Cottonwood and Cavendish, Idaho for the growing seasons of 2019-2020 and 2020-2021.

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Chapter 3: Investigating the Effect of Nitrogen Stabilizers and Increasing Nitrogen Fertilizer Rate on Nitrifying Bacteria and Archaea

3.1 Introduction

The Pacific Northwest (PNW) is an important region for agricultural production in the US. Wheat is the predominant crop grown in the region, producing 17% of the nation's wheat (Karimi et al., 2018). Other small grains, legumes, and canola are also grown in rotation with wheat. However, economic incentives, including commodity subsidies, farm programs, and global markets have pushed wheat production in the PNW (Kruger et al., 2017; Pan et al., 2017). Nitrogen is a critical fertilizer in wheat production and is a major factor influencing protein and winter wheat yield in the PNW (Wang et al., 2019). With heavy precipitation in fall to late spring, subsurface movement of water may cause significant loss of nitrate below the root zone through groundwater (Keller et al., 2008). Other factors such as nitrification, the conversion of ammonium/ammonia to nitrate by nitrifying microbes, also aid in the loss of nitrogen through leaching.

3.1.1 *Nitrogen Stabilizers Impact on Nitrifying Bacteria and Archaea*

To combat nitrogen loss through leaching, nitrogen stabilizers can be used to slow down the process of nitrification by inhibiting nitrifying organisms in the soil. Despite the potential benefits of using nitrogen stabilizers, there are questions about how these stabilizers impact the nitrifying bacteria and archaea populations. Several studies have been conducted in different types of soil that have mixed results on the population archaea and bacteria (Wolt, 2000; Liu et al., 2018; Clark et al., 2020; Hayden et al., 2021). Most studies agree with the fact that the application of nitrogen increase the population density of nitrifying bacteria without having an effect on the archaea populations (Okano et al., 2004; Taylor et al., 2012; Dai et al., 2013; Ouyang et al., 2017). In similar studies, the addition of nitrapyrin with the nitrogen fertilizer, either reduced or had no effect on the growth of archaea and bacteria (Fisk et al., 2015; Gu et al., 2018; Zhou et al., 2020). The Zhou et al study claimed that nitrapyrin had no effect on the ammonia-oxidizing archaea (AOA) while nitrapyrin inhibited ammonia-oxidizing bacteria (AOB). On the other hand, both Fisk et al. (2015) and Gu et al. (2018) saw no effect on the AOB after applying the nitrification inhibitor.

Studies that compare multiple nitrification inhibitors on nitrifying bacteria and archaea have also had contradicting results. A study led in 2020 on three typical paddy soils (black soil, red soil, and purple soil) found that across all three soils, AOA *amoA* gene copies were not depleted by the application of three types of nitrification inhibitors (dicyandiamide (DCD), 3,4-dimethylpyrazole-phosphate (DMPP), and nitrapyrin nitrapyrin (Zhou et al., 2020). In fact, for the DCD and DMPP treatments, AOA *amoA* gene copies numbers increased by day 28. However, the AOB *amoA* gene copies were significantly inhibited by all stabilizer treatments compared to urea alone except in the black soil paddy in which nitrapyrin had low inhibitory efficiency on AOB. Papadopoulou et al (2020) found that DCD and DMPP were most effective at inhibiting AOB while AOA and AOB had equal sensitivity to NP. The same study claimed that nitrapyrin had the most potency concerning AOA inhibition while nitrapyrin and DMPP were most effective against AOB compared to DCD.

3.1.2 *Nitrogen Fertilizer Rates Impact on Nitrifying Bacteria and Archaea*

Most studies support the theory that AOB *amoA* gene copy number increases with the addition of nitrogen fertilizers. However, the same cannot be said for AOA (Levičnik-Höfferle et al., 2012; Xu et al., 2012; Chen et al., 2013). A study observing the response of ammonia oxidizers to different fertilizer inputs found that fertilization did not increase AOA populations and that gene copies were only significantly affected by soil depth (Li et al., 2021). Other studies even found a decrease in AOA population size after the addition of inorganic fertilizers (Shen et al, 2011). A study on mineral fertilizer rate effect on AOA found that population size decreased after fertilizer treatments which were 75 kg/ha N and 75 kg/ha P_2O_5 in the form of urea and superphosphate, respectively (Fan et al., 2011).

3.1.3 *Objectives*

The population of nitrifying bacteria and archaea responsible for nitrification in agricultural soils are questionably impacted by nitrogen stabilizers and increasing nitrogen fertilizer rates. Growers in the PNW are interested in using nitrogen stabilizers to limit nitrogen fertilizer losses, but some are concerned about the impact on soil microorganisms. To address these concerns, a study was conducted to monitor the abundance of AOB and AOA after the

application of nitrapyrin mixed with UAN-32 at different nitrogen rates. Abundance of AOB and AOA was measured through the concentration *amoA* gene copy number.

3.2 Methods and Materials

3.2.1 *Experimental Design*

The experimental design for this study was a randomized complete block design with two treatments. One treatment consisted of five rates of nitrogen fertilizer (0, 56, 112, 168, and 224 kg N/ha) each with and without the second treatment Instinct® II (Corteva Agriscience) nitrogen stabilizer. The soft white winter wheat LSC Hulk was planted with four replications of each combination of treatments totaling forty plots at each location. The experiment was conducted at Cottonwood and Cavendish, ID during the growing years of 2019-2020 and 2020-2021. See chapter 2 for more details on study sites and trial design.

