Mineralization of Nitrogen in Liquid Dairy Manure during Storage

Yihuai Hu

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Jactone A. Ogejo, Chair
Zachary Easton
Biswarup Mukhopadhyay

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ABSTRACT

Loss of nitrogen (N) from dairy manure during storage is an issue of economic, environmental, and social concern for farming communities. The lost N 1) decreases the value of manure as a fertilizer and is an economic loss because supplemental inorganic N fertilizer is purchased to meet N needs on farms; 2) produces the potential pollution for water and air systems, thereby damaging the associated ecosystems; 3) causes challenges to human health. Thus, it is vital to manage and use N in an efficient and eco-friendly manner. N mineralization is a pathway in the N cycle, which converts organic N to inorganic N that is more susceptible to loss. The objective of this study was to conduct lab-scale experiments to assess the effects of temperature, manure solids content, using manure seed and autoclave sterilization operation at the start of storage, and storage time on the N mineralization and the associated microbial community during the storage of liquid dairy manure. Manure scrapped from the barn floor of a commercial dairy farm and diluted to make experimental stocks with high (46 to 78 g/L) and low (19 to 36 g/L) total solids (TS), to simulate what is typically transported to the manure storage pit was used. The manure was incubated in the laboratory at three temperatures (10, 20, and 30°C) for two storage periods (60 and 180 days). Manure samples were taken at different storage time for analyses. The results showed that temperature and using sterilization operation at the start of storage had significant effects on N mineralization for both storage periods ($p < 0.05$). The highest N mineralization rate occurred at 30℃, which rate constant (k) was 0.096 $week^{-1}$. While, the lowest N mineralization occurred at 10℃, and its corresponding k was 0.013 $week^{-1}$. The concentrations of mineralized N (Nm) with non-sterilized (R) manure were significantly higher than that with sterilized (R0) manure ($p < 0.05$). Compared to that with high TS (H) manure, the concentrations of Nm were significantly higher with low TS (L) manure after 180-d storage ($p < 0.05$). Raw manure augmented with manure seed (MS) had significantly higher Nm than the manure seed only (SO) ($p < 0.05$). In order to investigate the changes of microbial community in manure, samples were collected on days 0, 30, 90, and 180 for the 180-d storage experiment, and days 0, 30, and 60 for the 60-d storage experiment, and then manure DNA under different condition was successfully

extracted from collected samples and used for 16S rRNA sequencing. This study provided a more comprehensive understanding of the impact factors for manure storage, and was expected to clarify the relationship between N mineralization and the associated microbial community.

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GENERAL AUDIENCE ABSTRACT

Loss of nitrogen (N) from dairy manure during storage is rooted in the process of degradation via microbial activities. During storage of dairy manure, up to 60% of N can be lost to the environment (the air, rivers, groundwater, etc.), causing damages such as global warming and water pollution. However, it is challenging to manage and reduce the N lost during manure storage because of lack of comprehensive knowledge of the complex microbial activities in manure storage structures. Thus, the long-term goal of this study is to discern the interactions of the physical, chemical, and microbial processes that affect the N transformation. The generated information will help to mitigate/minimize the loss of nitrogenous gases during storage of dairy manure. The specific objectives included: 1) to evaluate the effects of selected factors (including storage time, temperature, manure solids content, using manure seed and sterilization operation at the beginning of storage) on N mineralization during storage of liquid dairy manure and determine the associated N mineralization rate; 2) to reveal the microbial communities in stored liquid dairy manure under different conditions (listed above). The outcome of this study could be used to refine N mineralization input parameter of manure storage submodules of the process-based models such as Manure DeNitrification-DeComposition model (Manure-DNDC) and Integrated Farm System Model (IFSM) with the goal to improve their accuracy of estimating or accounting for the fate or cycling of N in dairy manure during storage.

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Abbreviations

10/20/30	10, 20 or 30℃, respectively
C/N ratio	carbon-to-nitrogen ratio
H	high TS manure
k	N mineralization rate constant
L	low TS manure
MS	raw manure with manure seed
N	nitrogen
Nm	mineralized nitrogen (mg N/g VS)
Nr	reactive nitrogen
OrgN	organic nitrogen (mg N/g VS)
R	non-sterilized manure
R0	sterilized manure
SO	manure seed only
t	storage time
TAN	total ammonia nitrogen (mg N/g VS)
TCOD	total chemical oxygen demand (mg/g VS)
TN	total nitrogen (mg N/g VS)
TP	total phosphorus (mg P/g VS)
TS	total solids (g/L)
VS	total volatile solids (g/L)
VS/TS	the ratio between volatile solids and total solids

Chapter 1 Introduction

Nitrogen (N) is a crucial element and building block of components of living organisms, including proteins, nucleic acids, and other cellular constituents essential for sustaining all forms of life. N is an essential macronutrient required by plants, crops, and animals, and it comes in many forms, as described below. Even though N is the most abundant element in the atmosphere (78%), it is in the form of dinitrogen (N_2) gas. Most living organisms are unable to directly use the N_2, except for some archaea and bacteria with the ability to transform N_2 to reactive N via the process of N fixation. The N-fixation is a process achieved by free-living bacteria and archaea (i.e. diazotroph) or a result of symbiotic relationships between N-fixing microorganisms and eukaryotes, such as unicellular haptophyte algae, termites, bivalves, and legumes such as alfalfa, beans, peas, and soybeans (Kuypers, Marchant, & Kartal, 2018). In addition to N_2, many other organic and inorganic forms of N are present in the environment. Reactive forms of nitrogen (Nr) that supports growth (directly or indirectly) includes N compounds in the earth's atmosphere and biosphere, which are photochemically reactive, radiatively active, and biologically active (Galloway et al., 2004). The Nr includes inorganic reduced forms such as ammonia (NH_3) and ammonium ions (NH_4^+), inorganic oxidized forms such as oxides of N (NO_x), nitric acid (HNO_3), nitrous oxide (N_2O), nitrite (NO_2^-) and nitrate (NO_3^-) ions, and organic compounds such as urea, amines and proteins. The increased quantities of Nr in the atmosphere due to anthropogenic activities present challenges that impact the health and welfare of humans and ecosystems (Galloway, Cowling, Seitzinger, & Socolow, 2002). Small quantities of Nr in the atmosphere may provide beneficial effects (e.g., crop production increases where atmospheric Nr deposits appropriately), but at larger quantities may impact ecosystems negatively (Galloway et al., 2003; 2002). Specifically, larger amounts of Nr in the atmosphere influence the air quality and global climate, which, in turn, have adverse effects on human health and the environment (Galloway et al., 2002; 2004).

Dairy manure contains N that is typically used as a nutrient supplement for crop production (Kellogg, Lander, Moffitt, & Gollehon, 2000). Literature reports that during storage, up to 60% of N in dairy manure may be lost to the atmosphere through volatilization (Arogo, Westerman, Heber, Robarge, & Classen, 2006; NRC, 2003). The N loss from manure storage occurs via transformation (which includes a mix of microbial activities and biogeochemical

reactions) of organic N to volatile forms of inorganic N, such as NH_3, N_2O, NO, and N_2. The loss of these N forms lowers the value of manure as a fertilizer (NRC, 2003). What's more, emission of N_2O into the atmosphere leads to global warming (its global warming potential is 310 times that of carbon dioxides (CO_2)) via destroying the ozone layer, which enhances the detrimental impact of the UV (ultraviolet) sun rays (Wuebbles, 2009).

Additionally, it has been reported that the quantity of volatile N gases is related to the amount of volatile organic matter, total ammonia N (TAN = NH_4^+ + NH_3), temperature, pH, wind speed, the characteristics of the surface interfacing with the atmosphere, and the chemical and microbial activities in the manure (Arogo et al., 2006; Li et al., 2012; Rotz, Montes, Hafner, Heber, & Grant, 2014). N mineralization is the basis for subsequent nitrification and denitrification. Thus, a better understanding of N mineralization in stored manure can be used to refine the nutrient flow models such as Manure DeNitrification-DeComposition (Manure-DNDC) model (Li et al., 2012), Integrated Farm System Model (IFSM) (Rotz et al., 2014), Global Livestock Environmental Assessment Model (GLEAM) (Uwizeye et al., 2018), and Nutrient Flow Model (NFM) (Dijk, Leneman, & van der Veen, 1996). Furthermore, understanding the N mineralization can provide more information to guide N management on farms, and then help to find a way to reduce the loss of harmful N gases to the environment.

This study focuses on understanding the N mineralization process in dairy manure during storage with a long-term goal of revealing the complex relationships of the physical, chemical, and microbial processes that contribute to the N transformation. The knowledge generated will present producers and professionals interested in the subject with information to use to design and implement mitigation strategies, which can minimize the loss of nitrogenous gases during storage of dairy manure. The specific objectives were to assess:

1) the effects of selected factors (storage time, temperature, manure solids content, and using manure seed and sterilization operation at the start of storage) on N mineralization during the storage of liquid dairy manure, and evaluate the corresponding N mineralization rate.
2) the microbial communities in stored liquid dairy manure under the effects of selected factors in objective 1.

Chapter 2 Literature Review

2.1 The value of nitrogen

Nitrogen (N) is a key element and building block of components of each living organism, including proteins, nucleic acids, and other cellular constituents essential for sustaining all forms of life. N is a vital macronutrient required by plants, crops, and animals, and it comes in many forms, as described below. Although N is the most abundant element in the atmosphere (78%), it is in the form of dinitrogen (N_2) gas. Most living organisms cannot use the atmospheric N_2 directly, except for limited bacteria and archaea with the ability to convert N_2 to Nr. In general, the microorganisms that have the nitrogenase metalloenzyme can fix N_2 into NH_3 (Kuypers et al., 2018). Even though no nitrogen-fixing eukaryotes were found, many eukaryotes (e.g., unicellular haptophyte algae, animals including termites and bivalves, crop legumes such as alfalfa, beans, peas, and soy) live in symbioses with nitrogen-fixing microorganisms, support them to fix N (Kuypers et al., 2018). Besides N_2, other forms of N (organic and inorganic) are present in the environment. Galloway et al. (2004) compared the contributions of natural and anthropogenic activities on the transformation of the unreactive N_2 to the Nr for 1860 and the early 1990s, and predicted the global N budget in 2050. They found that Nr availability was greatly increased due to anthropogenic activities related to food production and energy production during the 200 years.

The increased Nr concentrations have effects on the health and welfare of humans and ecosystems (Galloway et al., 2002). For example, the utility of synthetic N fertilizers has the beneficial effect on human health by providing macronutrient (N) to increase the yield and nutritional quality of foods, and to meet dietary requirements and food preferences for population growth. The production and use of N fertilizers and fossil energy has increased the wealth and well-being of the population in many parts of the world. However, the high concentrations of Nr have some adverse effects on human health, including exposure to high concentrations of ozone, delicate particulate matter and N oxides (such as NO_2) resulting in respiratory and heart disease (Galloway et al., 2002).

In terms of environmental impact, a small amount of added Nr usually has a beneficial effect, but at a higher rate, a negative ecosystem impact is produced. Increased Nr input can

enhance productivity in the natural ecosystems which contain limited Nr (Galloway et al., 2003; 2002). However, higher Nr input rates often result in loss of biodiversity in terrestrial and aquatic ecosystems, an intrusion of N-loving weeds, and changes in beneficial soil abundance that alters ecosystem function. NOx atmospheric deposits from fossil fuel combustion and NHy from animal agriculture usually cause acidification of forests, soils, and freshwater aquatic ecosystems. Soil N saturation in terrestrial ecosystems increases the export of Nr to the downstream marine environment, leading to eutrophication of coastal ecosystems and, in some cases, to hypoxia (Galloway et al., 2002; 2004).

Air quality and global climate are also impacted by the increases of Nr, which harms human health and the environment. Increases in tropospheric ozone associated with N oxide emissions can cause ozone damage to crops, forests, and natural ecosystems, as well as susceptibility to pathogen and insect attacks. Ozone, other oxidants, and acid deposits can damage structural materials and artifacts (Galloway et al., 2002). The regional haze reduces the visibility of the landscape and the airport. The reduction in stratospheric ozone and global climate change may be due to increased greenhouse gas N_2O emissions from terrestrial and aquatic ecosystems associated with increased Nr inputs (Galloway et al., 2002; 2004).

Various forms of Nr circulate through biogeochemical pathways and are easily distributed through hydrological and atmospheric transport processes (Galloway et al., 2002). Thus, a single Nr molecule can cascade in various environmental systems and contribute to a variety of continuous effects. Besides, long-distance transport of Nr has a detrimental impact on countries far from sources (Galloway et al., 2002; 2004).

Small quantities of Nr in the ecosystem can provide beneficial effects, but at larger quantities may impact the ecosystems negatively (Galloway et al., 2003; 2002). Therefore, it is vital to properly manage N in the environment to maximize the value of N.

2.2 Nitrogen in sustainable agriculture

The USDA defines sustainable agriculture as a system that can sustain its productivity and its usefulness to society indefinitely, resource-saving, socially-supportive, commercially competitive, and environmentally sound (USDA, 2007). Under the law addressed by Congress in the 1990 “Farm Bill” (Law, 1990), “the term sustainable agriculture means an integrated system

of plant and animal production practices having a site-specific application that will, over the long term:

- satisfy human food and fiber needs;
- enhance environmental quality and the natural resource base upon which the agricultural economy depends;
- make the most efficient use of nonrenewable resources and on-farm resources and integrate, where appropriate, natural biological cycles and controls;
- sustain the economic viability of farm operations; and
- enhance the quality of life for farmers and society as a whole."

Based on the statements about sustainable agriculture mentioned above, nitrogen should be adequately managed, and if mismanaged, can lead to severe environmental issues. For example, as mentioned before, the increased amounts of N_2O can cause greenhouse warming. Nitrate is very mobile and easily dissolved in water, entering groundwater and surface waters such as ponds, rivers, and streams. High concentrated nitrates can be toxic to infants, causing anoxia, or internal suffocation. Nitrogen pollution can also lead to harmful algal blooms, which often create toxins that can kill fish and other animals. These toxins move up the food chain and endanger larger animals as well. Mismanagement of nitrogen can also result in environmental effects like acid rain and dead zones and hypoxia, which is a reduced level of oxygen in the water.

Meeting sustainable agriculture requirements needs a combination of environmental, economic, and social conditions. The conventional farming, which refers to agricultural systems including the utility of synthetic chemical fertilizers, herbicides, pesticides and other continuous additives, GMOs (genetically modified organisms), concentrated animal feeding operations, heavy irrigation, intensive tillage or concentrated monoculture production, has been developed since the late 19th century, and has become popular all over the world after the 1940s (Ethan, 2016, April 19). The conventional practice in production agriculture has focused on how to efficiently and effectively maintain and increase production to improve profitability. However, they may be harmful to the environment and human beings. For example, conventional farming uses a large quantity of synthetic chemical fertilizers and pesticides to increase the yield of crops.

The USDA (2013) reported it as “this method usually alters the natural environment, deteriorates soil quality, and eliminates biodiversity,” conventional agriculture could improve the efficiency of farming but achieved it at a significant cost to the environment. Meanwhile, the utility of pesticides is toxic to human beings (Igbedioh, 1991), and fertilizer runoff pollutes the water system (Halliday & Wolfe, 1991). Therefore, nowadays, we need sustainable agriculture, and to achieve requirements of sustainability, systematic consideration of environmental, profitability, and social issues are required.

2.3 Manure as a source of N for plants

The form of N that can be taken up by plants includes ammonia-nitrogen (NH_4^+-N), nitrite-nitrogen (NO_2^--N), and nitrate-nitrogen (NO_3^--N) (Breteler & Luczak, 1982; R. Haynes & Goh, 1978). Different plants require the different amount of N to meet their demand. Lack of N can cause plant growth retardation, but excessive N can lead to inhibition of plant development. Since N is sensitive to loss in many different processes, when N-source fertilizers are applied to the field, N may be released as gaseous forms and result in that the fertilizers cannot satisfy the needs of plants. Meanwhile, the released gaseous N will be transported to other fields where the plants do not need much N input, and inhibit their growth (Cameron, Di, & Moir, 2013).

Manure is a natural byproduct from livestock production and is a good source of plant available N (Jokela, 1992). It is natural and environment-friendly compared to chemical fertilizers. The N from manure is predominantly present in two forms – organic N (such as proteins) and inorganic N (such as NH_3). The fraction of each of these forms in manure varies depending on manure types, for example, in solid dairy manure there is more organic N compared to inorganic N; in liquid dairy manure the ratio of organic N to inorganic N is approximately 1:1 (Eghball, Wienhold, Gilley, & Eigenberg, 2002; Lorimor, 2000; Pettygrove, Heinrich, & Eagle, 2010).

In general, the forms of inorganic N include (1) NH_4^+-N, NO_2^--N and (2) NO_3^--N; and organic N includes (3) microbial biomass, (4) organic N which is potentially available for N mineralization (such as proteins, urea and uric acid), and (5) N which is unavailable for microbial utilization and mineralization (Cameron et al., 2013; Noonan, Zaman, Cameron, & Di, 1998). The inorganic N contributes to N loss by part (1) and (2) can be released and used rapidly in several weeks after application, and the part (3) and (4) will play the role of the substrate for long-term plant-absorbable N through the mineralization and nitrification processes. Based on the statements from Haynes and Naidu (1998), it was a traditional agricultural practice to apply organic fertilizers at agronomic rates for plant nutrient supply. It is known that the application of manure, in addition to providing nutrients, also has a beneficial effect on the physical properties of the soil. Since organic fertilizer has many nutrients and long-lasting effect, containing microorganisms, enzymes, etc., it can promote nutrition in the rhizosphere, preserve water, preserve fertilizer, regulate soil physical and chemical properties, improve soil buffering capacity (Huang et al., 2006), and improve the quality of agricultural products (Mozafar, 1994). Thus, developing the knowledge about how to effectively manage and fully use dairy manure will help farmers be more profitable and competitive, without damaging our environment.

2.4 Nitrogen mineralization in manure

The nitrogen cycle (Figure 2-1) is a significant biogeochemical cycle on the earth. In the cycle, N will be converted into multiple chemical forms with circulating among the atmosphere, terrestrial, and marine ecosystems. Among a series of processes, N mineralization is a vital process for most autotrophic creatures which can only consume inorganic N as the nutrient, since those organisms such as plants are unable to directly assimilate organic N compounds like urea, uric acid, nucleic acids, amino acids, and so on for their growth, with constructing enzymes and protoplasm (Osman, 2012). N mineralization is also a crucial process occurred in dairy manure. The definition of N mineralization is the decomposition (i.e., oxidation) of the chemical compounds in organic matter, by which the nutrients in those compounds are released in soluble, inorganic forms for plants to ingest (White, 2013). In dairy manure, the various forms and corresponding proportions of N elements are classified and shown in Figure 2-2. The total organic N can be further divided into particulate and soluble N, and the total inorganic N can be divided into NH_3 and nitrogen oxides. The Organic N can be transformed into inorganic forms

via N mineralization. Likewise, inorganic N can be transformed into organic forms by plant and microbe's uptake and N fixation. Pettygrove et al. (2010) report in dairy lagoon water, the proportion of l to inorganic N is around half to half, and the organic N can be separated into three parts: a) microbial nitrogen; b) excreted nitrogen from intestinal wall; c) structural nitrogen from the food of the cattle (Chadwick, John, Pain, Chambers, & Williams, 2000).

Roy, Misra, and Montanez (2002) reported that mineral N losses to the environment from fertilizer utility all over the world were over 36 million metric tons per year, worth over $11 billion, and with harmful environmental impacts as described before. They also estimated the mineral N consumption would be 96 million metric tons per year in 2030, based on the corresponding food demand were around 2800 million metric tons per year. Thus, properly managing manure and controlling/mitigating the mineralization and losses of N from manure would be very important for reducing environmental pollution and making economic profits.

