

ABSTRACT

DEVINEY, ALISON VICTORIA. Conserving Nitrogen in Liquid Swine Manure by Urease Enzyme Inhibition. (Under the direction of Dr. John J. Classen).

Urea has the highest fraction of nitrogen among common fertilizer materials. Liquid swine waste has potential as a source of urea if the urea can be preserved before it is hydrolyzed by urease enzyme. However, urease from fecal bacteria quickly catalyzes the conversion of urea to ammonia and carbon dioxide. Up to 80% of the ammonia nitrogen is then lost through volatilization, affecting air quality and impairing sensitive ecosystems by deposition. This nitrogen loss represents a source of environmental pollution as well as the loss of a valuable plant nutrient. Batch tests were performed using thymol, salicylic hydroxamic acid (SHAM), N-(n-butyl) thiophosphoric triamide (NBPT), and two pH adjustments as potential urease inhibitors in a 6% urea solution. Samples were analyzed weekly over six weeks for ammonia formation. Urea preservation was highest (> 99%) for the pH adjusted treatments of pH < 3 and pH > 11, followed by NBPT (90%), SHAM (84%) and Thymol (80%) versus an untreated control (14%). The experiment was repeated over two weeks with NBPT, pH < 3, and pH > 11 in urine collected from gestating sows. Preserved urea was 91%, 82% and 70% for pH > 11, pH < 3 and NBPT respectively.

The same three treatments were then evaluated in a pilot-scale farm system. Inhibitor was added to liquid waste from 30 swine as it was collected and recirculated beneath a solid-liquid separating system using a manure scraper. Excess treated wastewater was collected in a separate "product" storage tank. Analysis of samples from the product tank at the time of collection and again after a week of storage indicated that urea was best preserved and manure nitrogen conserved by the pH < 3 treatment.

During the course of the pilot study it became clear that a quick field method for analyzing ammonium in manure solutions was needed. Ammonium ion selective electrodes (AISEs) provide this type of analysis and are commonly used with surface and wastewaters, but little information exists on the use of an AISE for detecting ammonium in manure solutions. Therefore, an initial investigation into the effect of liquid swine manure on AISE response was conducted using acidified liquid waste collected from the pilot study and also dilutions of sow urine with and without added ammonium. The effects studied include urea hydrolysis by urease enzyme in phosphate buffer, low and high pH, sample stirring, ionic strength and interference from potassium ions. Although not conclusive, these investigations indicated that an AISE may be appropriate for measuring ammonium concentrations in liquid swine waste, but further study is

needed to determine how the effects of ionic strength and interferences can be mitigated to improve AISE response relative to standard lab analyses.

The overall conclusion of this thesis is that the conservation and recovery of nitrogen from swine manure solutions by urea preservation is possible and that treatment by acidification to $\text{pH} < 3$ is not only effective, this treatment has some distinct advantages over other methods of urease enzyme inhibition tested. These include ease of acid application by dosing through pH monitoring, and solution preservation by apparent microbial inactivation. Although not investigated in this study, the system design and material selection to accommodate the corrosive properties of extremely low pH may prove economically impractical at a farm-scale application. However, even acidification to $\text{pH} < 6.5$ ensures all ammonia is ionized and thus prevents volatilization, reducing environmental impacts and increasing potential for greater nitrogen recovery from manure solutions. Further study must be conducted to find an economical and practical recovery method of this conserved nitrogen that creates a storable and transportable fertilizer product.

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Conserving Nitrogen in Liquid Swine Manure by Urease Enzyme Inhibition

by
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DEDICATION

Meine liebe Mutti--danke schön für alles.

BIOGRAPHY

Alison Deviney was born in Knoxville, Tennessee as the first daughter of three to Don and Veronika Whitlow. She spent much of her adult life on “micro-farms” in Georgia, Tennessee and Alabama raising all manner of barnyard critters including cats, dogs, chickens, horses, pigs, cows, sheep, goats, and the occasional duck. She’s since settled down in Cary, NC with her husband, Todd, step-daughter, Milena, two crazy dogs, some tenacious koi, and four pet chickens. Although her bachelor’s degree is in Drawing and Painting she’s always loved science and learning and decided to return to college to pursue a degree in Engineering. Her hobbies include gardening, hiking, designing, and building stuff.

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CHAPTER 1: Literature Review

1. Introduction

Over the last fifty years the amount of annual global reactive nitrogen (Nr) creation has nearly doubled, due in greatest part to the intensification of agricultural production. Anthropogenic sources of Nr include Haber-Bosch production (100 Tg N/yr.), biological fixation from agriculture crop cultivation (31.5 Tg N/yr.) and fossil fuel combustion (24.5 Tg N/yr.) (Galloway et al., 2004). Thus, the majority of Nr atmospheric emissions in terrestrial ecosystems are also the result of agricultural processes, and the vast majority of these losses are in the form of ammonia volatilization as a direct (e.g., manure production) or indirect (e.g., feed crop fertilizer) result from animal husbandry (de Vries et al., 2013; Erisman et al., 2013; Galloway et al., 2004; McCubbin et al., 2002; Schlesinger & Hartley, 1992). The concentration of food animal production with the increasing number of head per farm as well as the tendency for centralization of farms around processing facilities has increased the regional impacts of this reactive nitrogen, upsetting sensitive ecological balances and negatively affecting human health (Aneja et al., 2008; Lopez-Ridaura et al., 2009; Sigsgaard & Balmes, 2017). Studies have therefore focused on reducing ammonia volatilization from agricultural sources (Petersen et al., 2016; Hou et al., 2015; Ndegwa et al., 2008). Although nitrogen partitioning in manure varies with species, typically half or more of manure nitrogen is excreted as urea in the liquid fraction (Li et al., 2012). Urea quickly hydrolyzes to ammonia when exposed to fecal urease enzyme. If the urea can be preserved, then not only will ammonia volatilization be significantly reduced, but the conserved nitrogen may then be recovered and recycled to feed crop production. Recycling recovered nutrients as fertilizer products could in turn lead to a reduction in the amount of additional reactive nitrogen being produced annually.

This thesis will focus specifically on the conservation and recovery of nitrogen from swine manure. However, the concepts and investigations of this research may be applied more broadly to other livestock species. Furthermore, nitrogen recovery is just one piece of a comprehensive sustainable manure management strategy. A complete system must treat manure as resource rather than a waste byproduct of livestock rearing, addressing energy, nutrient and water recovery and recycling while maximizing efficiency and minimizing environmental impacts.

2. Urease Inhibition in Swine Manure Liquid

2.1. The Reactive Nitrogen Problem

The nitrogen cycle (Figure 1.1) is part of the biogeochemical boundary laid out in Rockstrom's nine planetary boundaries, or metrics of environmental sustainability (Rockstrom, 2009). Nitrogen, one of the most abundant elements on Earth and a main component of protein, is essential for life. Nitrogen as N_2 is an inert gas that comprises more than 78% of the Earth's atmosphere.

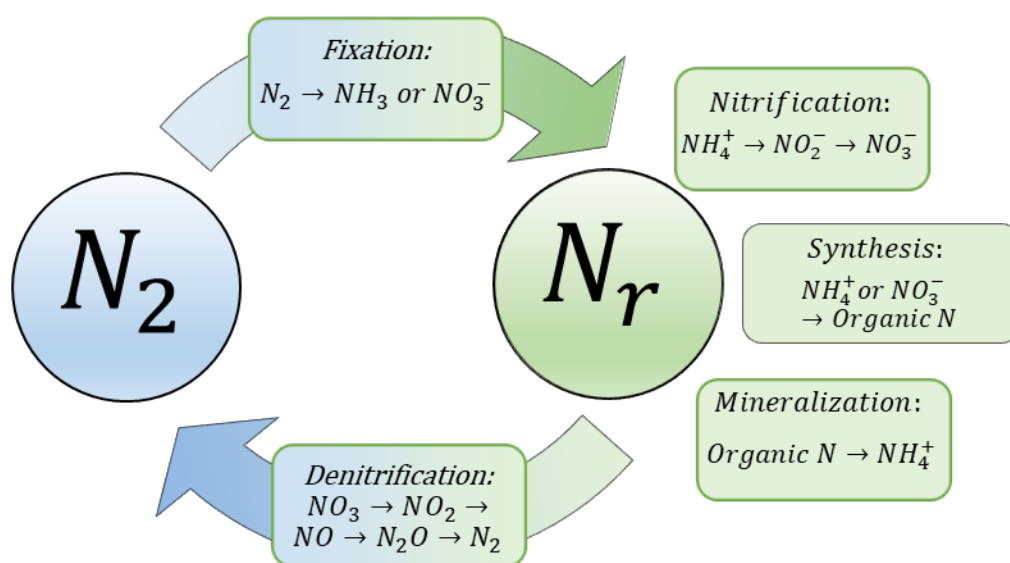


Figure 1.1: The Nitrogen Cycle: Non-reactive atmospheric dinitrogen is converted to and from reactive nitrogen through various anthropomorphic, natural and biological processes.

Reactive nitrogen (N_r), on the other hand, comprises the biologically and photochemically active compounds of nitrogen, including NH_3 , NH_4^+ , NO_3^- , NO_2^- , NO , N_2O , and organic-N. Prior to the industrial revolution, these two nitrogen forms remained fairly balanced, with biological nitrogen fixation roughly equaling denitrification (Ayres, 1997; Galloway et al., 2004). However, with the advent of the Haber-Bosch process, nitrogen fertilizer production has outpaced natural biological fixation as the primary source of N_r . As a result, fertilizer production combined with legume fixation (e.g. soy for feed crops) have more than doubled annual global N_r inputs without

accounting for an equal transformation back to N_2 (Galloway et al., 2004; Brasseur et al., 1999). Thus, anthropogenic activities have caused the nitrogen cycle to become unbalanced, particularly at regional scales, crossing the planetary boundary threshold. This current rate of change is likely unsustainable in the long-term.

Food animal production contributes in several ways to the growing nitrogen imbalance. Soy and corn fertilized by synthesized nitrogen are the staple feedstuffs for concentrated animal feeding operations (CAFOs). Even with the dramatic improvements in feed conversion ratios, more than 70% of consumed nitrogen ends up in the manure of most livestock species (Xiccato et al, 2005). Furthermore, manure from all livestock accounts for only about 5% of total crop fertilizer application in the U.S. (MacDonald et al., 2009), therefore this “leftover” nitrogen is generally not conserved beyond what is land applied after storage and treatment. This results in not only the need to create more reactive nitrogen for fertilizer, but in turn can lead to local and regional environmental degradation and negative health impacts for both animals and humans. The main source of reactive nitrogen pollution from livestock is in the form of ammonia emissions (Oenema et al, 2007).

The residence time of ammonia once airborne is relatively short. The majority is removed through dry deposition within a few kilometers of the source (Krupa, 2003) as illustrated in Figure 1.2 where deposition hotspots correlate to concentrations of swine and poultry farms in eastern North Carolina and swine and dairy farms in northern Iowa. Wet deposition can occur during precipitation events, or ammonia can react with acidic atmospheric species (e.g. HNO_3 , H_2SO_4) to form fine particulate matter ($PM_{2.5}$) (Aneja et al., 2008). Ammonia deposition contributes significantly to soil acidification and nitrogen-loading of surface waters, often leading to eutrophication (Sutton et al., 2008, Sherman et al., 2007; Hao et al, 2005). Sensitive ecological regions and coastal areas are particularly vulnerable to excess nitrogen deposition. Additionally, emissions from intensive swine operations have a documented negative effect on the health and quality of life of nearby residents (Thu et al., 1997; Wing & Wolf, 2000).

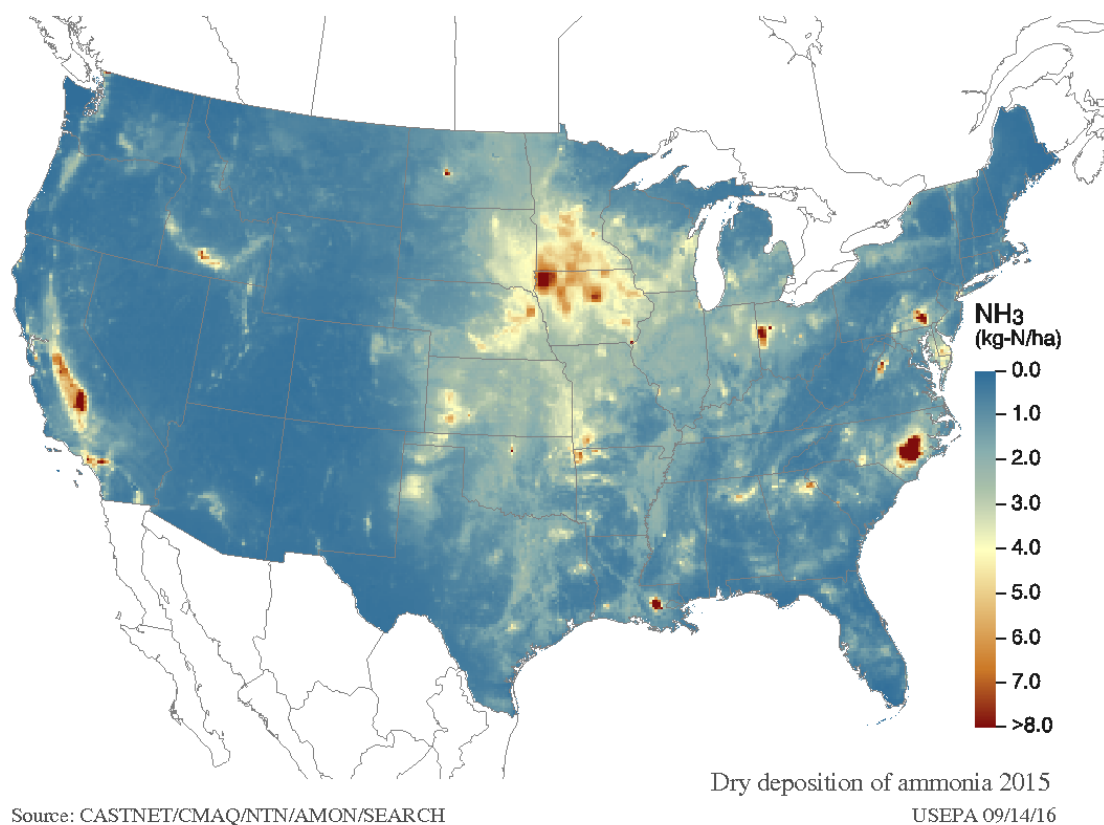


Figure 1.2: Dry deposition of ammonia (kg-N/ha) 2015. *National Atmospheric Deposition Program, 2016. National Atmospheric Deposition Program 2015 Annual Summary. NADP Data Report 2016-02. Illinois State Water Survey, University of Illinois at Urbana-Champaign, IL.*

2.2. Role of Swine Manure in Reactive Nitrogen Loading

2.2.1. Concentration of Swine Farms

The pork industry has experienced significant growth and changes in production over the past fifty years. Where once nearly half-a million farms raised swine in small herds along with crops and other livestock, today in the U.S. pigs are typically the sole livestock species on a farm and given an average 0.67 m² (7.2 ft²) of barn space per head to grow to a market weight exceeding 113 kg (250 lbs.) (Kliebenstein et al., 2005). In addition to this increased efficiency, market drivers and industry standardization have resulted in significant consolidation as well, with a 70% decrease in the actual number of hog farms while the number of hogs per farm risen from about 1,000 head to over 8,000 (McBride & Key, 2013). While many farms still manage all

aspects of swine rearing from farrow to finishing, producers have also begun to specialize in particular growth phases, such as sow farms or finishing farms. Furthermore, fewer hogs are taken to market these days, as most are contracted directly by companies who provide the pigs and feed to producers, standardizing their process even further. Finally, these industrial farms tend to congregate around central processing facilities, creating dense populations of swine in relatively small areas.

2.2.2. Current Swine Waste Management Practices

Today's CAFOs control all aspects of swine rearing for optimal growth, including environmental conditions, nutrient intake, and of course waste management. However, little progress has been made in the implementation of sustainable waste management practices.

Most swine farms currently use one of two manure management strategies for collection and storage. In northern states where winters are colder, pits beneath the slatted barn floors are deep enough to collect and hold manure for six months to a year. Late fall or early spring the whole slurry is then agitated and pumped into tankers to be land applied as fertilizer. In southern states where winters are warmer outdoor storage is preferred in the form of an anaerobic lagoon. There, the pits in the barns are shallow, and are flushed daily or weekly using recycled effluent from the lagoons. This diluted slurry is gravity-fed into the lagoon where the solids settle and are consumed by anaerobic bacteria. What remains forms a sludge layer that must be removed every 10 to 15 years. Meanwhile, the lagoon supernatant is used seasonally as nutrient-rich irrigation for field crops like hay.

One innovation that is slowly gaining traction is manure removal by belts or scrapers. This significantly reduces manure volume by not diluting with wash water, thereby reducing the size requirement for anaerobic digesters used for methane production. Furthermore, because the manure is typically removed every few hours from the barn, inhouse odor and emissions are also reduced (Hjorth et al., 2010; Parker & Lim, 2011).

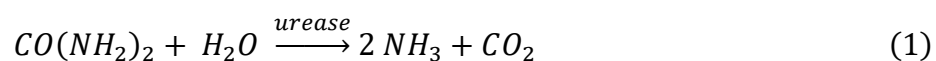
Some systems go a step farther and use a collection trough with scrapers or belts to separate the solids and liquids. While both fractions are still used as fertilizer product, the partitioning of nutrients with separation is such that most of the inorganic nitrogen is in the liquid fraction while

the organic nitrogen and the majority of the phosphorus remain with the drier solids (Lavoie et al., 2009; Phillippe et al. 2011).

2.2.3. Ammonia Formation in Swine Manure

The nitrogen in swine manure is partitioned fairly evenly between the solids and urine, with nearly all of the urine nitrogen in the urea form. Urea is an organic molecule that can be produced biologically or manufactured. In mammals, urea is synthesized in the liver to remove excess ammonia and subsequently excreted in urine. Biological ammonia results from protein metabolism; therefore, the amount of protein in the diet can affect the concentration of urea in urine (Blanco & Blanco, 2017). Since the advent of economical ammonia manufacturing using the Haber-Bosch process, urea has become one of the most abundantly produced chemicals globally. Urea easily dissolves in water but is relatively stable; the uncatalyzed hydrolysis of urea occurs so slowly in aqueous solutions the reaction has not been observed (Krajewska, 2009). Urea contains 46% nitrogen—the highest concentration of nitrogen by mass of a fertilizer product. Yet, despite more than 90% of manufactured urea being used as agricultural fertilizer, it is a ubiquitous chemical in its versatility and importance to numerous industries including cosmetics, transportation, medicine, and plastics (Edrisi et al., 2016).

Thus, a significant portion of ammonia in swine manure is produced through the enzymatic hydrolysis of urine urea (eq. 1). The rest comes from the microbial decomposition of proteins and amino acids in solids. A small percentage of ammonia is excreted directly.



In both waste management systems previously discussed, all of the waste including fecal solids, urine, spilled feed and water is collected in the pit beneath the barn floor. Because urine and feces are mixed, urease enzyme quickly hydrolyzes the urea. Urea hydrolysis tends to increase the pH of a manure solution. The pKa of ammonia is about 9.2 so at an alkaline pH ammonia volatilizes. Only a small percentage of ammonia is excreted directly, but since nearly half of the nitrogen in swine manure is in the urea form, both deep pit and shallow pit systems may lose up to 30% of manure nitrogen to ammonia volatilization just in the barn (USEPA, 2005).

Ammonia is also produced through the microbial decomposition of proteins and amino acids in fecal solids (mineralization), though this process takes much longer than enzyme-catalyzed urea hydrolysis. Therefore, the open lagoon will volatilize an additional 30-40% of manure nitrogen as solids are broken down (Arogo et al., 2003; Doorn et al., 2002).

These losses are further exemplified by the 2014 National Emissions Inventory Data from EPA which estimates that of the 2.1 million tons of ammonia emitted in 2014 from U.S. livestock manure, nearly 21% was contributed by swine operations (US EPA, 2017). Thus, in addition to negative environmental impacts, ammonia emissions represent a substantial economic loss in nutrient value. In 2017, U.S. prices for anhydrous ammonia fertilizer averaged \$500 per ton (Schnitkey, 2017). If all nitrogen lost as ammonia was conserved and recovered during the manure collection and handling process, it could represent nearly a quarter-million dollar added value annually to the swine industry, as well as offsetting a significant portion of synthetic fertilizer use in feed crop production. Further economic benefit could be realized through reduced environmental and health costs associated with ammonia emissions. Therefore, it makes both environmental and economic sense to conserve manure nitrogen for recovery and use.