3.2.2 *Sampling*

Soil samples were collected in the soft white winter wheat plots in the fall, seven weeks after planting, and in early spring when the soil temperature was between 0 and 4^{o}C from three sites within each plot for all treatments and replications outlined in the previous section. Cores were removed from the center of the row to depth of 0 to 15 cm, homogenized and placed on ice for transportation back to the laboratory. Soil was stored in a 30 ml centrifuge tube at -20^{o}C.

3.2.3 *Generation of Standards for Quantitative PCR*

To create bacterial and archaeal *amoA* gene copy standards for qPCR, DNA was extracted from two soil samples following the DNeasy® PowerSoil® Pro Kit (Qiagen Hilden, Germany) protocol. Bacterial and archaeal *amoA* genes were PCR amplified. For bacterial *amoA* gene amplification, primes used were amoA-1F (5’ GGGGTTTCTACTGGTGGT-3’) and amoA-2R (5’CCCCTCKGSAAAGCCTTCTTC-3’) with a fragment length of 491 bp (Rotthauwe et al., 1997). For archaeal *amoA* gene amplification, primers used were Arch-amoAF (5’-STAATGGTCTGGCTTAGACG’3) and Arch-amoAR (5’-GCGGCCATCCATCTGTATGT-3’) with a fragment length of 635 bp (Francis et al., 2005). Each 20 µl PCR reaction contained 4 µl 5x PCR buffer, 1.2 µl of 2 mM dNTPs (Thermo Fisher

Scientific, Grand Island, NY, USA), 1.3 µl of each forward and reverse prime (10 µM stock solution), 0.2 µl of GoTaq DNA Polymerase (Promega, Madison, WI, USA), 1 µl of template DNA, and 11 µl of PCR water. DNA was amplified using an Eppendorf Ag Thermocycler (Eppendorf, Hamburg, Germany) with settings as follows: 1 cycle of 94°C for 3 min; 30 cycles of 94ºC for 30 s, 52 ºC for 30s, and 72 ºC for 30s; 1 cycle of 72 ºC for 5 min; and hold indefinitely at 15°C. Resulting PCR products were run on a 1% electrophoresis gel with ethidium bromide (10 mg/ml) at 100 V for 1 hr adjacent to a 100 bp ladder (100 bp+ Gene Ruler, Thermo Fisher Scientific, Grand Island, NY, USA) with negative and positive controls to confirm the presence of amplified DNA.

Transformations were completed for both the bacterial and archaeal samples using the TOPO® TA Cloning® Kit with One Shot® TOP10 Chemically Competent *E. coli* (Life Technologies). Cloning was completed as described in the kit protocol. Rather than shaking the transformed cells at 37ºC for 1 hr, cells were mixed gently every 15 min for 1 hr during incubation at 37ºC. Selective Luria-Bertani (LB) plates (5 g Bacto-Tryptone, 2.5 g Bacto-Yeast Extract, 2.5 g NaCl, 7.5 g agar, 0.25 g kanamycin) were prepared. After the LB medium had solidified, 40 µl X-gal stock solution (40 mg X-gal, 2 ml dimethylformamide) and 4 µl of IPTG stock solution (200 mg isopropylthio-β-D-galactoside, 1 mL sterile water) were spread evenly on each plate. For each bacterial, archaeal, and control transformation, 10 µl of cells were spread on one prewarmed selective LB plate and 50 µl on an additional four prewarmed plates. For the 10 µl volume of transformation, 20 µl of S.O.C. medium was added to the plate at the same time to ensure even spreading. Colonies were then incubated overnight at 37ºC. After overnight incubation, white colonies were screened from the blue colonies on the archaeal and bacterial LB plates. The white colonies were transferred to another LB medium plate containing 50 µg/ml kanamycin with 20 mg/ml X-gal, and 200 mg/ml IPTG. For each set of clones, 25 colonies were transferred by sterile toothpick to the same selective plate and were incubated overnight at 37ºC. The second overnight cultures were screened for white colonies from the blue colonies again then placed in culture tubes containing 3 ml of LB broth with 50 µg/ml kanamycin (5 g Bacto-Tryptone, 2.5 g Bacto-Yeast Extract, 2.5 g NaCl, 0.25 g kanamycin) using a sterile toothpick. Culture tubes were placed in a 37ºC incubator and shook at about 150 rpm overnight.

A well-established plasmid mini prep extraction protocol (Sambrook et al., 1989) was used to isolate the plasmid DNA from the overnight cultures grown in the LB broth. The isolation began by placing 1 ml of the overnight culture into an Eppendorf tube and centrifuging at 15,000 rpm with a Thermo IEC Micromax RF Microcentrifuge 3593 (Thermo Electron Corporation, Milford, MA, USA) for 30 seconds. The supernatant was aspirated off and the pellet resuspended in 200 µl of Solution I (10 ml 0.5 M EDTA, 490 ml sterile water). 400 µl of Solution II (0.8 g NaOH, 1 ml 10% SDA, 99 ml sterile water) was added along with 300 µl of Solution III (122.7 g KOAc, 71.5 ml acetic acid). The tubes were mixed by inversion. Samples were then centrifuged at 16,000 rpm for 10 minutes at room temperature. 750 µl of supernatant was the carefully removed to another tube and 750 µL of isopropanol was added. Each sample was centrifuged again at 16,000 rpm for 5 minutes at room temperature. Supernatant was aspirated off and pellets were washed twice with 70% EtOH and dried. After drying, pellets were resuspended in 50 µl of sterile water. DNA was then PCR amplified, and presence confirmed using electrophoresis as previously described.