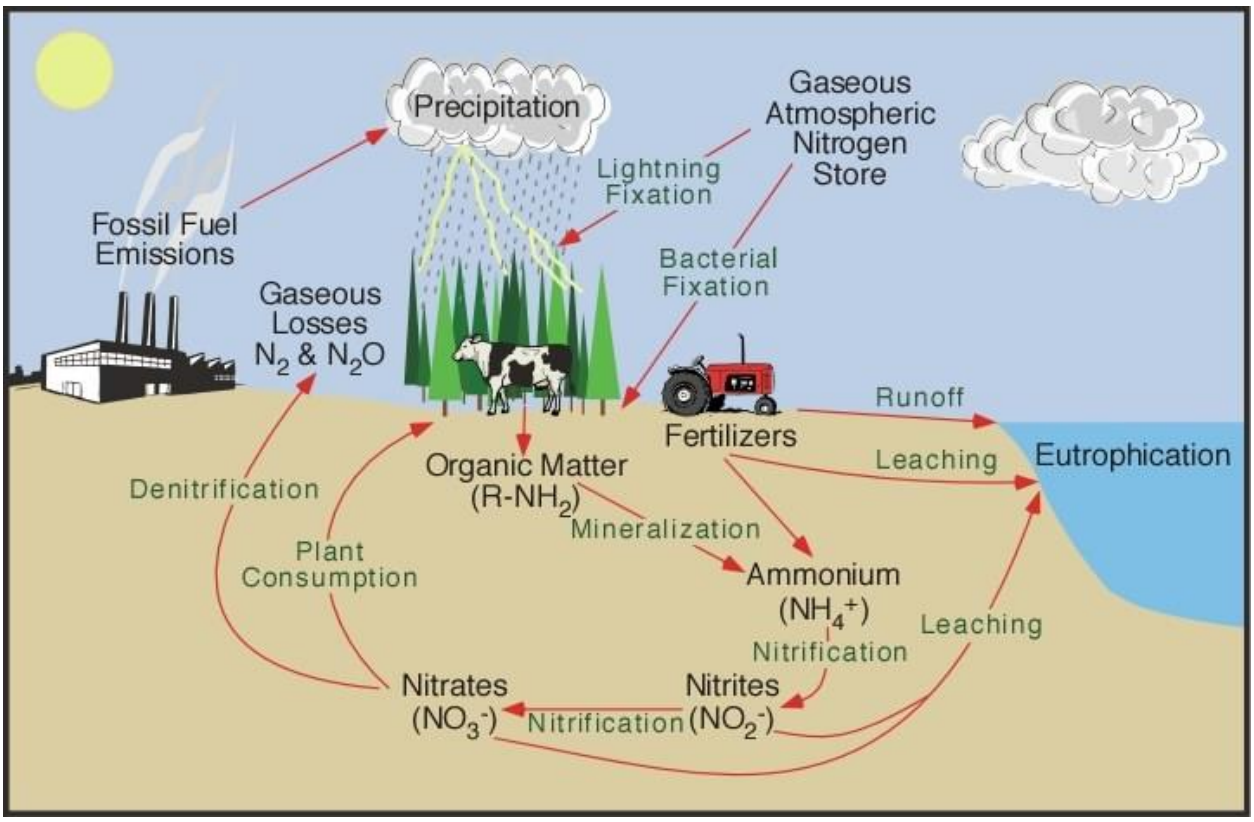


Figure 2-1 Nitrogen cycle (Pidwirny, 2006)

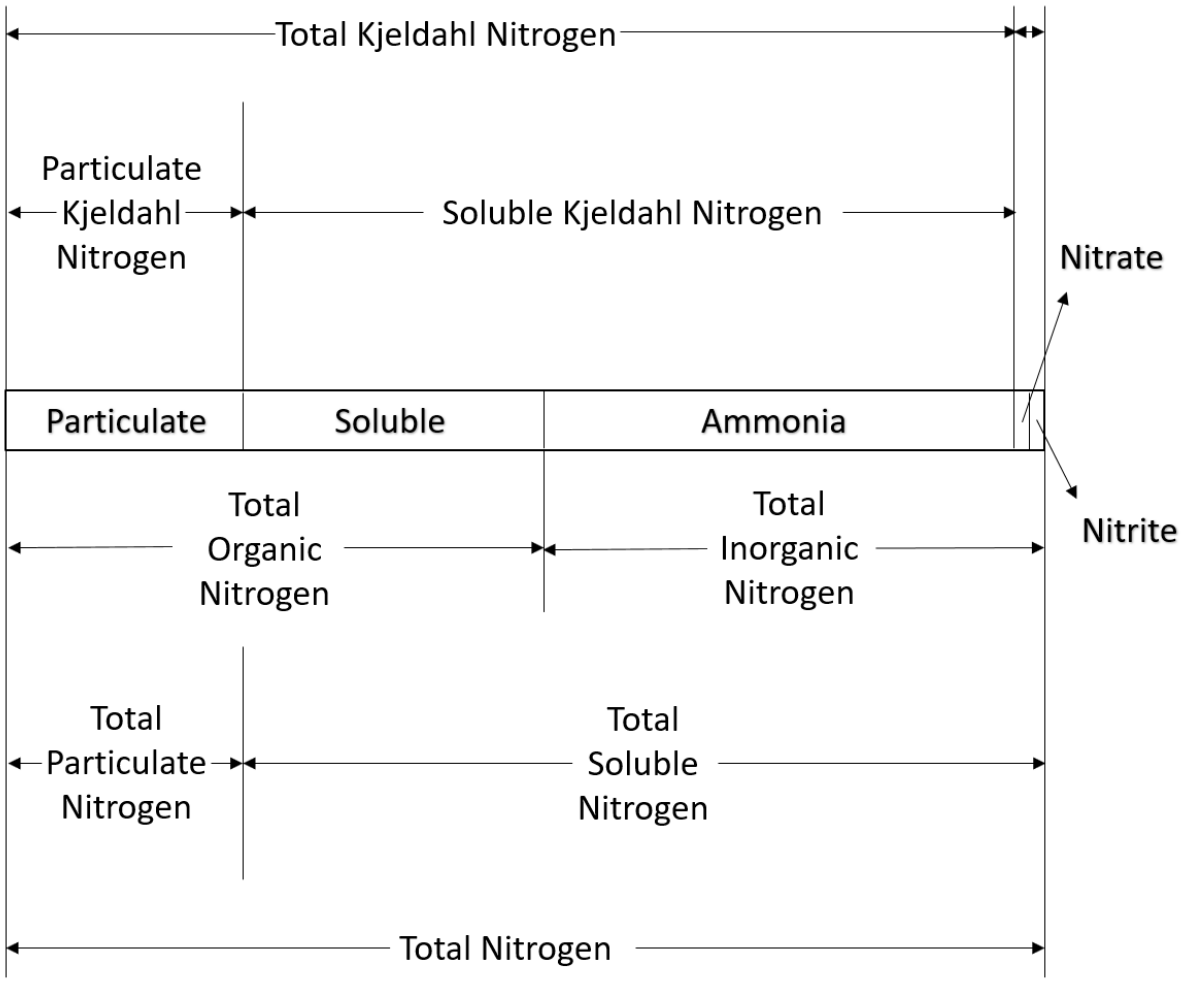


Figure 2-2 Different forms of nitrogen in dairy manure

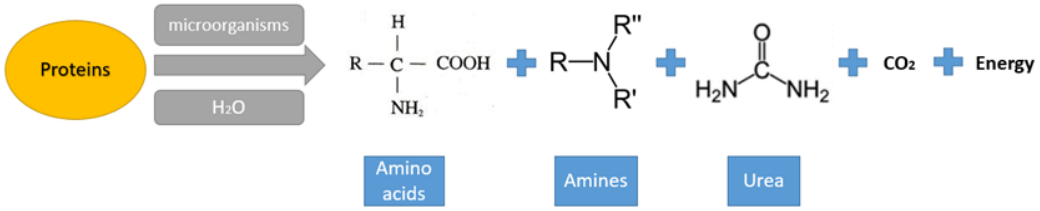
2.5 The mechanism of inorganic N supply process

The inorganic N supply process plays a vital role in providing plants with an absorbable source of nitrogen. It comprises a series of microbial and enzymatic processes that transform organic N to inorganic forms (Zaman, Di, & Cameron, 1999 a; Zaman, Di, Cameron, & Frampton, 1999 b). It involves N mineralization and nitrification, and the N mineralization contains aminization and ammonification (Bolan, Saggar, Luo, Bhandral, & Singh, 2004). The mechanisms of these processes were reviewed and discussed below.

2.5.1 Aminization

During aminization microorganisms (primarily heterotrophs) break down macromolecules of organic N compounds such as complex proteins to simpler forms of organic

N such as amino acids, amino sugars, amides, amines, and nucleic acids (Cai, Chang, & Cheng, 2017; Zaman et al., 1999 a) as described in the general (Equation 2-1) below.

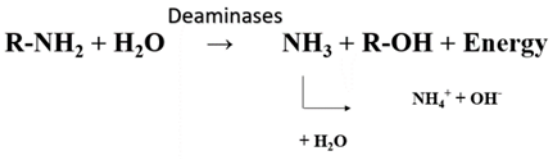


(Equation 2-1)

Proteolytic activity (i.e., aminization, the equation shown above), catalyzed by proteases which are secreted by microorganisms, produces organic compounds having lower molecular weight, such as amino acids, amides, amines (Zaman et al., 1999 b). In soils, the aminization process occurs in natural environmental conditions and can be stimulated by the abundant presence of proteinaceous and carbonaceous organic materials (Zaman et al., 1999 a; Zaman et al., 1999 b).

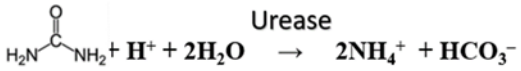
2.5.2 Ammonification

The process of transforming organic N to NH_3/NH_4^+-N is called ammonification, mediated by many kinds of bacteria, fungi, actinomycetes, other microorganisms, and some animals. The bacteria (e.g., *Bacillus, Clostridium, Proteus, Pseudomonas, and Streptomyces*) that accomplish the process are called ammonifying bacteria or ammonifiers (Osman, 2012; Prakash, Mousumi, & Prasad, 2012; Van Elsas, Trevors, Jansson, & Nannipieri, 2006). In details, ammonification is the transformation of amino groups ($-NH_2$) and other low molecular weight nitrogen-containing organic compounds and byproducts from aminization to NH_3 and NH_4^+ salts. The total amount of NH_3 and NH_4^+ is commonly called total ammonia nitrogen (TAN). The ammonification process is achieved by deaminases, within or outside the microbial cells, primarily the cells of autotrophs, with the liberation of NH_4^+ (Cai et al., 2017; Zaman et al., 1999 a). The reaction (Equation 2-2) shows below.



(Equation 2-2)

Ammonification process involves a gradual simplification of complex compounds. The action of enzymes produced by microorganisms is mainly hydrolytic and oxidative under aerobic conditions (Zaman et al., 1999 a; Zaman et al., 1999 b). Urea hydrolysis, the oxidation of the simple organic compound urea ($CO(NH_2)_2$, is one of the most elementary ammonification reactions (Cai et al., 2017), and it is also a rapid pathway (compared to proteins convert to NH_3) for accumulating inorganic N in dairy manure (Extension, 2011). It is achieved by the action of a microbial enzyme named urease, which produces two units of NH_4^+ by oxidizing one unit of urea. The reaction (Equation 2-3) shows below.



(Equation 2-3)

2.5.3 Nitrification

The process of conversion of NH_3 to $NO_2^-$and then to NO_3^- is known as nitrification. It contains two sub-steps. The first one is conducted by ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA), which oxidize NH_4^+ to NO_2^- (Di et al., 2010). The equation of this reaction shows below.

$$\frac{1}{6}NH_4^+ + \frac{1}{4}O_2 = \frac{1}{6}NO_2^- + \frac{1}{3}H^+ + \frac{1}{6}H_2O$$

(Equation 2-4)

The second step of nitrification is accomplished by nitrite-oxidizing bacteria (NOB), which convert nitrite to nitrate (Cai et al., 2017). The reaction equation shows below.

$$\frac{1}{2}NO_2^- + \frac{1}{4}O_2 = \frac{1}{2}NO_3^-$$

(Equation 2-5)

Since the bacteria and archaea accountable for nitrification are susceptible to acidity, the nitrification process cannot be conducted at significant rates in acidic conditions. This is why plants of acidic habitats must be capable of utilizing ammonium as their source of nitrogen nutrition (de Graaf, Bobbink, Roelofs, & Verbeek, 1998).

2.6 Factors affecting nitrogen mineralization in manure

Many studies have reported on methods of manure N mineralization in the last several decades, but their results were not consistent, making it difficult to use them for verifying

process-based models. Their inconsistent results perhaps attributed to the high variability in quality and quantity of manure, inappropriately relating the N mineralization to environmental factors and manure characteristics, and non-inclusion of microbial activities responsible for the N mineralization. This study tries to clarify these aspects.

The previous studies have identified characteristics of manure, environmental factors, and microbial activities as factors that affect N mineralization in manure under both laboratory (Guntiñas, Leirós, Trasar-Cepeda, & Gil-Sotres, 2012; Mohanty et al., 2011) and in-situ field conditions (Colman & Schimel, 2013; Eghball et al., 2002; Manzoni & Porporato, 2009). Although there was much evidence showing that N mineralization was affected by the environment (Eghball, 2000; Watts, Torbert, & Prior, 2007; Whalen, Chang, & Olson, 2001; Zaman & Chang, 2004), it is still hard to thoroughly interpret the relationships between N mineralization and the environment (Cookson, Cornforth, & Rowarth, 2002; Eghball, 2000; Whalen et al., 2001); (Bagherzadeh, Brumme, & Beese, 2008). Some studies indicated that N mineralization was often affected by several factors such as the types of manure, the methods of manure application, organic matters (Chae & Tabatabai, 1986; Thompson & Meisinger, 2002), moisture and temperature (Dalias, Anderson, Bottner, & Coûteaux, 2002; Dewes, 1996; T Griffin, Honeycutt, & He, 2002; Zaman & Chang, 2004), and microorganisms and their activities (Bagherzadeh et al., 2008; Cookson et al., 2002; Van Kessel, Reeves, & Meisinger, 2000). The management of N in manure without considering these factors may lead to inefficient use of nitrogen and adversely affect the environment.

2.6.1 Manure characteristics

Thompson and Meisinger (2002) reported the N mineralization would be affected by the various characteristics of manure from different animal species. The impact on N mineralization varies with the changes of chemical characteristics of manure, e.g., the C/N ratio of manure is one of the representative indicators to predict the N mineralization (Paul, 2014). In general, organisms decomposing organic matters require a C/N ratio around 8:1 to build new cells and maintain their activities. For example, when the manure has a C/N ratio which is lower than 8:1, organisms may acquire N from other sources, leading to immobilize N from the environment (Gale et al., 2006). Besides, the C/N ratio in manure may result in nearly half of variations in N mineralization (Chadwick et al., 2000; Gonçalves & Carlyle, 1994; TS Griffin, 2007).

What's more, the density, total suspended solids (TSS), and organic N of manure affect the N mineralization in liquid manure (Chae & Tabatabai, 1986). Rochette, Angers, Chantigny, Gagnon, and Bertrand (2006) also reported that the N mineralization rate is faster in liquid manure compared to solid manure under field conditions. In general, liquid manure has a more substantial proportion of inorganic N (mainly TAN) than that of solid manure, which can be a benefit for the initial stages of mineralization to obtain more N (Calderon, McCarty, & Reeves, 2005; Sommer, Petersen, Sørensen, Poulsen, & Møller, 2007).

2.6.2 Environmental factors

The temperature is a vital factor for manure N mineralization processes (Agehara & Warncke, 2005; Dalias et al., 2002; Dewes, 1996). Almost all microbial processes, including N mineralization, are temperature dependent. Many studies, both for the laboratory and field experiments, indicated that the higher temperatures during incubation would stimulate net manure N mineralization because of increasing microbial enzymatic activities (Cookson et al., 2002; Dalias et al., 2002; Eghball, 2000; Melillo et al., 2002). W. Wang, Smith, and Chen (2003) and C. Wang, Wan, Xing, Zhang, and Han (2006) reported that at lower temperatures, since the demand of microbes for the bioavailable organic N was small, the relationship of accumulating net mineralized N during incubation was almost linear associated with the incubation time. However, when temperatures increased, because mineralized organic matters were consumed faster, the correlation between net mineralized N and the incubation time would be curvilinear. For manure N mineralization in soil, the temperature from 25 to 35 °C may result in optimum N mineralization (Nicolardot, Fauvet, & Cheneby, 1994; Stark & Firestone, 1996).

The effects of pH on nitrogen mineralization were less comprehensively studied than the effects of temperature, and the results in different kinds of literature were inconsistent. Dancer, Peterson, and Chesters (1973) reported soil pH had effects on ammonification and nitrification. Their results showed pH slightly influenced the rates of ammonification, and significantly affected the rates of nitrification. Curtin, Campbell, and Jalil (1998) measured N mineralization in soils by aerobic incubation and found there was no statistical relationship between pH and the parameters of the first-order kinetic equation of N mineralization. Thus, they concluded that the pH did not directly connect to the rate constant (k) and potentially mineralizable N (N_0) used in the N mineralization equation. However, with increasing the pH, N mineralization was

stimulated, and raising pH to 7.3-7.4, the mineralized N was 2-3 times compared to the untreated samples. The effects of pH was attributed to labile organic matter released with pH increasing. However, Cheng et al. (2013) evaluated the effects of pH on N mineralization of forest soils using the ^{15}N tracing technique and calculated by the numerical model FLUAZ. They found that with pH increasing, the net N mineralization rate was decreased due to that the rate of NH_4^+ immobilization was faster than the gross N mineralization rate.

2.6.3 The microbial activities

Some literature reported that microbial activities were another factor to affect N mineralization. Bengtsson, Bengtson, and Månsson (2003) conducted a laboratory experiment to evaluate N mineralization of forest soil, indicated that N mineralization rates in soils were more related to the respiration rate and ATP content than to the C/N ratio, and found that leaching of nitrate from soils in forests might considerately depend on the microbial density and activities. Zaman et al. (1999 b) reported the relationships of enzyme activities and the soil microbial biomass to N mineralization and nitrification rates using an incubation technique. They found gross N mineralization rates were positively correlated with microbial biomass and enzyme activities in soils which were treated with dairy shed effluent.

2.7 Approaches for estimation of manure N mineralization

Numerous approaches used to determine N mineralization in manure and agricultural soils are mainly divided into two types - laboratory and field methods (Cabrera, Kissel, & Vigil, 1994; Chae & Tabatabai, 1986; Dou, Toth, Jabro, Fox, & Fritton, 1996; Gilmour & Skinner, 1999; C. Honeycutt et al., 2005; Van Kessel & Reeves, 2002). These current experimental methods (both laboratory and field) for estimating the net amount of mineralized N are somewhat flawed and unstable, and considering the combination of multiple methods can make the experimental results more reliable (Benbi & Richter, 2002).

Laboratory approaches were used to assess or quantify N-mineralization under an ideal or controlled environment, including chemical extraction (Ros, Hoffland, Van Kessel, & Temminghoff, 2009), microbial activities (Dahnke & Johnson, 1990; Pettygrove et al., 2003), electro-ultrafiltration filtration method (Dou et al., 1996) and so on. Laboratory incubation, which is the most common method for estimating N mineralization, has been used in many

studies (Castellanos & Pratt, 1981; Chae & Tabatabai, 1986; Eneji, Honna, Yamamoto, Saito, & Masuda, 2002; Morvan, Nicolardot, & Péan, 2006; Van Kessel & Reeves, 2002; 2000). The incubation technique has been reported to overestimate the N mineralization rate that occurs under field conditions (Cabrera & Kissel, 1988; C. W. Honeycutt, 1999; Sistani, Adeli, McGowen, Tewolde, & Brink, 2008). The reason for overestimating the rate of N mineralization may be that the incubation occurred at a temperature of 22-25 ℃ (C. Honeycutt et al., 2005; Van Kessel & Reeves, 2002), which is an ideal condition for microorganisms conduct the N mineralization and other associated processes.

