In-Storage Psychrophilic Anaerobic Digestion of Swine Manure

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DEDICATION

This document is dedicated to my grandfather Dr. Charles Alfred Sankey, whose thesis from 1930 provided inspiration (Sankey, 1930)[1], and whose advice was invaluable.

[1]Sankey C.A. (1930) The mechanism of the action of sulphurous acid on lignin and related compounds., Chemical Engineering, McGill University, Montreal.

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ABSTRACT

If a robust anaerobic digestion process could be shown to develop in a covered manure storage tank then In-Storage Psychrophilic Anaerobic Digestion (ISPAD) could be used as a treatment system for the liquid manure resulting from intensive pork production in the Canadian climate. The most critical issue influencing the success of ISPAD is the acclimation of the microbial community in the manure to the ambient (psychrophilic) conditions. To evaluate the effectiveness of an acclimated anaerobic digestion process, the kinetics of both carbohydrate and protein degradation must be defined, such that the extent of both methane and ammonia production in the system may be determined. Finally, the impact of treatment by ISPAD on the fertilizer value of manure must be assessed.

To address these issues, the composition of manure samples from a full-scale ISPAD installation in Quebec was analyzed in the laboratory, the activity and effectiveness of the microbial community were assessed in batch cultures with various substrates at several temperatures, and finally the extent of ammonia volatilization from the manure was determined in wind tunnel simulations of land application.

The results of these analyses indicated that the manure microbial community in an ISPAD installation did acclimate to its psychrophilic operating conditions. The assessed kinetics suggested that robust co-existing psychrophilic and mesophilic populations degraded carbohydrates, releasing 63 % of the manure's potential methane for use as fuel. In contrast, protein degradation remained mesophilic, though long retention of manure in the system compensated

for the slow reaction rate, resulting in 65 % of manure proteins transformed to plant available ammonia/ammonium nitrogen (TAN), which was conserved in the ISPAD tank. Because ammonia volatilization following land application was only 53 % of that lost by conventionally stored manure, the ISPAD treated manure could supply up to 21 % more plant available TAN.

It is therefore concluded that ISPAD can be an effective treatment system for swine manure in the Canadian climate, releasing methane for use as fuel and conserving nitrogen for use as fertilizer.

ABRÉGÉ

Si le lisier peut développer une population robuste de microbes anaérobies en entreposage sous une couverture étanche, le concept de Digestion-Anaérobie-Psychrophile-En-Stockage (DAPES) peut alors être un traitement efficace et abordable pour les effluents d'élevages intensifs de porcs soumis au climat canadien. L'acclimatation des communautés microbiennes anaérobies aux conditions psychrophiles est donc l'élément essentiel qui détermine le développement du traitement sous le concept de la DAPES. La cinétique microbienne de dégradation des hydrates de carbones et des protéines est un indice du niveau d'acclimatation psychrophile et du potentiel de production du bio-méthane et de la minéralisation de l'azote par la DAPES. Finalement, ces procédés ont un impact direct sur la valeur fertilisante du lisier à la sortie de la DAPES.

Dans le but de mesurer cette acclimatation et ce potentiel de production de biogaz, des lisiers furent obtenus d'un système DAPES installé au Québec depuis trois ans, et ceux-ci furent caractérisés par analyses chimiques. L'activité microbienne de ces échantillons de lisiers DAPES fut aussi mesurée par des essais de culture en séries avec divers substrats et sous trois différentes températures, soit 8, 18 et 35 °C. Finalement, le potentiel de perte d'azote par la volatilisation de l'ammoniac lors des épandages au sol des lisiers DAPES fut aussi mesuré.