Clones with the proper *amoA* gene inserts were confirmed by sequencing. Eight samples of the bacterial and archaeal *amoA* gene PCR products were purified using 1 µl ExoSAP-IT PCR Product Cleanup Reagent (Applied Biosystems, Thermo Fisher Scientific, Grand Island, NY, USA) and 2.5 µl of PCR product in a 1.5 ml centrifuge tube. Each tube was incubated at 37^{o}C in a dry bead bath for 15 minutes then incubated another 15 minutes at 80 ^{o}C. Once the samples cooled to room temperature, 14 µl of PCR water was added to the tubes. To prepare the DNA sequencing samples, *amoA* gene sequencing reactions were made using 1 µl of PCR product, 2.5 µl of 3.2 pmol stock solution forward primers (amoA-1F or Arch-amoAF), and 9.5 µl of PCR water to a final volume of 12.5 µl. Sequencing was performed by Elim Biopharmaceuticals, Inc. (Hayward, CA, USA). Using Basic Local Alignment Search Tool (BLAST) through the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi), sequences were analyzed and compared to authenticated *amoA* gene bacterial and archaeal sequences. One sample from each organism was chosen to become the standard control for qPCR.

The two clones chosen for bacterial and archaeal standards were grown at 37^{o}C overnight on prewarmed LB plates containing 50 µg/ml kanamycin with 20 mg/ml X-gal, and

200 mg/ml IPTG. Once the *E. coli* colonies were large enough, each sample was scraped with a sterile toothpick and placed in a 250 ml flask containing 25 ml of LB broth with 50 µg/ml kanamycin. Cultures were placed in a 37°C incubator and shook at about 150 rpm overnight. DNA plasmid was extracted from the overnight culture using the QIAprep® Spin Miniprep Kit (Qiagen Hilden, Germany) per the manufacturer's instructions. Multiple extractions of DNA plasmid from each culture were then pooled to acquire a greater concentration of DNA. Presence of DNA plasmid was confirmed using electrophoresis as previously described. Length of the plasmids with vector inserted for bacterial clones was 4,422 bp and for archaeal clones was 4,566 bp. DNA copy number was found using a nanodrop. Concentration of each sample was measured three times then averaged. Copy number was found using the following equation: $number\ of\ copies = \frac{Xng \times 6.0221 \times 10^{23}\ molecules/mole}{\left(N \times 660\frac{g}{mole}\right) \times 1 \times 10^{9} ng/g}$. Plasmid DNA was diluted to create standard concentrations for qPCR that included 10-fold serial dilutions from 5 to 5 x 10^6 copies for each organism.

3.2.4 *DNA Soil Extraction*

Soil samples collected from the field and stored in centrifuge tubes at -20°C were thawed and a representative sample was extracted as previously described. Once the DNA was extracted, samples were cleaned with polyvinylpolypyrrolidone (100 mg PVPP/1ml sterile water). For each sample, 50 µl of suspended PVPP mixture was dispensed into 2 ml Eppendorf tubes using cut pipet tips. The PVPP was then centrifuged for 3 minutes at 10,400 rpm. Water from the pellet was discarded and 50 µl of the DNA extract was mixed by flicking the tubes. DNA samples were incubated with the cleaned PVPP for 3 min at room temperature then centrifuged at 10,400 rpm for 3 min. The supernatant containing the cleaned DNA was transferred to a new 1.5 ml tube. This final centrifugation step was repeated to remove any residual PVPP. Due to the high concentration of DNA in the samples, all samples were diluted at 1:10 prior to qPCR.

3.2.5. *Real-time qPCR*

Real-time qPCR was performed to quantify the bacterial and archaeal *amoA* gene copies using the ready-to-use FastStart Essential DNA Green Master mix (Roche Molecular

Systems, Incorporated Pleasanton, California) with the Light Cycler 96® Instrument (Roche Diagnostics Corporation Indianapolis, Indiana). Reactions were made following the FastStart Essential DNA Green Master kit protocol with the specific nitrifying archaea and bacteria primers as previously mentioned. The quantity of DNA extracted from the soil samples used in the unknown reaction samples was 1µl. Standard reactions contained 5 µl of the stock solutions of known the copy numbers. The parameters for quantifying bacterial *amoA* gene copies were as follows: 1 cycle at 95^{o}C for 600 s; 45 cycles at 95^{o}C for 30 s, 56^{o}C for 30 s, 72^{o}C for 30 s; 1 melting cycle at 95^{o}C for 10 s, 65^{o}C at 60 s, 97 ^{o}C for 1 s; and 1 cooling cycle at 37^{o}C for 30 s. The parameters for quantifying archaeal *amoA* gene copies were the same with the exception of using an annealing temperature of 52^{o}C for 60 s during the amplification phase of the reaction (Fisk et al., 2015). Analysis of data was conducted using the Light Cycler 96® software 1.1. Absolute quantity of the samples was measured and analyzed as well as Tm calling to verify the PCR product.

3.2.6. *Quantitative and statistical analysis*

Data from the study were analyzed using SAS version 9.4 (SAS Institute, Inc., Cary, NC). Bacterial and archaeal *amoA* gene copy numbers were subjected to analysis of variance (ANOVA) using a generalized linear mixed model (PROC GLIMMIX). Bacterial and archaeal *amoA* gene copy number from both locations were analyzed separately, but years were analyzed together. Normal random effects were assumed. Significant differences between treatments were identified using differences of least squares means with an alpha value of 0.05. Normality and homogeneity of variance assumptions were determined with PROC UNIVARIATE and PROC GLIMMIX respectively. The data were analyzed using the statistical model outlined below:

$$y_{ijkl} = \mu + r_i + \alpha_j + w_{ij} + \beta_k + (\alpha\beta)_{jk} + \gamma_l + (\alpha\gamma)_{jl} + (\beta\gamma)_{kl} + (\alpha\beta\gamma)_{jkl} + s_{ijkl}$$