As for the field methods, they are more related to N mineralization in soils or manure compost with soils, instead of manure only. The in-situ field methods, which can be used for determining net N mineralization of manure under in-situ condition, are much different from the laboratory approaches. The in-situ field methods include isotope nitrogen-15 (^{15}N) method, covered cylinder, buried bag, soil tests (residual profile NO_3^--N test, pre-sidedress NO_3^--N test, etc.), and ion exchange resins (Barraclough & Puri, 1995; Geens, Davies, Maggs, & Barraclough, 1991). For example, anion exchange resin was shown to accurately estimate N released from manure fertilization during the growing season in stands of Pinus radiata near Canberra, Australia (Raison, Connell, Khanna, & Falkiner, 1992). Some researchers (Brye, Norman, Nordheim, Gower, & Bundy, 2002; Eghball, 2000; TS Griffin, 2007; C. Honeycutt et al., 2005) used anion exchange resin method to simulate natural soil condition, since natural soil has the ability to capture nitrate ions, which is similar to this method. Their results demonstrated that anion exchange resin was a reliable method for measurement of N mineralization. This method has broadly been used in many studies such as in arctic soils (Giblin, Laundre, Nadelhoffer, & Shaver, 1994), deserts and dryland agroecosystems (Kolberg, Rouppet, Westfall, & Peterson, 1997; Lajtha, 1988), forests (D Binkley, Aber, Pastor, & Nadelhoffer, 1986; Dan Binkley & Matson, 1983), grassland (Hook & Burke, 1995), moist and fertilized agricultural soil (Brye et al., 2002), and with manure, compost and organic soil amendments (Eghball, 2000; Hanselman, Graetz, & Obreza, 2004). However, in general, the results of many field methods are unstable and easily influenced by many dynamics and soil factors (Khan et al., 2007). Therefore, a number of scientists have tried to connect N mineralization to several factors, including total nitrogen, C/N ratio, volatile solids, soluble organic nitrogen, organic matter content, moisture, temperature, pH, and so on, to eliminate the impacts on the results of field methods.

Many models have been utilized to estimate and describe N mineralization dynamics (Beauchamp, Reynolds, Brasche-Villeneuve, & Kirby, 1986; Benbi & Richter, 2002; Ferrara & Avci, 1982). These models can be categorized including 1) simple functional models to simulate the amount of net mineralized nitrogen; 2) mechanistic models with a projection of microbial biomass processes to predict long-term cycles of carbon and nitrogen. I only review the first group of models in my study. Simple functional models do not consider the basic process which influences N mineralization. Models simulate the net N mineralization without separately considering the ammonification and nitrification processes. The parameters used in the models are acquired from lab-scale incubation tests, which is through plotting N mineralization results to the time of incubation. The single-fraction model of N mineralization was described by Stanford and Smith (1972). They defined N mineralization potentials of soils as the quantity of soil organic N susceptible to mineralization at a rate of mineralization (k) according to first-order kinetics:

$$\frac{dN}{dt} = -kN$$

(Equation 2-6)

where:

- N is the amount of potentially mineralizable nitrogen (ppm N)
- t is the specified periods of time (weeks)
- k is the rate constant of N mineralization ($week^{-1}$)

Integration of the equation above between time t_0 and t:

$$N_t = N_0 \cdot exp(-kt)$$

(Equation 2-7)

where

- N_0 is the initial amount of substrate or the potentially mineralizable N (ppm N)
- N_t is the amount of substrate at time t (ppm N)

The equation can be substituted by $N_t=(N_0-N_m)$, where N_m is the N mineralized in time t:

$$N_m = N_0[1 - exp(-kt)]$$

(Equation 2-8)

Many researchers (Deans, Molina, & Clapp, 1986; Molina, Clapp, & Larson, 1980; Nuske & Richter, 1981) reported that more than one fraction of organic N might be directly mineralized in soil organic N, each with its specific rate of decomposition. Their model described net N mineralization by dividing the mineralizable soil organic N into different fractions, each of which is then assumed to mineralize according to first-order kinetics:

$$N_m = \sum_{i=1}^{n} N_{o_i}[1 - \exp(-k_i t)]$$

(Equation 2-9)

where

- i is a specific N fraction, n represents the total amount of fractions
- N_{0i} is the potentially mineralizable N in the i-th fraction (ppm N)
- t is the specified periods of time (weeks)
- k_i is the mineralization rate constant for the i-th fraction ($week^{-1}$)

However, the first-order model for estimating N mineralization is not always accurate and still has potential errors. Smith, Schnabel, McNeal, and Campbell (1980) reported that the extractions of mineral N during incubation should include estimation of TN leached or some reason for excluding the amounts of organic N leached, and when considering values of TN leached instead of values of mineral N leached alone resulted in significant differences in predictions of N mineralization potentials (N_0) and mineralization rate constant (k). They indicated that using transformed data and ignoring organic N leached would result in serious errors when determining N_0 and k. Some studies also suggested that in the absence of air-drying, net N mineralization could be described by zero-order kinetics (Addiscott, 1983; Houot, Molina, Clapp, & Chaussod, 1989; Tabatabai & Al-Khafaji, 1980):

$$N_m = Kt$$

(Equation 2-10)

where

- Nm is the N mineralized in time t (ppm N)
- t is the specified periods of time (weeks)

- K is the mineralization rate constant (ppm N·week^{-1})

Similarly, Mary, Beaudoin, Justes, and Machet (1999) showed that when a small portion of organic N was mineralized, the in-situ mineralization kinetics were linear, and when a larger portion was mineralized, the in-situ mineralization kinetics were curvilinear.

2.8 Challenge of inconsistency between the model results and the experimental results

Commonly used approaches for estimating N mineralization in manure storage include emission factors, mass balance, direct measurement, and models. VanderZaag, Jayasundara, and Wagner-Riddle (2011) reported lack of adequate and reliable information about N losses from full-scale manure storage tanks. This lack of information may be attributed to the cost and challenging undertaking and difficulties related to conducting direct measurement (NRC, 2003; Heber et al., 2009). Associating measured and modeled data is also a key challenge. Muck, Guest, and Richards (1984) reported measurements of N losses from two (top and bottom loaded) earthen manure storage pits receiving dairy manure for periods of around one year. Nitrogen losses from bottom loaded pits (3% - 8%) were much lower than from top loaded pits (29% - 39%). However, these results did not properly match with a previously developed model (developed based previous data excluding the data shown above) established by Muck and Steenhuis (1982). The model simulated a consistent nitrogen loss of 3% - 60% from top loaded pits and around 15% from bottom loaded pits under all conditions. Some reports expected that the rate of N loss from slurry tanks would be influenced by ambient temperature, manure pH, loading rate, and wind speed (Li et al., 2012; Muck & Steenhuis, 1982; Olesen & Sommer, 1993). Therefore, minimal losses would occur at temperatures below freezing or with manure pH less than 6 and an increased loss rate with temperature and pH (Li et al., 2012; Muck & Steenhuis, 1982). Some previous work (Massé, Masse, Claveau, Benchaar, & Thomas, 2008; Umetsu et al., 2005; Wood, VanderZaag, Wagner-Riddle, Smith, & Gordon, 2014) estimating aerial pollutants at laboratory or pilot scales related emissions to manure characteristics and environmental factors, but the results are not consistent, making it difficult to use them for verifying process-based models. These inconsistent results perhaps are attributed to the high variability in quantity and quality of manure, an inappropriate association of the quantities of volatile compounds to environmental factors and manure characteristics, and non-inclusion of microbial activities

responsible for the formation of the volatile compounds. This study aims to ascertain these challenges.

Because of the many factors influencing mineralization, predicting mineralization patterns during the storage of dairy manure accurately is a challenge. It is well understood that mineralization is a microbial mediated process which is not only affected by substrate characteristics but by temperature, pH, and microbial activities. A better understanding of the key affecting factors and their interactions on net N mineralization in manure storage will facilitate our understanding of manure N availability and management, as well as mitigate/minimize the loss of nitrogenous gases and protect our environment and health. Hence, there is a need to discern the complex interactions of the physical, chemical, and microbial processes that affect the nitrogen transformation during the storage of dairy manure, to provide more precise estimation of mineralized manure N.

Chapter 3 Materials and methods

3.1 Manure source and collection

The manure used in this study was collected from the barn floor of a commercial dairy farm located in Franklin County, VA. At this farm, manure was scraped from the floor and moved to an earthen manure storage pit. The manure used as seed (defined as manure seed) was collected from earthen pit for manure storage of that farm. Upon collection, the manure and seed were placed in 20 L (5 gals) plastic buckets, capped and transported to the Byproduct Management Laboratory, Biological Systems Engineering Department, Virginia Tech. Once in the lab, the manure was processed for the various experimental conditions for the storage studies as described below.

3.2 Experimental plan and manure preparation

Two incubation experiments were conducted for two storage periods, 180 and 60 days, respectively. The manure solids content, storage temperature, using sterilization operation and seeding at the beginning of storage were evaluated during the storage periods. One set of experiments was setup to compare N mineralization in non-sterilized and sterilized manure with high and low TS concentrations during 180-d storage period. Another set of experiment were setup to compare N mineralization in non-sterilized and sterilized raw manure mixed and with manure seed and manure seed only for 60-d storage period. Each set of experiments was conducted at three storage temperatures (10°C, 20°C and 30°C).

Manure collected from the farm was thoroughly mixed and portions drawn to make samples with high (H) and low (L) TS. Samples of manure designated H, were diluted to achieve TS between 46 g/L to 78 g/L, and samples designated as L were diluted to TS between 19 g/L to 36 g/L. Once the samples were prepared, 200 mL well-mixed aliquots were drawn and placed into 250 mL Erlenmeyer flasks for storage. The flasks were further divided into two batches, one to be autoclaved and the other not. The autoclaved batch (designated R0) of Erlenmeyer flasks was plugged with cotton balls and sealed with foil and then sterilized in the Autoclave under high-pressure saturated steam at 121°C for 60 min. The other batch of Erlenmeyer flasks (designated as R) was plugged with cotton balls without sterilization. The flasks from both

batches were subjected to storage at a pre-set temperature (10, 20 and 30℃, designated as 10/20/30 respectively). The low temperature incubator (Model 307, Fisher Scientific, Dublin, Ohio) for 10℃ storage, the incubator (CLASSIC C25KC, New Brunswick Scientific Co., Inc., Edison, New Jersey) for 20℃ storage, and the incubator (Innova 4400, New Brunswick Scientific Co., Inc., Edison, New Jersey) for 30℃ storage were used in the study.

The 60-d storage study was aimed to test if the storage time, temperature, sterilization operation, and adding manure seed had effects on N mineralization during storage. The sample preparation for testing the storage time, temperature and using sterilization operation at the start of storage was the same as the 180-d storage experiment. To test the effect of manure seed, two types of manure samples were set. The samples of the experimental group were the mixture of raw manure and manure seed. 150 mL raw manure and 50 mL manure seed were mixed and incubated in one 250 mL Erlenmeyer flask, designated as MS. The samples of the control group were manure seed (200 mL manure seed incubated in each 250 mL Erlenmeyer flask) without raw manure, designated as SO. The sample preparation for testing the storage time, temperature and using sterilization operation at the start of storage was the same as the 180-d storage experiment. In other words, compared to 180-d storage experiment, the 60-d storage experiment replaced the H and L manure samples with MS and SO manure samples. Other operations and incubation processes were the same as the 180-d storage experiment.

All the designations of treatments mentioned above will be used to indicate the samples in the following statements. For example, 30-H-R groups refer to the non-sterilized samples with high TS incubated at 30℃; 20-L-R0 groups refer to the sterilized samples with low TS incubated at 20℃; 20-MS-R groups refer to the non-sterilized samples of raw manure with manure seed incubated at 20℃; and 10-SO-R0 groups refer to the sterilized samples of manure seed only incubated at 10℃.

3.3 Sample collection and analysis

Samples were collected on days 0, 15, 30, 60, 90, 120, 180 for the 180-d and 0, 15, 30, 45, 60 for the 60-d storage experiments. During each sampling event, two flasks of each treatment were taken from the incubator and composited, and then poured into 500 mL plastic bottles for storage. Then three aliquots were taken from the composited sample for analysis. For

every parameter analyzed, three 20 mL aliquots of manure were taken to use. The samples were analyzed for total solids (TS), volatile solids (VS), and pH according to the standard method for wastewater analysis (American Public Health Association, 2012). The pH was measured using the IDS pH combined electrode (SenTix® 940-3, Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany). The total Chemical oxygen demand (TCOD) and the total phosphorus (TP) were analyzed using a HACH DR/2500 Spectrophotometer (HACH Odyssey, Loveland, Colo.) based on the HACH procedure similar to 4500-P in the standard methods for wastewater analysis (American Public Health Association, 2012). The total nitrogen (TN) for those samples were measured following the Quikchem® Method 10-107-04-1-A (Wendt, 2000). The TAN for those samples were measured following the QuikChem® Method 10-107-06-5-J (Egan, 2015).

3.4 Mineralized nitrogen and nitrogen mineralization rate

3.4.1 Calculating mineralized nitrogen

The mineralized N was defined as the quantity of N transformed from organic into inorganic form in a given time. Only organic N was considered as the mineralizable form. The mineralizable N was obtained as the difference between TN and inorganic N. Inorganic N was assumed to be equal to the TAN. All oxidized forms of N (nitrate and nitrite) were assumed to be negligible. The N concentration was reported as mg N/g VS. Thus, organic N was calculated using Equation 3-1 shown below:

$$OrgN(t) = TN(t) - TAN(t)$$

(Equation 3-1)

where

- OrgN(t) is the concentration of organic N after t days of storage (mg N/g VS)
- TN(t) is the average total N concentration after t days of storage (mg N/g VS)
- TAN(t) is the average total ammonia N concentration after t days of storage (mg N/g VS)

The mineralized N (Nm) at each time step was calculated as the difference between organic N at the beginning of the experiment and organic N at the sampling time (Equation 3-2)

$$Nm(t) = OrgN(0) - OrgN(t)$$

(Equation 3-2)

where

- Nm(t) is the mineralized N concentration after t days of storage (mg N/g VS)
- OrgN(0) is the concentration of organic N at the beginning of the storage period (mg N/g VS)
- OrgN(t) is the concentration of organic N after t days of storage (mg N/g VS)

3.4.2 Calculation and analysis of nitrogen mineralization rate constant

A nonlinear regression approach described by Stanford and Smith (1972) was adopted and used in the N mineralization calculations. The mineralization rate was assumed to follow first-order kinetics represented (Equation 3-3):

$$dOrgN/dt = -kN$$

(Equation 3-3)

where

- OrgN is the concentration of organic N (mg N/g VS)
- t is the storage time (weeks)
- k is the N mineralization rate constant ($week^{-1}$)

The storage times were converted to weeks to use in Equation 3-3.
Integrating Equation 3-3 between time t_0 and t, yields:

$$OrgN(t) = OrgN(0) \times \exp(-kt)$$

(Equation 3-4)

where

- OrgN(0) is the initial amount of organic N (mg N/g VS)
- OrgN(t) is the amount of organic N at time t (mg N/g VS)

Substituting the relationship Nm(t) = OrgN(0) - OrgN(t) into Equation 3-4 and simplifying Equation 3-5, whose slope is the negative value of mineralization rate constant k when $ln(1 - Nm(t)/OrgN(0))$ is plotted against t:

$$ln\left(1-\frac{Nm(t)}{\text{OrgN}(0)}\right)=-kt$$

(Equation 3-5)

3.5 DNA extraction

The DNA was extracted from manure using modified methods developed for soils described by St-Pierre and Wright (2014), Hess et al. (2011), and Yu and Morrison (2004). In this study, the QIAamp Fast DNA Stool Mini Kits (#51604, QIAGEN, Hilden, Germany) were used. DNA was extracted from samples collected on days 0, 30, 90, and 180 and 0, 30, and 60 for the 180-d and the 60-d storage experiments, respectively. Briefly, the manure samples were beaten in a mix of 0.1 mm and 1.0 mm disruption beads (#9830 and #9832, respectively, Research Products International, Mt. Prospect, IL) to lyse the microbial cells within the inhibitex buffer, which separated inhibitors from DNA. The DNA from the lysate was bound to the silica membrane, and any remaining inhibitors and contaminants were removed by washing steps, and then DNA was eluted from the membrane. For each sample, approximately 0.2 to 0.5 g of manure was weighed and added into a 2 ml nuclease-free centrifuge tube. The added weight was based on the dry matter content of the sample, i.e., the more dry matter content, the less manure added. Then 1 ml of inhibitex buffer was added to each tube with sample. The mixture in each tube was vortexed for 1 min at full speed (3000 rpm) on the vortex mixer (M37615, Barnstead/Thermolyne, Dubuque, Iowa). After vortexing, the tube was incubated at 90°C in a water bath (Model 188, Precision Scientific, Chicago, Illinois) for 5 min. After incubation, the samples were vortexed for 15 sec, centrifuged for 1 min at 16,000 x g (IEC MicroCL 21R, Thermo ELECTRON CORPORATION, Osterode, Germany). After centrifuging, the supernatant was transferred into a new 2 ml nuclease-free centrifuge tube and the vortex and centrifuge steps were repeated. Approximately 4 to10 µl RNase A (depending on the quantity of the samples) was added into the tube. The tube was incubated for 3 min at 37°C in an incubator (CLASSIC C25KC, New Brunswick Scientific Co., Inc., Edison, New Jersey). The tube was centrifuged for 3 min at 16,000 x g and an aliquot of 600 µl of the supernatant was drawn and added into a new 2 ml tube along with 25µl proteinase K and 600µl AL buffer. The mixture was vortexed for 15 sec and incubated at 70°C in the water bath for 10 min. After incubation, 600µl of 100% ethanol was added to the tube and vortexed for 15 sec. An aliquot of 600 µl of the lysate (treated by proteinase K and 100% ethanol) was added to the labeled QIAmp spin column and centrifuged

for 1 min at 16,000 x g. The centrifuging step was repeated until all lysate was loaded into the column. After that, the column with DNA was placed in a new collection tube, and 500 µl AW1 buffer was added to the column. The column with the collection tube was centrifuged for 1 min at 16,000 x g. Then the column was placed in a new collection tube, and 500 µl AW2 buffer was added to the column. The column with the collection tube was centrifuged for 3 min at 16,000 x g. After centrifuging, the column was placed into a new 2 ml nuclease-free centrifuge tube, and 50 µl eluent buffer (ATE Buffer) was added into the column. The mixture was incubated for 2 min at room temperature and centrifuged at 16,000 x g for 1 min. And then the column was re-eluted with 30 µl eluent buffer, incubated at room temperature for 1 min and centrifuged at 16,000 x g for 1 min. The extracted DNA was in the 2 ml centrifuge tube with eluent buffer. All the extracted DNA samples were stored at -20°C in a freezer before being used for 16S rRNA analysis. The concentrations of extracted DNA were determined by use of a NanoDrop Lite Spectrophotometer (ND-LITE, Thermo Fisher Scientific, Wilmington, DE).