2.2.4. Reducing Ammonia Volatilization

Limiting ammonia emissions from manure has become a focal point of livestock waste management research across all major species, including poultry, dairy and swine. This approach is critical in addressing the immediate environmental impacts of reactive nitrogen and can help conserve that nitrogen for later recovery. One possible method of conservation is to prevent the formation of ammonia from urea by inhibiting the urease enzyme. Because enzyme-catalyzed hydrolysis is virtually spontaneous, this approach is most practical if applied at the time of deposition when urea nitrogen is first exposed to fecal urease enzyme.

A mechanical method to reduce urea hydrolysis is source separation, where solids and liquid are separated in the barn, usually at the point of deposition. Some farms use this method primarily for the advantages of overall manure volume reduction and a drier solid product for field application. However, source separation has the additional benefit of reducing odors and ammonia volatilization (Parker & Lim, 2011). There is also a redistribution of plant

macronutrients where phosphorus and potassium remain mainly in the solids while much of the nitrogen is in the liquid fraction (von Bernuth et al., 2005).

Von Bernuth et al. (2005) tested a separation system using a scraper over a V-shaped concrete base beneath the slatted floor of a finishing barn. The liquid fraction (urine and spilled drinking water) collected in the center channel of the sloped floor and was pumped to a holding tank, while the solid fraction (feces and spilled feed) were pushed off by the scraper to a collection point. The study objectives included what effect the system had on ammonia and hydrogen sulfide emissions. Hydrogen sulfide remained below the 0.5 ppm detectible limit while ammonia concentration remained below 5 ppm for 97% of the study period. By comparison, Blunden et al. (2008) measured an average ammonia-nitrogen concentration greater than 8 ppm in a pull-plug barn, where manure collected in a shallow pit filled with a foot of water and was emptied weekly. Maximum concentrations in this system reached 14 ppm. Hydrogen sulfide emissions in the pull-plug barn generally remained below 0.5 ppm except in winter when they averaged 0.67 ppm.

Koger et al. (2014) tested a separation system in the form of a conveyor belt installed beneath the slatted barn floor that would catch the solids but had a slightly arched surface so that liquid would run off into side gutters. The liquid was channeled to a sealed container to prevent ammonia loss. The solids were allowed to sit on the belt to dry and then conveyed daily to a collection bin. Moisture content and weight of the solids, volume of the liquid, methane, and ammonia gases in the barn and odor at the exhaust fans were each measured. The average solids moisture content was half of what was reported in a conventional barn with minimal odor. Ammonia and methane emissions were reduced by 73% on average. Odor was approximately 60% of a conventional system at the exhaust fan. Although initial costs are higher, Koger et al. (2014) concluded that a belt separation system provided significant improvements in manure handling and environmental air quality in swine barns.

These studies show that source separation can be an effective method for reducing ammonia emissions in the barn. However, part of the reason for this is the quick removal of waste from the barn. Some exposure of urea to urease enzyme still occurs and so ammonia will continue to form during handling and storage. Predicala et al. (2007) observed an increase in ammonia

emissions using a scraper separation system. This could be due to the lack of manure dilution and exposure of manure solids and liquids to increased air circulation, encouraging volatilization from urea hydrolysis.

2.3. The Urease Enzyme

Urease is a protein and an enzyme produced by plants, bacteria and fungi with the specific function of hydrolyzing urea to carbamate and ammonia in the presence of water. It is one of the most efficient enzymes known, catalyzing this reaction almost instantly at a rate 10¹⁴ times faster than would occur naturally (Karplus et al., 1997). The carbamate then quickly and spontaneously degrades into another ammonia and carbon dioxide. Thus, for every mole of urea hydrolyzed, two moles of ammonia and one mole of carbon dioxide are released. While urease can hydrolyze other substrates—typically analogs of urea and phosphoric acid amides and esters—the reaction rate is so much slower than with urea that these substrates are sometimes employed as urease inhibitors (Krajewska, 2009; Zerner, 1991).

Urease enzyme is unique not only for its high proficiency, but also because of its similarity across all sources from plants to fungi to bacteria. Although the enzyme may vary in number and type of subunits depending upon source, all ureases share a strong similarity in amino acid sequencing that is substantially different from other enzymes (Amtul et al., 2002). Additionally, all ureases are metalloenzymes, meaning they contain two nickel ions in the active site necessary for the coordination and hydrolysis of substrate (Krajewska, 2009). Many of these ureases have been isolated for laboratory use. The activity of urease is defined by enzyme units (U). One enzyme unit is the amount of enzyme required to catalyze one micromole of substrate per minute at a specified pH and temperature (Harrison et al., 2015).

2.4. Methods of Inhibiting Urease

The primary role of urease for organisms is to make available the nitrogen in urea as a biological nutrient (Amtul et al., 2002). Both natural and synthetic urea can be hydrolyzed by urease enzyme. Because of the virulent nature of some bacteria, urease inhibition has been of interest in the medical community as a method of controlling disease or regulating urinary pH to prevent encrustation and kidney stone formation (Jones et al., 2005; Macegoniuk, 2013; Tabak

et al., 1996). In crop science, the addition of a urease inhibitor is commonly used with urea fertilizer to reduce ammonia formation after application and slow the mineralization process. This does, in effect, create a “time-release” fertilizer, so that surface-applied nitrogen can be incorporated into the soil as well as giving crops time for uptake. This delay helps reduce nutrient loss through volatilization and leaching as well as preventing ammonia toxicity in young plants (Bremner et al., 1991).

In livestock manures, air quality, odor control, and nutrient conservation are the primary reasons for much of the study of urease inhibition (Hagenkamp-Korth et al. 2015; Ndegwa et al., 2008; Varel, 1997;). These studies generally focus on the use of inhibitors to prevent ammonia volatilization. However, preserving urea in the barn and during storage could help maximize potential nitrogen recovery. Table 1.1 lists potential urease inhibition methods currently used with medicine, soil, or manure and grouped by type. Each type is explained in further detail below.

Table 1.1: Urease Inhibitors by type/mechanism of action

Inhibitor	Application	References
<i>Phosphoramides</i>		
PPD	soil, manure	Amtul et al. (2002); Varel (1997)
CHPT	manure	Varel (1997)
NBPT	soil, manure	Creason et al. (1990); Bremner, et al. (1991); Qui-Xiang et al. (1994); ; Manunza et al. (1999); Varel & Wells (2007); Krajewska (2009); Goos (2013)
<i>Hydroxamic Acids</i>		
AHA	medicine	Manunza et al. (1999); Amtul et al. (2002); Jones et al. (2005); Krajewska (2009)
SHAM	medicine	Hassan et al. (1997)
<i>Natural Derivatives</i>		
DMO, pitch, terpenes	soil	Modolo et al. (2015)
thymol	soil	Patra et al. (2009)
	medicine, manure	Tabak et al. (1996); Varel & Wells (2007)
<i>Acidification</i>		
	manure, soil	Fidaleo & Lavecchia (2003); Fangueiro et al. (2015)
H ₂ SO ₄	manure	Dai & Blanes-Vidal (2013); Petersen et al. (2016)
<i>Alkalization</i>		
Ca(OH) ₂	human urine	Randall et al. (2016)

2.4.1. Phosphoramides

Phosphoramides are commonly used as urease inhibitors to slow the rate of urea nitrogen fertilizer hydrolysis after land application. These are competitive slow-binding inhibitors that attach to the enzyme's active site, preventing urea from engaging with the urease (Krajewska, 2009). Of several possible chemical species of thiophosphoric amides studied for urease inhibition, N-(N- Butyl) Thiophosphoric Triamide (NBPT) has consistently proven to be the most effective and persistent (Bremner, et al., 1991; Goos, 2013). Interestingly, it is the oxygen analog of this compound that is the actual inhibitor (Creason et al., 1990). The rate of conversion to this oxon-analog increases in the presence of oxygen aeration or hydrogen peroxide (Qui-Xiang et al., 1994).

For livestock application, Varel (1997) conducted one of the preliminary experiments using urease inhibitors typically applied to soils to prevent urea hydrolysis in manure slurries. He tested two chemical relatives of NBPT: phenyl phosphorodiamidate (PPD) and cyclohexylphosphoric triamide (CHPT). The inhibitors were tested on fresh manure collected from dairy cows and finishing swine using either a single application or weekly applications. The single treatment was effective for 4-11 days while the weekly application was more effective for long-term urease inhibition. Different weekly doses of PPDA were also tested at 10, 40 and 100 mg/L, effectively preserving 72, 92 and 92% of urea, respectively, over 28 days. When inhibitor application was stopped, urea was completely hydrolyzed by 21 days in the dairy slurry and 35 days in the swine slurry. Later, Varel and Wells (2007) conducted field trials using a single treatment of NBPT in swine pits and reported that it had a short-term effect on urea preservation lasting 6-10 days, suggesting a weekly application might be necessary for continuous urease inhibition.

2.4.2. Hydroxamic Acids

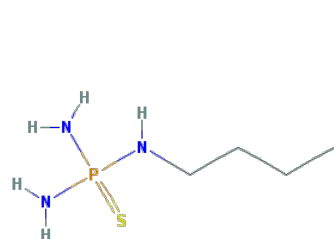
In her review of ureases, Krajewska (2009) noted that hydroxamic acids are well known as metalloenzyme inhibitors. Due to their relatively nontoxic nature hydroxamic acids have been frequently studied in medical applications. Like NBPT, hydroxamic acids are competitive slow-binding inhibitors to urease enzyme, though not as strongly binding to the active site as NBPT (Manunza et al., 1999). Jones et al. (2005) tested urease inhibitors as a way to prevent alkalization of urine solutions and thus reduce calcium and magnesium encrustation on medical devices. They showed acetohydroxamic acid (AHA) to be the most effective urease inhibitor of three tested, reducing encrustation by 93% over solutions with no inhibitor present. There is also evidence that AHA actually has a greater affinity for bacterial urease than plant urease (Amtul et al., 2002). This indicates that despite the similarities in structure, certain differences in ureases may play a role in their behavior with different inhibitors. Another derivative of interest is salicylhydroxamic acid (SHAM), a commonly available compound often used in the treatment of urinary tract infections. Hassan et al. (1997) observed that the effectiveness of SHAM as a urease inhibitor is dependent upon both the concentration of urease and the concentration of inhibitor.

2.4.3. Plant Oil Derivatives

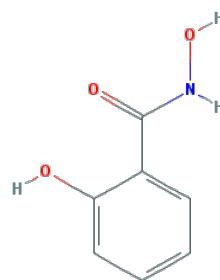
In response to the high cost and potentially negative environmental impacts of synthetic urease inhibitors, various plant derivatives have been investigated as possible natural sources for urease inhibition. Modolo et al. (2015) conducted a review of natural substances studied, including members of the Allium family (e.g. garlic, leeks), members of the Brassica family (e.g. cabbage, Brussel sprouts), spices (e.g. cumin), herbs (e.g. oregano), and *Camelia sinensis* (tea leaf), among others. While their review spanned many applications for urease inhibition, they found a number of possible options for agricultural use. For example, the application of urea mixed with polyphenol extracts temporarily inhibited urease activity at the soil surface to prevent ammonia volatilization but was subsequently broken down by microbial activity once washed into the subsurface soil layer (Fernando & Roberts, 1976). Modolo et al. (2015) acknowledged, however, future challenges include identifying the effective constituents of natural extracts, establishing the mechanism of action, and scale up for commercial production.

Patra et al. (2009) also tested urea coated with natural inhibitors to determine their effect on urea hydrolysis and nitrification in sandy loam soil. Four treatments were used: dementholized oil (DMO), pitch, terpenes (menthol mint oil), and dicyandiamide (DCD)—a commercial time-release chemical. Two concentrations of each inhibitor were tested (0.5% and 1.0%) and compared with a control (untreated soil) and urea-only treatment. All levels and treatments had some effect on urease, reducing ammonia emissions by 14% to 34% over the urea-only application. The DMO was most effective at 0.5% with 29% reduction but the terpenes were more effective at the 1.0% level with a 34% reduction. All treatments also inhibited nitrification, although the 1.0% level was the more effective. In all cases the natural inhibitors were more effective than DCD. A useful follow up to this study would be the application of these inhibitors to manure fertilizer. Use of livestock manure over commercial fertilizer would further promote Patra et al.'s (2009) argument for natural products.

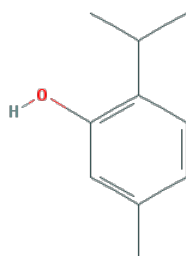
Extracts of thyme and cinnamon have been shown to have significant urease inhibitory effect against *H. pylori* bacteria with aqueous thyme extract the most effective at inhibiting both the bacteria's growth and urease enzyme activity (Tabak et al., 1996). Thymol, a polyphenol in thyme oil, has also been used in livestock manure studies. Though predominantly considered an antimicrobial, Varel and Wells (2007) used thymol to determine its effect on odor, ammonia volatilization and overall bacteria growth in manure. Thymol produced a significant reduction in coliform bacteria colony forming units in the slurry and total elimination of *Campylobacter*, an infectious bacterium. This resulted in up to a 93% reduction in methane and a 76% reduction in overall gas emissions. A 3.0 g/L treatment also eliminated 100% of odor causing volatile fatty acids. Varel and Wells (2007) noted an insecticidal benefit of plant oils derivatives like thymol for fly control. It is unclear from the research whether thymol had a direct impact on urease enzyme activity (Modolo et al., 2015). It seems plausible that thymol could indirectly inhibit the production of bacterial urease through its bactericidal properties.



a) N-(n-butyl) thiophosphoric triamide $C_4H_{14}N_3PS$



b) salicylhydroxamic acid



c) thymol $C_{10}H_{14}O$

Figure 1.3: 2D structure and chemical formulas of selected urease inhibitors.

Source: PubChem, <https://pubchem.ncbi.nlm.nih.gov>

2.4.4. pH adjustment

Although the research was related to soil pH rather than livestock manure, Frankenberger and Johnson (1982) conducted experiments that could be used to determine a starting point for fecal urease inhibition study. They assayed the activity of several soil enzymes including urease in three different soil types over a pH range from 1 to 13. The urease enzyme assay indicated activity was permanently inhibited at pH below 4 to 8 and above 9 to 12 depending on the soil type. This suggests that the conditions for urease inhibition are affected by factors other than pH. The authors also noted that soil properties which may have impacted enzyme activity include cation exchange capacity (CEC), surface area and organic matter content. This is relative to

manure which also contains a high percentage of organic matter and a mixture of anions and cations in solution which could have similar effects as soil CEC. The influence of pH was considered reversible near the optimal value for enzyme activity but irreversible at the low and high end of the ranges presented. This study is valuable to the understanding of the effect of pH on urea hydrolysis in livestock manure but only from the perspective of the similarities between soil and manure. The authors also note that enzymes in solutions (such as slurries) are more sensitive to temperature than in soils.

Acidification of swine slurry is a well-known and commonly practiced method of reducing ammonia emissions (Fangueiro et al., 2015; Ndegwa et al., 2008; Petersen et al., 2016). Because the pKa of ammonia is about 9.2, in a slurry pH below 7, most of the ammonia exists in the aqueous ionic phase as ammonium (NH_4^+) instead of the gaseous phase (NH_3). Urease activity is pH dependent with an optimal pH for most ureases near neutral, while the rate of reaction declines significantly at pH 4 (Bhowmick & Jagannadham, 2006; Fidaleo & Lavecchia 2003). Therefore, acidifying slurry can also inhibit urease activity.

In a review of slurry acidification, Fangueiro et al. (2015) determined that pH is most commonly reduced by the addition of sulfuric acid. Acidification can also be achieved by the addition of a hydrolysable salt (e.g. aluminum chloride) or fermentable materials that stimulate the production of organic acids. It should be noted that the acidification and manure handling process can have an effect on slurry composition as well as other gas emissions such as methane or hydrogen sulfide, which generally increases as pH is reduced (Dai & Blanes-Vidal 2013; Fangueiro et al., 2015; Petersen et al., 2016).

Another approach to urease inhibition using pH adjustment was tested by Randall et al. (2016) using calcium hydroxide ($\text{Ca}(\text{OH})_2$) to stabilize urea in human urine, which is similar in composition to swine urine. $\text{Ca}(\text{OH})_2$, commonly known as lime, was selected because it attains a pH near 12.5 at saturation, is inexpensive and readily available. An advantage of using an alkaline solution for urease inhibition is the precipitation of phosphate, magnesium and calcium, adding the potential for additional nutrient recovery. Randall et al. (2016) cautioned that chemical hydrolysis of urea to ammonia begins to occur above pH 13 and at temperatures above 40°C. However, at high pH any ammonia present in solution is mainly in the gas form and will

volatilize causing nitrogen loss. Because saturation pH of $\text{Ca}(\text{OH})_2$ rises as temperature decreases these conditions may limit dewatering and recovery methods.

2.5. Preserving Urea by Urease Inhibition

The concept of inhibiting urease enzyme activity to reduce ammonia emissions from swine manure has been previously explored. However, there is no apparent literature regarding whether inhibitors can preserve urea for an extended period of time to allow for the accumulation, processing and storage of a nitrogen-rich fertilizer product. Several factors must be considered when choosing an inhibitor for this purpose:

- What impacts, if any, does the inhibitor have on the final product?
- What is the availability of the inhibitor?
- How can the inhibitor be applied?
- How long do the inhibition effects last in swine urine?

This thesis will consider these questions in the selection and testing of urease inhibitors for the purpose of preserving urea in liquid swine manure. Determining an inhibitor's effectiveness over time can be done by measuring the change in ammonium concentration of a test solution. The formation of ammonia will quickly occur if urea is being hydrolyzed but more slowly if ammonification of organic nitrogen is occurring through microbial processes.

Therefore, to determine if urease inhibition is effective, the concentration of ammonia in solution should be monitored. This can be done by collecting and acidifying samples to $\text{pH} < 2$ and submitting for lab analysis by spectrophotometry. To determine the urea concentration in these samples they must first be preserved at $\text{pH} < 2$ and a portion submitted for lab analysis of ammonia, then neutralized and treated with urease enzyme to hydrolyze any urea present, and finally preserved again for a second spectrophotometric analysis of ammonia. The urea concentration can then be estimated by the difference in ammonia before and after hydrolysis.

A significant time savings could be realized if there was an accurate method for measuring ammonium concentration in manure solutions in the field. One possible option for field analysis is to use an ammonium ion selective electrode (AISE). Therefore, this thesis will also explore whether this sensor can be used for accurate and repeatable measurement of ammonium concentration in swine manure solutions.

3. Analyzing Ammonium and Urea Concentrations

Several methods exist for determining ammonia and urea concentrations in solution. Most common are those listed in the Standard Methods 4500-NH₃ (APHA, 2012). For ammonia determination, these methods generally require multiple sample preparation steps including filtration, distillation and dilution, treatment with colorimetric reagents and finally analysis either manually or with automated equipment. While two methods for an ammonia-gas sensing electrode are included in the standard methods, there is not a standard method for using an ammonium ion selective electrode (AISE).

Field methods for determining total ammoniacal nitrogen (TAN) are often required for analysis of environmental waters, municipal wastewater, and aquaculture waters where ammonia is toxic to aquatic life. Zhou and Boyd (2016) compare various field methods of TAN analysis in aquaculture waters, including water analysis kits using Nessler or salicylate methods, an ammonia-gas electrode and an AISE. Although they found of these field methods, the salicylate kit gave the best precision and accuracy at the lower concentrations measured, this method is more expensive than the electrode methods and required disposal of hazardous waste due to the reagents used. While the AISE measurements were impacted by the potassium and sodium concentrations of the shrimp water tested, these effects decreased with an increase in TAN concentration. This indicates that it is the ratios of ions in solution that causes interference in an AISE. While aquaculture waters typical have TAN concentrations < 5 mg/L, manure solutions typically present TAN concentrations two orders of magnitude higher or more, particularly after urea hydrolysis has occurred.

With regard the measurement of urea, the analysis of blood serum or urine urea is a common procedure in the medical field, while agricultural and environmental applications require urea analysis of fertilizer, food products (e.g., milk, wine), or surface water quality. Urea may be measured either directly via colorimetric or fluorometric methods, or indirectly by enzymatic degradation (Francis et al., 2002; Hojjatie & Abrams, 2015). Most direct methods require the addition of reagents to alter sample color or chemistry for flow injection analysis (FIA) or high performance liquid chromatography (HPLC). Handheld refractometers have been

developed for measuring urea in diesel exhaust fluid (e.g. Atago #4518 PAL-Urea, Bellevue, WA) although this type of analysis is designed for a pure urea solution.