Where y_{ijkl} is the expected response for the *i*th block/replicate, the *j*th nitrogen treatment, and the *k*th nitrogen stabilizer treatment; μ is the grand mean; α_j and β_k are the fixed effects of the *j*th nitrogen treatment and the *k*th nitrogen stabilizer treatment respectively; r_i is the random effect of the *i*th block/replicate, NID(0, σ_r^2); w_{ij} is the random whole plot error term, NID(0, σ_w^2); $(\alpha\beta)_{jk}$ is the fixed interaction between nitrogen and nitrogen stabilizer

treatments; γ_l is the fixed effect of the *l*th year; $(\alpha\gamma)_{jl}$ is the fixed interaction between nitrogen treatment and year; $(\beta\gamma)_{kl}$ is the fixed interaction between nitrogen stabilizer treatment and year; $(\alpha\beta\gamma)_{jkl}$ is the random three-way interaction, NID(0, $\sigma_{\alpha\beta\gamma}^2$); and s_{ijk} is the random split plot error term, NID(0, σ_s^2).

3.3 Results

3.3.1 *Nitrogen Stabilizer Impact on Nitrifying Bacteria and Archaea.* The response of bacterial and archaeal *amoA* gene copy numbers to the nitrogen stabilizer treatment were measured to observe potential negative impact on nitrifying organism population to Instinct® II. The gene copy number for *amoA*-producing archaea and bacteria did not differ between treatments in the fall sampling at Cottonwood, ID (Fig. 3.1). However, in the spring, plots treated with nitrogen stabilizer had a significantly higher (p-value of 0.0498) archaea gene copy number. The bacterial gene copy number in the spring was reduced by 12% in plots treated with nitrogen stabilizer, although this difference was not significant (*p*-value of 0.0605). The gene copy number for bacteria was higher in the spring compared to the fall while gene copy number was similar at both sampling times for archaea. In Cavendish, ID, the nitrogen stabilizer did not impact gene copy number of archaea, although the copy number was about 29% higher in the fall compared to the spring (Fig. 3.2). Bacterial gene copy number was similar between treatments in the fall, but in the spring, plots treated with nitrogen stabilizer had a significantly lower gene copy number (*p*-value = 0.0008). The gene copy number was also substantially higher in the spring compared to the fall for bacterial *amoA* at Cavendish.

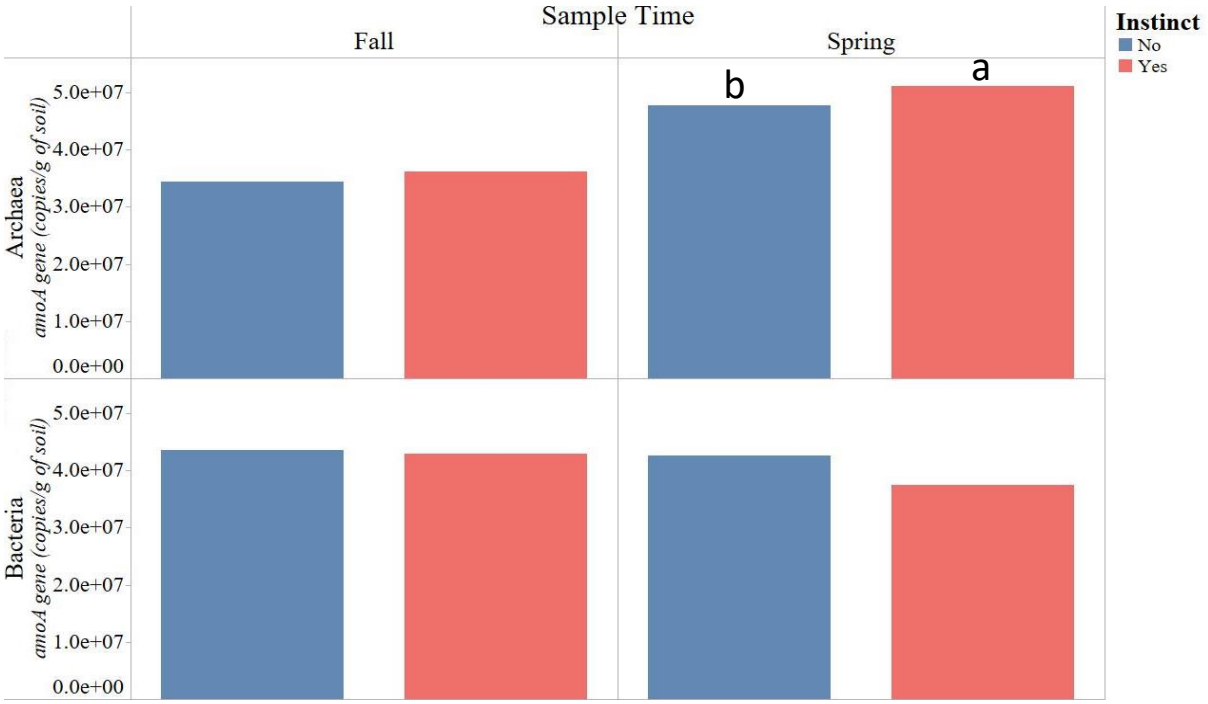

Figure 3.1. Archaea and bacteria *amoA* gene copy number following nitrogen stabilizer treatment in 2020 and 2021 at Cottonwood, ID. Means presented for each bar with different letters are significantly different ($p \leq 0.05$).