3.6 DNA gel electrophoresis

The extracted genomic DNA sequences were subjected to agarose gel electrophoresis. The DNA gel electrophoresis was a modified method described by JoVE (2019) for isolating and identifying DNA fragments by size. The DNA fragments of different lengths were loaded into an agarose porous gel. The agarose was a carbohydrate from red algae. Since the DNA nucleotides contained the negatively charged phosphate groups, after applying an electric field, the loaded fragments migrated through the gel towards the anode. The DNA ladder, which was a collection of fragments or bands of known sizes, were loaded into the gel as well. Since larger DNA fragments were more challenging to migrate through the gel than smaller fragments, after the gel run was completed, the presence of target DNA was determined through comparing the positions of the DNA sample to the DNA ladders.

3.6.1 Melting agarose and casting the gel

A 1% (w/v) gel was made up of 1 g of agarose and 100 mL of TAE buffer. The TAE buffer was made up of Tris-acetate buffer, at pH around 8.3, and EDTA, which sequestered divalent cations. The appropriately weighed agarose and running buffer were added to a flask, and the buffer volume was not more than one-third of the flask volume. The mixture of agarose

and buffer was melted by the microwave oven. Every 30 sec, the flask was taken out from the microwave oven, and the contents were well mixed. The step was repeated until completely dissolving the agarose. The 3 μL of 0.5 mg/ml Ethidium Bromide (EB) was then added into the solution. The EB is an aromatic compound used between individual base pairs or inserts of DNA and gives the DNA intense orange fluorescence under the UV light. When the agarose was cooling, the gel mold was prepared by placing the gel tray in a casting apparatus. The molten agarose was poured into a gel mold, allowed to harden at room temperature, and then put in the gel box to use.

3.6.2 Setting up and Running the Gel

The DNA samples were mixed with the gel-loading dye, which was made at a 6X concentration and helped the DNA be visualized and loaded into the wells. The gel-loading dye also helped the DNA to be determined the extent to which the sample migrated during the running. The power supply was set to the 130 volts for the short gel and 170 volts for the long gel, and in order to cover the gel surface, the running buffer was added into the gel box. The wires of the gel box were connected to the power supply. Since negatively charged DNA would move toward the anode (in red), which was positive, the bottom of the gel box was connected to the anode. The lid of the gel box was removed, and the DNA samples were slowly and carefully loaded into the gel. The 1kb DNA standard ladder (N3232L, NEB, Ipswich, MA) was loaded along with the DNA samples. And then the lid was replaced, and the power was turned on. The gel was running until the dye migrated to the appropriate distance.

3.6.3 Visualizing Separated DNA Fragments

The power supply was turned off, and the lid was removed from the gel box when the electrophoresis was completed. The gel tray with the gel was removed from the box, and the excess buffer was removed from the gel surface. A paper tower was used to absorb the remaining buffer from the gel tray. The gel was removed from the gel tray, and was exposed to the UV light to visualize the DNA fragments. The DNA fragment appeared as an orange fluorescent band, and a photo of the gel was taken. At the end of the gel electrophoresis, the gel and the buffer were properly disposed of according to the institutional regulations.

3.7 16S rDNA PCR (polymerase chain reaction)

The 16S rRNA genes (rDNA) were amplified by using universal primers 27f (5'-AGA GTT TGA TCM TGG CTC AG-3'; positions 8 to 27) and 1525r (5'-AAG GAG GTG WTC CAR CC-3'). Every PCR mix (25 µl) included 1 µl of DNA, 0.5 µl of primers 27f and 1525r, respectively, 0.5 µl of deoxynucleoside triphosphates (dNTPs) (N0447L, New England Biolabs® Inc., Ipswich, MA), 2.5 µl of 10X standard *Taq* reaction buffer (M0273, New England Biolabs® Inc., Ipswich, MA), 0.125 µl of *Taq* polymerase (M0273S, New England Biolabs® Inc., Ipswich, MA), and 19.875 µl of nuclease-free water. The DNA thermal cycler (T100^{TM}, BIO-RAD, Singapore) used for PCR was programmed as follows: The conditions consisted of an initial denaturation at 95°C for 3 min; 34 cycles at 95°C for 30-sec denaturation, 53°C for 30-sec annealing, and 68°C for 90-sec extension; and a final extension step consisting of 68°C for 5 sec. The PCR products were electrophoresed followed the same procedures in Chapter 3.6.

3.8 16S rRNA sequencing and analysis

All the extracted DNA sequences were sent to the Argonne National Lab for 16S rRNA sequencing. At the Argonne National Lab, the V4 region of the 16S rRNA gene (515F-806R) was amplified using the Earth Microbiome Project barcoded primer set, adapted for the Illumina MiSeq by adding nine extra bases in the adapter region of the forward amplification primer that support paired-end sequencing. The primers also included the Illumina flowcell adapter sequences. The forward amplification primer also contained a twelve base barcode sequence that supports pooling of up to 2,167 different samples in each lane (Caporaso et al., 2010; Caporaso et al., 2012). Each 25ul PCR reaction contained 12ul of MoBio PCR Water (Certified DNA-Free), 10ul of 5 Prime HotMasterMix (1x), 1ul of Forward Primer (5uM concentration, 200pM final), and 1ul of template DNA. The conditions for PCR were also followed: 94°C for 3 minutes to denature the DNA, with 35 cycles at 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s; with a final extension of 10 min at 72 °C to ensure complete amplification. Amplicons were quantified using PicoGreen (Invitrogen) and a plate reader. Once quantified, different volumes of each of the products are pooled into a single tube so that each amplicon is represented equally. This pool is then cleaned up using the UltraClean® PCR Clean-Up Kit (MoBIO), and then quantified using the Qubit (Invitrogen). After quantification, the molarity of the pool is determined and diluted

down to 2nM, denatured, and then diluted to a final concentration of 6.75pM with a 10% PhiX spike for sequencing on the Illumina MiSeq.

The sequence data received from the Argonne National Lab will be analyzed using the Quantitative Insights into Microbial Ecology 2 (QIIME 2) platform (Bolyen et al., 2018). The sample sequences will be fetched using the Barcode, and then the Barcode and primer sequences will be removed. The sequences of poor quality will be removed as well. The clean sequences will be clustered into OTU (Operational taxonomic units) using QIIME 2 software at 97% similarity, and the abundance information for each OTU in each sample will be statistically analyzed. Phylogenetic diversity analysis will be evaluated in terms of alpha and beta diversity indices using phyloseq package in R (McMurdie & Holmes, 2013). Alpha diversity will be calculated from species richness using Chao1 (Chao, 1984) and species evenness using Shannon Index (Hill, 1973). Microbial community differences between samples or Beta diversity will be computed using UniFrac (Lozupone & Knight, 2005), Jaccard (Jaccard, 1901), and Bray Curtis (Bray & Curtis, 1957) algorithms.

3.9 Statistical analysis

Analysis of variance (ANOVA) was conducted using the JMP® Pro 14 (SAS Institute Inc., Cary, NC, 2019), to examine the effects of storage time (6 storage periods for 180-d storage and 4 storage periods for 60-d storage) and temperature (3 temperatures) on N mineralization. Each storage period had 3 observations and each temperature had 6 observations for 180-d and 4 observations for 60-d storage. The observations were assumed to be normally distributed and there was no interaction between storage time and temperature. Tukey's honestly significant difference (HSD) test was used to discern differences in means. The level of significant difference for all effects and comparisons was set at $p \leq 0.05$.

The paired t-test was used to examine the effects of total solids (for 180-d storage), adding manure seed (for 60-d storage), and using sterilization operation. For testing the effects of total solids, the H and L non-sterilized manure under same temperature after same storage period was paired, and the difference of corresponding Nm for each pair was calculated. Then the t-test was used to compare the mean difference of Nm to 0. The assumption of the paired t-test included that the differences between pairs were normally distributed. The null hypothesis was

that the mean difference of Nm between paired observations was 0. If the mean difference was not 0, the null hypothesis was rejected. If the mean difference of Nm of H and L manure was large than 0, the HTS was more beneficial to N mineralization, and vice versa. To test the effect of manure seed, the Nm of non-sterilized MS and SO samples under same temperature after same storage period was paired. To test the effect of sterilization operation, the Nm of sterilized and non-sterilized manure under same temperature after same storage period was paired. And then the MS and SO, and R and R0 manure were also examined by the paired t-test following the same process.

Chapter 4 Results

4.1 Manure characteristics

The characteristics of manure (raw and sterilized) used in the 180-d storage experiments are shown in Tables 4-1 and 4-2, and the characteristics of raw manure mixed with manure seed and manure seed only (non-sterilized and sterilized) used in the 60-d storage experiments are shown in Tables 4-3 and 4-4. The average values of the pertinent manure characteristics used in the 180-d storage experiment ranged from 19 to 78 g/L for the TS; 16 to 66 g/L for the VS; 6.3 to 8.3 for the pH; 34 to 87 mg N/g VS for the TN; 0.4 to 8 mg N/g VS for the TAN; 2 to 9 mg P/g VS for the TP; and 1,247 to 1,685 mg/g VS for the TCOD. The ranges of the average values of the manure characteristics used in the 60-d storage experiment were from 49 to 143 g/L for the TS; 40 to 131g/L for the VS; 6.2 to 9.8 for the pH; 20 to 32 mg N/g VS for the TN; 3 to 15 mg N/g VS for the TAN; 8 to 12 mg P/g VS for the TP; and 1,099 to 1,341 mg/g VS for the TCOD. In general, the average values of characteristics of the H and MS manure samples were higher than those of the L and SO manure samples except for the pH and TAN. The pH of H, L, MS, and SO manure samples were comparable, but an increase of manure pH was observed after sterilization in most cases.

The TAN of SO samples was generally higher than that of MS samples. Compared to the SO samples, which were from an earthen storage pit and already mineralized for several days, the MS samples with more raw manure (without mineralization to release TAN from organic N) resulted in higher TAN. Meanwhile, higher temperatures, such as sterilization operation, are known to accelerate the release of CO_2, and resulting in increasing pH (Gerlach, Lambrecht, & Oelßner, 2019).

The characteristics of dairy manure from other parts of the world reported in literature were compiled and compared to the results of this study (Table 4-5). The data was collected from different countries, including Canada, China, Denmark, Estonia, Holland, and the United States. There were some similarities and differences in manure characteristics for all the dairy manure from different countries. For example, the concentrations of TS and VS (Table 4-5) from those countries listed above were different, ranging from 27 g/L to 266 g/L and 10 g/L to 137 g/L, respectively; the TN and TAN concentrations (Table 4-5) were also different from each

other, ranging from 28 mg N/g VS to 175 mg N/g VS and 4 mg N/g VS to 77 mg N/g VS, respectively. Not like the TS, VS, TN, and TAN, almost all manure samples had similar pH, ranging from 6.3 to 8.4; and several concentrations of TP and TCOD were comparable to the results of this study, e.g., the TP concentrations from Denmark, Estonia, and Iowa were similar to the TP in this study; the TCOD from China and Holland were close to the concentrations of TCOD observed in this study. Overall, the dairy manures from different locations have their unique characteristics, and the main reasons include the followings: 1) the size, species, sex, and age of dairy cows impacts on the manure compositions (ASABE, 2010); 2) the dairy cattle feed composition has effects on the manure characteristics (Sørensen, Weisbjerg, & Lund, 2003); 3) housing, bedding and rearing management influence the characteristics of manure (Eghball & Power, 1994). Thus, the models related to N cycling in dairy farms need more data from different conditions to make them more robust and reliable.

Table 4-1 The average characteristics of non-sterilized (R) raw manure for 180-d storage experiment

Characteristics	*Storage temperature (°C)*					
	10	***20***	***30***	***10***	***20***	***30***
	----------*High TS*----------			----------*Low TS*----------		
TS (g/L)	46	71	72	21	28	28
VS (g/L)	39	60	61	17	25	25
pH	6.8	7.4	7.3	6.8	6.3	6.3
TN (mg N/g VS)	71	45	42	66	50	50
TAN (mg N/g VS)	3	6	5	8	4	4
TP (mg P/g VS)	8	9	7	7	2	2
TCOD (mg / g VS)	1,685	1,366	1,364	1,414	1,291	1,291

Table 4-2 The average characteristics of sterilized (R0) raw manure for 180-d storage experiment

Characteristics	*Storage temperature (°C)*					
	10	***20***	***30***	***10***	***20***	***30***
	----------*High TS*----------			----------*Low TS*----------		
TS (g/L)	66	75	78	19	36	36
VS (g/L)	57	64	66	16	33	33
pH	7.6	8.3	8.3	8.2	6.3	6.3
TN (mg N/g VS)	48	43	40	87	34	34
TAN (mg N/g VS)	0.4	5	4	5	1	1
TP (mg P/g VS)	6	8	9	8	2	2
TCOD (mg / g VS)	1,282	1,268	1,307	1,919	1,247	1,247

Table 4-3 The average characteristics of non-sterilized (R) raw manure and manure seed for 60-d storage experiment

Characteristics	*Storage temperature (℃)*					
	10	*20*	*30*	*10*	*20*	*30*
	----------Raw manure with manure seed----------			*----------Manure seed only----------*		
TS (g/L)	111	109	109	49	49	49
VS (g/L)	101	98	98	40	40	40
pH	6.2	6.3	6.3	7.5	7.7	7.7
TN (mg N/g VS)	24	29	29	32	32	32
TAN (mg N/g VS)	4	4	4	14	15	15
TP (mg P/g VS)	8	11	11	8	10	10
TCOD (mg / g VS)	1,150	1,099	1,099	1,232	1,115	1,115

Table 4-4 The average characteristics of sterilized (R0) raw manure and manure seed for 60-d storage experiment

Characteristics	*Storage temperature (℃)*					
	10	*20*	*30*	*10*	*20*	*30*
	----------Raw manure with manure seed----------			*----------Manure seed only----------*		
TS (g/L)	143	133	133	71	55	55
VS (g/L)	131	121	121	59	45	45
pH	6.4	6.5	6.5	9.6	9.8	9.8
TN (mg N/g VS)	22	26	26	20	23	23
TAN (mg N/g VS)	3	3	3	4	6	6
TP (mg P/g VS)	10	10	10	12	12	12
TCOD (mg / g VS)	1,146	1,282	1,282	1,341	1,201	1,201

Table 4-5 The characteristics of manure from this study and literature

Location	*TS (g/L)*	*VS (g/L)*	*pH*	*TN (mg N/g VS)*	*TAN (mg N/g VS)*	*TP (mg P/g VS)*	*TCOD (mg/g VS)*	*C/N ratio*	*Source*
Alma, Ontario, Canada	92	65	7.3	28	15	-	-	18.2	Maldaner, Wagner-Riddle, VanderZaag, Gordon, and Duke (2018)
Ames, IA, USA	105	91	6.9	28	8	6	321	-	Wu-Haan, Burns, Moody, Grewell, and Raman (2010)
Ilmatsalu, Estonia	61	47	-	100	62	11	-	-	Pitk, Palatsi, Kaparaju, Fernández, and Vilu (2014)
Dayton, VA, USA	46	23	7.0	126	62	20	-	-	Shen, Ogejo, and Bowers (2011)
Pingdu, Shandong, China	266	137	8.4	36	-	-	-	20.2	X. Li, Shi, Yang, Xu, and Guo (2019)
Prairie du Sac, WI, USA	27	10	6.8	175	77	-	-	8.1	Holly, Larson, Powell, Ruark, and Aguirre-Villegas (2017)
Xi'an, Shaanxi, China	160	124	7.7	85	4	4	737	8.2	Yun et al. (2019)
Chatham, VA, USA	75	60	7.6	54	22	9	1,330	-	Collins, Ogejo, and King (2012)
Denmark	95	75	-	61	24	12	-	-	Sommer et al. (2007)
Holland	91	73	-	53	23	-	1,521	-	El-Mashad, Van Loon, Zeeman, and Bot (2005)
VA, USA	72	61	7.3	42	5	7	1,364	-	30-H-R samples from 180-d storage experiment in this study
VA, USA	109	98	6.3	29	4	11	1,099	-	30-MS-R samples from 60-d storage experiment in this study

4.2 The manure characteristics at different storage period

4.2.1 The TS and VS

The TS concentrations, VS concentrations, and VS/TS of the incubated manure for the 180-d storage simulation experiment are shown in Figures 4-1, 4-2 and 4-3, respectively. In general, the TS concentration of non-sterilized H and L manure (Figure 4-1 A & C) at 20 and 30℃ decreased with the storage time. The decrease in TS of the 10-L-R group was much less than that of the former groups. For sterilized manure (Figure 4-1 B & D), the TS concentrations of 20-L-R0 and 30-L-R0 groups decreased with the storage time as non-sterilized manure, but the TS concentrations of 10-H-R0, 10-L-R0, and 20-H-R0 did not have obvious change over time. All the VS concentrations had the same trend as the TS concentrations, but the extent of changes in the VS concentrations was different from that of TS. As the VS/TS shown in Figure 4-3, the VS/TS of non-sterilized manure at 20 and 30℃ decreased more than that of sterilized manure. The highest decrease of VS/TS was occurred in the groups of non-sterilized manure stored at 30℃. The VS/TS of sterilized manure and non-sterilized manure at 10℃ has no obvious changes over storage time.

The TS concentrations, VS concentrations, and the VS/TS of manure for the 60-d storage experiment are shown in Figures 4-4, 4-5 and 4-6, respectively. The highest decrease of TS concentration was seen in the group of 10-SO-R0, while the highest decrease of VS concentration occurred in the group of 30-MS-R. The concentration changes of TS and VS in 60-d storage experiment was not as large as those in the 180-d storage experiment, and the VS/TS in all groups in 60-d test did not have obvious change compared to that in 180-d test.