The most common indirect way to measure urea concentration is the urease method (Fawcett & Scott, 1960). In this method the difference in ammonia nitrogen concentration before and after urea hydrolysis with urease enzyme is measured, with this difference being used to calculate the initial urea concentration. While this enzymatic method is typically coupled with a colorimetric ammonia-measuring method, this study proposes to couple the urease method with an AISE to determine urea concentrations in liquid manure.

3.1. Ammonium Ion Selective Electrodes

Ion selective electrodes (ISEs) are potentiometric sensors used for determination of a specific electrolyte concentration in solution (Bakker & Pretsch, 2008). ISEs are one of the most common and well-studied analytical instruments available, in part due to their simplicity of design, use and portability (Pretsch, 2007). While the most well-known and frequently used ISE sensor is the pH probe, ISEs can detect dozens of inorganic cations and anions in addition to some organic and neutral analytes (Bühlmann et al. 1998).

The basic components of an ISE sensor include a reference electrode, an ion selective electrode, and a voltmeter. Each electrode, or half-cell, may exist as an individual probe but often ISEs are combination electrodes where both the reference and sensor are located in the same housing. An ISE functions on the principle of electrical conductivity of ions in solution. Each electrode is surrounded by an electrolyte filling solution that does not interact directly with the analyte. This allows the junctions between the filling solutions and the electrodes to have a constant potential. The sum of these internal junction potentials is referred to as E_0 , represented by the intercept of the Nernst equation (Eq. 1). E_0 is the constant reference potential, s is the slope of the linear relationship between the total potential generated (E) and the log of the ion activity (a_i).

$$E = E_0 + s \log(a_i) \quad (1)$$

The ion selective electrode contains a membrane infused with a selective ionophore that, when immersed in a sample solution, reversibly binds to the ion of interest so that only the activity of that ion is measured. For the past several decades, either nonactin alone or some mixture thereof has been the only practical ammonium ionophore available (Bühlmann et al. 1998; Suzuki, et al., 2000). Nonactin, though preferential to ammonium does not completely exclude ions of similar size and charge, particularly potassium and sodium.

The membrane potential is the product of the slope (Eq. 2) and the log of the ion activity (Eq. 1). R is the gas constant (8.314 J/K-mol) and F is the Faraday constant (96,485 C/mol). A conversion from natural log to logarithm of the ion activity is also included in the slope.

$$s = 2.303 \frac{RT}{z_i F} \quad (2)$$

Thus, for ammonium which has a charge number (z_i) of +1, the ideal slope should be 59.1 mV at 25°C. The potential that is measured by the voltmeter (E) is the sum of the reference potential and the membrane potential that develops as a result of the ion activity, which equals the ion concentration under ideal conditions. It is important to note the temperature dependence of the slope, which is why calibration solutions should be at a temperature similar to sample solutions. While the Nernst equation is applicable under ideal conditions, in practice these conditions rarely exist. Ions interact with each other in solution and this interaction is described via the activity of the ion. Activity is therefore influenced by the charge and concentrations of other ions in solution, which is measured as ionic strength (Eq. 3). Therefore, the higher the ionic strength of a solution, the lower the activity of the analyte, and thus the lower the response of the AISE.

$$I = \sum_i C_i z_i^2 \quad (3)$$

Furthermore, as previously mentioned, ionophores are not perfectly selective for the ion of interest. Other ions that may bind to the membrane are referred to as interfering ions and when present in a solution matrix, their effect on the AISE response must be considered. This is

accomplished with a modified version of the Nernstian equation known as the Nicolsky-Eisenman equation (Eq. 4) (Fry & Langley, 2001).

$$E = E_0 + s \log \left(a_i + \sum_n k_{in}^{pot} (a_n)^{\frac{z_i}{z_n}} \right) \quad (4)$$

In the Nicolsky-Eisenman equation the sum of the activity of the interferents ($a_n^{z_i/z_n}$) multiplied by their selectivity coefficients (k_{in}^{pot}) is added to the activity of the analyte when calculating the total potential. This then accounts for the portion of the response that can be attributed to the interferents. The selectivity coefficient (SC) is unique to each interferent and may be determined empirically by measuring the change in analyte activity to the addition of a known amount of the interferent. Umezawa et al. (2000) have generated a list of SCs for numerous ISEs collected from literature and based upon the type of ionophore used, as well as the method by which the SC was determined. From this list it is evident that, depending on the mix of nonactin used and the membrane composition, the primary interferents for ammonium are potassium and sodium but may also include lithium, calcium, magnesium, rubidium, cesium, and hydrogen ions.

Although AISEs are available in a variety of configurations, the most common are combination electrodes (housing both the reference and sensing electrode together) and either a PVC membrane with a liquid junction or a solid-state membrane. PVC membranes may be permanent or replaceable, and the inner electrolyte may be sealed or fillable. Reference electrodes may have a double junction; that is an internal and external junction to prevent interaction between the sample and reference filling solution. Today's sensors are often designed to work with a specific meter, which may use information collected from additional sensors such as pH or temperature to automatically apply corrections for improved accuracy. Multisensor bulkheads, often encased in protective housings—are used for in situ applications to transmit or log data in real time. Most also offer readings in either mV, mass or molar concentration. Due to propriety membrane composition, algorithms and other specifications, modern instruments offer different ranges of protection, precision, pH, temperature, and even

noted interferents for AISEs. Some manufacturers will provide SCs for interferents based on specified conditions (Table 1.2).

An AISE may potentially be used to determine urea concentrations in manure solutions. One particular advantage of the wide range of detection is the ability to accommodate the change in ammonia concentration from urea hydrolysis by urease with little or no dilution. AISEs have been used in the development of biosensors to measure urea concentrations by immobilizing urease enzyme to the ammonium sensor membrane (Dindar et al., 2011; Kuralay et al., 2006). These urea biosensors, though subject to the same conditions and interferences as a standard AISE, also share the same advantages in ease of sample preparation and quick analysis time. Although not yet commercially available, if a method for using an AISE with the urease method is established, the same principles could be applied to this type of urea biosensor in manure solutions in the future.

Table 1.2: Specifications of some ammonium ion selective electrodes

<i>Manufacturer and Model</i>	<i>Range, mg/L</i>	<i>pH Range</i>	<i>Temp °C</i>	<i>Interferences</i>	<i>Reference</i>
<i>Aqua TROLL 600 0033700</i>	0 to 10,000 ± 10%	N/A	0 - 40	Cs, K, Tl, pH, Ag, Li, Na	https://in-situ.com/products/accessories/aqua-troll-600-water-quality-ion-selective-electrode-sensors/
<i>ASI 43-Series</i>	0.03 to 18,000	2 - 7	0 - 50	pH<2, Li, Na, K, Cs, Mg, Ca, Sr, Ba	http://www.asi-sensors.com/index.php?option=com_opencart&Itemid=207&route=product/product&product_id=36
<i>EDT direction 3051</i>	0.9 to 9,000	0 - 8.5	5- -50	Ca, K, Na	http://www.edt.co.uk/Combination-Ammonium-Ion-Selective-Electrode-3051
<i>Hach #LXV440.99.1001 2</i>	0 to 1,000 ± 5%	5 - 9	2 - 40	K (reference included)	https://www.hach.com/aise-sc-ise-ammonium-probe/product-details?id=14667082652
<i>Nico 2000 Ltd ELIT 8051</i>	0.03 to 1,800	0 - 8.5	0 - 50	Na, K, Mg, Ca, Li	http://www.nico2000.net/analytical/ammonium.htm#specs
<i>Oakton WD-35802-03</i>	0.1 to 18,000	4 - 10	0 - 50	K	http://www.novatech-usa.com/Products/ISE-Electrodes-and-Solution-Kits/WD-35802-03
<i>Omega ISE-8712</i>	0.1 to 18,000	4 - 10	0 - 50	K	https://www.omega.com/manuals/manualpdf/M3965.pdf
<i>Vernier NH4-BTA</i>	1 to 18,000 ± 10%	4 - 7.5	0 - 40	K	www.vernier.com/files/manuals/nh4-bta.pdf
<i>YSI Pro Series 1004</i>	0 to 200 ± 10%	N/A	0 - 30	N/A	https://www.ysi.com/File%20Library/Documents/Brochures%20and%20Catalogs/YSI-Professional-Plus-Spec-Sheet-W14-04-0812.pdf
<i>YSI TruLine #400371</i>	0.02 to 18,000 ± 2%	1 - 12	0 - 40	H, Na, K, Li	www.ysi.com/File%20Library/Documents/Manuals/Ammonium-ISE-Manual-YSI-FEB2016C.pdf

3.2. Current uses of AISEs

The ability to perform real-time analysis without altering sample chemistry makes AISEs desirable for a variety of freshwater and wastewater applications (Crespo, 2017; Winkler et al., 2004). In-situ environmental water analysis has prompted development of more robust AISEs under adverse conditions such as depth-related pressure (Athavale et al., 2015) or use in activated sludge (Kaelin et al., 2008). This is accomplished through advancements in ion-selective electrode design using solid-state membrane construction and cleaning strategies for membrane fouling. Common for water quality analysis is the use of multiparameter sondes that allow not only the ability to measure multiple parameters at once but also the use of sophisticated algorithms to compensate for potential interferences in ISE responses. Additionally, since freshwater ammonium concentrations are often < 5 mg/L, efforts have also focused on improving the lower limits of detection of AISEs (Crespo, 2017; Radu et al., 2007).

Livestock manures, on the other hand, generally have ammonium concentrations above 100 mg/L and often in excess of 1,000 mg/L depending on the state of dilution, urea hydrolysis and microbial activity that has taken place (Table 1.3). The high ammonia concentration and variability in manure both within and among species poses a unique challenge for measuring ammonium with an ISE. Sommer et al. (1992) compared an AISE to other standard methods to determine the TAN in swine and cattle slurries. The slurry samples were diluted 100x to compensate for the effect of the high ionic strength on ammonium activity. The AISE was an F2322 ammonium half-cell electrode (Radiometer A/S, Copenhagen) used in conjunction with a calomel (Hg_2Cl_2) reference electrode. Results were compared with manual distillation-titration (MDT) at concentrations from approximately 2,000 mg TAN/L to 6,000 mg TAN/L. No temperature compensation was mentioned. Results were similar to the MDT method when AISE measurements were corrected to TAN by addition of NH_3 as calculated from solution pH. The authors viewed this as a disadvantage since the solution pH must be known, although current AISEs are typically used with a pH sensor and often calculate the $\text{NH}_3\text{-N}$ concentration for the user.

Table 1.3: Manure Characteristics of Different Sources

Livestock Source	Parameter All values in mg/L (mean \pm std. dev)					
	TKN-N	TAN-N	K	Ca	Mg	Na
finisher swine ^[a]	1,470 \pm 40	366 \pm 12	880 \pm 30	200 \pm 10	74 \pm 4	220 \pm 10
sow urine ^[b]	3,450 \pm 130	161 \pm 9	1,880 \pm 10	95 \pm 4	100 \pm 1	950 \pm 4
sow urine ^[b]	3,590 \pm 20	148 \pm 3	2,090 \pm 40	150 \pm 4	130 \pm 2	750 \pm 20
swine fresh manure ^[c]	5,700 \pm 2,000	3,600 \pm 1,100	3,600 \pm 1,600	3,400 \pm 1,400	800 \pm 170	800 \pm 630
dairy fresh manure ^[c]	5,190 \pm 1,100	961 \pm 198	3,420 \pm 1,000	1,970 \pm 600	850 \pm 170	730 \pm 210
beef fresh manure ^[c]	5,970 \pm 1,190	1,943 \pm 99	3,690 \pm 940	2,180 \pm 1,360	840 \pm 270	530 \pm 400
sheep fresh manure ^[c]	10,400 \pm 2,900	2,890	7,900 \pm 2,600	4,830 \pm 2,070	1,160 \pm 360	1,620 \pm 560
layer fresh manure ^[c]	13,300 \pm 3,100	3,320 \pm 670	4,820 \pm 1,030	17,200 \pm 7,400	1,790 \pm 550	1,520 \pm 510
broiler fresh manure ^[c]	13,200 \pm 2,200	3,360 \pm 120	4,840 \pm 810	3,940	1,450	1,450
turkey fresh manure ^[c]	14,000 \pm 2,300	4,040 \pm 370	5,040 \pm 1,690	11,200 \pm 6,020	1,290 \pm 130	1,170 \pm 210
dairy liquid slurry ^[d]	2,630 \pm 1,070	1,100 \pm 80	2,390 \pm 1,040	1,250 \pm 710	600 \pm 340	370 \pm 200
swine liquid slurry ^[e]	3,170 \pm 1,790	2,010 \pm 310	1,820 \pm 1,050	940 \pm 740	330 \pm 270	350 \pm 220
swine lagoon liquid ^[f]	560 \pm 270	460 \pm 30	480 \pm 260	120 \pm 60	40 \pm 30	170 \pm 90
dairy lagoon liquid ^[g]	580 \pm 310	380 \pm 50	650 \pm 400	300 \pm 180	160 \pm 100	200 \pm 100

[a] wastewater collected from a previous study by authors using a solid-liquid separating scraper system

[b] collected from lactating sows in farrowing crates

[c] feces and urine as voided, values based on unit conversions of original data (Barker et al., 1994; Barker & Overcash, 2007)

[d] 6-12 mos. accumulation of manure, milking center wastewater, average storage surface rainfall surplus (Barker et al., 1994)

[e] 3-6 mos. accumulation of manure, urine, excess water usage, storage surface rainfall surplus (Barker et al., 1994)

[f] manure, excess water usage, average annual lagoon surface rainfall surplus (Barker et al., 1994)

[g] manure, milking center wastewater, average annual lagoon surface rainfall surplus (Barker et al., 1994)

Zhou and Boyd (2016) performed a similar study comparing the precision and accuracy of different analytical methods for the determination of TAN in aquaculture waters. The AISE selected for this study was a Radiometer Analytical half-cell model ISE25NH4 with a PVC membrane, detection range of 0.06 to 20,000 mg/L NH₄-N, and pH range of 3-8. Low (specific conductance 60-120 μ S/cm) and medium hardness (200-5,000 μ S/cm) freshwater sample

locations and one location of saline water (2-6 g salinity/L) were tested. For each of these water samples, low (0.03-0.25 mg/L), medium (0.3-0.7 mg/L) and high (0.8-2.0 mg/L) TAN concentrations were tested.

Samples were spiked with 0.3 mg/L of ammonia nitrogen and spike recovery of the AISE was compared to the salicylate method, which was previously selected among other lab methods for accuracy. The mean accuracy of the AISE response differed from the salicylate method in percent spike recovery only for the low concentration-low hardness test. However, the AISE mean measurements were significantly different for all of the water samples except for the low hardness-high concentration sample, and the difference was 2.7 to 8 times higher in the saline solution. This difference was believed to be the result of high concentrations of potassium and sodium in the saline water, although this effect decreased as TAN concentration increased. The precision of the AISE was considered reasonably good. In their discussion, Zhou and Boyd (2016) reiterated the advantages of using an AISE with regard to lower cost, no concern for sample color or turbidity, no need to dispose of hazardous waste, and high concentration ranges that eliminate the need for sample dilution.

These studies indicate that the primary deterrents to using an AISE with manure solutions are high ionic strength and the presence of interfering ions in high concentrations. These factors are well-accepted and indeed, little literature exists where an effort has been made to understand how an AISE might work in a complex solution such as manure with these high ion concentrations.

3.3. A methodology for measuring ammonium and urea in manure solutions

A methodology for field analysis of ammonium and urea in liquid swine manure using an AISE may be developed in two phases, with the second phase contingent upon the outcomes of the first. The first phase begins with the series of experiments conducted in this study to determine the accuracy of an AISE when measuring some liquid manure solutions. As previously stated, the main confounding factor to using an AISE in manure is the high concentrations of dissolved ions present. This leads to a reduction in ion activity and therefore a reduced response by the sensor due to high ionic strength of the solution, but also an increased response due to interference from some of those ions which the AISE interprets as ammonium, with the most significant

interferent being potassium. Although the ionic composition of different manures is generally similar, as shown in Table 1.3, the concentrations and ratios of these components varies widely both within and across species, over time due to microbial activity, and with dilution or treatment of material. Due to the research focus of this thesis, the initial investigations into the development of a methodology for using an AISE in manure will focus on swine manure solutions. Upon successful analysis the method may applied to manures from other livestock species.

Studies have examined mitigating the matrix effects of complex solutions with some success. Sommer et al. (1992) used an AISE to measure TAN in pig and cattle slurries by diluting the solutions 100x to prevent the ionic strength effects. When the ammonia concentration was calculated based on solution pH and added to the ammonium concentration of the solution, they found the AISE method to be relatively accurate when compared with standard methods . No mention was made in this study concerning interference from other ions such as potassium, so it may be that enough TAN was present that this was not an issue.

Deyhimi et al. (2008) presented a mixed solution method (MSM) for determining selectivity coefficients for an AISE in a mixed electrolyte system. In this method, SCs are measured for each interferent in the presence of all other interferents at once. Compared with the commonly used separate solution method (SSM), MSM indicated a 5x and 12x increase in the SCs of the divalent cations Ca^{2+} and Mg^{2+} respectively, although these would still exert virtually no influence on AISE response at relative concentrations in most manure solutions. K^+ and Na^+ exhibited similar influence with both SSM and MSM, with K^+ continuing to have the most significant impact on AISE response with a SC of 0.126. This means that for an 8:1 ratio of K^+ to NH_4^+ the concentration of NH_4^+ -N measured by the AISE should approximately double. The MSM method demonstrated not only the effect of interfering ions on the response for ammonium, but the effect of mixed solutions on the interferents themselves.

These matrix effects would be expected to increase with ionic strength, as interactions between increasing concentrations of ions in solution become more complex, and are influenced as well by solution temperature and pH. Because an AISE is only able to measure the free ammonium ions, these interactions reduce the activity, or availability of ions to interact with the sensing

membrane and therefore reduce the AISE's response. The reduction in activity can be significant. An ionic strength of 0.01 M would produce a 10% reduction in response, while an ionic strength of 0.5M would produce a 34% reduction in response (Rundle, 2000).

4. Summary and Research Objectives

Although there is considerable literature available regarding the inhibition of urease enzyme from both plant and bacterial sources, these studies seem focused primarily in either the medical or soil science fields. In medicine the primary concern is disease control by limiting growth of urease producing bacteria. In agriculture the focus is mainly on preventing ammonia volatilization from soil after urea fertilizer application. Only a few studies have looked at using urease inhibition to reduce ammonia emissions in animal housing. In these, environmental benefits of urease inhibition are recognized by improved air quality. However, economic benefits may also be realized in the conservation of nitrogen, particularly if it is in the non-volatile urea form.

This review presents a starting point for selecting potential inhibitors for study in swine manure and wastewater solutions. The following chapters will examine inhibitor effectiveness in urea and urine solutions, as well as a pilot scale study for inhibitor application and effectiveness over time.

The research objectives of this study were two-fold: 1) to determine whether urea nitrogen can be preserved in liquid swine waste by inhibiting urease enzyme activity and 2) to determine how a complex manure solution such as sow urine affects an ammonium ion selective electrode response. The primary study tested five urease inhibition methods (NBPT, thymol, SHAM, and two pH adjustments) via batch testing in urea solution and urine to determine their effectiveness. From these tests three inhibition methods were applied to a pilot scale study using a solid-liquid separation system to isolate and treat liquid manure produced by 30 pigs in a barn setting. As the result of a need realized during the primary study, a secondary study was initiated to determine whether an ammonium ion selective electrode is an appropriate instrument for measuring TAN and urea concentrations in swine manure solutions. Based on the results of these investigations, future research questions may include:

- What is the optimal inhibitor dosing?
- Are there any additional benefits or undesired effects associated with the use of an inhibitor (e.g., does the inhibition method affect hydrogen sulfide or other gaseous emissions)?
- Does reducing urease contact with urea by mechanical separation of manure solids and liquids increase urease inhibitor effectiveness?
- How much nitrogen can be recovered from inhibitor treated liquids and what does that recovery look like?
- Is the process economically feasible?

This thesis will contribute to a better understanding of the benefits and challenges associated with urea preservation in swine manure through urease enzyme inhibition. Additionally, observations of the effects of swine manure solutions on AISE response presented herein offer a starting point for the development of a methodology for using an AISE and the urease method to analyze ammonium and urea concentrations in liquid swine manure.