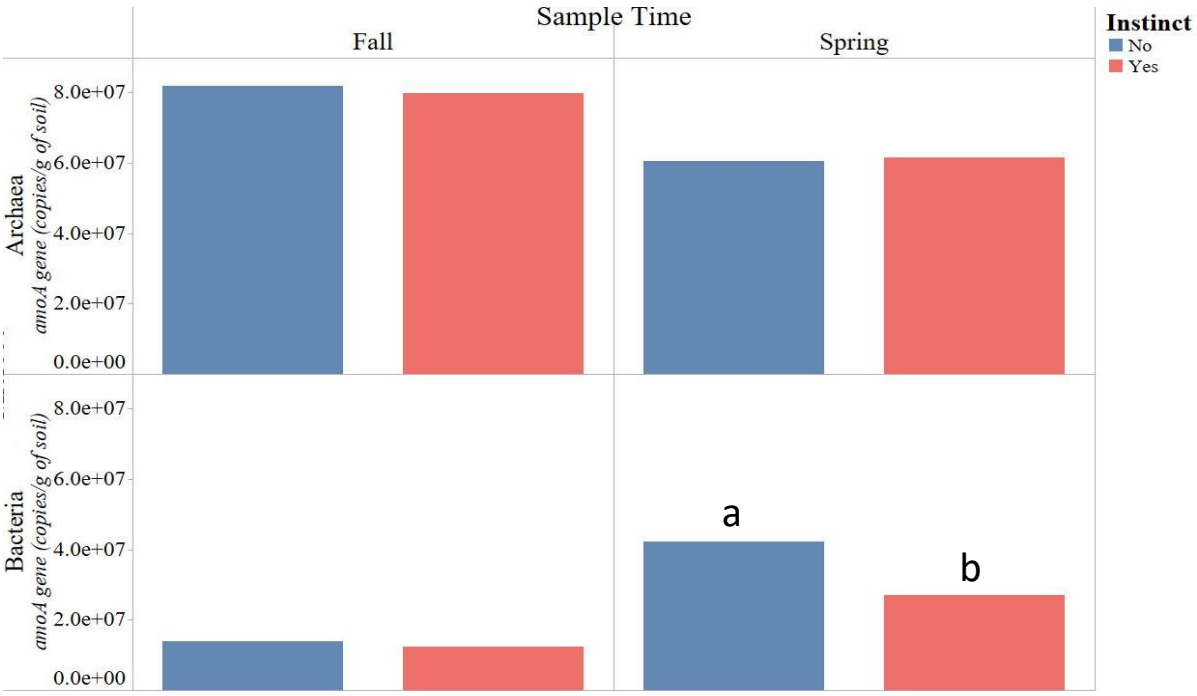

Figure 3.2. Archaea and bacteria *amoA* gene copy number following nitrogen stabilizer treatment in 2020 and 2021 at Cavendish, ID. Means presented for each bar with different letters are significantly different ($p \leq 0.05$).

3.3.2 *Nitrogen Rate Impact on Nitrifying Bacteria and Archaea.* Bacterial and archaeal *amoA* gene copy number was measured to track the change in population density with increasing nitrogen fertilizer rate. In both Cottonwood and Cavendish, there was a significant impact of nitrogen fertilizer rate on bacterial gene copy number for the spring sampling time with *p*-values of 0.0028 and 0.0193, respectively (Figures 3.3 and 3.4). As the nitrogen fertilizer rate increased, the bacterial gene copy number increased. Similar to the nitrogen stabilizer treatment, there was an increase in bacterial gene copy number in the spring compared to the fall at Cavendish. There was no impact of nitrogen rate on bacteria in the fall. Gene copy number for archaea was not significantly impacted by nitrogen fertilizer rate in either the spring and fall for both locations.

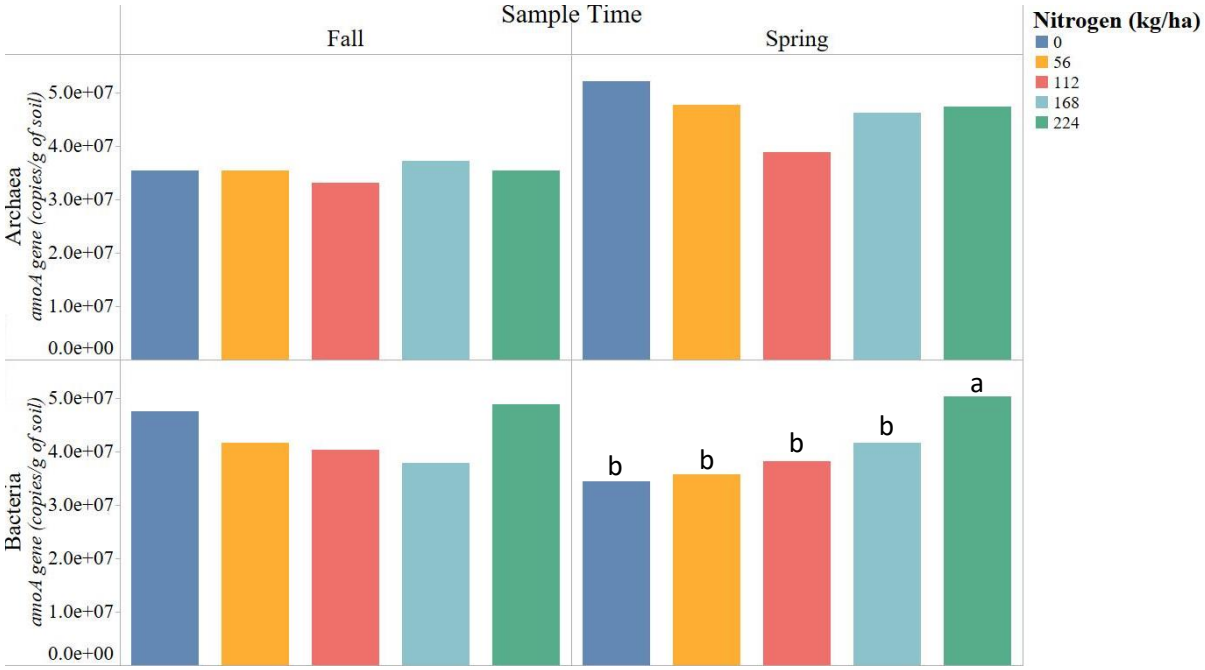

Figure 3.3. Influence of nitrogen fertilizer rate on archaeal and bacterial *amoA* gene copy number during 2020 and 2021 at Cottonwood, ID. Means presented for each bar with same letters are not significantly different ($p \leq 0.05$).