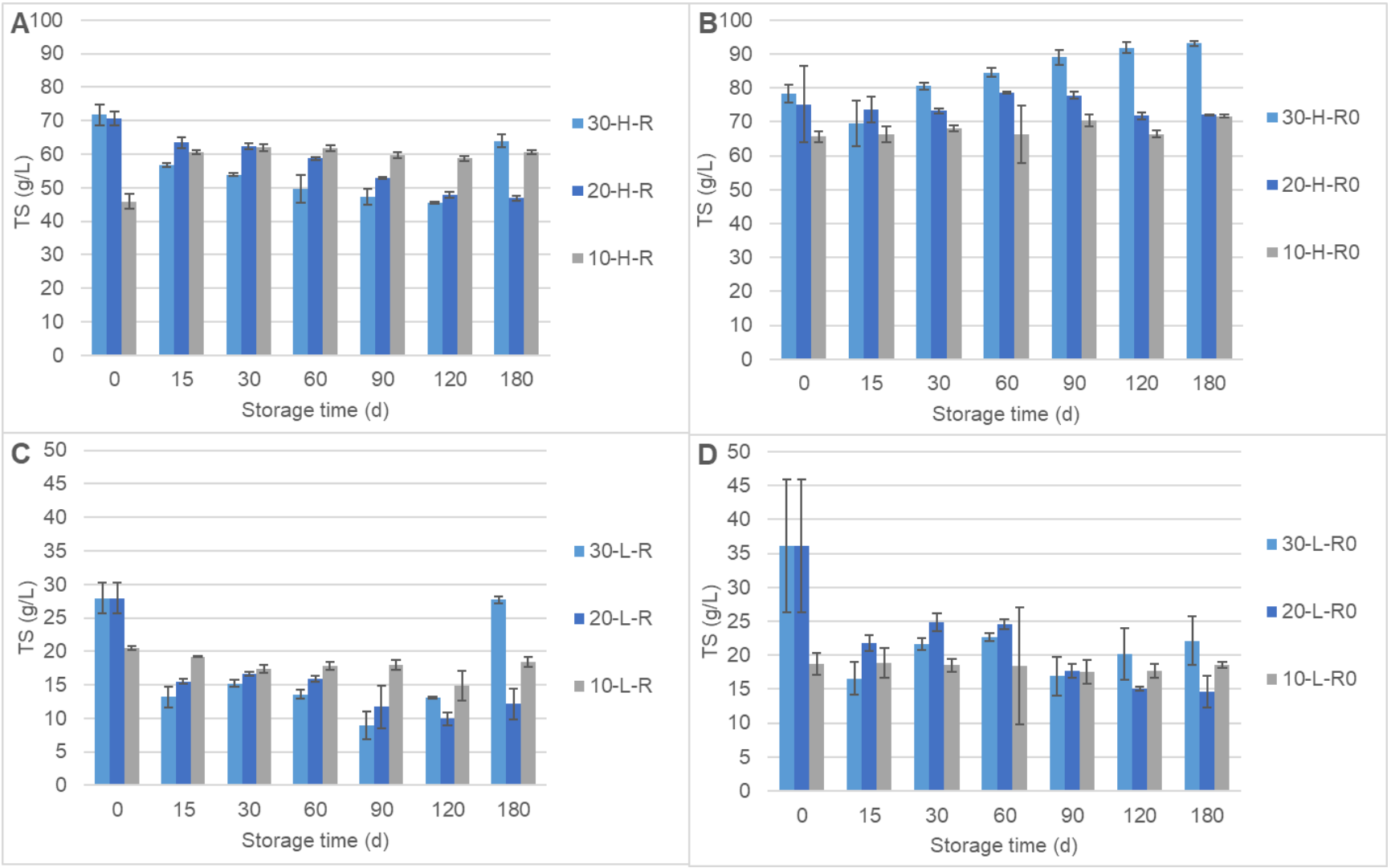


Figure 4-1 The TS concentrations of manure during 180-d storage period
(A: The TS of high TS and non-sterilized samples stored at 30/20/10℃; B: The TS of high TS and sterilized samples stored at 30/20/10℃; C: The TS of low TS and non-sterilized samples stored at 30/20/10℃; D: The TS of low TS and sterilized samples stored at 30/20/10℃. The error bar indicates the standard deviation)

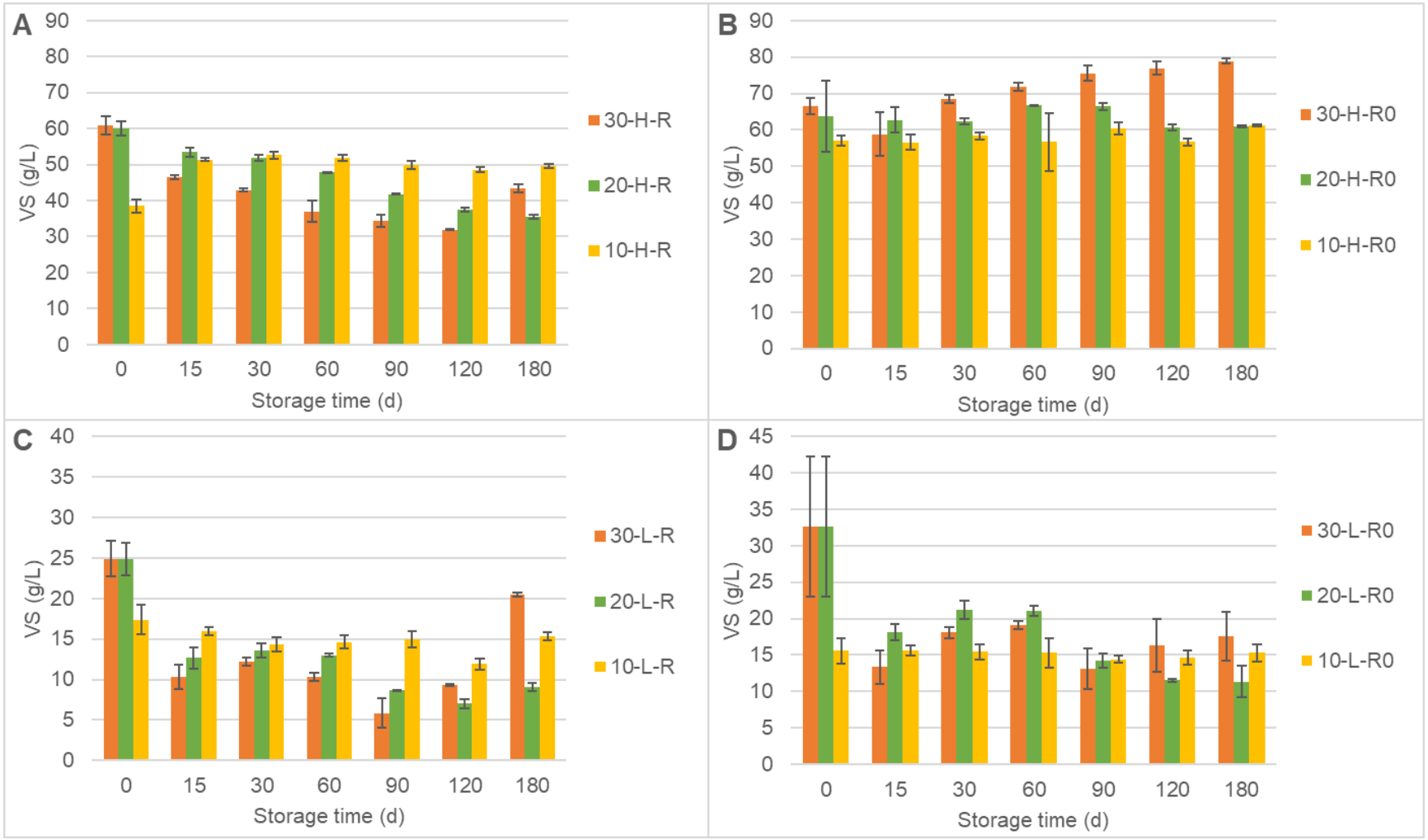


Figure 4-2 The VS concentrations of manure during 180-d storage period
(A: The VS of high TS and non-sterilized samples stored at 30/20/10℃; B: The VS of high TS and sterilized samples stored at 30/20/10℃; C: The VS of low TS and non-sterilized samples stored at 30/20/10℃; D: The VS of low TS and sterilized samples stored at 30/20/10℃. The error bar indicates the standard deviation)

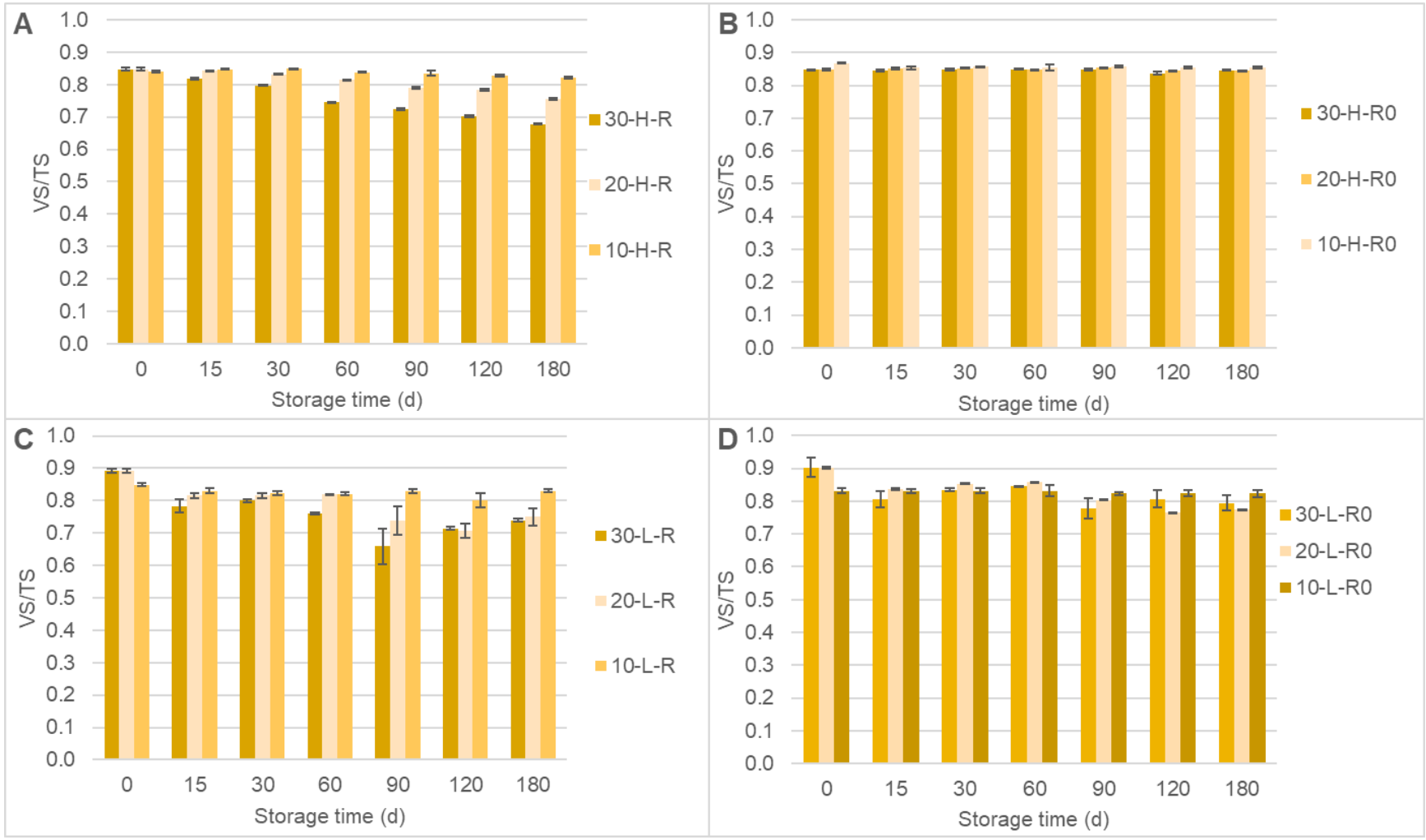


Figure 4-3 The VS/TS of manure during 180-d storage period
(A: The VS/TS of high TS and non-sterilized samples stored at 30/20/10℃; B: The VS/TS of high TS and sterilized samples stored at 30/20/10℃; C: The VS/TS of low TS and non-sterilized samples stored at 30/20/10℃; D: The VS/TS of low TS and sterilized samples stored at 30/20/10℃. The error bar indicates the standard deviation)

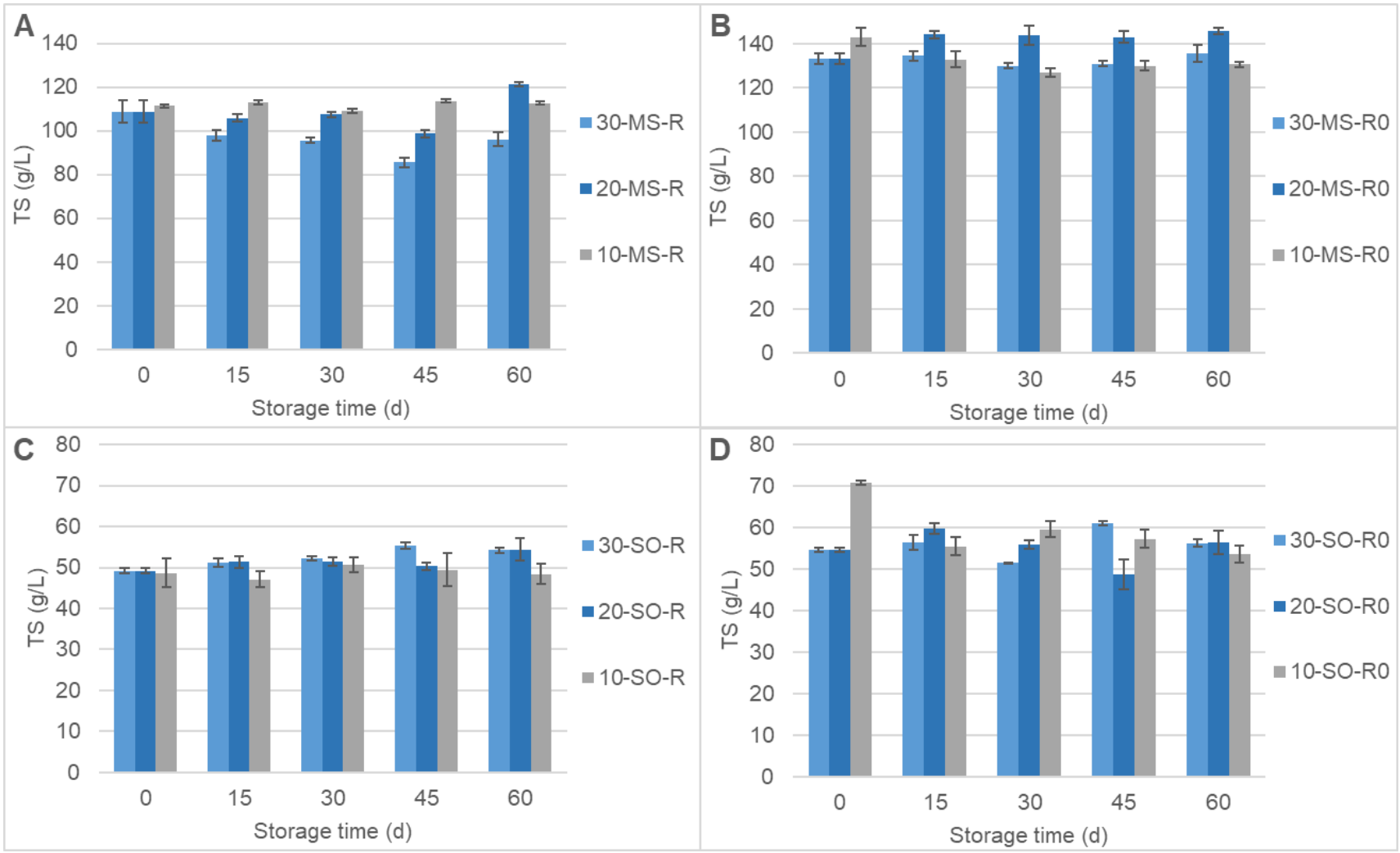


Figure 4-4 The TS concentrations of manure during 60-d storage period
(A: The TS of non-sterilized raw manure with manure seed stored at 30/20/10℃; B: The TS of sterilized raw manure with manure seed stored at 30/20/10℃; C: The TS of non-sterilized manure seed only stored at 30/20/10℃; D: The TS of sterilized manure seed only stored at 30/20/10℃. The error bar indicates the standard deviation)

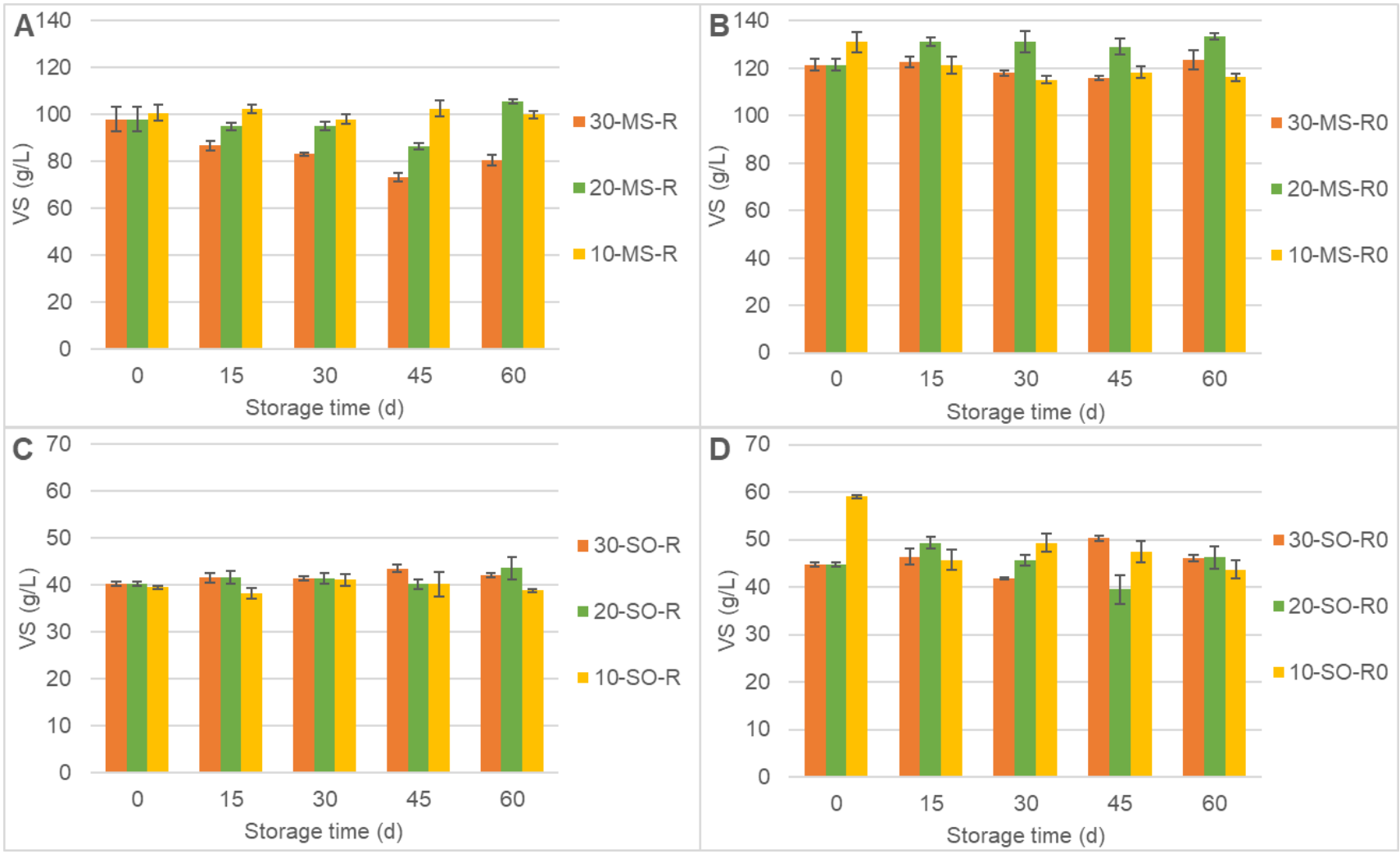


Figure 4-5 The VS concentrations of manure during 60-d storage period
(A: The VS of non-sterilized raw manure with manure seed stored at 30/20/10℃; B: The VS of sterilized raw manure with manure seed stored at 30/20/10℃; C: The VS of non-sterilized manure seed only stored at 30/20/10℃; D: The VS of sterilized manure seed only stored at 30/20/10℃. The error bar indicates the standard deviation)