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CHAPTER 2: Urea Nitrogen Preservation in Swine Urine by Urease Inhibition

1. Introduction

Domestic livestock production contributes over 40% of the total global atmospheric ammonia (Schlesinger & Hartley, 1992). A third to half of this comes from animal housing and manure storage with the rest split between animal grazing and land application of manure (Beusen et al., 2008). Ammonia is the main form of nitrogen lost from manure, produced through the enzymatic hydrolysis of urine urea or the microbial decomposition of proteins and amino acids in fecal solids. A small percentage of ammonia is excreted directly.

At alkaline pH ammonia volatilizes, although residence time once airborne is short relative to other emissions like methane or carbon dioxide (Brasseur et al., 1999). This means these emissions tend to have greater impact on the regional ecosystems where they are emitted. Ammonia is either removed quickly by deposition or reacts with acidic atmospheric species (e.g. HNO_3 , H_2SO_4 , HCl) to form secondary organic aerosols such as ammonium nitrate, ammonium sulfate and ammonium chloride which contribute to fine particulate matter ($\text{PM}_{2.5}$). $\text{PM}_{2.5}$ causes atmospheric haze and negative health effects through inhalation. As these particles are deposited to soil or surface water, the decomposition of ammonium salts by nitrifying bacteria forms nitrate and hydrogen ions, leading to acidification and eutrophication (Aneja et al., 2008, Donham, 2000; Hao et al, 2005; Sutton et al., 2008). High concentrations of ammonia in confined animal housing can cause negative respiratory and immunological responses and reduced production (Donham, 2000; Jones et al, 1998; Murphy et al, 2012). Furthermore, ammonia emissions contribute to the formation of fine particulate matter, causing both acute and chronic human health problems in farm workers and in surrounding communities (Borlee et al, 2017; Donham et al, 2002; Paulet and Jacob, 2014). Due to the short-lived nature of atmospheric reactive nitrogen, ammonia emissions have also historically garnered little attention for their impact on global climate change. However, Erismann et al. (2011) noted numerous direct (e.g. aerosol formation) and indirect (e.g. changes in methane production) impacts on greenhouse gasses by reactive nitrogen. Additionally, the nitrogen cycle, listed as one of nine planetary boundaries, or metrics of environmental sustainability, is already considered to have exceeded sustainable limits (de Vries et al., 2013).

The control of ammonia emissions by the inhibition of urease enzyme has been explored in previous research. A lab study by Hagenkamp-Korth et al. (2015) suggests a single urease inhibitor application directly to dairy barn floors reduced ammonia emissions an average of 48% over the test period of 4 to 6 days. In another livestock application, an antimicrobial plant oil, Thymol and a commercial soil urease inhibitor product, Agrotain (containing N-(n-butyl) thiophosphoric triamide) were applied to swine manure pits, temporarily inhibiting urea hydrolysis for 6 to 10 days (Varel and Wells, 2007).

However, most agricultural applications focus on limiting urease activity using soil amendments to reduce ammonia volatilization in fields when urea fertilizer is applied (Ni et al., 2014; Soares et al., 2012; Watson et al., 2008). While many urease studies focus on reducing ammonia emissions, few have considered the additional benefit of urea preservation for nitrogen recovery. If the urea in fresh urine can be preserved at the source it would prevent the volatilization of ammonia that causes adverse effects on livestock, humans and the environment. This recovered urea can then be applied to crops as a concentrated source of nitrogen, possibly even replacing a substantial portion of the urea nitrogen manufactured for this purpose.

In this study, fsoiive inhibition methods were tested, including two pH adjustments, N-(n-butyl) thiophosphoric triamide (NBPT), salicylic hydroxamic acid (SHAM), and thymol. NBPT is commonly used as a soil urease inhibitor and has been shown to be very effective at slowing the rate of urea nitrogen fertilizer hydrolysis after application (Bremner, et al., 1991; Goos, 2013). SHAM is a metalloenzyme inhibitor and thus effective on urease which contains two nickel atoms in its active site. SHAM has been used in medical applications to control urinary tract infections but its effectiveness is known to be dose dependent (Hassan et al., 1997). NBPT and SHAM are competitive inhibitors that attach to the enzyme's active site, preventing urea from engaging with urease (Krajewka, 2009, Manunza et al., 1999). Thymol is a plant oil derivative with potent antimicrobial properties that has been previously studied for urease inhibition though it's mode of action in that regard is largely unknown (Modolo et al., 2014; Varel & Wells, 2007).

Finally, both acidification and alkalization will be tested as methods of urease inhibition. It has been well-studied that urease activity is limited to a range of pH values. Frankenberger & Johanson (1982) observed that urease stability in soil varied with pH, with activity ranging across

3 soil types from pH 4 to 10. Fidaleo & Lavecchia (2003) conducted a kinetic analysis of enzymatic urea hydrolysis from pH 4 to 9, also observing that the rate of urea hydrolysis is pH dependent. Hellström et al. (1999) were able to stabilize urea in source separated urine for more than 100 days at pH < 5 with a one-time dose of 60 meq/l H₂SO₄. Randall et al. (2016) were also able to stabilize fresh urine by addition of Ca(OH)₂ to a saturation pH of 12.5.

Our study investigated these methods of inhibiting urease activity in swine urine with the goal of preserving as much urea content as feasible during storage, processing and transport. Based on the cited studies, we determined which of several urease inhibitor treatments were most effective in preserving the urea content in urine. We then tested the effectiveness of those treatments with an in-barn pilot-scale system for treating the wastewater recovered from a solid-liquid separating scraper system.

2. Materials and Methods

Jack bean urease was purchased from Sigma-Aldrich Corporation (St. Louis, MO), catalog no. U1500 with labeled activity of 40,318 units/gram. Per the manufacturer's data sheet, one unit (U) of urease will liberate 1.0 μmol of ammonia from urea per minute at pH 7.0 and 25°C. Urease enzyme was prepared fresh for each experiment by mixing the urease powder with 0.4 M sodium phosphate buffer solution adjusted to pH 7.0 (Dai & Karring, 2014) for a unit activity of 32 U urease per ml of phosphate buffer. NBPT was obtained in the form of the commercially available product Agrotain Advanced 1.0 (Koch Agronomic Services, LLC, Wichita, KS). Urine was collected from lactating sows in farrowing crates during the morning feeding. This urine was collected in clean buckets, mixed in a 20-L carboy placed in ice, then used fresh for experiment 1 and with NBPT in experiment 2. A second urine collection was chilled and used fresh for the pH adjustments urine in experiment 4. Concentrated sulfuric acid (95-98% w/w), sodium hydroxide pellets (≥ 97% assay) and all other chemicals used were reagent-grade purchased through Fisher Scientific (Hampton, NH).

Lab analysis of ammonia and total nitrogen were conducted using Standard Methods 4500-NH₃: segmented flow automatic analyzer and 4500-N: persulfate digestion with automatic analyzer (APHA, 2012).

Statistical analysis of results from lab analysis of TKN and TAN were performed using JMP version Pro 13.2.1 (SAS Institute Inc., Cary, NC) and R version 3.4.3 (R Foundation for Statistical Computing, Vienna, Austria) software packages. All samples were acidified to $\text{pH} < 2$ with concentrated sulfuric acid (H_2SO_4) prior to analysis.

2.1. Swine urine vs. urea solution

A urease enzyme solution was prepared at a concentration of 32 U urease enzyme per 1 ml 0.4 M phosphate buffer. A 1 M urea solution was prepared by mixing 60.06 g crystallized urea with deionized water for a total volume of 1 L. Three 100 ml aliquots of the urea solution and of urine were each pipetted into three 250 ml Nalgene containers. Each of the 100 ml urine and urea solution replicates was mixed with 9 ml of the urease phosphate buffer and split into 6 samples of >10 ml each in individual 20-ml lidded glass vials. The final urease enzyme concentration was 3 U urease per ml test solution. One sample jar of each replicate of the urine with urease or urea solution with urease was immediately acidified with H_2SO_4 to $\text{pH} < 2.0$ for initial nitrogen analysis (TKN and TAN). A subsequent sample from each replicate was acidified to $\text{pH} < 2$ at 1, 2, 3, 14 and 22 hours and submitted to the lab for TKN and TAN analysis.

2.2. Treatments for urease inhibition in 1 M urea solution

Five urease inhibitor treatments were tested, including two pH adjustments, NBPT, SHAM, and thymol. Values of $\text{pH} < 3$ and $\text{pH} > 12$ were selected as outside the range of urease activity in soil per Frankenberger & Johanson (1982). For the pH adjustments, 100 ml of 1 M urea solution was titrated with either 1N H_2SO_4 to $\text{pH} < 3$ or 0.5N NaOH to $\text{pH} > 12$.

Agrotain Advanced 1.0, a commercial preparation containing 30% NBPT w/v, was diluted 100:1 to facilitate accurate measurement. Based on the manufacturer's recommendation for application with urea, 2 ml of the diluted Agrotain were added to 100 ml urea solution for a final estimated concentration of 62.5 mg/L NBPT.

SHAM was prepared in two concentrations by adding 0.2527 g and 0.5043 g to a 0.1 M tris buffer solution at $\text{pH} 7.5$. Five ml of each concentration was then added to 100 ml of urea solution for nominal SHAM concentrations of 50 and 100 mg/L.

A solution was mixed containing 0.4997 g thymol in 10 ml ethanol; three ml of the thymol solution was added to 100 ml of the urea solution for a nominal concentration of approximately 1.5 g/L thymol in urea solution.

After inhibitor treatment was added, each 100 ml urea solution was dosed with 9 ml of the urease buffer solution. Each of three replicates was then pipetted in > 10 ml aliquots into 20-ml glass vials and sealed. One set of replicates for each treatment was submitted weekly for TKN and TAN analysis.

2.3. pH range test

For this experiment 2.5 L of 1 M urea solution were divided into 100 ml aliquots and dispensed into (24) glass jars with lids for storage. Five ml of urease phosphate buffer solution was pipetted into each of 24 20-ml lidded glass vials. The pH in each vial was then adjusted by titration with 0.5N NaOH or 1N H₂SO₄ to produce three replicates each of nominal pH values 4, 5, 6, 7, 8, 9, 10, and 11. One vial of pH adjusted urease solution was mixed into each jar of 100 ml urea solution for a urease dosing level of 1.6 U urease per ml urea solution. The final pH value of each replicate was noted. After incubating for 7 days at room temperature, the pH in each glass jar was tested before a 10 ml sample was taken for lab analysis of TKN and TAN. The pH was checked again at 14 days, although no additional samples were taken for lab analysis.

2.4. NBPT and pH adjustments in swine urine

The inhibitory effect of NBPT, pH < 3, and pH > 12 on urease activity was tested in swine urine. NBPT was tested in urine at the same time as the 1 M urea solution and the urine replicates were prepared the same way as the urea solution replicates. The pH adjustments were conducted with the second batch of freshly collected urine. A control sample of the urine was acidified to pH < 2 and submitted for lab analysis of TKN and TAN. Urine was dispensed into two beakers of 150 ml each and 7.5 ml of urease buffer solution was mixed into each beaker for a urease dosing concentration of 1.6 U urease per ml urine. Each beaker was split into 50 ml aliquots dispensed into lidded glass jars. Three jars were titrated to pH < 3 and three jars were titrated to pH > 12. Samples from each vial were submitted at 7 and 14 days for TKN and TAN analysis and pH was measured.

2.5. Pilot Study

The pilot study was conducted in one room of the Swine Research Unit of North Carolina State University, Raleigh, NC. Thirty pigs approximately eight weeks old with an estimated weight of 21-23 kg were divided into three pens in groups of ten. Water via nipple drinkers and dry feed were provided free choice throughout the study.

A stainless steel pan was suspended beneath the slatted floor of the pens to collect all solid and liquid waste produced by the pigs, including feces, urine, spilled feed and water. The pan had a 1% slope to a center trough that collected and recirculated the liquid waste stream. The solids were scraped from the pan using a Hog Slat brand pit scraper system modified with rubber material along the scraping edge to fit the pan slope and improve scraper performance. The solids were scraped into a 946-L (250-gallon) poly tank with an 80-mm (3-inch) drain every 4 hours during the day; the scraper was not run at night.

A skid installed outside of the pit contained the recirculation and collection system illustrated in Figure 2.1. The pH electrode was installed in a 64-L polypropylene tank used for inhibitor mixing and recirculation of the wastewater. Another 64-L (17-gallon) polypropylene tank for inhibitor dosing was installed and connected to a pH measuring and dosing system (Hanna Instruments, model BL 7916). A 946-L vertical polypropylene product storage tank collected excess liquid that reached the overflow port on the recirculation tank. Samples were taken from the product tank at 24 to 48 hour intervals throughout each inhibitor test.

In-line 91.94-W (1/8-HP) recirculation pumps (Little Giant model TE-5-MD-HC) were used to recirculate the liquid through the collection pan trough. A collection port for sampling was placed in the recirculation system immediately after wastewater left the pan and a second collection port was placed in the bottom of the product tank.

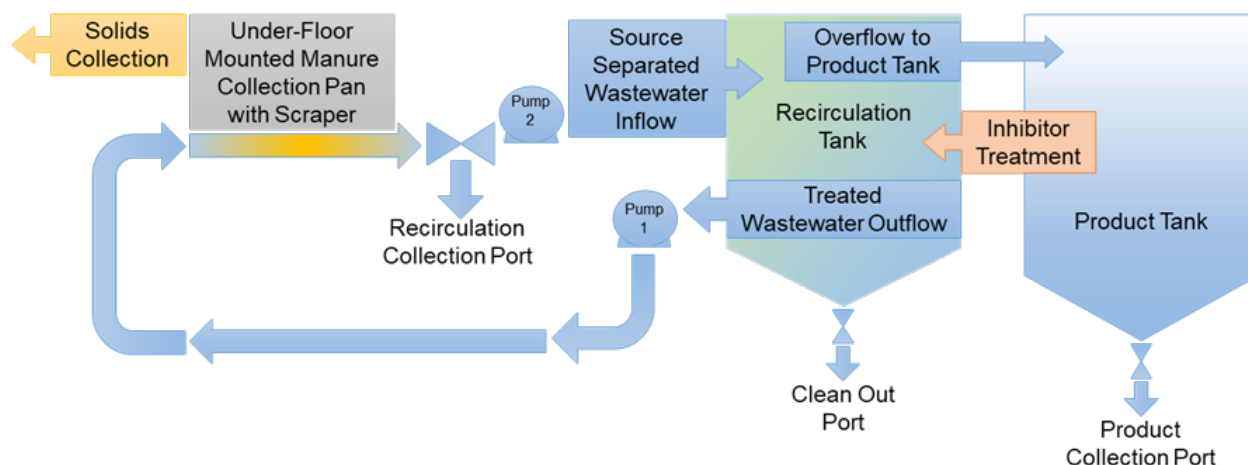


Figure 2.1: Schematic of the wastewater recirculation, treatment and collection system used in the pilot scale project.

2.5.1. NBPT

A preliminary lab study was conducted to determine the optimal NBPT dosage for the pilot study. Four concentrations of NBPT (6.25 mg/L, 31.3 mg/L, 62.5 mg/L, 125 mg/L) and an untreated control were tested in triplicate for TAN formation in wastewater collected from the recirculation system of the pilot study. Agrotain Advanced 1.0 was diluted 100x for a manageable concentration at low volumes. The diluted Agrotain was then added at a rate of none (control), 0.2, 1, 2, and 4 ml to 100 ml aliquots of the wastewater and sealed. The solutions were allowed to sit at room temperature for one week, then 35 ml of each sample was submitted for lab analysis of TKN and TAN.

Based on the results from the lab experiment and the concentration of NBPT used in literature (Varel and Wells, 2007), an estimated dosing rate of 82.6 mg/L NBPT was applied to the recirculating tank daily, with total application based on estimated daily volume of wastewater collected. Three week-long NBPT trials were conducted when the pigs were 15, 20, and 22 weeks of age. The first trial was dosed at 50 ml Agrotain (~16 mg NBPT) per day for the first four days and not dosed for the last two days. The second and third trials were dosed at 100 ml Agrotain (~ 31 mg NBPT) per day each day of the trial. Samples (500 ml) were collected from the product tank every 24 to 48 hours after the start of each trial, acidified to pH < 2 and submitted for TKN and TAN analysis. Product volumes were recorded daily.

2.5.2. Acidification

Three week-long acidification trials were conducted when the pigs were 11, 16, and 18 weeks of age. Concentrated H_2SO_4 (95-98% w/w) was placed in the dosing tank and the dosing pump was set to maintain a constant pH of 2.5 for the duration of each trial. The volumes of acid used and product collected were recorded for each trial. A fourth acidification trial was conducted to collect enough product for future concentration testing. Samples (500 ml) were collected from the product tank every 24-48 hours after the start of each trial, acidified to $\text{pH} < 2$ if needed and submitted for TKN and TAN analysis. Product volumes were recorded daily.

2.5.3. Alkalization

Two trials for high pH were conducted when the pigs were 12 and 13 weeks old. Concentrated NaOH (18 M) was placed in the dosing tank and the dosing pump was set to maintain a constant pH of 12.0 for the duration of each trial. The mass of base used and the product collected were both measured. Two 500 ml samples were collected for each trial from the product tank, acidified to $\text{pH} < 2$, and submitted for TKN and TAN analysis. Product volumes were recorded daily.

3. Results and Discussion

3.1. Urease enzyme activity in urine and urea solution

Figure 2.2 illustrates the change in ammonia concentration of fresh sow urine and a 1 M aqueous urea solution over a 22-hour test period due to urea hydrolysis by added jack bean urease. Possible contamination of the urine with fecal bacteria may have resulted in additional urease enzyme in the urine, increasing the initial rate of urea hydrolysis. However, overall the formation of ammonia trended similarly between urine and urea solution over time suggesting that the urea solution could be an acceptable substitute for urine when testing urease activity.

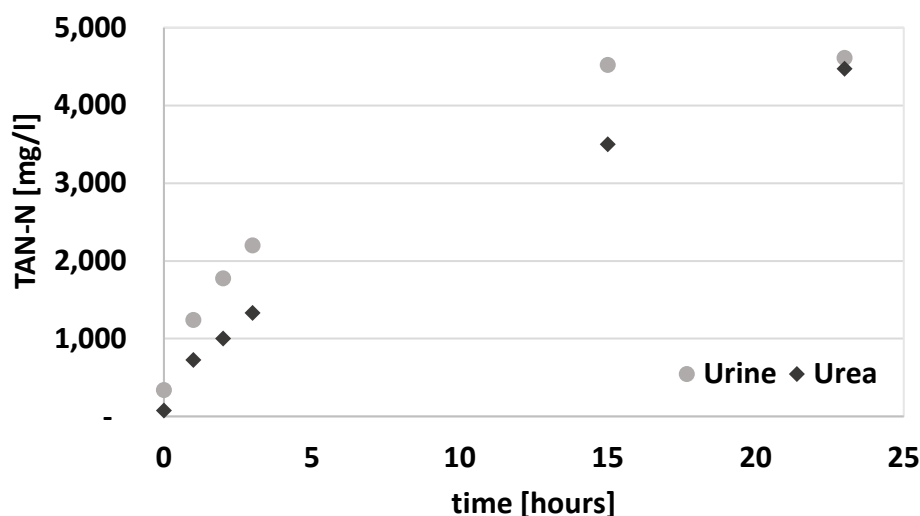


Figure 2.2: Effect of jack bean urease in sow urine and 1 M urea solution.

3.2. Treatments for urease inhibition in 1 M urea solution

Experimental results in Figure 2.3 show that the pH adjustments had the highest inhibitory effect on urease activity with < 1% TAN formation, followed by NBPT, SHAM at 50 mg/L, thymol and SHAM at 100 mg/L. Although these results suggest that at pH > 12 nearly all urease activity is inhibited, Figure 2.4 shows some loss of TKN for this treatment, as well as all other treatments except pH < 3. TKN represents the total organic and ammoniacal nitrogen in the system. In a pure urea solution all TKN is initially in the urea form, therefore any TAN formation in an alkaline solution will lead to ammonia loss through volatilization. The amount of ammonia lost is dependent upon both the solution pH and the ammonium concentration. This loss of ammonia is represented as the difference between initial and final TKN. Loss of TKN is apparent in the other treatments and the control, which all had an average pH above 9.2, the pKa of ammonia. Quantifying these losses and adding them to the total TAN remaining in solution indicates urea-N preservation by urease inhibition for the treatments as follows: pH < 3 (>99%), pH > 12 (93%), NBPT (85%), SHAM at 50 mg/L (83%), SHAM at 100 mg/L (63%). There was approximately 9% urea-N remaining in the control solution.