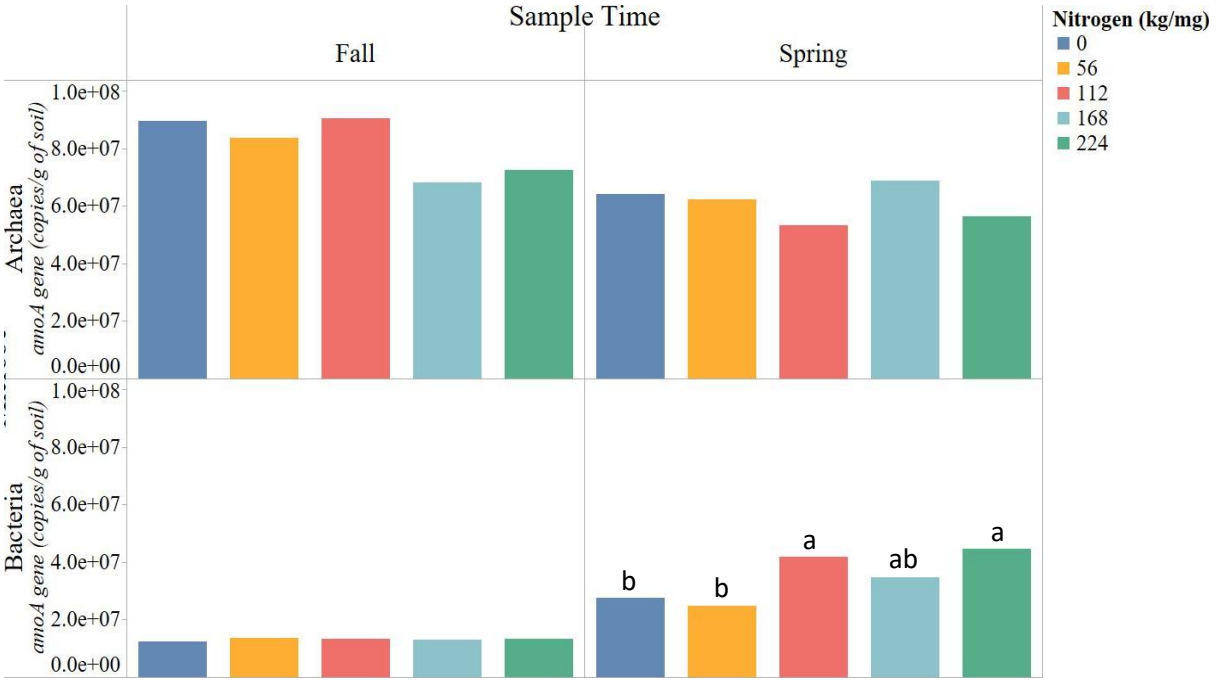

Figure 3.4. Influence of nitrogen fertilizer rate on archaeal and bacterial *amoA* gene copy number during 2020 and 2021 at Cavendish, ID. Means presented for each bar with same letters are not significantly different ($p \leq 0.05$).

3.4 Discussion

In this study, the impact of nitrogen stabilizers and nitrogen fertilizer rates on populations of ammonia oxidizing bacteria and archaea was examined. The effect of the addition nitrogen stabilizers, specifically nitrapyrin, with nitrogen fertilizer on nitrifying organisms in unclear. Additionally, studies exploring how *amoA* gene abundance of ammonia-oxidizing bacteria and archaea respond to nitrogen addition vary considerably. Nitrifying soil archaea and bacteria *amoA* gene copy numbers were quantified under five nitrogen fertilizer rates with and without the nitrification inhibitor Instinct.

For the nitrification inhibitor treatment, AOA populations at Cottonwood were higher in the spring samples compared to the fall. There also were significantly greater AOA gene copy numbers in the spring in plots treated with the nitrogen stabilizer. The converse was true for AOA populations at Cavendish in that spring gene copy numbers were lower than fall numbers. This suggests that conditions were more conducive for nitrification in the spring and that there was a greater potential for conversion of ammonium in the spring compared to the

fall. However, the gene copy number of AOA was not influenced by nitrapyrin. While the results were somewhat mixed, there was not much response of AOA populations to the treatments. Tao et al. (2021b) found that AOA were resistant to nitrapyrin, and that abundance was greater than AOB after nitrapyrin treatment. Somestudies even suggest that nitrapyrin is not effective towards AOA (Cui et al., 2013; Shen et al., 2013). The opposite was said for Tao et al. (2021a) and Gu et al. (2018) were they found that nitrapyrin addition significantly inhibited AOA growth and gene abundance.