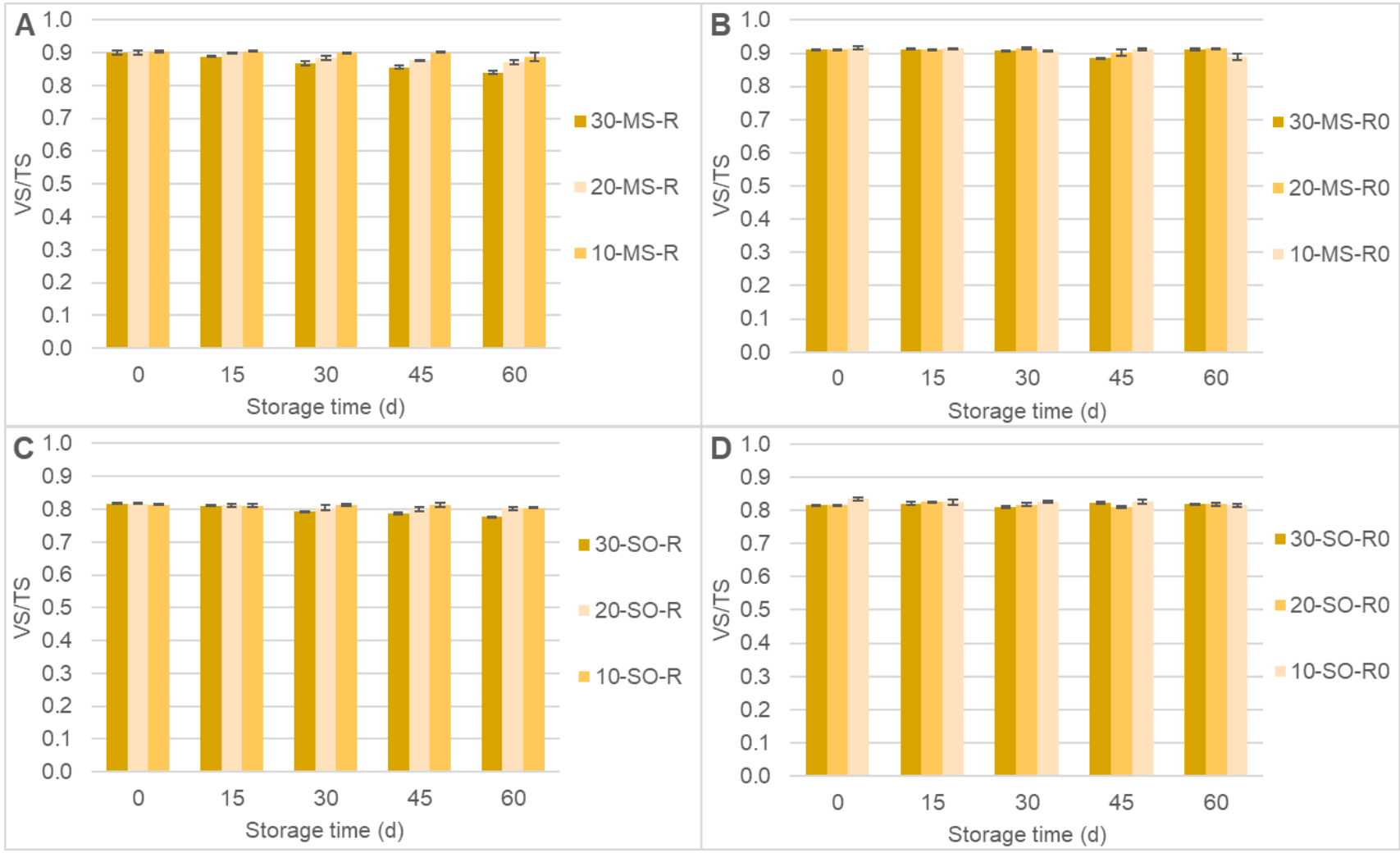


Figure 4-6 The VS/TS of manure during 60-d storage period
(A: The VS/TS of non-sterilized raw manure with manure seed stored at 30/20/10℃; B: The VS/TS of sterilized raw manure with manure seed stored at 30/20/10℃; C: The VS/TS of non-sterilized manure seed only stored at 30/20/10℃; D: The VS/TS of sterilized manure seed only stored at 30/20/10℃. The error bar indicates the standard deviation)

4.2.2 The pH

The pH of manure during 180-d storage is shown in Figure 4-7. Overall, the pH increased with the storage time at all temperatures for non-sterilized, H and L manure. No obvious increase in pH was observed for sterilized manure groups of 10-H-R0, 10-L-R0, 20-H-R0, and 30-H-R0, except for the 20-L-R0 and 30-L-R0 that increased as non-sterilized manure.

The pH of manure samples during 60-d storage are shown in Figure 4-8. For MS manure (Figure 4-8 A), the pH was close in magnitude for groups 30-MS-R0, 20-MS-R0, 10-MS-R0. Not like the sterilized manure at all temperatures and the sterilized and non-sterilized manure stored at 10℃, the pH of non-sterilized manure stored at 20 and 30℃ started increasing after 30 days of storage, and reached up to 8.4 after 60 days of storage; and the pH of non-sterilized manure was higher at 30℃ compared to that at other temperatures. Similar to the MS samples, pH of the SO (Figure 4-8 B) manure did not change considerably during the storage.

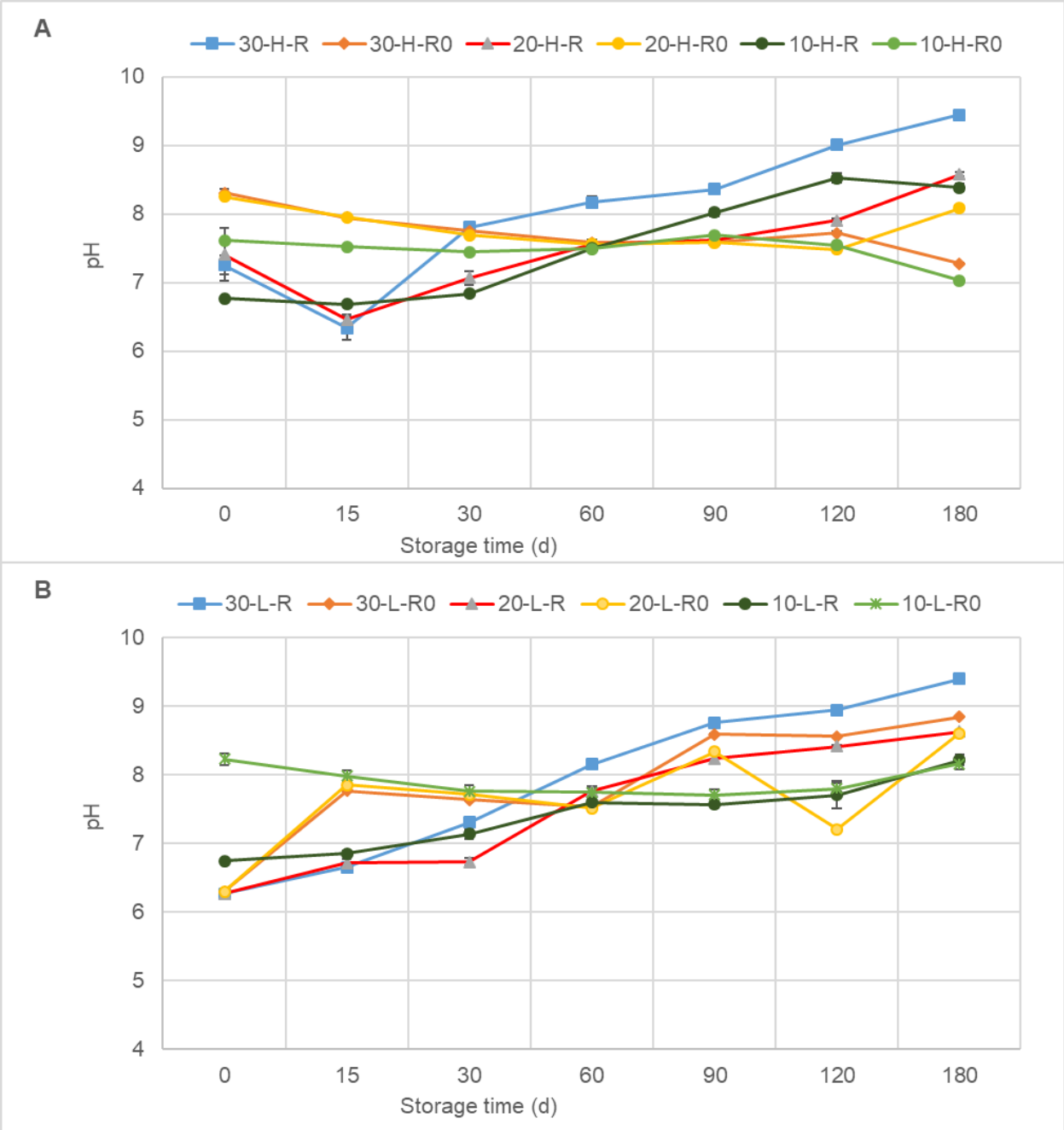


Figure 4-7 The pH of manure during 180-d storage period
(A: The pH of high TS manure stored at 30/20/10℃; B: The pH of low TS manure stored at 30/20/10℃; The error bar indicates the standard deviation)

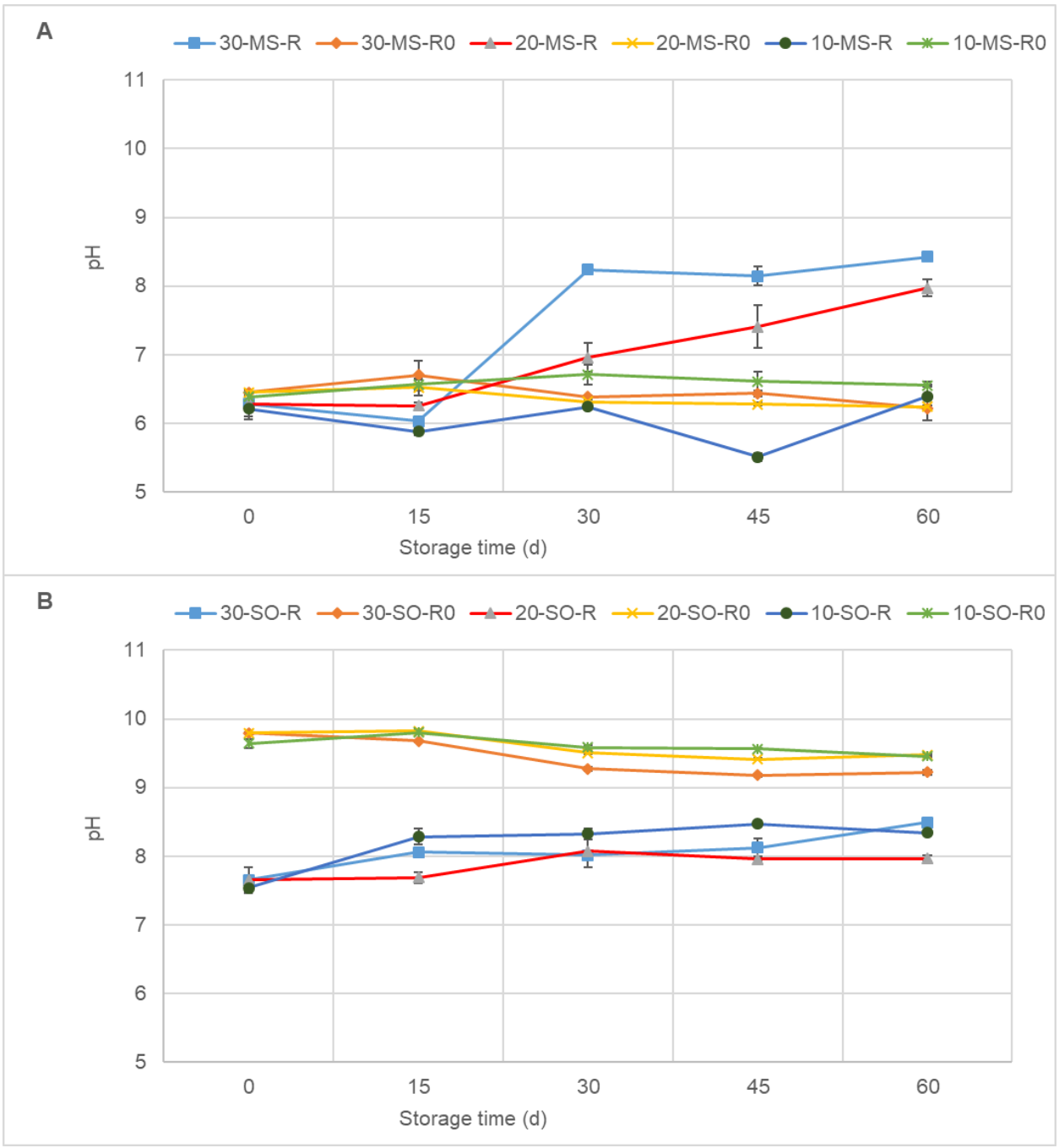


Figure 4-8 The pH of manure during 60-d storage period
(A: The pH of raw manure with manure seed stored at 30/20/10℃; B: The pH of manure seed only stored at 30/20/10℃; The error bar indicates the standard deviation)

4.2.3 The TCOD

The TCOD concentrations of manure during 180- and 60-d storage periods are shown in Figures 4-9 and 4-10, respectively. Overall, the TCOD concentrations decreased during the storage, except for manure stored at 10℃ in 180-d storage period. The ranges of TCOD concentrations for 180-d and 60-d storage periods were from 454 mg/g VS to 2,704 mg/g VS and 309 mg/g VS to 1,447 mg/g VS, respectively.

4.2.4 The TP

The TP concentrations of manure during 180- and 60-d storage are shown in Figures 4-11 and 4-12, respectively. Since phosphorus is not volatile, it stays in the dairy manure during storage and treatments. Organic forms of P stay as a part of microorganisms in dairy manure, such as ATP, phospholipids, and DNA, and the reactive P dissolved in the solution. Thus, theoretically, no change in TP should occur during storage. Overall, the TP concentrations of most manure groups remained stable during 180-d storage. For manure groups of 30-H-R, 30-L-R, 30-L-R0, 20-L-R, and 10-H-R, the TP concentrations increased on day 180 for the 180-d storage period. This is possibly due to the reduction in moisture content during the storage time (Bernal, Navarro, Roig, Cegarra, & Garcia, 1996). For the 60-d storage period, all the TP concentrations of manure decreased or remained constant, which is the same as the expectation.

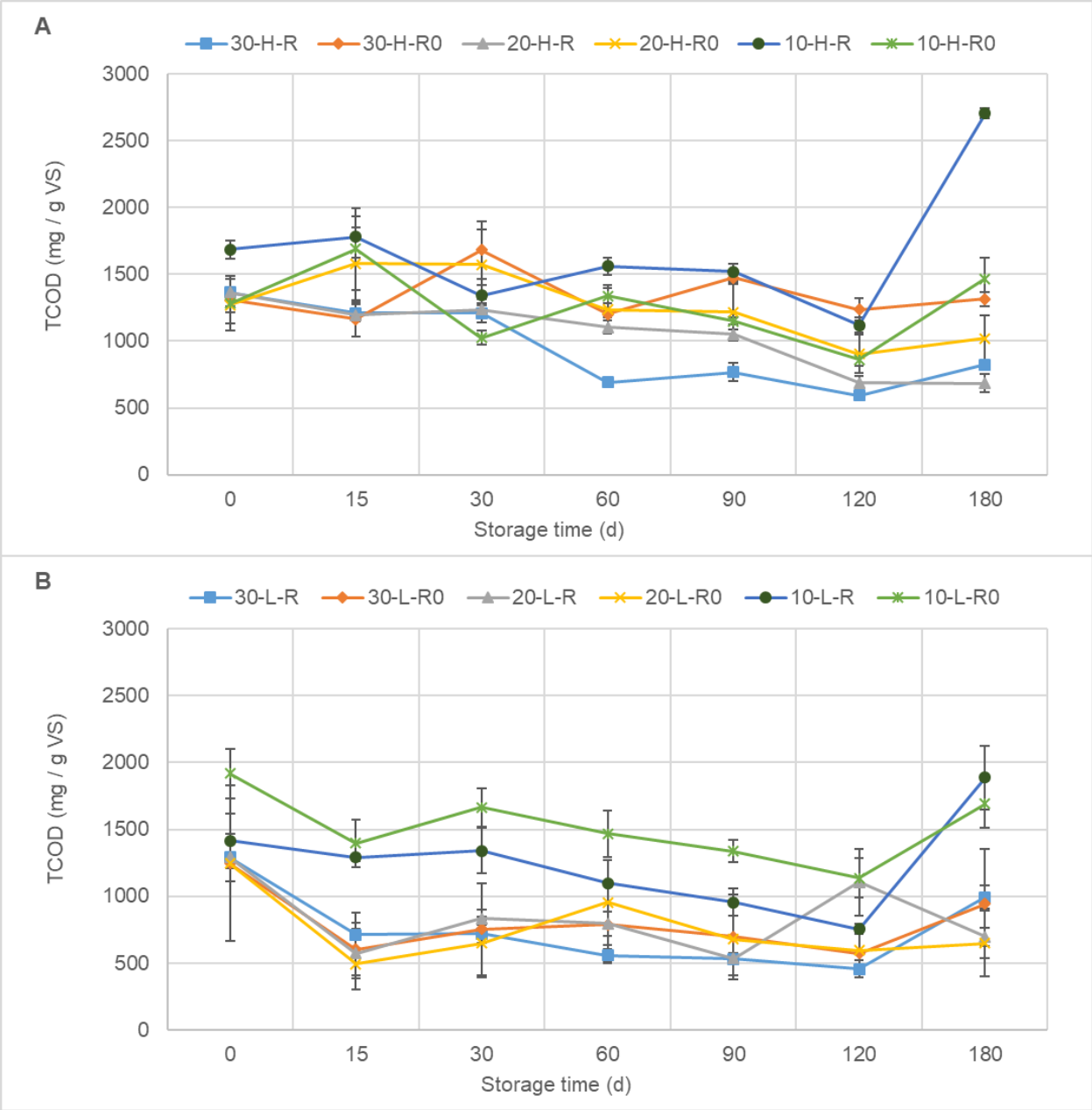


Figure 4-9 The concentrations of TCOD of manure during 180-d storage period (A: The TCOD of high TS manure stored at 30/20/10℃; B: The TCOD of low TS manure stored at 30/20/10℃; The error bar indicates the standard deviation)

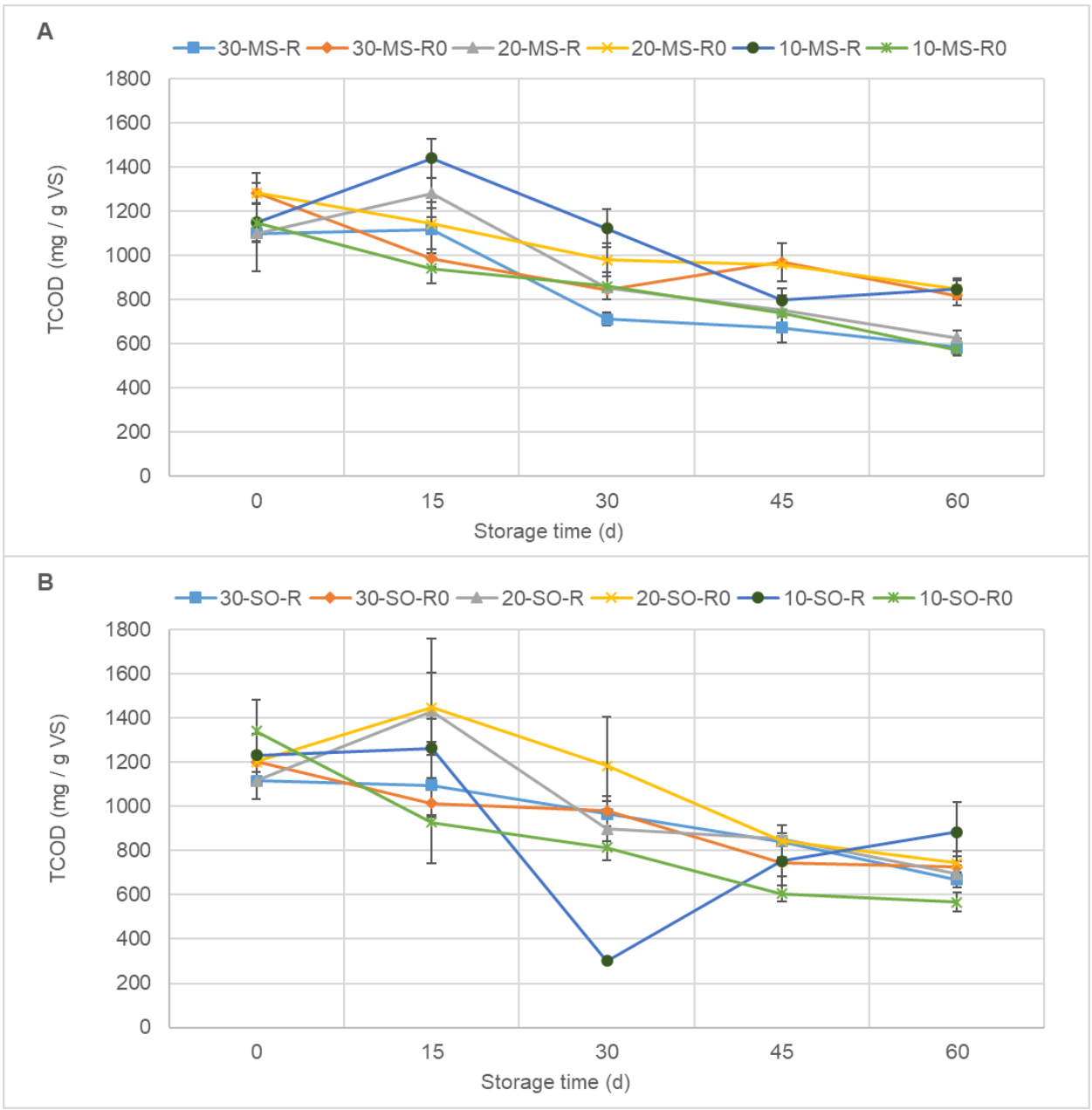


Figure 4-10 The concentrations of TCOD of manure during 60-d storage period
(A: The TCOD of raw manure with manure seed stored at 30/20/10℃; B: The TCOD of manure seed only stored at 30/20/10℃; The error bar indicates the standard deviation)