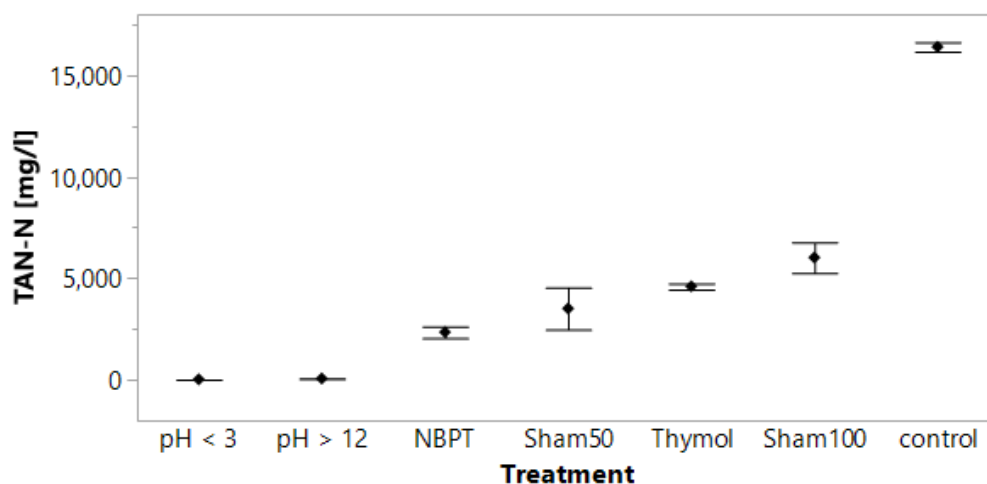


Figure 2.3: Average TAN formation across treatments over a six-week period. Error bars = 1 standard error from mean.

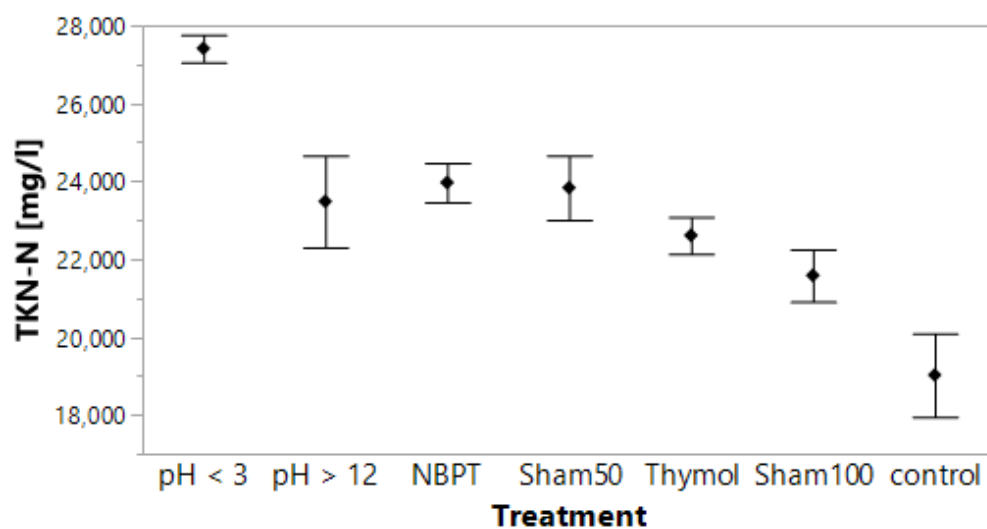


Figure 2.4: Average TKN (mg/L) across treatments over a six-week period. Error bars = 1 standard error from mean.

3.3. pH range test

Testing a range of nominal pH values in urea solution between 4 and 11, it was observed that urease enzyme remained active over a 2-week period across all values, with the highest activity observed at pH 7.0 (Figure 2.5). These results are consistent with literature.

An interesting phenomenon was observed with one replicate of the pH 4 treatment set. The

initial pH of one sample was titrated < 3 and brought back to pH 3.56 with 2 drops of NaOH in the urease buffer with a final pH of 3.93 after the pH-adjusted urease buffer was added to the urea solution. This sample had a pH of 4.42 at the end of the first week and 5.33 at week 2. This is the only sample that demonstrated significant inhibition of urease activity in the pH range test. This suggests that while urease exhibits variable activity between pH 4.0 and 11.0, a pH < 3 denatured the enzyme such that it did not fully regain function when pH was adjusted upward again. This seems in line with previous research that indicates that jack bean urease loses both activity and structural integrity when acidified below pH 6.0 but completely loses enzymatic activity at pH 2.0 (Bhowmick and Jagannadham, 2006).

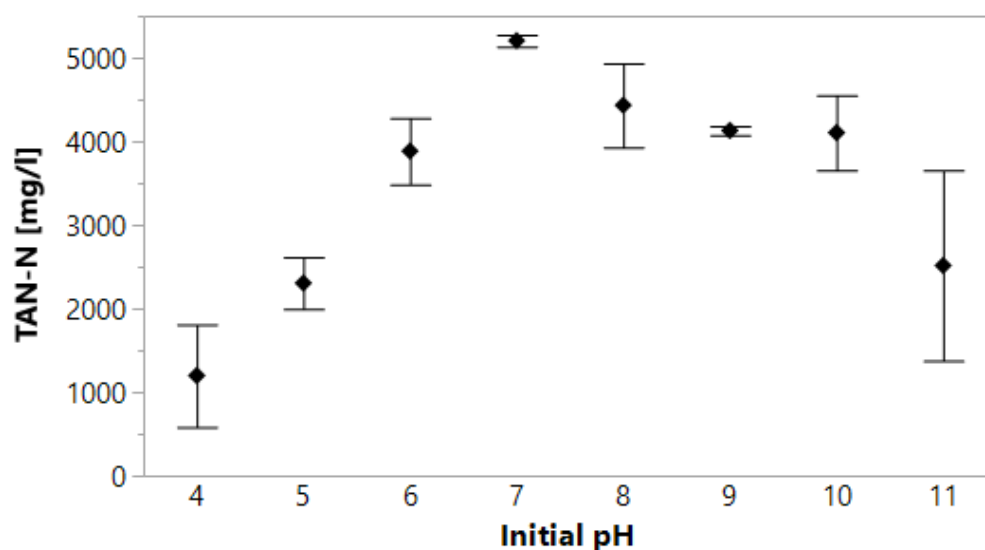


Figure 2.5: TAN formation in 1 M urea solution observed across all pH levels at one week. Initial solution characteristics: pH 7.05, TAN 2.4 mg/L, TKN 28,700 mg/L. Final pH across all samples = 9.7. Error bars = 1 standard error from mean.

3.4. NBPT and pH adjustments in swine urine

The final experiment of the preliminary analysis tested the inhibitory effect of NBPT, pH < 3 and pH > 12 on jack bean urease added to fresh sow urine. Figure 2.6 illustrates the change in TAN as a percentage of total nitrogen. While Tukey's comparison of means indicated no statistical difference between the two pH treatments ($p=0.13$), two of the three low pH replicates showed higher TAN formation by the second week, as suggested by the larger standard error.

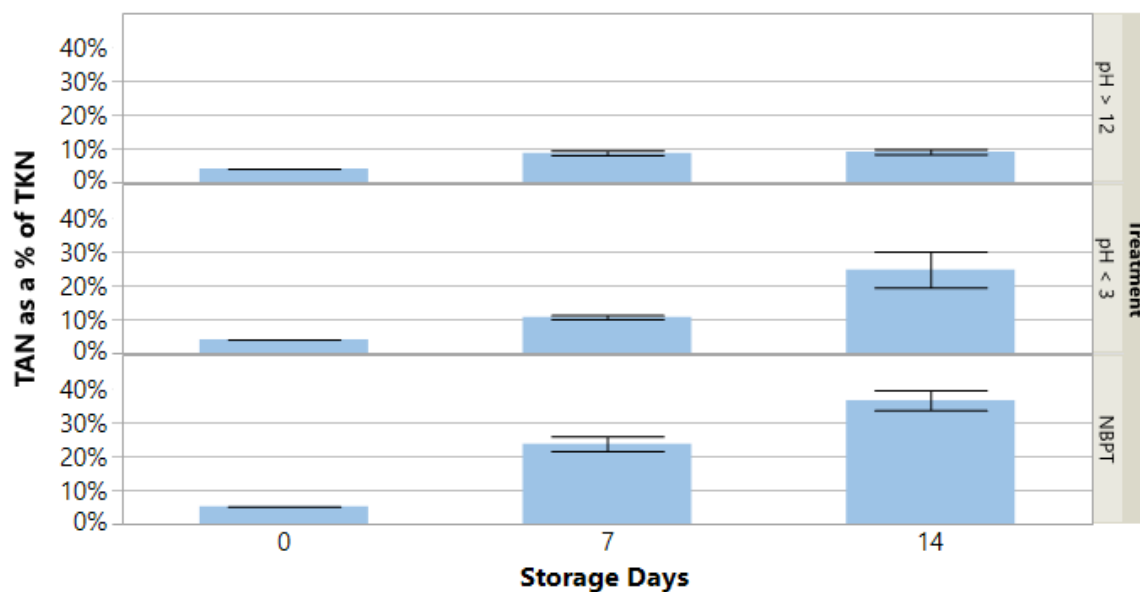


Figure 2.6: Comparison of the change in TAN as a percentage of total nitrogen across all inhibition treatments in urine. Error bars = 1 standard error from mean.

The results of the individual reps for the low pH treatment are shown in Figure 2.7, because the data appear to suggest the actual threshold for urease inhibition may lie below pH 3. Although additional study would be required to confirm this in urine, the hypothesis is supported by Bowhmick and Jagannadham (2006) who indicated that jack bean urease only loses all activity at pH 2.0.

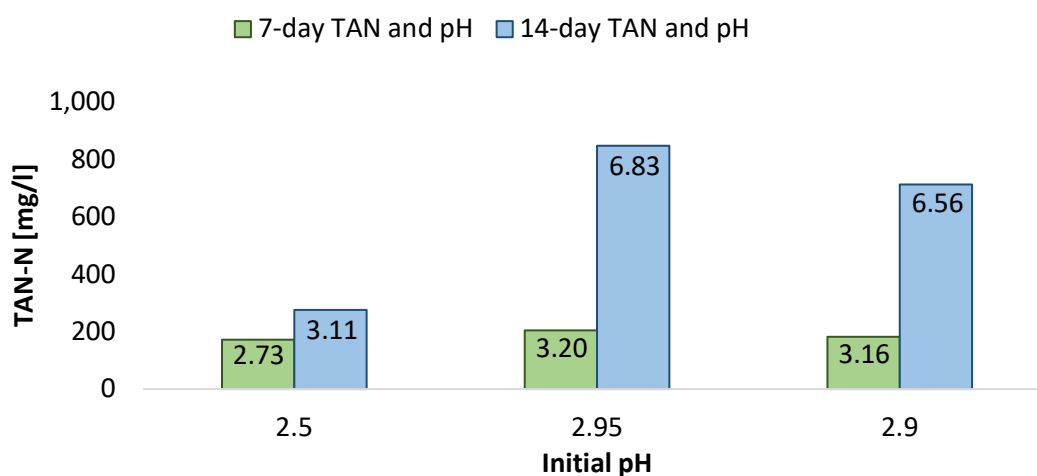


Figure 2.7: TAN and pH of the three low pH replicates in urine at 7 and 14 days. Solution pH is indicated on the bars.

Statistical analysis was not done on data for the high pH values because some loss of TAN likely occurred due to volatilization of ammonia and this loss was not measured.

All three urine samples at high pH exhibited precipitation shortly after treatment while none of the low pH samples showed any precipitation (Figure 2.8). This precipitate is likely struvite, or some similar mineral formed from ions present in the urine and represents a possible method for recovering additional nutrients such as phosphorus (Randall et al., 2016). Analysis to determine the precipitate composition was outside the scope of this study.

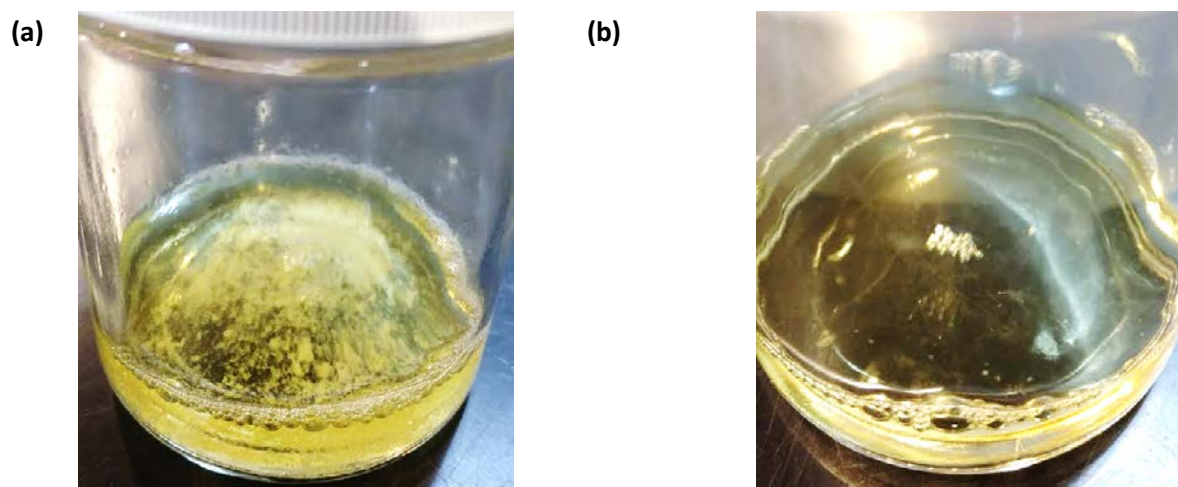


Figure 2.8: Photos of (a) a urine sample treated with NaOH to pH > 12 and (b) a sample treated with H₂SO₄ to pH < 3 approximately 1 hour after treatment.

3.5. Pilot study

Two types of inhibitor effects were observed during the pilot study: how much effect inhibition treatment had at the time of application on TAN formation, and the amount of TAN formation during sample storage after collection. Figure 2.9 illustrates that mean initial TAN formation was highest during NBPT application and lowest when pH > 12 was maintained. However, a Tukey's test indicates no statistical difference between the low and high pH treatments ($p=0.46$). Thus, given the difficulties explained below regarding the high pH treatment, acidification to pH < 3 remains the optimal choice for urease inhibition treatment in recirculating wastewater during collection.

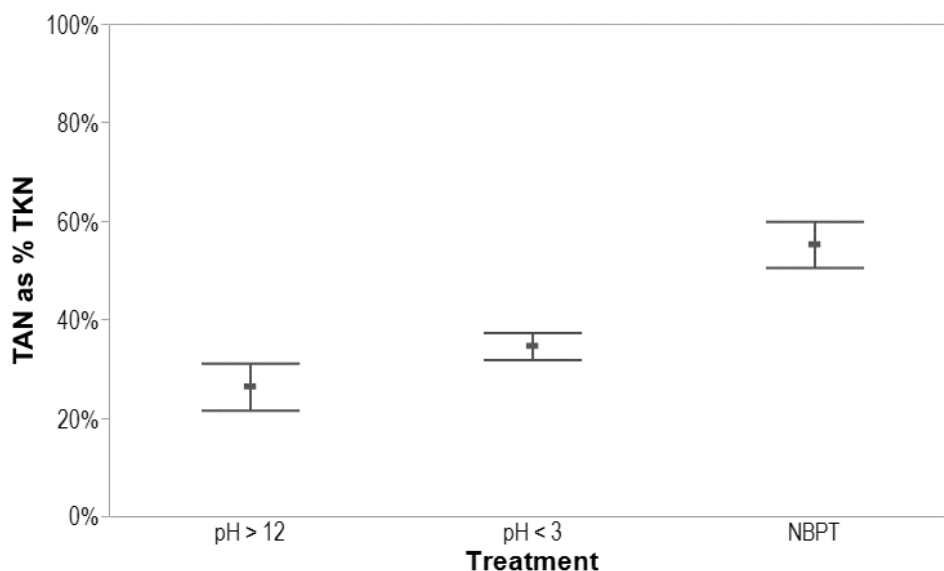


Figure 2.9: Effectiveness of urease inhibition treatment represented by TAN concentration as a percentage of total nitrogen (TKN) in wastewater at time of collection; some TAN formation due to fecal urease exposure prior to treatment.

Several challenges were encountered in the pilot study, which was conducted from April through July. Urea concentration was not consistent across all trials due to changes in pig size, drinking patterns and microbial activity as influenced by seasonal weather. Furthermore, the composition of the wastewater in the pilot scale system contained not only urine produced by the pigs, but also spilled water and some solids from feed and feces as the liquid traversed the pan from the point of deposition to the center channel for collection. These differences represent the type of dynamic system challenges that could be encountered in a full-scale farm installation.

3.5.1. NBPT

Prior to initiating the pilot studies of NBPT, a batch lab study was conducted to compare the effectiveness of different doses in source separated swine wastewater (Figure 2.10) collected from the pan. A comparison with Dunnett's Method suggests that the inhibitory effect does not change appreciably between concentrations of 31 and 125 mg/l when compared with the original dose of 62.5 mg/l ($p=1.0$ and $p=.83$, respectively). However, due to the short-term inhibition seen by Varel and Wells (2007) when adding NBPT at a rate of 83 mg/l, this was the

chosen application rate for the recirculating system. Thus, Agrotain Advanced 1.0 was applied at a rate of 50 ml per day for the 1st trial and 100 ml per day during the 2nd and 3rd trials. Although these rates were based on expected product accumulation and intended to be 82.6 mg NBPT per liter, for most days accumulation was less than anticipated and so dosing on a per liter volume was generally above 100 mg/l NBPT. However, the results illustrated in Figure 2.11 do not appear to indicate a consistent trend between dosing concentration and urease inhibition as indicated by the change in TAN over time. Storage time for samples ranged from 7 to 12 days. Samples were stored at room temperature in sealed 500 ml bottles with little headspace. Because NBPT does not have antimicrobial properties, some ammonia formed could be the product of anaerobic bacteria activity in the samples. Still, the average pH remained near neutral and this is an indication of urease inhibition as previous tests have shown that even a small amount of urea hydrolysis will increase pH above 9.

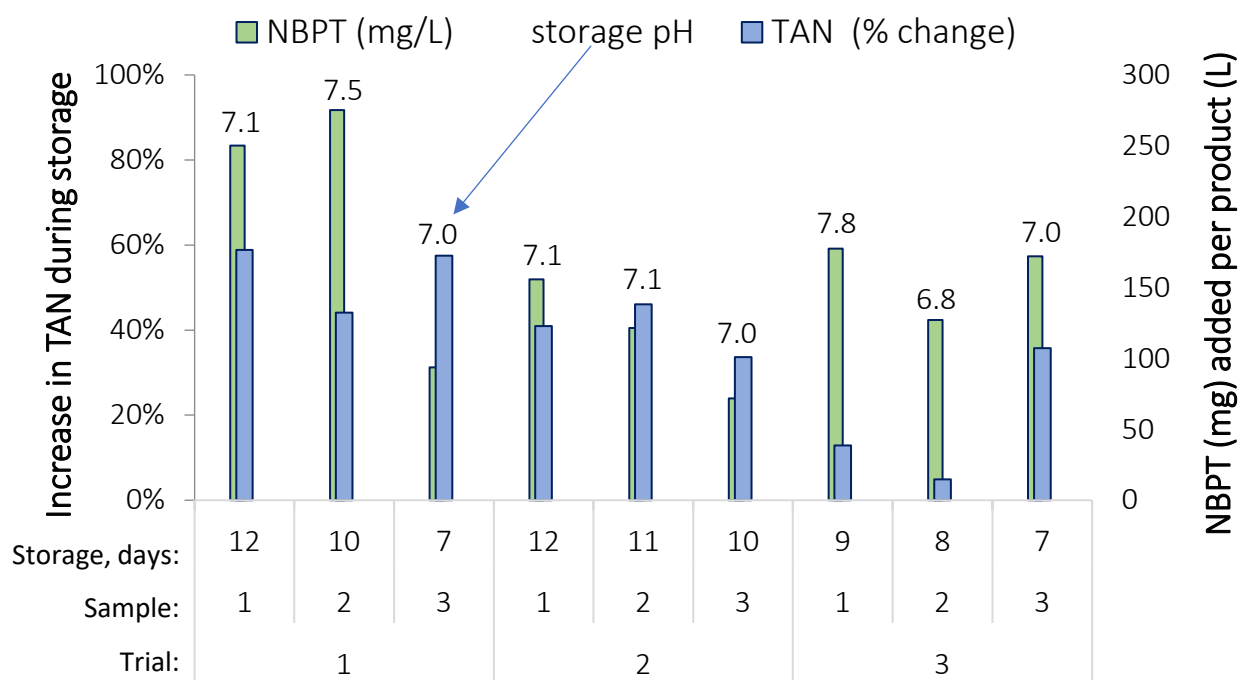


Figure 2.10: The change in TAN as a % of TKN after one week at room temperature for different concentrations of NBPT in untreated wastewater. Error bars = 1 standard error from mean.