For global populations, there was a large increase in bacteria copy number in the spring compared to the fall at Cavendish. However, at Cottonwood bacterial populations were similar. This is consistent with other studies in which populations behaved differently at the locations (Schuster et al., 2006; He et al., 2007; Nicol et al., 2008; Huang et al., 2021). In this current study at both locations, there was a decrease in AOB gene copy number in plots treated with a nitrification inhibitor, although this difference was significant only at Cavendish. This same decrease in AOB gene copy number is seen in multiple studies where AOB gene copy was reduced by nitrapyrin addition (Cui et al., 2013; Hayden et al., 2021; Tao et al., 2021a; Tao et al., 2021b; Ramotowski and Shi, 2022). The soil in both the Tao et al. (2021a and 2021b) experiments were calcareous (2% calcium carbonate, sand to clay texture), fluvisols (very young soil, coarse sand to heavy clay texture), and ferralsols (deeply weathered, clay soil). Other studies tested AOB abundance after nitrapyrin addition in multiple textures of soil including heavy clay, sand, and clay loam with high organic matter (Cui et al., 2013; Hayden et al., 2021). All of the soils in these experiments expect for the clay loam/black had a decrease in AOB gene copy abundance after nitrapyrin application.

Much of this inconsistency can be explained by the soil environment and characteristics. For example, in the Zhou et al. (2020) study, nitrapyrin may have had a low effectiveness on the inhibition *amoA* gene copies in the high soil organic matter (SOM) of black soil because of the high absorption rate of nitrapyrin to the SOM causing low availability. Zhang et al. (2020) observed a similar phenomenon in that as the SOM decreased, there was a corresponding decrease in adsorption of applied stabilizers. They observed that black soil (45 g/kg of SOM), had the greatest adsorption capacity of nitrapyrin at different temperatures.

Volatilization can also occur with high soil moisture when stabilizers are applied to the surface (Wolt, 2000).

In most cases, the gene copy number for both AOA and AOB was similar in the spring or higher in the spring relative to the fall. The exception was at Cavendish where a decrease in AOA gene copy number was observed in the spring compared to the fall. This may be explained by AOA's higher affinity for NH_3 than AOB, making AOB a stronger competitor in the presence of increased substrate concentration (Hatzenpichler et al., 2008; Marten-Habbena et al., 2009; Schleper, 2010; Prosser and Nicol, 2012; Beeckman et al., 2018). Therefore, AOB populations would readily oxidize the ammonium in inorganic fertilizers and be stimulated while AOA abundance would be suppressed or inhibited (He et al., 2012; Norman and Barrett, 2016). When examining inorganic ammonium quantities in Chapter 2, when ammonium levels were high, the AOA gene copy number decreased in the spring samples at Cavendish.

The lack of significant response of AOB to the nitrification inhibitor treatment in Cottonwood compared to Cavendish may be explained by the differences in soil characteristics at the two sites. Before planting in the fall, 60 cm deep soil samples were taken to document the nutrient concentration, pH, and OM of the soil before applying fertilizer. Cottonwood soil tests demonstrated higher OM content at this location than Cavendish as well as more residual nitrogen and potassium. Average pH was similar at both locations. The higher percentage of OM in the soil and the diminished negative response of bacterial *amoA* gene copy number to nitrogen stabilizer treatment at Cottonwood may support the claim that nitrapyrin is highly absorbed by OM and therefore inhibitory effectiveness is decreased (Jacinthe and Pichtel, 1992; Wolt, 2000) However, these observations could be due to other environmental factors such as soil texture, soil pH, rainfall events, historic management practices, competition from other microbial communities, and residual nutrients present in the soil before planting (Di and Cameron, 2011; Kleineidam et al., 2011; Shi et al., 2016; Gu et al., 2018; Fu et al., 2018; Chen et al., 2019; Fan et al., 2019).

The AOA gene copy number was not significantly influenced by nitrogen fertilizer rate at either location. However, there was a significant increase in AOB gene copy number with increasing nitrogen rate at both locations, with the 224 kg/ha nitrogen rate resulting in the

highest AOB gene copy number. In a previous study, Shen et al. (2011) observed the effect of increasing nitrogen loading levels (0 to 64 g N/m^2 per year) on AOA and AOB abundance in a semiarid grassland. They found that AOB abundance increased with higher nitrogen loading rates after the addition of 4 g N/m^2 per year. In the same study, the AOA *amoA* gene copy numbers remained unchanged after the addition of urea. The only exception to the change of AOA gene copy number was the treatment of 64 g N/m^2 per year which significantly lowered the gene copy numbers (Shen et al., 2011). Li et al. (2021) also saw an increase in AOB abundance after the addition of inorganic NPK which agrees with other studies (Wang et al., 2014; Xue et al., 2016).

To add to the lack of knowledge and uncertainty on soil AOA, scientists are questioning not only what family to categorize species of AOA but which phylum of archaea that AOA belong to. In 2008, a third archaeal phylum was proposed for mesophilic crenarchaeota, the Thaumarchaeota (Brochier-Armanet et al., 2008). These mesophilic crenarchaeota lack the typical hyperthermophilic crenarchaeal characteristics (Brochier-Armanet et al., 2008). Interestingly, the current distinct signature of archaea organized in the proposed phylum Thaumarchaeota is the ability to oxidize ammonium (Stieglmeier et al., 2014).

The AOA and AOB populations were investigated after the application of nitrapyrin primarily because of the concern among growers that the nitrification inhibitor could negatively impact the abundance of these nitrifying bacteria. The investigation showed that AOA are not impacted by nitrapyrin or the addition of nitrogen fertilizer. On the other hand, the data shows that AOB are sensitive to nitrapyrin. However, it could be argued that the stimulation of nitrogen fertilizer on AOB abundance could be stronger than the deleterious effect of the nitrapyrin. From these observations, a negative impact on the soil microbial community structure could be debated, though the effects are marginal.