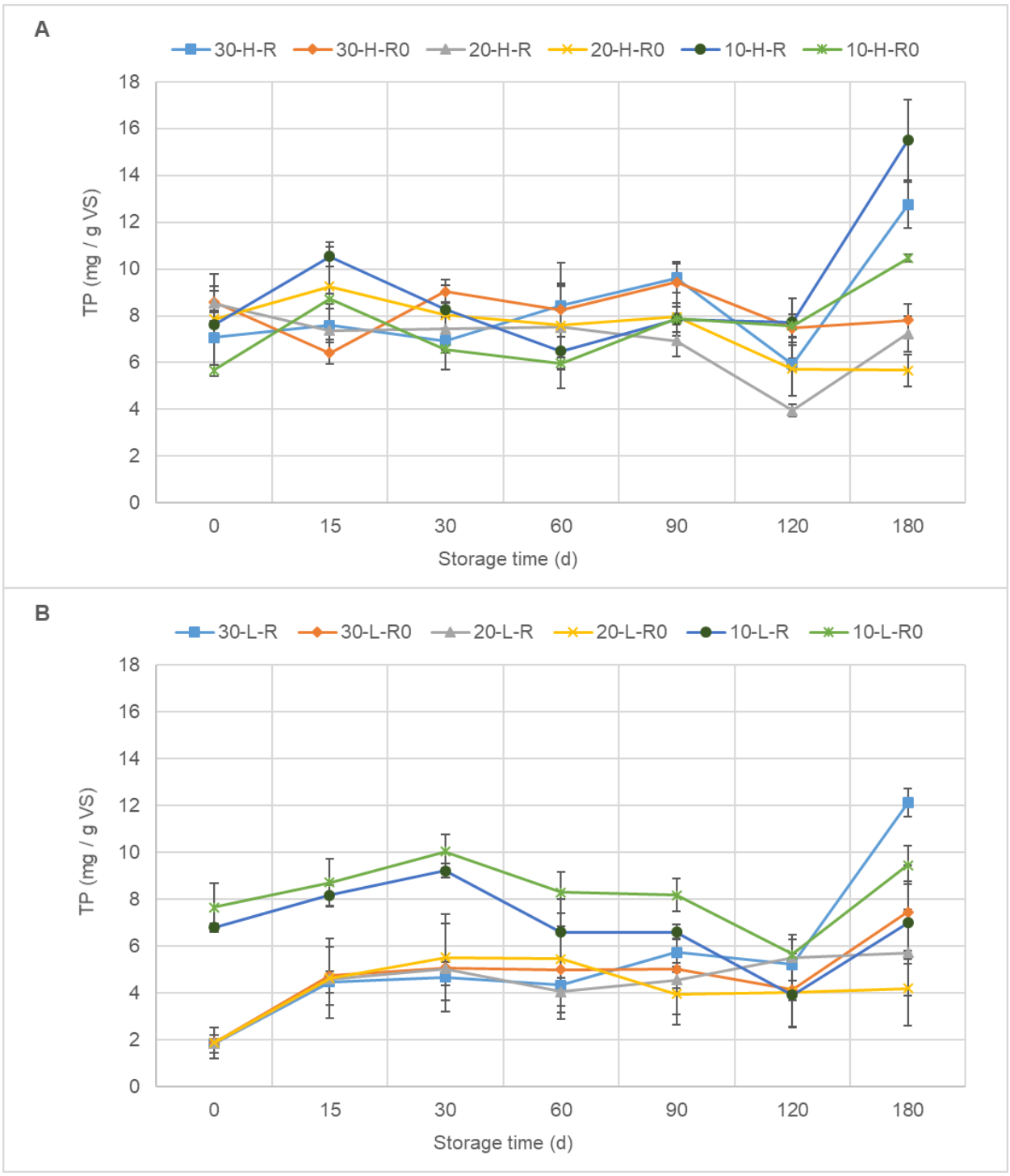


Figure 4-11 The concentrations of TP of manure during 180-d storage period
(A: The TP of high TS manure stored at 30/20/10℃; B: The TP of low TS manure stored at 30/20/10℃; The error bar indicates the standard deviation)

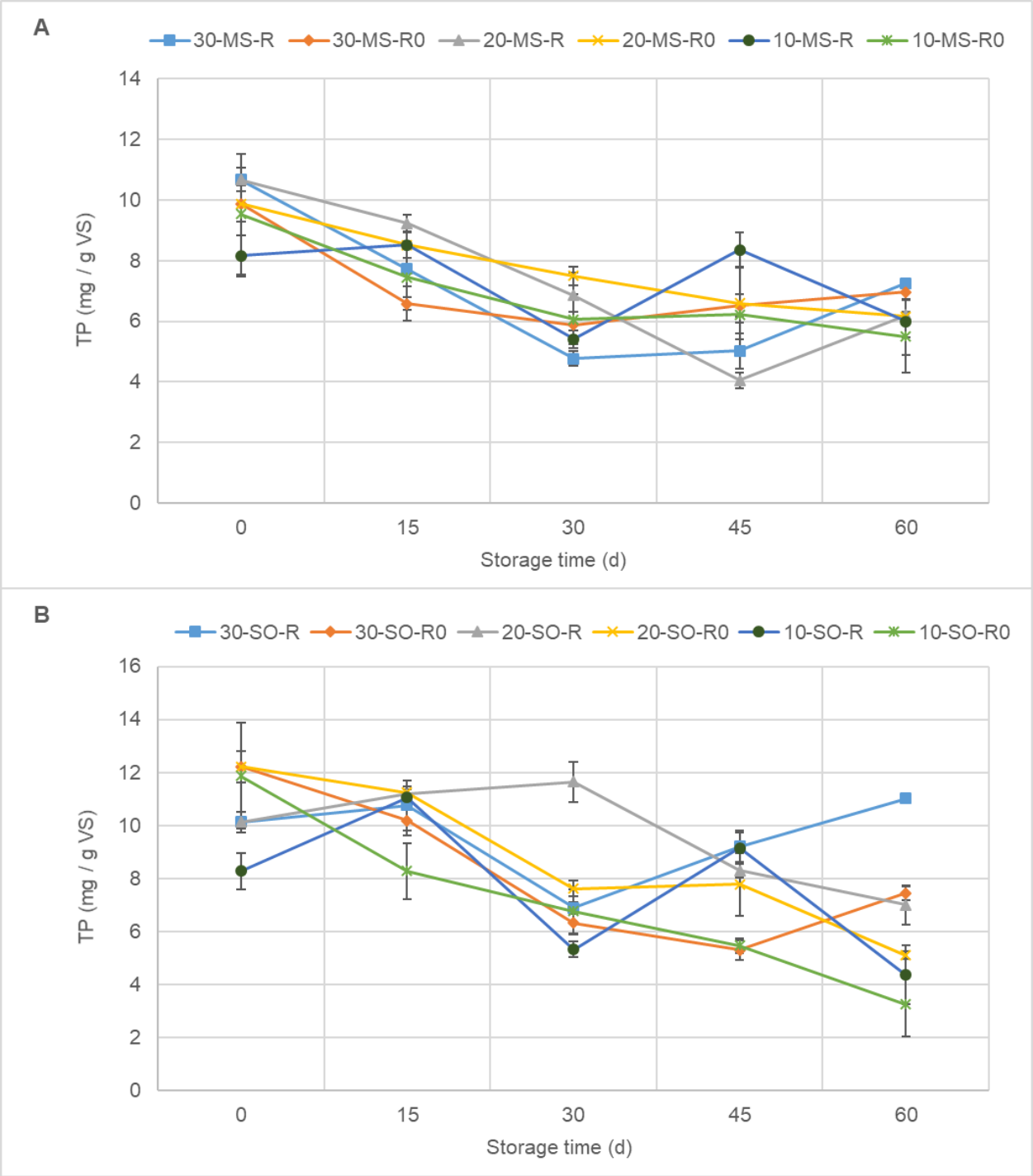


Figure 4-12 The concentrations of TP of manure during 60-d storage period
(A: The TP of raw manure with manure seed stored at 30/20/10℃; B: The TP of manure seed only stored at 30/20/10℃; The error bar indicates the standard deviation)

4.3 Nitrogen mineralization

4.3.1 180-d storage experiment

The Nm during the 180-d storage period is shown in Figure 4-13. The storage time and temperature had significant ($p < 0.05$) effects on the Nm during the 180-d storage period. Comparing the Nm revealed similarities in concentrations of the Nm at 20 and 30℃ but they were significantly different ($p < 0.05$) from the Nm at 10℃. Nitrogen mineralized after 30, 120, and 180 d of storage were significantly ($p < 0.05$) higher than other times samples during the storage periods. The TS content and sterilization operation showed that both factors had significant ($p < 0.05$) effects on the Nm. Specifically, Nm in the L manure samples were higher ($p < 0.05$) than that in the H manure, and the Nm in non-sterilized manure was significant ($p < 0.05$) higher than that in sterilized manure.

4.3.2 60-d storage experiment

The Nm during the 60-d storage period is shown in Figure 4-14. Similar to the results in 180-d storage experiment, the temperature had a significant ($p < 0.05$) effect on the Nm, while there was no evidence ($p > 0.05$) to conclude that the storage time had any effect on the Nm. The Nm at 20 and 30℃ were similar but significantly ($p < 0.05$) different from the Nm at 10℃. In addition, using manure seed at the beginning of storage had a significant ($p < 0.05$) effect on the Nm, and the MS manure samples had higher Nm than the SO manure samples. For the manure stored at 20 and 30℃, the sterilization operation had a significant ($p < 0.05$) effect on the Nm, and the non-sterilized (R) manure had higher Nm compared to the sterilized (R0) manure. However, for the manure stored at 10℃, there was no significant difference on the Nm between sterilized and non-sterilized manure.

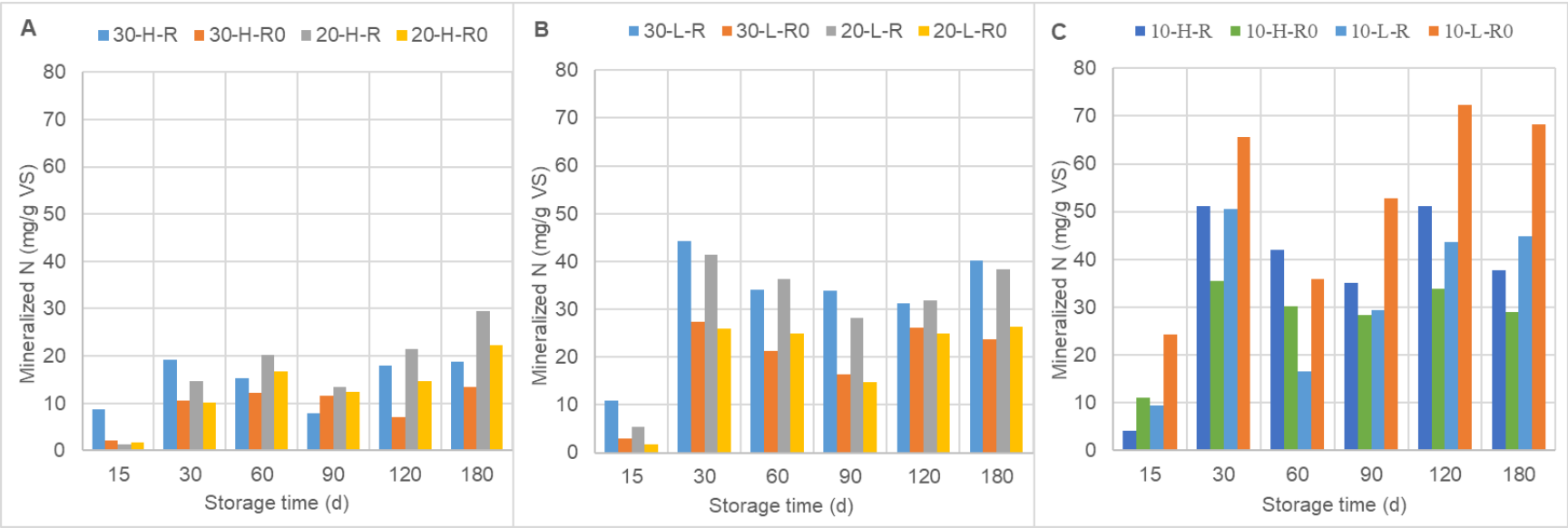


Figure 4-13 The concentrations of mineralized nitrogen (Nm) in raw manure during a 180-d storage period at different temperatures (A: The Nm of high TS manure stored at 30 and 20℃; B: The Nm of low TS manure stored at 30 and 20℃; C: The Nm of high and low TS manure stored at 10℃; The error bar indicates the standard deviation)

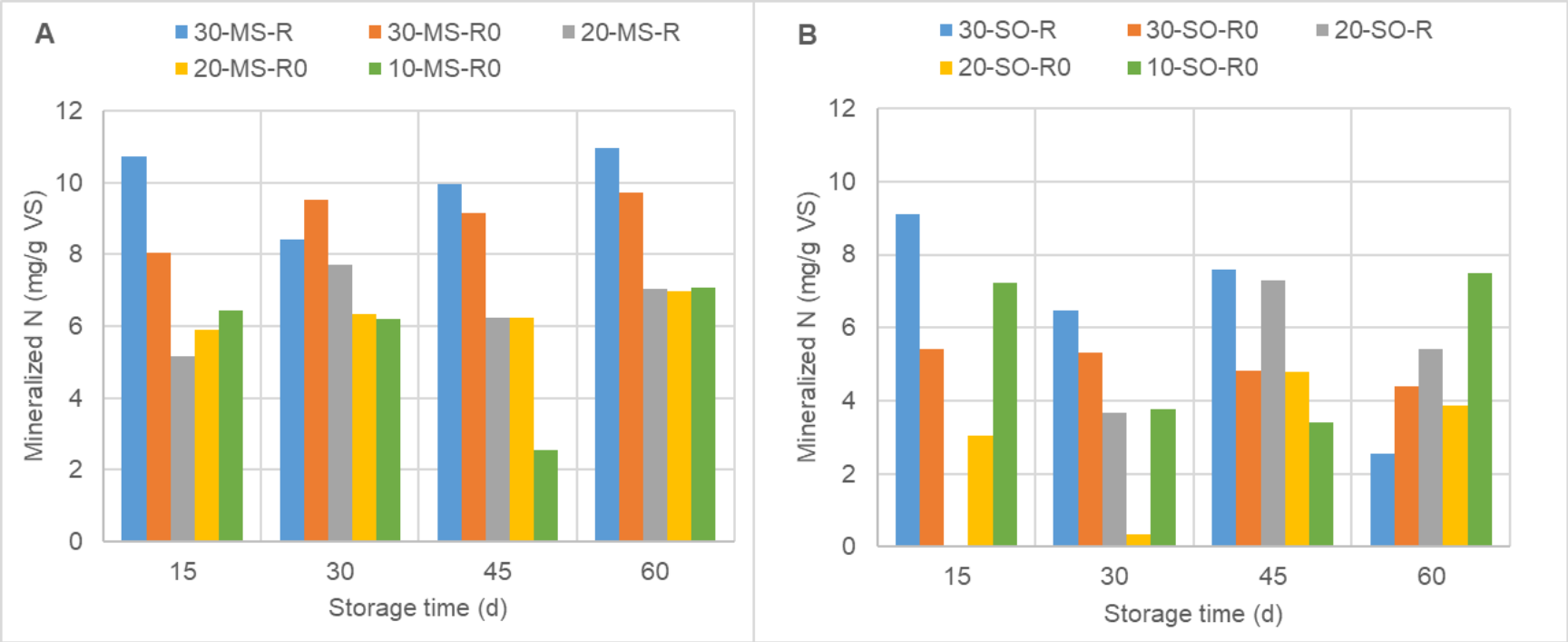


Figure 4-14 The concentrations of mineralized nitrogen (Nm) in raw manure and manure seed during a 60-d storage period at different temperatures (A: The Nm of raw manure with manure seed stored at 30/20/10℃; B: The Nm of manure seed only stored at 30/20/10℃; The error bar indicates the standard deviation)

4.4 Mineralizable nitrogen and the N mineralization rate constant (k)

Theoretically, the potential mineralizable N is equal to the initial quantity of organic N in the manure, OrgN(0). N mineralization is the process that the OrgN(0) is transformed into inorganic forms of N by the microbial activities (Ros, Temminghoff, & Hoffland, 2011). In this study, the OrgN(0) concentrations ranged from 33 to 82 and 15 to 25 mg N/g VS for the 180- and 60-d storage experiments, respectively. The mineralization rate constant k obtained using (Equation 3-5) are presented in Tables 4-6 and 4-7. In this study, the highest k values were 0.096 $week^{-1}$ (30-L-R) and 0.082 $week^{-1}$ (30-MS-R) for the 180- and 60-d storage experiments, respectively, and the lowest values of k for those two experiments were 0.021 $week^{-1}$ (30-H-R0) and 0.013 $week^{-1}$ (10-MS-R), respectively. Overall, the manure samples stored at higher temperature had higher k, and the highest k occurred in manure stored at 30℃; the non-sterilized manure stored at 20 and 30℃ had higher k compared to the corresponding sterilized manure; and the k values of L manure were higher than that of H manure.

Table 4-6 N mineralization rate constant k ($week^{-1}$) in 180-d storage

Treatment		*Storage Temperatures (℃)*		
		10	***20***	***30***
Non-sterilized manure	***High solids***	0.056	0.052	0.035
	Low solids	0.068	0.084	0.096
Sterilized manure	***High solids***	0.060	0.035	0.021
	Low solids	0.081	0.077	0.071

Table 4-7 N mineralization rate constant k ($week^{-1}$) in 60-d storage

Treatment		*Storage Temperatures (℃)*		
		10	***20***	***30***
Non-sterilized manure	***Raw manure w/ seed***	0.013	0.050	0.082
	Manure seed only	0.039	0.055	0.062
Sterilized manure	***Raw manure w/ seed***	0.050	0.051	0.079
	Manure seed only	0.068	0.035	0.052

In addition, when calculating the k based on the (Equation 3-5), the unit of $N_m(t)$ and OrgN(0) is mg N/g VS. The unit can be converted to mg N/L by multiplying corresponding VS (g/L). It is easy to find the VS (g/L) can be removed as a common factor from the numerator and denominator when calculating k. Therefore, it can be concluded that the value of k is independent of the unit of N_m and OrgN.