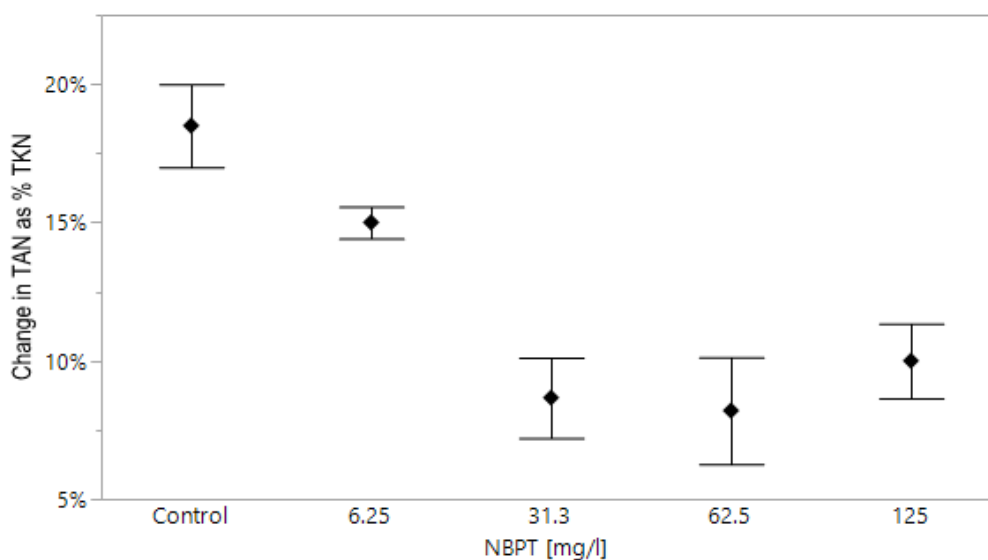


Figure 2.11: Comparison of change in wastewater TAN and storage pH with dose of NBPT added during 3 pilot study trials.

3.5.2. pH > 12

Product tank samples taken during the high pH trials indicated relatively little additional ammonia production over time (Figure 2.12). Reduction in total nitrogen (TKN) across all samples after storage indicates volatilization occurred. Because the nitrogen is lost as ammonia, this suggests that the storage TAN would be higher had volatilization not occurred. Therefore, while it appears that urea hydrolysis is inhibited at high pH, some ammonia formation did occur and was subsequently lost during storage.

The high pH trials were scheduled to run one week each but both ended prematurely (3 and 4 days respectively) when the NaOH solution in the dosing tank ran out earlier than expected. This was likely due to mineral precipitation that had previously been observed in the lab tests with swine urine. Because those were batch tests, there was little apparent effect on pH. However, in the continuous system as cations consumed hydroxide and precipitated this lowered the alkalinity of the wastewater solution. Without this buffering capacity the dosing pump struggled to maintain the 12.5 pH set point. This, combined with the tendency of

ammonia to volatilize at pH values above 9.2 suggests that high pH treatment is not a practical method of urease inhibition for manure wastewater solutions.

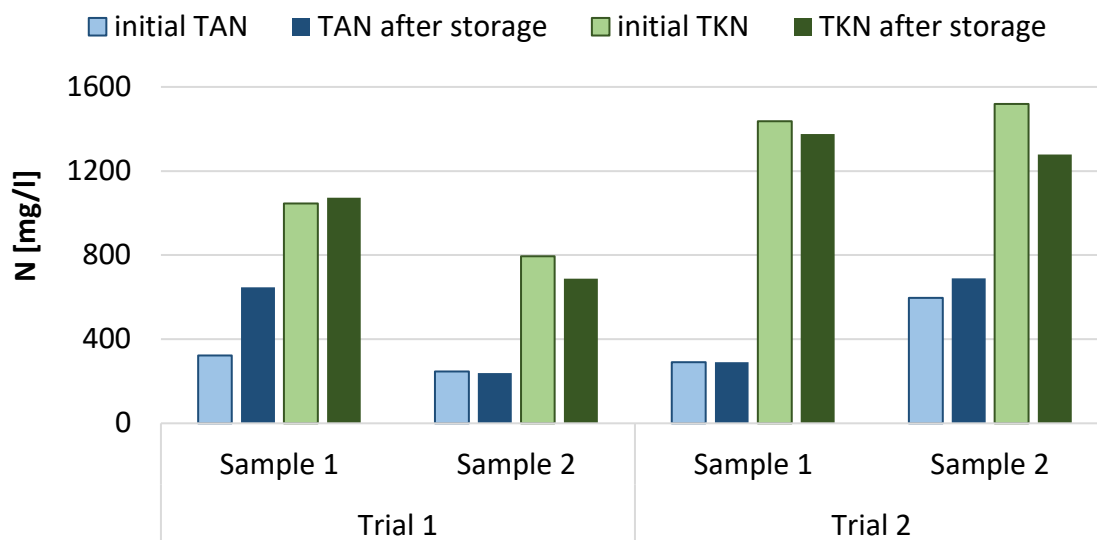


Figure 2.12: Difference in TAN and TKN of high pH samples from product tank of the pilot study. TKN generally decreases during storage due to ammonia volatilization.

3.5.3 pH < 3

Four low pH trials were conducted during the study period, with samples from three trials analyzed after a storage period of at least seven days. Treatment was applied by the dosing pump to the recirculation system with a target pH of 2.5 using 36 M (95%) H₂SO₄. The first trial was ended by a power outage that stopped the recirculation system. The following three trials were conducted over 6, 7 and 6 days, respectively. Samples were collected from both the recirculation system and the product tank every 24 or 48 hours during each trial and analyzed for TKN and TAN. Using one-way ANOVA, Figure 2.13 shows no statistically significant change in either ammonia ($p=0.79$) or TKN ($p=0.95$) between collection and storage analysis of samples from the product tank taken during the low pH tests (no storage samples were submitted for trial 4). Despite differences in total nitrogen and ammonia formation due to pig growth, water consumption and urine exposure to fecal urease, TAN as a percentage of TKN ranged from 24% to 53% with an average of 34%.

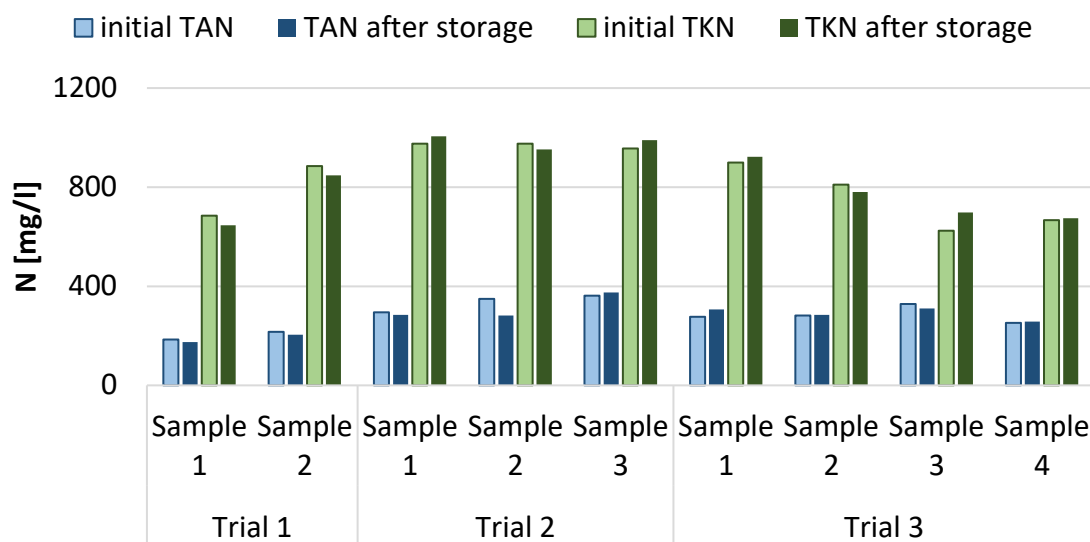


Figure 2.13: Difference in TAN and TKN of samples taken from product tank during 3 low pH trials in pilot study.

3.5.4. Neutralization of acidified samples

Four samples from the first low pH trial (2 from the recirculation system and 2 from the product tank) were neutralized to approximately pH 7 and allowed to sit at room temperature for one week before being submitted for lab analysis of TKN and TAN. In each case ammonia increased in the neutralized samples, but total nitrogen decreased (Figure 2.14). At pH greater than about 6.5 enough ammonia exists in the gaseous NH_3 form to exhibit measurable losses by volatilization. It is likely that the decrease in TKN is the result of ammonia volatilization. Bhowmick and Jagannadham (2006) have shown that while urease enzyme may be partially inhibited by protein unfolding at low pH, it can still retain activity to pH 4. They also show that acid-induced unfolding in jack bean urease is reversible in the presence of anions such as Cl^- , SO_4^{2-} and PO_4^{3-} . These anions are present in swine urine. The results from this experiment suggest that the enzyme is not completely denatured at low pH and may resume activity similar to its native state when pH is neutralized. This test was not repeated with the high pH samples because it seems unlikely based on pilot-scale tests that this is a feasible technique for urease inhibition in practice. Solutions were not tested for microbial activity.

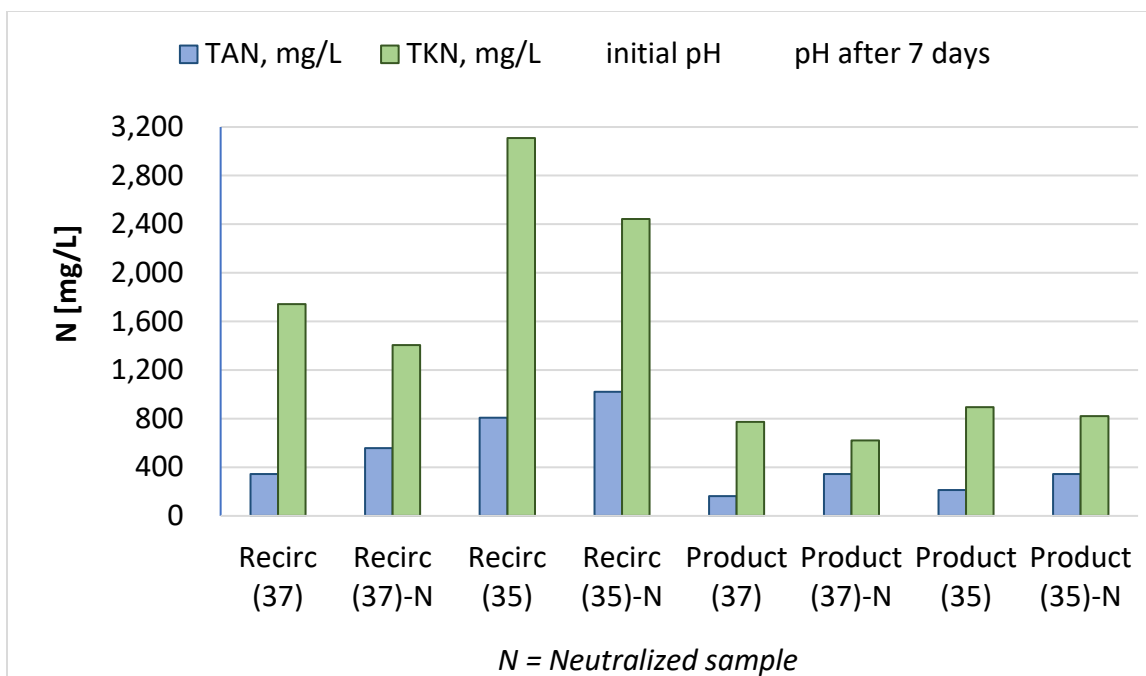


Figure 2.14: Comparison of TAN and TKN in stored samples from low pH trials before and after neutralization to pH 7.

4. Conclusion

Acidification of swine wastewater to a pH < 3 at the time of liquid collection was the most practical and effective urease inhibitor tested in this study, significantly reducing the rate of ammonia production by preventing urea hydrolysis by urease enzyme. Although time to inhibitor application impacted the amount of initial TAN formation from fecal urease contact with urea, the low pH still prevented volatilization of any ammonia that was formed during the collection process. Advantages to using acidification to inhibit urease include the ease of application using a pH sensor and dosing pump. Additionally, the low pH appears to limit microbial activity which would aid in preservation over time by preventing the mineralization of organic nitrogen in solution. One challenge to using this inhibition method in a full-scale system is the corrosiveness of acid and low pH on equipment and concrete, as well as the danger of handling concentrated acid solutions. Although only H₂SO₄ was tested in this study, it might be worth looking at other acids for effect on urease based on availability and dosing.

While increasing pH > 12 did provide good inhibition of urease, in the pilot study this method ultimately proved impractical in the recirculation system as it was designed. Perhaps if

accommodation was made for the mineral precipitation that occurred, this method might be effective at not only preserving urea but also recovering other nutrients such as phosphorus.

The availability of NBPT as a common agricultural chemical for soil urease inhibition makes it an appealing option, even though the inhibitory effects were somewhat less than the pH adjustments. This method may still be valuable for short-term storage of a few days, particularly if dosing could be automated in some fashion based on volume accumulation.

The main issue that follows with any method used is how to recover the urea from solution. Despite the conservation of nitrogen by urea preservation demonstrated in this study, the final product is still too dilute for long-term storage or transportation. Volume reduction remains a major challenge. Whether this occurs by removing the nutrient from solution, or by removing water to concentrate nutrients depends upon both the end product desired and the economic viability of the process. Regardless, the indirect social and environmental benefits of preventing ammonia pollution must be considered in addition to the direct financial costs and product added value if the true worth of nutrient recovery from livestock manure is to be realized.

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CHAPTER 3: Ammonium Ion Selective Electrode Response in Swine Wastewater and Urine Solutions

1. Introduction

Rising awareness of environmental effects of excess reactive nitrogen from concentrated livestock operations is driving research to find ways to reduce manure ammonia emissions (Hou et al., 2015; Panetta et al, 2005; Petersen et al., 2016). The majority of these emissions are caused by the hydrolysis of urea in liquid manures by urease enzyme produced by fecal bacteria. Addition of antimicrobial agents (Varel and Wells, 2007), acidification of liquid manures (Fangueiro et al., 2015; Hjorth et al., 2015) and urease inhibition (Hagenkamp-Korth et al., 2015; Varel, 1997) are several options for mitigating ammonia emissions during the manure management process.

Quantifying the effectiveness of these efforts to reduce ammonia volatilization necessitates the measurement of ammonia concentrations in manure. Standard Methods (APHA, 2012) lists several options under Method 4500-NH₃ for measuring ammonia. Ammonia concentration and potential interferences affect the choice of method used, but generally these methods are time and labor intensive, requiring some measure of sample preparation or preservation, access to specialized laboratory equipment, and transport of samples to a lab for analysis. The methodologies often have low limits of detection (< 5 mg/L), require distillation or dilution and the addition of potentially hazardous reagents, titrants and indicator chemicals to perform, and are therefore destructive to the sample.

Only the ammonia selective electrode method (APHA, 2012) can be used for concentrations up to 1,400 mg NH₃-N/L without dilution. This method still requires the addition of strong base to pH > 11 so that all ammonia will be in the unionized form. The NH₃ then diffuses across the gas permeable membrane of the sensor for a potentiometric measurement. Sensors must be calibrated in standards that have the same temperature and level of dissolved species to compensate for potential interferences. Dilution is only necessary to bring the NH₃-N concentration within the linear range of a particular sensor's calibration curve. An electrode sensor offers a significant advantage both due to its high detection range when measuring the

potentially large change in ammonia-nitrogen during the hydrolysis of urea by urease enzyme to calculate the concentration of urea in a solution (aka the urease method), as well as its ability to be used *in-situ*.

An alternative to the ammonia selective electrode is an ammonium ion selective electrode (AISE). Because the AISE measures the concentration of ionized ammonia (ammonium) in solution it can typically operate across a wider pH range than the ammonia-gas sensor, from a pH as low as 0 to as high as 10. The log of the acid dissociation constant of ammonia (pKa) is 9.24 at 25°C. At this temperature and equivalent pH, half of the ammonia in solution exists in the ionized ammonium form and the other half as dissolved ammonia gas. Although pKa varies with solution temperature, if the ammonium concentration and pH are known, total ammonia for the solution can be calculated. Less than 5% of ammonia exists in the gaseous form at pH values below 6 at 25°C, so this calculation step may be negligible. Also, using an AISE may reduce the processing required for sample analysis, thereby reducing potential error introduced by handling, storage, chemical application, filtration, distillation and dilution.

Ion selective electrodes are well-studied analytical devices and their advantages and limitations are generally known, particularly those that use liquid-junction PVC membranes. The membrane is impregnated with an ion-transporting substance, or ionophore. The ionophore most often used in AISEs is nonactin, and it is not singularly selective for ammonium but merely preferential, with a tendency to bind to a lesser degree with other “interfering” ions. The amount of interference each of these ions exerts can be described by an empirically determined selectivity coefficient (SC). However, per Pretsch (2007), once SCs have been determined for these relevant ions, a fully predictable response to any mixture containing those ions may be expected. This suggests that, with appropriate corrective measures, an AISE may be a useful tool for measuring ammonium in manure solutions. The AISE has several advantages over standard laboratory methods, including low cost, portability, rapid and generally non-destructive sample analysis, and generally no special sample preparation required within the detection range of the instrument. However, in addition to potential interfering ions, other factors may affect the accuracy of these instruments, including sample ionic strength, pH and temperature, as well as instrument calibration status and age. There are many manufacturers and types of AISEs

available, therefore to achieve the best results possible the specific features of the particular instrument must be recognized and understood.

Swine manure solutions are highly variable, and their composition depends on factors including animal growth stage, feed composition, manure collection, dilution with drinking or wash water, treatment, and storage time. Swine manure solutions include pure urine, whole slurry with or without dilution, mechanically separated liquids, anaerobic digester effluent, and anaerobic lagoon supernatant. Analyzing the characteristics of these solutions through literature and lab analysis (Table 3.1) illustrates the variability that might affect the use of an AISE for ammonium and urea analysis.

Table 3.1: Swine Manure Characteristics of Different Sources

Livestock Source	Parameter <i>All values in mg/L (mean ± std. dev)</i>					
	TKN-N	TAN-N	K	Ca	Mg	Na
finisher swine ^[a]	1,470 ± 40	366 ± 12	880 ± 30	200 ± 10	74 ± 4	220 ± 10
sow urine ^[b]	3,450 ± 130	161 ± 9	1,880 ± 10	95 ± 4	100 ± 1	950 ± 4
sow urine ^[b]	3,590 ± 20	148 ± 3	2,090 ± 40	150 ± 4	130 ± 2	750 ± 20
swine fresh manure ^[c]	5,700 ± 2,000	3,600 ± 1,100	3,600 ± 1,600	3,400 ± 1,400	800 ± 170	800 ± 630
swine liquid slurry ^[d]	3,170 ± 1,790	2,010 ± 310	1,820 ± 1,050	940 ± 740	330 ± 270	350 ± 220
swine lagoon liquid ^[e]	560 ± 270	460 ± 30	480 ± 260	120 ± 60	40 ± 30	170 ± 90

[a] wastewater collected from a previous study by authors using a solid-liquid separating scraper system

[b] 2 separate collections (different groups) from lactating sows in farrowing crates

[c] feces and urine as voided, values based on unit conversions of original data (Barker, 1994; Barker, 2007)

[d] 3-6 mos. accumulation of manure, urine, excess water usage, storage surface rainfall surplus (Barker, 1994)

[e] manure, excess water usage, average annual lagoon surface rainfall surplus (Barker, 1994)

Some assumptions must be made for the development of an AISE methodology for manure solutions. For example, while it is important to establish the accuracy of an AISE relative to standard lab analysis, determining the effectiveness of nitrogen conservation measures means being able to accurately detect a change in ammonium concentration. Therefore, precision and repeatability may be more attainable goals if the effect of interferents can be accommodated by an empirically derived correction or calibration technique. Also, while it may prove unnecessary or even impossible to determine the exact individual effects of interferences such as potassium

due to the overall ionic strength of a solution (Rundle, 2011), it may be more practical to determine whether there is a consistent cumulative effect of interferents. Finally, due to the variability in solutions it will still be necessary to determine by a standard method the general relationships of ion concentrations in a solution. However, once this is established for a particular source, an AISE method could be a useful alternative to lab analysis for observing changes in TAN concentrations.