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Chapter 4: Conclusion and Further Research

Nitrogen fertilizer loss prevention for fall applications can be difficult especially in systems where spring application can be nearly impossible due to wet conditions. There are multiple ways that nitrogen is lost to the environment such as gaseous loss of nitrogen to the atmosphere as nitrous oxide or ammonia and through groundwater by leaching or horizontal runoff. In high rainfall zones or heavily irrigated fields, nitrate loss through leaching can be a major source of nitrogen loss. A remedy to this problem is to reduce nitrification, nitrogen transformation of ammonium to nitrate by nitrifying organisms in the soil. Nitrification can be inhibited through nitrogen stabilizers which slow the process of nitrification by bacteria for a period of time. However, there has been speculation on the effectiveness of nitrogen stabilizers to improve crop quality and on the impact nitrogen stabilizers may have on the microbial community. To verify nitrification inhibitors efficacy, trials need to be established in different environments to track nitrogen movement through the soil as well as its effect on crop performance. Additionally, potential impact on microbial health needed to be monitored through nitrifying bacterial and archaeal populations.

Through field trials conducted during the 2019-2020 and 2020-2021 growing seasons, nitrate and ammonium soil concentrations were measured during the cropping year throughout the soil profile after the application of five nitrogen rates, each with and without a nitrification inhibitor. Soil nitrate and ammonium concentration increased with increasing nitrogen rate. Ammonium concentrations were significantly greater when an inhibitor was used than without, but only in the top 0 to 15 cm and occasionally at the 15 to 30 cm depth. Nitrate concentrations were significantly reduced by the inhibitor but only to the 30 cm depth as well.

Crop performance was monitored after the five rates of nitrogen fertilizer with and without nitrogen stabilizer. Nitrogen rate treatment had a large impact on all agronomic data that was recorded, with significantly higher yields and test weight with increasing nitrogen rate. Despite reduced nitrification when nitrogen stabilizers were present, there was not a yield benefit when using the nitrogen stabilizer for any of the trials and other agronomic measurements were not consistently impacted. Test weight was significantly lower and protein was significantly higher only at Cavendish for the HRW following the application of

nitrification inhibitor, although the differences were small. Plant biomass and total nitrogen in the grain were significantly higher when using the inhibitor, but only at Cavendish for the SWWW.

Nitrifying archaeal and bacterial populations were evaluated after the application of the five nitrogen fertilizer rates with and without nitrogen stabilizer. Archaeal populations for the fall and spring sampling were similar in Cottonwood and Cavendish across the nitrogen rates. Bacterial populations were unaffected in fall samples, but at both locations in the spring bacterial populations increased with increasing nitrogen rate. The nitrification inhibitor treatment did not seem to influence the archaea populations, but it did impact the nitrifying bacterial population. Bacterial *amoA* gene copy number in the spring was lower at Cottonwood when an inhibitor was used and significantly decreased at Cavendish compared to the bacterial populations without inhibitor. From these observations, nitrification inhibitors do affect the soil microbial community by impeding nitrifying bacterial growth. However, this impact may be small compared to other factors that influence the soil microbial community. For example, the addition of nitrogen fertilizer caused a greater shift in ammonia-oxidizing bacterial populations than the nitrogen stabilizer. More research needs to be done to ensure that soil health and the microbial community structure are not adversely affected by this change in nitrifying population.

This thesis focused on the specific nitrification inhibitor Instinct® II mixed with UAN 32 nitrogen fertilizer. However, farmers in the region also use anhydrous and urea nitrogen fertilizers. Additionally, there are many other nitrogen stabilizers on the market that can be applied with these other types of nitrogen fertilizers. These data suggest that the application of nitrogen stabilizers with UAN 32 in this region may not be economical for growers to apply. More research needs to be done in various environments and climatic conditions to identify the proper regions in which these products will have the best performance. Other trials in the region can also be conducted to find the best combination of fertilizer and stabilizer to ensure improved crop performance and economic gain.

Appendix A: Soil Sample Tests

Table A.1. Pre-plant soil test for Cottonwood, Idaho for 2020 growing season.

Depth (cm)	NO3-N (kg/ha)	NH4-N (kg/ha)	Sulfur ppm	pH	Soluble Salts (mmhos/cm)	Organic Matter (%)	P (bic) ppm	K (bic) ppm	P (ace) ppm	K (ace) ppm
0	100	16	3	5.3	0.28	3.78	17	216	2.3	172
15	63		4							
30	44		4							
45	34									
Total	241	16								

Table A.2. Pre-plant soil test for Cavendish, Idaho for 2020 growing season.

Depth (cm)	NO3-N (kg/ha)	NH4-N (kg/ha)	Sulfur ppm	pH	Soluble Salts (mmhos/cm)	Organic Matter (%)	P (bic) ppm	K (bic) ppm	P (ace) ppm	K (ace) ppm
0	17	10	2	5.4	0.09	3.32	19	129	2.8	107
15	10		2							
30	15		3							
Total	42	10								

Table A.3. Pre-plant soil test Cottonwood, Idaho for 2021 growing season.

Depth (cm)	NO3-N (kg/ha)	NH4-N (kg/ha)	Sulfur ppm	pH	Soluble Salts (mmhos/cm)	Organic Matter (%)	P (bic) ppm	K (bic) ppm	P (ace) ppm	K (ace) ppm
0	12	12	2	5.9	0.14	4.44	12	150	1.8	114
15	7		2							
30	5		5							
45	6									
Total	30	12								

Table A.4. Pre-plant soil test Cavendish, Idaho for 2021 growing season.

Depth (cm)	NO3-N (kg/ha)	NH4-N (kg/ha)	Sulfur ppm	pH	Soluble Salts (mmhos/cm)	Organic Matter (%)	P (bic) ppm	K (bic) ppm	P (ace) ppm	K (ace) ppm
0	12	12	3	5.5	0.11	3.69	13	107	1.8	73
15	5		3							
30	6		3							
45	6									
Total	29	12								