4.5 Analysis of extracted DNA and 16S rDNA

The extracted DNA (genomic DNA) samples were analyzed via agarose gel electrophoresis, and selected results are shown in Figure 4-15. The band present above the 10.0 kb position based on the ladder were genomic DNA of manure samples. A few bands below the 10.0 kb position were likely due to sheared DNA fragments. Some of the extracted samples did not exhibit bands in the gel, and the reasons could be 1) low concentration of DNA; 2) DNA degradation during or after extraction. There were also smears in some lanes of the gel, which could come from proteins or RNA contaminants. The concentrations of extracted DNA samples were from 0.7 ng/μL to 175.5 ng/μL as determined by use of a NanoDrop. Most values of A260/A280 ratio for extracted samples were around 1.8, which indicated that the samples were made up of "pure" DNA. However, for several samples A260/A280 values were out of the range of pure DNA. A value of A260/A280 ratio over 1.8 indicated that the sample might contain a significant amount of RNA contaminants.

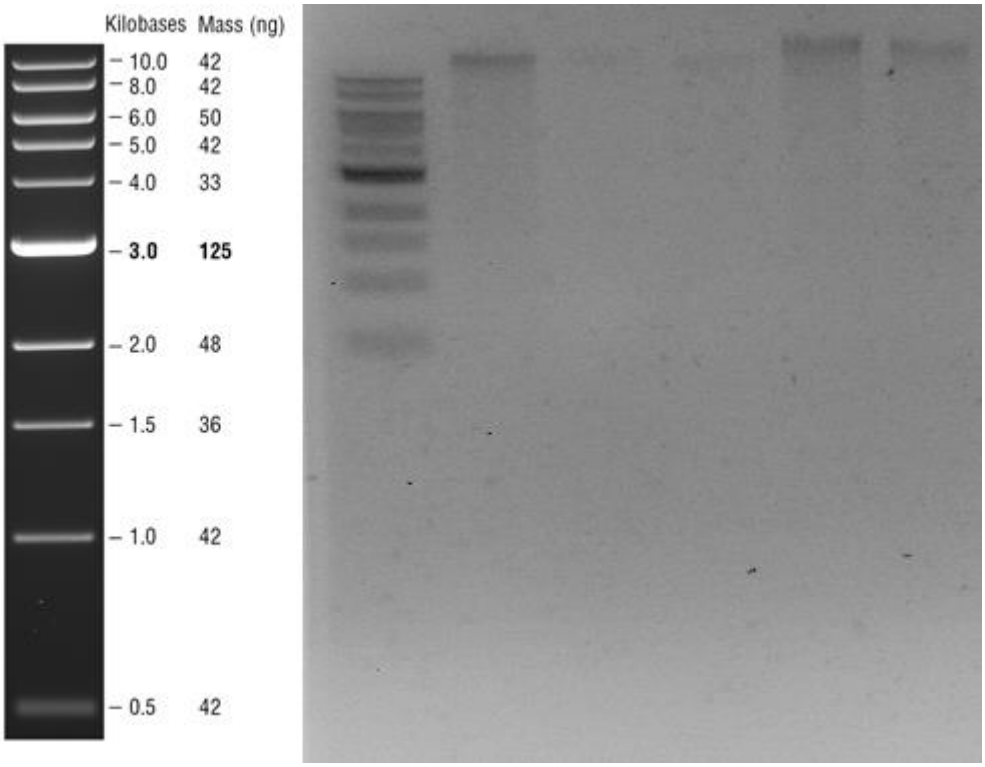


Figure 4-15 Selected results of genomic DNA from gel electrophoresis

Gel electrophoresis for extracted samples was not the most accurate way to check the presence of the genomic DNA, since there were several reasons discussed above causing a lack of band in the gel. Thus, 16S rDNA PCR was considered as a more accurate method to

determine the existence of genomic DNA in extracted samples. The PCR mixtures were run in the gel, and several selected results are illustrated in Figure 4-16. The expected size of a 16S rDNA amplicon is 1.5 kb and bands of this size were found in the PCR mixtures. However, there were still a few samples for which this band was not seen in the gel, and the reason could be the contaminants such as humic acids in samples inhibited the PCR reaction (Opel, Chung, & McCord, 2010). For this reason, for a limited number of samples, 16S rDNA fragments were amplified. The samples showing bands were qualified to be sent for 16S rRNA sequencing.

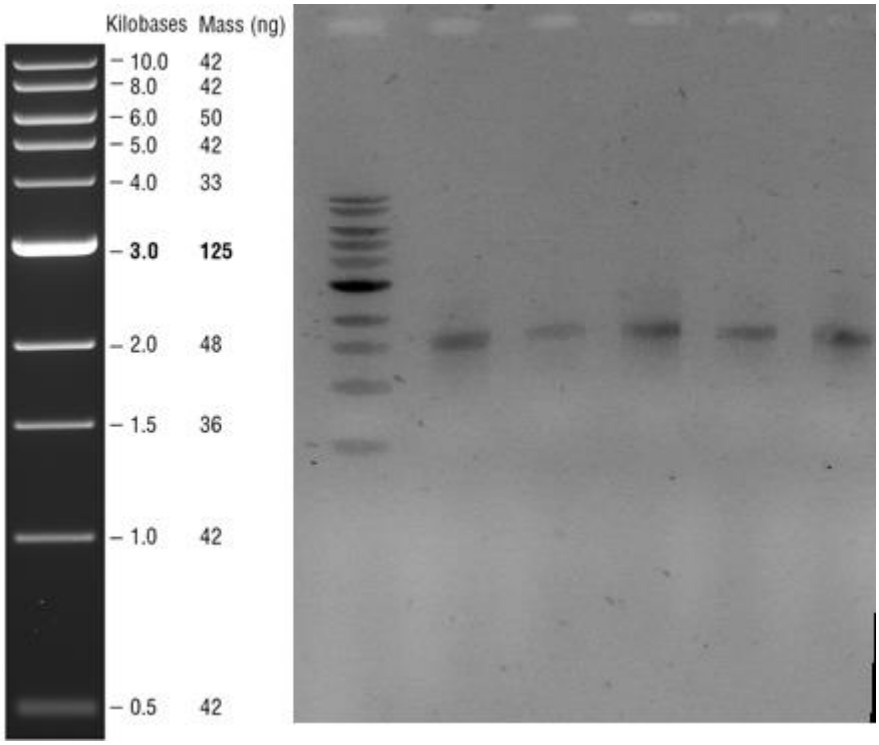


Figure 4-16 Selected results of 16S rDNA from gel electrophoresis

4.6 16S rRNA sequencing

4.6.1 Determination of sampling-depth value

After filtering out the low-quality sequences, the total number of valid sequences will be determined. And then the sample sequences will be split according to the Barcode label. After de-redundating the initial sequences, the 16S rRNA gene Unique Reads will be obtained and clustered into OTU for species classification at 97% similarity. The abundance information of each sample in different OTUs will be obtained. The data will be extracted by random sampling, and the curve will be drawn by the number of selected sequences and the number of OTUs they

can represent, that is, the dilution curve (Rarefaction). The rarefaction reflects the sampling depth of the samples and can be used to assess whether the amount of sequencing is sufficient to cover all taxa.

4.6.2 Analysis of diversity of archaeal and bacterial communities

4.6.2.1 Alpha-diversity analysis

Alpha-diversity index refers to the species diversity in a single sample. The Chao1 index predicts the species of microorganisms in the sample based on the measured number and relative proportion of the Tags and OTUs. The pedigree diversity reflects the phylogenetic diversity of characteristics of the species composition. The Shannon index is a diversity index that combines both OTU abundance and OTU uniformity. The larger the Shannon index and the phylogenetic diversity index are, the richer the species in the sample are and vice versa.

4.6.2.2 Beta-diversity analysis

Beta diversity analysis is used to compare the differences in species diversity between a pair of samples. UniFrac compares species community differences between samples through phylogenetic information. The calculated results can be used as an index to measure Beta diversity. It considers the evolutionary distance between species. The larger the index is, the greater the difference between samples is. Based on the species distribution of the samples, the unweighted UniFrac distance (only the species category differences are considered in each sample) and the weighted UniFrac distance will be calculated (the differences in species category between samples and the richness of species in each category are taken into account). And then the principal component analysis is performed on the distance matrix between samples to make the principal coordinate analysis of Beta diversity.

4.6.3 Analysis of the composition of archaeal and bacterial communities

4.6.3.1 The archaeal and bacterial phylum levels

The QIIME2 software will be used to analyze the composition and structure of the microbial community at the phylum levels. For each sample, the highest representations of archaeal and bacterial phyla, the lowest representations of archaeal and bacterial phyla, and their

relative abundances will be determined. In the composition of archaeal and bacterial phyla, the dominant and non-dominant phyla will be determined in each sample.

After comparing archaeal and bacterial phylum levels in each sample, the comparison among all the samples will also be conducted. The differences in compositions of archaeal and bacterial phyla and their relative abundances among different samples will be compared, and the corresponding dominant and non-dominant phyla will be compared as well. The comparison can be used to indicate how the archaeal and bacterial communities change in phylum levels during the storage of liquid dairy manure.

4.6.3.2 The archaeal and bacterial genus levels

Similar to the work in the last section, the composition and structure of archaeal and bacterial genus levels will be analyzed in each sample, and the highest representations of archaeal and bacterial genera, the lowest representations of archaeal and bacterial genera, and their relative abundances will be determined. Similarly, in the composition of archaeal and bacterial genera in each sample, the dominant and non-dominant genera will be determined.

Likewise, the comparison of archaeal and bacterial genus levels among all the samples will be carried out. The differences in compositions of archaeal and bacterial genera and their related abundances among different samples will be identified, and the corresponding dominant and non-dominant genera will be also compared. The results can reveal the commonality and distinction of archaeal and bacterial communities in manure samples at different storage time. What's more, the correlation of archaeal and bacterial community change and N mineralization will be uncovered and interpreted.

Chapter 5 Discussion

In this study, the effects of storage time, temperature, manure TS, adding manure seed and using sterilization operation at the beginning of storage on N mineralization during the storage were investigated and the corresponding N mineralization rate constants were calculated. The relevant measured parameters of manure characteristics were recorded, and all the information is discussed below.

Both of the VS concentrations and the ratio of VS to TS (VS/TS) can be used to represent the organic content of the manure (Chastain, Frase, & Moore, 2006). However, since the VS concentration in one manure sample was different from that in others, comparing VS concentrations was inappropriate. The VS/TS is a better indicator to describe the changes of the organic matters content in dairy manure, which can eliminate the influence of differences in the VS concentrations of manure samples at the beginning of storage. The higher VS/TS, the more organic content is contained in the manure. For the VS/TS of manure during 180-d storage period (Figure 4-3) and 60-d storage period (Figure 4-6), the decrease may be caused by the consumption of organic matters by microbial activities during storage. The magnitude of reduction in VS/TS could be used to indicate the extent of decomposition by microorganisms (Chastain et al., 2006). Therefore, it was speculated that a rapid decrease of the VS/TS indicated a rapid process of N mineralization, which was further proved by the results of manure group of 30-L-R with a rapid decrease of VS/TS (from 0.89 - 0.74) and the highest N mineralization rate constant k during 180-d storage. The VS/TS of some sterilized manure had no obvious change, which may be related to the death of microorganisms by sterilization operation. The enzyme activity of microorganisms was inhibited at low temperature, which resulted in no obvious change of the VS/TS of manure stored at low temperature (More, Daniel, & Petach, 1995). From the results, it found that the treatments with no obvious change in VS/TS resulted in low values of N mineralization rate constant. While, the increasing of the TS and VS concentrations in some manure groups may be caused by evaporation of moisture during the storage period (Alvenäs & Jansson, 1997).

The pH increases during the storage could be due to 1) the increased amount of NH_3/NH_4^+; 2) the evolution of CO_2; 3) the decrease of volatile fatty acids (VFA) content mediated by biological process or volatilization. In this study, the results showed pH increased

along with the N mineralization. It was indicated that the change of pH was related to N mineralization rate constant. While, for sterilized manure samples, almost bacteria and archaea were killed, and related enzymes were inactivated at the beginning of the storage, the microbial processes such as ammonification and degradation of proteins and VFAs were hard to occur. Because of this reason, the pH of those manure groups (30-H-R0-, 20-H-R0, and 10-H-R0) did not have obvious change, and the corresponding N mineralization rate constant was relatively low.

In this study, the results showed storage time only had effect on N mineralization in 180-d storage experiment, no change along with storage time in 60-d storage experiment. The storage time may be not long enough to cause a change under the condition in 60-d storage experiment. Furthermore, the results show there was no significant difference of the Nm at 20 and 30℃, which may be related to the complexity of manure, resulting in absence of homogenization of samples, may be, more replications were needed in future study. In this study, the Nm rate for 10-H-R was higher than that for the 30-H R, which was unexpected. The reasons for this unexpected situation might include 1) the loss of NH_4^+ as ammonia gas due to the increasing of pH; 2) the N mineralization in this study was the net mineralization of N, which was the difference between gross N mineralization and N immobilization. N immobilization is defined as the transformation of inorganic N compounds (such as NH_3, NH_4^+, NO_2^-, and NO_3^-) into organic forms (Jansson & Persson, 1982). As the temperature increases, the N mineralization rate increases, the N immobilization rate increases as well, while, the net mineralization rate may not increase, or even decrease. More experiments are needed to explore the relationship between N mineralization and immobilization, and the complex association of N mineralization and relevant parameters (Van Kessel & Reeves, 2002).

The raw manure with manure seed (MS) had higher Nm and N mineralization rate compared to the manure seed only (SO). Compared to the MS samples, SO samples had less organic N, which was used as carbon sources and energy to conduct N mineralization by archaea and bacteria. The VS/TS of SO samples (around 0.8) were lower than that of MS samples (around 0.9), which could also be used to support this result. While, the reason why the L manure had higher Nm than the H manure was still not so clear. One of the reasons may be the higher initial VS/TS of L manure (around 0.9) than that of H manure (around 0.85).

Some previous work had investigated the sterilization effect on N mineralization in soils, and the results showed that sterilization operation effectively eliminated the microbial populations of the soils (Wolf, Dao, Scott, & Lavy, 1989), but after sterilization, the N mineralization still occurred (Powlson & Jenkinson, 1976). These results from literature confirmed the results of this study, and the N mineralization after sterilization can be connected to the microbial community changes in related samples and lead to relationship of N mineralization, sterilization and microbial community.

The N mineralization rate constant (k) reported in this study were generally dissimilar to those reported by other studies, which may be due to the using of different media. For example, Stanford and Smith (1972) reported the most reliable estimated N mineralization rate constant k was 0.054 ± 0.009 week^{-1} for most of the soils. Campbell, Jame, and Winkleman (1984) reported that the k ranged from 0.0510 to 0.2280 week^{-1} for virgin and cultivated Western Canadian prairie soils incubated at 35℃. The k in soils amended with animal manure ranged from 0.0013 week^{-1} for horse manure to 0.067 week^{-1} for chicken manure (Chae & Tabatabai, 1986). Eghball (2000) reported that the k for non-composted beef cattle feedlot manure ranged from 0.011 to 0.23 week^{-1} (0.0167 to 0.036 day^{-1}). The differences in the N mineralization rate depends on the treatment, N sources, moisture, and temperature (Guntiñas et al., 2012), and the k values determined under laboratory conditions are typically different from those determined under field conditions. The highest k occurred in manure samples stored at 30℃ proved the manure had higher N mineralization rate under higher temperature, which was consistent with the previous statement. However, there were also several k values not matching my expectation, such as the k of non-sterilized, H manure at 10℃ was higher than the k of non-sterilized, H manure at 20 and 30℃. This is due to the N content in each dairy manure sample is highly variable and cannot be predicted through simple relationship between environmental and manure characteristics parameters.

Chapter 6 Conclusion

During the storage of liquid dairy manure, in general, the concentrations of TS and VS for sterilized (R0) samples increased, because of 1) low microbial activities resulting in low consuming of organic matter; 2) volatilization resulting in reducing of water. For non-sterilized (R) samples, the VS/TS decreased, which was due to due to the consumption of organic matter by microorganisms. As for the pH, it increased in R samples due to the production of NH_3/NH_4^+, the evolution of CO_2, and disappearance of VFAs; and the pH of most R0 samples decreased because of the lack of N mineralization and production of VFAs. This study investigated the influence of temperature, solids content, adding manure seed and using sterilization operation at the beginning of storage, and storage time on the N mineralization during the storage of liquid dairy manure. The results showed that temperature had a significant ($p < 0.05$) effect on N mineralization in both storage periods, with the highest ($k = 0.096$ week^{-1}) and lowest ($k = 0.013$ week^{-1}) N mineralization rates occurring at 30 and 10℃, respectively. The sterilization operation also had a significant ($p < 0.05$) effect on N mineralization for both experiments. The non-sterilized manure had higher concentrations of Nm and N mineralization rate compared to the sterilized manure. Manure TS and storage time had significant ($p < 0.05$) effects on N mineralization during the 180-d storage period. The mineralization rate was significant higher with manure samples of low TS than that of high TS. The highest concentrations of Nm were present at 30-d, 120-d, and 180-d of storage. The manure seed had significant ($p < 0.05$) effect on N mineralization during storage, with the MS manure had higher Nm than that of SO manure. Dairy manure was highly variable during the N mineralization process and no simple correlation between environmental conditions and manure characteristics related to N mineralization had been found to predict the change of N in dairy manure during storage. The results in this study can be used for supporting more complex models to accurately estimate the N mineralization in dairy manure.

The results of 16S rRNA sequencing will provide information about the composition of manure microbial (archaea and bacteria) populations and their relative representations. The knowledge of the microbial communities identified can be used to derive information about the mechanism of N transformation occurring in the storage units. The results will also be helpful to illustrate and document the dynamics and activities of the microbial communities which connect

to N transformations and loss during manure storage. The presence and abundance of related microorganisms, with the changes of manure characteristics, can be applied to generate more robust models to predict N loss and greenhouse gas emission from stored manure. The outcome of this study will also help refine N mineralization input parameter of manure storage submodules of the process-based models such as Manure DNDC and IFSM with the goal to improve their accuracy of estimating or accounting for the fate or cycling of N in dairy manure during storage.

Chapter 7 Summary

7.1 Intellectual merit

This study provided a comprehensive assessment of bacteria-mediated N mineralization during liquid dairy manure storage to improve N management on dairy farms. The dynamics of microbial communities and their relationships with the N mineralization process occurring in stored manure will be revealed using 16S rRNA based microbial community analysis. The effects of temperature, manure TS, adding manure seed and using sterilization operation on N transformation and loss will be assessed by linking the changes of manure characteristics. The effort will address critical knowledge gaps and rationally support decision tools for the evaluation of ecosystem services to control and mitigate N loss while protecting environmental health.

7.2 Broader impacts

The results of this study will show the value of a genome-enabled systems biology approach to inform the development of process-based models that will allow accurate predictions for nutrient losses and greenhouse gas emissions from manure storage systems. The study is helpful to understand the root cause of the losses — microbial degradation, to come up with meaningful and strategic biological-based management practices to reduce loss. The results will also provide new information on microbial communities in manure during storage. Those results in the short term will be used by agricultural researchers, engineers, ecologists, and ecosystem modelers to improve their understanding of manure gas losses and improve the tools for estimating and mitigating N losses from manure. In the long term, the information developed will be used by agricultural educators and dairy farmers to improve manure management on farms to conserve and use N more efficiently, thereby saving money and reducing damage to the environment.

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