The hypothesis for the development of an AISE method for liquid manure is that an AISE can be used to reliably and repeatedly measure the ammonium concentration in manure solutions. This study will look at the response of an AISE in two types of solution: swine wastewater preserved by a low pH treatment and sow urine. Additionally, changes in response due to dilution or the addition of ammonium will be examined for their effect on AISE accuracy relative to standard spectrophotometric analyses. The goal is to determine how ionic strength, the ratio of interfering potassium ion to ammonium, or possible interactions between the two impact the accuracy of an AISE relative to spectrophotometric analysis. Future study may then explore how this data can be used for the development of a methodology to compensate for interferences when using an AISE sensor.

2. Materials & Methods

2.1. Materials

A Professional Plus meter with a 1-meter Quatro cable multiparameter sonde (YSI, Inc., Yellow Springs, OH) was used for the analyses. The bulkhead of the Quatro cable was fitted with a conductivity-temperature sensor (YSI #005560), a pH ISE sensor (YSI #605101) and an ammonium ISE sensor (YSI #605104). This type of multi-sensor sonde is designed primarily for field surface water and wastewater applications such as streams, aquaculture or treatment plants. The conductivity sensor is a 4-electrode cell with a range 0-200 mS/cm and accuracy $\pm 0.5\%$ of reading or 0.001 mS/cm, whichever is greater. The pH sensor is a glass combination electrode with a range 0-14 units and accuracy ± 0.2 units. The ammonium sensor has a PVC membrane with a range 0-200 mg/L, and accuracy $\pm 10\%$ of reading or 2 mg/L, whichever is greater. All sensors were calibrated to manufacturer's standards daily prior to use.

Lab analyses were conducted by the North Carolina Department of Agriculture and Consumer Services agronomic services laboratory in Raleigh, NC (hereafter referred as “lab”). Phosphorus, sulfur and metals were determined using Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) after closed vessel nitric acid digestion in a microwave digestion system. Total nitrogen concentration (TKN) was determined by oxygen combustion gas chromatography with an elemental analyzer. Ammonia-N is determined by modified Berthelot reaction with an autoflow spectrophotometric analyzer (NCDA, 2018).

Urease enzyme (U1500) with an activity of 38,000 U/g was purchased from Sigma-Aldrich (St. Louis, MO). One unit of enzyme will hydrolyze 1 μmol of urea in 1 minute. All other chemicals used were reagent-grade and purchased through Fisher Scientific Co., LLC (Pittsburg, PA). Urine was collected from lactating sows in farrowing crates using clean buckets, then mixed in a 20-L carboy and dispensed into 500-mL polypropylene bottles before freezing within 2 hours of collection. These bottles were thawed in 4°C refrigeration a minimum of 48-hours prior to use. Samples were removed from refrigeration and allowed to reach room temperature on the day of use. Statistical analysis was performed using JMP software, version Pro 13.2.1 (SAS Institute Inc., Cary, NC).

2.2. Methods

One bottle of urine was thawed and mixed by inversion, then divided into three sample containers and submitted untreated but under refrigeration to the lab for analysis and processed within 48 hours.

The following tests were conducted to compare ammonium ion selective electrode (AISE) response with lab analysis to determine the effect and interaction of ionic strength and potassium ions in the solutions on the AISE’s accuracy.

2.2.1. PRAB sample analysis

Swine wastewater solution treated to $\text{pH} < 3$ from the final trial of a pilot scale urea preservation study was shipped to PRAB (Kalamazoo, MI), a manufacturer of pressure distillation units, for test processing. Prior to shipment a 500 mL sample of this liquid was collected and

refrigerated. PRAB returned 2.9 mL of concentrated wastewater and 100 mL of distillate from their test process for analysis.

During the preparation of these PRAB samples for independent lab analysis to determine the composition, TAN and urea concentrations, the opportunity was taken to test an AISE response in the samples before and after urea hydrolysis.

Urease enzyme with an activity of 38 U/mg was prepared at a concentration of 8 U/mL in a 400 mM phosphate buffer solution titrated to pH 7.0. A portion of the 500 mL wastewater sample taken from the original feedstock had an estimated combined organic nitrogen and ammonia concentration (TKN) of 1,400 mg/L based on previous analysis at the time of collection. The solution was diluted 10x with deionized (DI) water to bring it within the 0-200 mg/L NH₄-N range of the AISE. This initial dilution was measured with the Pro Plus sensors and temperature, pH, ammonium, ammonia, conductivity and total dissolved solids (TDS) were recorded. A second 10x dilution was performed using the urease buffer solution to hydrolyze the urea in the wastewater. Measurements from the Pro Plus sensors in the hydrolyzed dilution were recorded. The samples were acidified to pH < 2 and remeasured with the sensors prior submission to the lab for lab analysis.

The experiment was repeated with the wastewater concentrate. However, due to the high concentration and extremely low volume of the sample (2.9 mL), the concentrate was diluted in several steps. The initial 13.8x dilution was made with DI water to 40 mL. From the 40 mL, 25 mL was further diluted to 44.1x with DI water to 80 mL and measurements recorded. The remaining 15 mL was diluted to 55.2x with urease phosphate buffer solution to 60 mL. The AISE response for this dilution was outside of the sensor's range so a second dilution to 92.4x was performed, adding DI water to 100.5 mL and measurement were recorded. The hydrolyzed portion of the concentrate was then acidified with concentrated H₂SO₄ to a final dilution of 97.8x and measurements recorded again prior to submission to the lab analysis.

To maintain the same overall concentration before and after hydrolysis, half of the distillate was diluted 1.5x with DI water and measurements recorded with the Pro Plus sensors. The second half of the distillate was diluted 1.5x with urease phosphate buffer solution and after hydrolysis measurements were taken with the Pro Plus sensors. All samples were titrated with

concentrated sulfuric acid (H_2SO_4) to $\text{pH} < 2$ for preservation prior to submission to the lab analysis.

2.2.2. Effect of pH on AISE response in urea solution

All measurements were logged at 15-second intervals by the YSI Pro Plus meter, and by hand at noted intervals during titration. A burette was filled with 1N H_2SO_4 and 100 mL of 100 mg/L calibration standard was placed in a 150-mL beaker to be titrated. Measurements of temperature, pH, ammonium concentration, ammonia concentration and conductivity were recorded. The standard was then titrated down to pH 2 in increments of approximately 0.5 to 1 pH units at a time, stirring gently with sensors to mix. The volume of acid and sensor readings were recorded between each titration.

After titration with acid, the ammonium sensor calibration was checked, and the sensor recalibrated. A burette was then filled with 0.5N NaOH and the initial level recorded. The titration was repeated to pH 11 with volume of base added and sensor measurements recorded at intervals of approximately one pH unit. Ammonium sensor calibration was again checked at the end of the titration. Two replications of this experiment were conducted.

2.2.3. Effect of stirring vs. not stirring on AISE response in swine urine

An experiment was conducted to determine the effect of constant stirring vs. static flow on the AISE sensor performance. Sow urine was diluted to ensure the ammonium reading would be within the 0-200 mg/L range of the sensor. Approximately 150 mL of diluted urine, 100 mg/L ammonium standard and 1 mg/L ammonium standard were poured into separate 250-mL beakers. A stir bar was added to each beaker. The data logger was started, and the sensors were placed in a solution for 10 minutes, either stirring or not stirred based on a randomly generated treatment assignment. The assignment included two repetitions of either stirring or not stirring for each solution for a total of 12 samples per test. The sensors were rinsed and blotted dry between samples. Two replications of the experiment were conducted.

2.2.4. Ionic strength vs. interfering ion ratios in swine urine

An experiment was conducted to observe the effects and interactions of solution ionic strength and different potassium to ammonium-nitrogen ratios in sow urine. Lab analysis

indicated concentrations of approximately 160 mg/L TAN and 1,877 mg/L potassium in a sample of sow urine for a potassium to TAN ratio of 11.6. Urine (200 mL) was pipetted into a 250-mL beaker. Three dilutions (5x, 20x, and 100x) of urine were made by pipetting 40 mL, 10 mL, or 2 mL of urine into 200 mL volumetric flasks and making up with deionized (DI) water. These dilutions were each poured into 250-mL beakers. Fifty mL of a 117.6 g/L solution of ammonium chloride (NH₄Cl) was mixed to use as a concentrated source of ammonium for spiking samples. A 100 mg/L ammonium standard was used for calibration checks.

The sensors were rinsed and blotted dry between all samples and calibration checks. The sensors were placed in each solution, stirred gently and then allowed to sit undisturbed for five minutes while AISE stabilized. Measurements of temperature, conductivity, pH, ammonium and ammonia were recorded in 15 second intervals by data logger and by hand at the end of the five minutes. The solutions were spiked with the concentrated NH₄Cl according to Table 3.2. A calibration check was performed between the dilutions. Three replications of the experiment were conducted. The undiluted urine was not spiked because this would have exceeded the AISE's maximum range of 200 mg/L NH₄-N. A second spike was not added to the 5x dilution because this also would have exceeded the AISE's range.

Table 3.2: Volume of concentrated NH₄Cl added to change the ratio of K:NH₄

	Dilution:	1x	5x	20x	100x
K:NH ₄ Ratio	11.6		<i>initial ratio--no spike</i>		
	2.0	.	1 mL	249 μL	50 μL
	0.5	.	.	908 μL	180 μL

3. Results & Discussion

3.1. PRAB sample analysis

The accuracy range for the YSI Pro Plus ammonium sensor is 0-200 mg/L ± the greater of 10% or 2 mg/L. Due to a high TAN concentration (1,470 mg/L), a swine wastewater was diluted 10x to bring the TAN concentration within the range of the YSI Pro Plus AISE. Two primary interferences with AISE readings are high ionic strength which suppresses an AISE response and high ratios of free interfering ions to ammonium in solution, which increases response, or apparent

concentration of ammonium. Lab values were all evaluated after acidification, so these values were used to compare to both the acidified and non-acidified post-hydrolysis measurements taken by the AISE.

A comparison of TAN as measured by the AISE and lab analysis in Figure 3.1 indicates that the sensor's measurements averaged within 5% of lab values for the 10x diluted wastewater before and after urea hydrolysis. Also, the response initially averaged 5% lower than the lab analysis but was 5% higher after hydrolysis, possibly due to the 300-fold increase in Na^+ from the sodium phosphate in the urease buffer solution. However, after the samples were acidified for preservation, the average difference rose to 13%. This indicates that H^+ may exert a similar influence on other interfering ions, although the increase in ionic strength as measured by conductivity was very large and thus a decreased response would have been expected. All of the TAN values for the concentrate exceeded the AISE's upper limit of 200 mg/L and were 33% to 44% below lab values. An ISE response curve tapers past its upper detection limit due to membrane saturation (Rundle, 2000), therefore this response is expected.

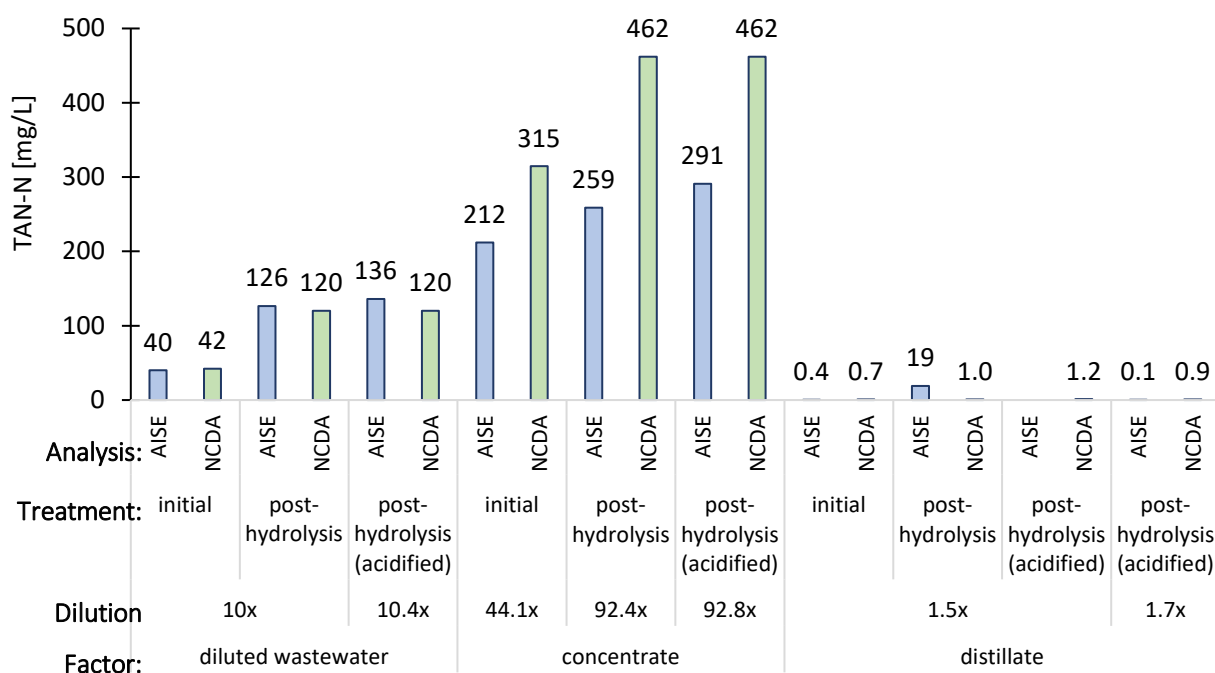


Figure 3.1: Comparison of AISE and lab analysis of different concentrations of low pH-treated wastewater before and after urea hydrolysis by urease enzyme addition.

The measurements of the very low concentrations in the distillate were also lower than lab values though still within the ± 2 mg/L accuracy range of the AISE. An exception is for the post hydrolysis unacidified measurement of distillate which was an order of magnitude higher than the corresponding lab value. This seems to indicate that at low ammonium concentrations the sensor is particularly sensitive to interferences from both ionic strength and interfering ions.

Table 3.3 lists the average ratio of interfering ions to ammonium in the PRAB sample solutions, based on lab analysis. The potassium concentration is 3x higher than ammonium and therefore should have caused roughly a 30% increase in the AISE response. Yet an increased response was not seen in any of the initial (non-hydrolyzed) solutions measured. Rather, the AISE value was lower than the lab analysis for each, suggesting ionic strength was exerting a greater overall influence. Although the ratio of K:NH₄-N should have shifted in favor of ammonium after urea hydrolysis, the 10x wastewater dilution and the distillate both saw increases in the AISE response relative to the lab analysis. This was probably due to the potassium-phosphate buffer used in preparing the urease enzyme and indeed Table 3.3 indicates an increase in the post hydrolysis ratios for the 10x and distillate solutions. Thus, a different method of urease preparation may be desirable in future work.

Table 3.3: Ratio of ammonium to interfering ions in the PRAB sample solutions

<i>SELECTIVITY COEFFICIENT^[1]:</i>		0.1	0.00006	0.0002	0.002
SOURCE	Treatment	K:NH ₄ -N	Ca:NH ₄ -N	Mg:NH ₄ -N	Na:NH ₄ -N
10X WW	initial	3.0	0.7	0.3	0.9
10X WW	hydrolyzed	5.6	0.2	0.1	83.6
CONCENTRATE	initial	1.8	0.2	0.2	0.9
CONCENTRATE	hydrolyzed	1.2	0.1	0.1	14.0
DISTILLATE	initial	15	8	2	115.7
DISTILLATE	hydrolyzed	314	4	1	6371.5

[1] Source: "Technical Specifications for the Ammonium Ion-Selective Electrode ELIT 8051"
http://www.nico2000.net/ise_specs/ammonium.pdf. Selectivity coefficients are presented for comparison only and are not necessarily accurate for the solutions tested.

The average pH of the solutions in Figure 1 were: pH 3 for the initial 10x wastewater dilution, pH 6.8 for post-hydrolysis solutions and pH < 3 for acidified solutions. Although the pH range for

the Pro Plus AISE is pH 4-10, it is not clear how pH below 4 or above 10 impacts the AISE response, and whether this influenced the sensor's readings. Therefore, further investigation into the effect of pH on the AISE response was conducted.

3.2. Effect of pH on AISE response in urea solution

A 100 mg/L standard solution was titrated through a range of pH levels between 2 and 11 to determine the effect of pH on the AISE's accuracy. Because the Pro Plus meter calculates both ammonium and ammonia concentrations in solution, these two readings were added for a total ammoniacal nitrogen (TAN) concentration. The TAN concentration was then adjusted for the volume added by the titration, standardizing the final measurement to the initial concentration of 100 mg/L.

Figure 3.2 illustrates that sensor readings, though decreasing, remained within the range of accuracy from the initial pH 5.9 down to pH 4. Below pH 4 the readings became more variable until near pH 3 when they consistently fell below lower limit. This agrees with YSI's pH range of 4-10 for the Pro Plus ammonium ISE. From pH 4 to 2, the conductivity of the solution quickly increased from 3,000 to 7,000 $\mu\text{S}/\text{cm}$, indicating a large increase in ionic strength which correlates to the suppressive effect that was seen in the AISE's response. Titration in the alkaline direction with a starting point of approximately pH 8 saw this suppressed response continued, and accurate values were not recorded until near the upper limit of the listed range of pH 10. At pH 11 most sensor readings were still within the AISE's accuracy range although this pH exceeds YSI's listed pH range for the sensor.

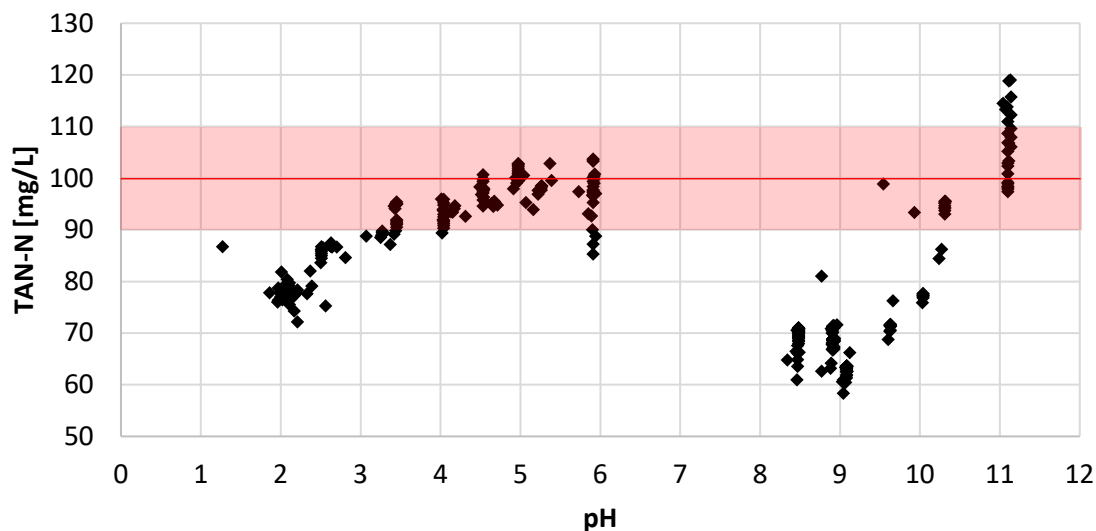


Figure 3.2: Total ammonia concentration in solution titrated from pH 2 to pH 11, adjusted for volume addition. AISE range of accuracy highlighted. Initial standard pH = 5.9.

The time required for the readings to stabilize increased by more than 10 minutes as pH increased, even when placing the sensor in fresh standard after completing the low titration. A second test titrating with base before acid was not performed for comparison. However, an increased response time is an indication that an AISE either requires reconditioning or may be nearing the end of its useful life. This may be an indication that a very high ionic strength has a negative impact on the sensor membrane and therefore the sensor should be evaluated and reconditioned if necessary after exposed to these conditions.

3.3. Effect of stirring vs. not stirring on AISE response in swine urine

Arguments have been made for and against stirring when measuring a solution with ISEs. In general, stirring may decrease response time by decreasing the thickness of the boundary layer between the bulk of a sample solution and a nondiffusive membrane (Morf, 1981). The glass bulb of a pH sensor is an example of a nondiffusive membrane, which is likely why gentle movement is often recommended when measuring pH. However, Morf (1981) notes that with a PVC-membrane such as that used in an AISE, the internal steady-state is attained much more slowly relative to the external equilibration at the membrane surface. Therefore stirring is less likely to have a noticeable impact on response time.

Another argument for stirring is to ensure homogeneity of the solution being measured. However, since an AISE is measuring free ammonium in solution, again this is unlikely to have a significant impact on the sensor's response. Stirring can impact solution temperature however, particularly from the friction generated by a magnetic stir bar. Depending on the solution and ion being measured, this temperature change may be enough to impact measurement if not compensated for.

The YSI Pro Plus sensor array used in these experiments is designed for field use which may include moving streams, aquaculture ponds or waste treatment systems. Many of these scenarios involving moving fluid so a test was conducted to determine if a solution should be allowed to remain static or stirred when measuring $\text{NH}_4\text{-N}$. The results of this test are given in Figure 3.3. A 2-sample students t-test indicates no significant difference between measurements taken in a static or stirred solution by the YSI Pro Plus ammonium sensor ($P = 0.973$).

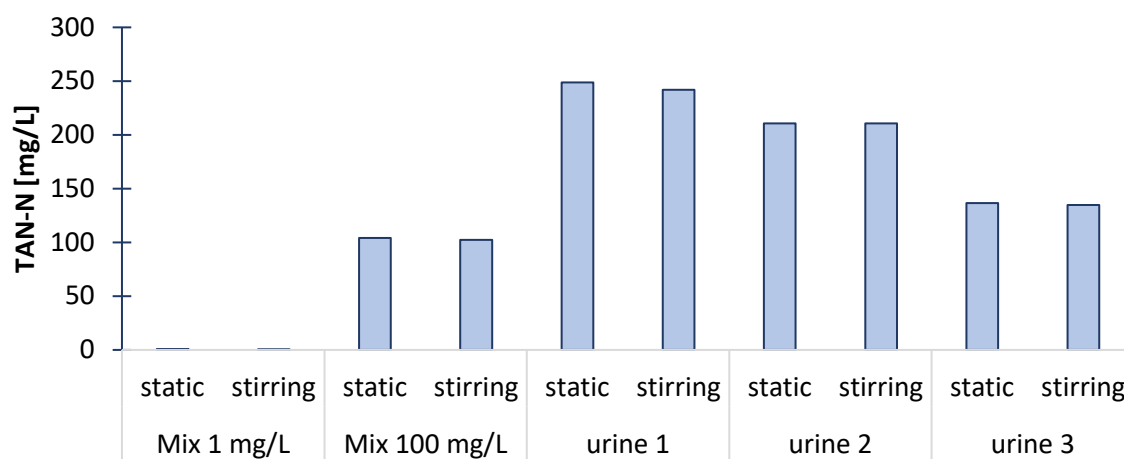


Figure 3.3: Comparison of AISE response to static or stirred treatment of solutions, with the maximum value attained over a 10-minute measurement.

3.4. Ionic strength vs. interfering ion ratios in swine urine

The final experiment compared the effects of ionic strength and ratios of potassium to ammonium in sample solutions. Four dilutions of sow urine (1x, 5x, 20x, and 100x) and three ratios of $\text{K}:\text{NH}_4\text{-N}$ (11.6, 2.0 and 0.5) were tested. As ionic strength of a solution increases, the activity of free ions in solution decreases, therefore high ionic strength should cause a reduction in the AISE response. Dilution decreases ionic strength but does not affect the ratio of interfering

ions to ammonium so the suppressive effect of ionic strength is expected to become less noticeable. Conversely, because interfering ions in sow urine do exist in relatively high ratios (Table 3.1), this causes the AISE to interpret these interferences as additional ammonium and therefor increases the response. Since potassium was the most influential interferent present, samples were spiked with ammonium to reduce the K:NH₄ ratio, which would then be expected to reduce the influence of interfering ions on the sensor. This change would generally not be expected to have an impact on ionic strength except at very high ammonium concentrations.

The Pro Plus meter corrects for ionic strength in solutions with a salinity up to 2 ppt. The only sample tested above this threshold was the undiluted urine, with a salinity of 6.1 ppt. Salinity and specific conductance (*Sp. Cond.*) are both calculated by the meter using the conductivity sensor. To correlate these readings to ionic strength (*I*), the Russell Equation (Eq 1) was used (Russell, 1976). Due to the complexity of urine composition, this equation was only used to provide an estimate and was not expected to provide an exact value for the ionic strength of the samples.

$$I (M) = 1.6 \times 10^{-5} \times Sp. Cond. \left(\frac{\mu S}{cm} \right) - 1.5 \times 10^{-4} \quad (1)$$

To understand how the corrected concentration values provided by the meter compare to the uncompensated potentiometric readings from the AISE, the Nernst Equation (Eq 2) was used to calculate the uncorrected ammonium activity in mg/L. A linear calibration curve was derived for each run of the experiment. The slope *s* and intercept *E*₀ from fitted linear equations were then inserted into the Nernst equation, along with the mV reading from the AISE as *E*. From this the assumed activity *a* of ammonium in the solution could be derived.

$$E = E_0 + s \log(a) \quad (2)$$

Under ideal conditions (i.e., standard temperature and pressure), ammonium activity in a pure solution would equal the concentration. In real world applications, conditions are rarely ideal, and if there are interfering ions present their influence and activity must also be taken into account. The amount of influence the interferent *j* exerts when measuring an analyte *i* is expressed by a selectivity coefficient, *K*_{ij}^{pot}. For example, a *K*_{ij}^{pot} of 0.1 will increase response 10% when equal concentrations of interferent and analyte are present. The Nicolsky equation (Eq. 3)

includes these effects, where a_i is the activity of the ion of interest, to which is added the product of the interfering ion activities a_j , their charge numbers z , and their selectivity coefficients K_{ij}^{pot} . This equation indicates that the effects are additive for all interferents in solution.

$$E = E_0 + s \log \left(a_i + \sum K_{ij}^{pot} (a_j)^{\frac{z_i}{z_j}} \right) \quad (3)$$

To compensate for ionic strength in the Nernst equation values, activity coefficients (γ) were calculated based on ionic strength using the Davies equation (Eq. 4). The activity calculated by the Nernst equation was then divided by this activity coefficient to yield an adjusted concentration.

$$\log_{10}(\gamma) = -0.51z^2 \left\{ \frac{I^{\frac{1}{2}}}{1 + I^{\frac{1}{2}}} - 0.3I \right\} \text{ for } I < 0.5 \text{ M} \quad (4)$$

Neither the calculated (Nernst, Davies) nor the measured (AISE) ammonium concentrations have a correction for potassium interference. Rundle (2011) suggested a selectivity coefficient of 0.1 or 10% for potassium when measuring ammonium, so this was applied to the Davies calculation (Davies w/SC). By comparing these four different methods of analysis (AISE, Nernst, Davies, and Davies w/SC) to the lab values (Figure 3.4), the complexity of the interplay between ionic strength and interfering ions on an AISE became apparent. While the AISE meter had the best overall accuracy, each of the calculations displayed differing strengths depending on the solution parameters. For example, concentration as calculated using the uncorrected Nernst equation was most accurate at the 20x dilution ($I = 0.039 \text{ M}$) with the lowest K:NH₄-N ratio of 0.5, showing no statistical difference from the lab value in a student's t-test. On the other hand, the Davies w/SC method had the best accuracy at the 5x dilution with a K:NH₄-N ratio of 2.0, again showing no statistical difference. This solution had both a higher ionic strength (0.063 M) and interfering ion ratio, both of which are corrected for with the Davies w/SC. Only the AISE showed no statistical difference from lab values in the undiluted (1x) urine which had both the highest ionic strength at 0.171 M (6.35 ppt salinity, 3x the meter's stated limit) and K:NH₄-N ratio of 11.6. Conversely, the AISE was least accurate at the 100x dilution ($I = 0.008$) and lowest K:NH₄-

N ratio of 0.5, while all other calculation methods showed no statistical difference from the lab values, regardless of applied correction.

Overall, it appeared that the AISE meter, which uses proprietary algorithms and input from conductivity, temperature and pH to calculate an ammonium concentration, was fairly accurate within its stated range of accuracy and at moderate (0.03-0.06 M) ionic strength. However, it did seem to overcompensate for ionic strength, yielding concentrations lower than lab values except for those dilutions with very high (11.6) K:NH₄-N ratios.

Like the meter, the Nernst equation—which does not compensate for either ionic strength or interfering ions—showed no statistical difference from lab values for moderate ionic strength and potassium ratios. While least accurate at high ionic strength and potassium ratios, the uncorrected Nernst values were not statistically different from lab values at the highest dilution (100x) and lower (0.5 and 2) potassium ratios. When ionic strength only was compensated for (Davies), values were consistently higher than lab values for all but the lowest concentration (100x) and lower (0.5 and 2) potassium ratios, which were not statistically different. This is expected because the upward influence of interfering ions on AISE response remains. Therefore, a potassium selectivity coefficient (K_j^{pot}) of 0.1 was selected from literature (Rundle, 2011) and applied using Equation 3. The result is a significant over-reduction of ammonium concentration for the 11.6 ratios. At the 2.0 ratios the ionic strength and potassium corrections seem to balance out with final values similar to lab values which, in some instances, are not statistically different. At the 0.5 ratios however, ionic strength seems to come into play again. When $I = 0.39$, the Davies w/SC values is significantly higher than the lab value, but when $I = 0.008$ there is no statistical difference.

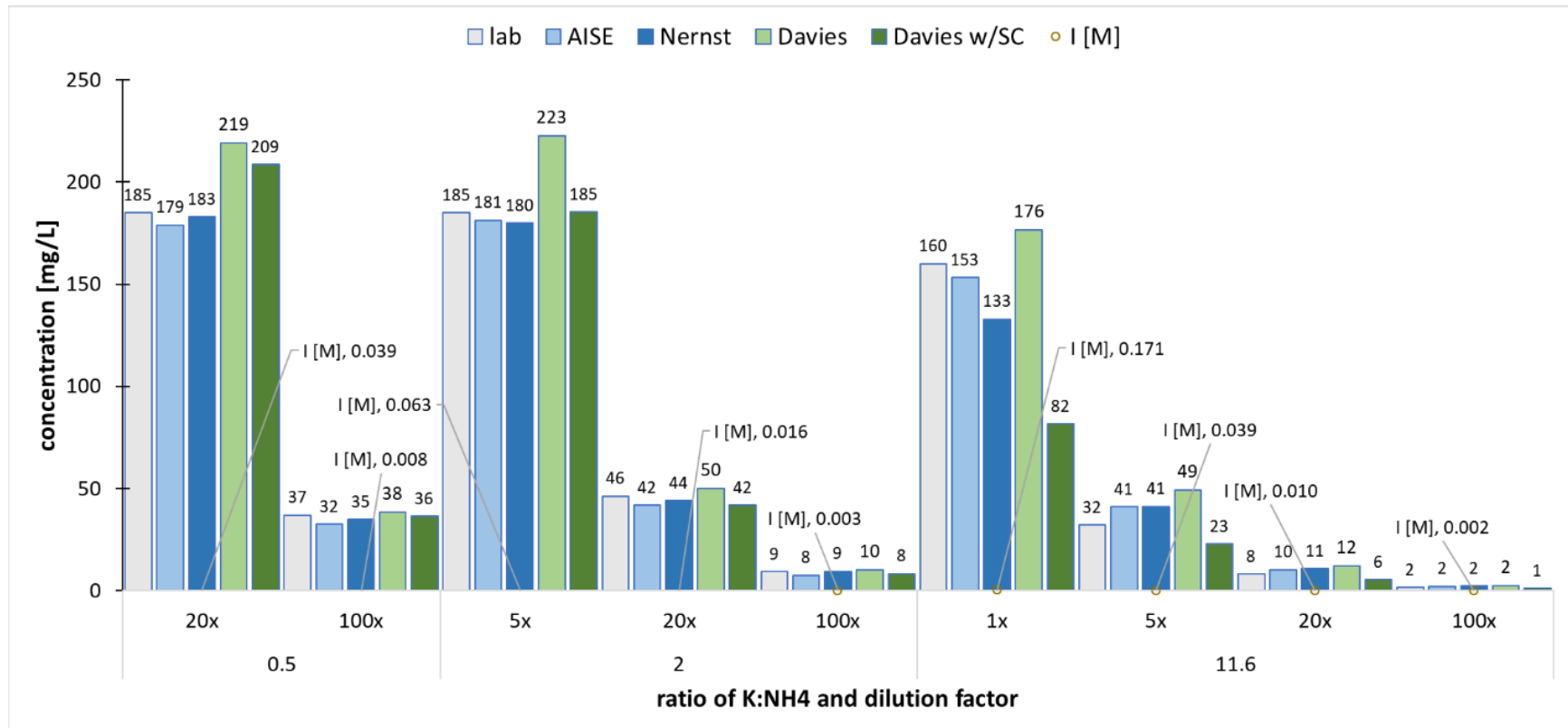


Figure 3.4: Comparison of NH₄-N from lab analysis to concentrations as calculated by AISE meter, by Nernst equation using AISE mV output, and by Nernst equation using Davies activity correction for ionic strength, without and with additional correction for potassium interference with a selectivity coefficient of 0.1. Values above bars are ammonium concentrations.

To adjust the K:NH₄-N ratios, solutions were spiked with concentrated ammonium chloride. The undiluted urine (1x) was not spiked because additional ammonium would have exceeded the AISE's maximum range. The change in ammonium detected or "recovered" by each method of analysis appears to be influenced by both ionic strength and interfering ions. At the highest ionic strength (0.063 M) and potassium concentration (2.0) the Davies w/SC analysis which compensates for both was the most accurate, averaging within 6.5% of the actual change in concentration. Yet at higher ionic strength (0.039 M) and low potassium concentration (0.5) both the AISE meter and the Nernst equation yielded recovery values very near lab values while the ionic strength corrected methods overcompensated by 20% or more. At very low ionic strength (0.003 M and 0.008 M) all methods were within 10% of the actual change (except for the AISE at the 100x – 2.0 level which averaged 35% lower), with the Davies corrected and Davies w/SC corrected values averaging within about 1% of the actual value for the lowest potassium ratio.

Table 3.4: Recovery of ammonium spikes to sow urine dilutions for analysis based on AISE Pro Plus meter, the Nernst equation calculated using AISE mV readings, the Nernst equation with a correction for ionic strength using an activity coefficient calculated by Davies equation and the Davies corrected values with an additional correction for interfering K⁺ ions using a selectivity coefficient of 0.1; error indicates one standard deviation above or below the mean, n=3.

Dilution / Ratio:	5x – 2.0	20x – 2.0	20x – 0.5	100x – 2.0	100x – 0.5
Ionic strength [M]:	0.063	0.016	0.039	0.003	0.008
Change in NH ₄ -N [mg/L]:	153	38.3	138.7	7.7	27.7
AISE	140 ± 8.3	32 ± 1.4	137 ± 8.6	5 ± 0.3	25 ± 1.0
Nernst	139 ± 11.4	33 ± 1.8	139 ± 6.6	7 ± 2.4	25 ± 2.3
Davies	173 ± 14.3	38 ± 2.1	169 ± 8.6	8 ± 2.5	28 ± 2.5
Davies w/SC	163 ± 12.0	36 ± 1.4	167 ± 8.3	7 ± 2.3	28 ± 2.1

4. Conclusions

This study presents a first step in analyzing how a complex liquid manure such as sow urine affects an AISE response and how that response is interpreted by the method used to estimate ammonium concentration in solution. While the results generally followed the trends expected from literature, the accuracy of the method used to determine the actual concentration of ammonium in solution or to detect a change in ammonium concentration is dependent upon the solution composition and ionic strength. These influences can be altered by dilution and should be investigated further.

Next steps in the development of an AISE methodology include 1) using potassium addition to determine more precisely how potassium interference affects AISE readings in manure solutions, 2) testing calibration techniques such as the addition of ionic strength adjustment buffer and/or potassium spikes in calibration solutions to control for interferences in sample measurement, and 3) testing methods of calibration, sample preparation and concentration calculations for applicability on other manure solutions such as whole slurry, lagoon or digester effluent.

Although any calibration adjustment techniques will raise the lower detection limit of an AISE, this is unlikely to be an issue since manure solutions generally have much higher ammonium concentrations than the surface waters or wastewaters typically measured with these sensors, even when urea hydrolysis is inhibited. However, it should also be noted that the sensor used in this experiment, although specifically designed for field use, has a particularly limited detection range relative to other AISEs available. Additionally, the ongoing development of more selective ionophores and more robust membrane materials will also have an impact on the sensitivity of AISEs and the influence of interferences. Therefore, any methodology developed for measuring ammonium concentration in liquid manure solutions using an AISE should be adjusted to the parameters of the particular sensor used.

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CHAPTER 4: Conclusions and Future Study

Conservation and recovery of nutrients from manure is vital to the sustainability of the livestock industry. Current manure management practices, particularly in the Southeast U.S., focus on disposal rather than treating manure as a useful byproduct of animal husbandry. Such practices lead to social hardships from odor and health impacts, environmental degradation, and major economic losses. These losses include potential energy production from methane recovery, crop fertilizer from conserved plant nutrients, and also the costs associated with ecological remediation, human illness and corresponding legal ramifications. This is a complex and persistent problem that has evolved with the intensification and specialization of the agriculture industry, where traditional multi-species multi-crop farms were consolidated into industrial livestock production operations centralized around meat packing plants.

While this intense concentration exacerbates the negative impacts of manure management practices functioning only as waste disposal plans, it does provide an opportunity to economize energy and nutrient recovery through strategic location of processing facilities, thereby turning waste into a potential commodity. Still, there are barriers to adopting sustainable technologies, not the least of which is the high cost of installation and maintenance. To this end research continues to focus on finding practical and economical ways to make manure management sustainable. Although energy production is beginning to gain traction with installations like the directed biogas facility in Duplin County, NC, this optimization effort has been particularly challenging on the nutrient recovery side.

A major hindrance is the massive volume of manure that is produced. Even with the most efficient livestock species more than half of the feed consumed is excreted. There is often not enough cropland in the immediate vicinity of production to utilize the nutrients in the manure by direct application. When there is enough land, seasonal demands require that manure be stored for months at a time, during which losses still occur through volatilization. These are the situations generally encountered during the handling and management of swine manure.

Therefore, the goal when developing manure nutrient recovery technology is multifold. A system must be able to 1) prevent nutrient loss, 2) recover nutrients in a concentrated, storable and transportable product, and 3) be economically viable at the scale to which it is applied. The

results of the research in this thesis address the first point. We have successfully shown that urea nitrogen can be preserved through the application of urease inhibitors to liquid swine manure. Furthermore, when acidification is the method of inhibition, ammonia nitrogen is also conserved.

With the pilot system we realized however, the challenge involved in treating liquid manure at the point of deposition. The mechanical separation of solids and liquid as a primary step has two advantages: urea exposure to fecal urease is limited and overall volume is reduced by eliminating the need to flush barns. However, separating belts and scrapers are best applied to new construction. A practical and economical retrofit of separating systems to existing structures is still needed.

Recirculating the liquid to maintain a constant level of inhibition worked very well in the pilot study. Although only operated one week at a time for inhibitor trials, the system required little to no attention except for foaming issues which might be managed with anti-foaming agents or agitating the liquid surface. A full-scale manure scraper combined with recirculating treated wastewater would be the follow-up case study, but this appears to be an effective option for reducing manure volume and conserving nitrogen.

The next step is to address the second goal of creating a useful fertilizer product. Even with a solid-liquid separation system and no dilution with flush water, the volume of liquid produced is still too large to manage without further reduction. Analysis of concentrate from an initial test of vacuum distillation indicated this process has potential to reduce liquid manure volume up to 90%. Future study should continue to test the vacuum distillation process with different levels of acidification. The purpose would be to find an optimal combination of nitrogen conservation, preservation by limiting microbial activity, and volume reduction, all relative to the costs associated with system materials, chemical and energy inputs, and process time.

These last considerations of course relate to the final goal of economic viability. Scale is important—right now most systems are considered for use only at the farm level and therefore put the burden on the producer to finance, install, operate, and maintain the system. Here economy of scale could help by expanding across multiple farms by either 1) centralizing product processing or 2) creating a service industry specific to the need. Aside from basic economics of

cost and return, as important to widespread adoption is the risk associated with unproven technology, which results from a lack of full-scale farm studies of these innovations. Without some sort of regulatory incentive or insurance against failure it is very, very difficult to motivate a change of the order required to make a substantial difference. The studies presented in this thesis are small pieces contributing to the puzzle at large that is coming together to build a sustainable future for livestock production.