Les résultats des ces travaux en laboratoire indiquent que les populations microbiennes anaérobies se sont effectivement acclimatées aux conditions psychrophiles. Les cinétiques microbiennes indiquent la présence de deux

populations robustes, dont une psychrophile et l'autre mésophile, qui peuvent décomposer 63 % des hydrates de carbone. Cependant, la dégradation des protéines demeure mésophile, bien que la longue rétention du lisier dans le système contrebalance le taux moins élevé du processus. Par conséquence, 65 % des protéines sont transformées en azote ammoniacal (AA), conservé dans le réservoir. Suivant l'épandage sur terre agricole, la volatilisation de cet AA équivalait à seulement 53 % du même perdu après épandage d'un lisier extrait d'entreposage conventionnel. En conséquence, les cultures recevraient jusqu'à 21 % plus d'engrais azoté provenant de lisier DAPES.

La recherche conclut alors que le procédé DAPES peut fournir un traitement efficace pour les lisiers de porcs sujets au climat canadien, produisant la méthane pour utilisation combustible, et conservant l'azote pour fertilisation.

Chapter 1. Introduction

1.1 Problem Statement

An increasing number of Canadian watersheds are affected by intensive livestock production; these are dominated by pork and dairy in Ontario and Quebec, and beef in Alberta (Hofmann and Beaulieu, 2006b). Some of the greatest intensities are found in the province of Quebec, where 38 % of total livestock is housed in high-density regions, with more than 70 animal units per square kilometre. This includes 60 % of the province's pigs, of which the province has the largest inventory in the country (Beaulieu and Bedard, 2003). In these watersheds, the storage and subsequent land application of manure is an important non-point source of pollution (Berka et al., 2001). Stored and land-applied manure affect the condition of the soil, water and air in the receiving environment, as well as livestock, wildlife and humans in the region. These effects interact with one another and with local conditions such as climate, soils and topography, making it important to address mitigation in a comprehensive manner (Skinner et al., 1997).

Storage tanks are used to accumulate manure and allow the timing and quantity of land application to be managed (Beegle et al., 2000). However, stored liquid manure undergoes some anaerobic digestion which releases greenhouse gases, primarily methane, to the atmosphere. In the Canadian climate, up to 23 % of the potential methane in the manure may be lost in this way (Park et al., 2006). In addition, up to 60 % of the total ammonia/ammonium nitrogen (TAN) in manure may be volatilized from storage facilities (Muck and Steenhuis, 1982).

When stored manure is applied to cropland, up to 68 % of the remaining TAN can be lost through volatilization (Huijsmans et al., 2003). Volatilized ammonia is transported by wind to be deposited by rain on plants, land and water bodies, which contributes to acidification. Additional processes, such as surface run-off and leaching, carry aqueous forms of nitrogen, along with phosphorus and other contaminants, to ground and surface waters (Hooda et al., 2000).

While some of these impacts may be mitigated by the timing and technique of manure application, treatment processes that either change the manure's composition or separate its components are increasingly recommended as elements of farm-based or regional nutrient management (Martinez et al., 2009). For the liquid manure systems used by high-density pork producers, treatment by anaerobic digestion has shown promise. Anaerobic digestion is a microbial process that occurs naturally in water-based solutions without added oxygen. The anaerobic microbial community consumes organic matter and produces biogas composed primarily of methane and carbon dioxide. This process can be used to reduce the solids content of manure and the gaseous emissions to the atmosphere, while the biogas is captured and used as fuel for associated applications such as heating or electricity generation (Burton and Turner, 2003).

Anaerobic digestion systems have been an integral part of municipal and industrial wastewater treatment systems for over a hundred years, and some efforts to apply the technology at the farm scale have been successful (Goodrich and Schmidt, 2002). However, the technology is not widely used due to the high initial cost of industrially-designed systems and the need for specially-trained

operators to manage the process which can be unstable and unreliable at the agricultural scale (Friman, 1984; Scruton, 2004). With these issues in mind, the concept of In-Storage Psychrophilic Anaerobic Digestion (ISPAD) was developed.

ISPAD begins with an in-ground concrete manure storage tank of the type commonly used in Canada. The selection and use of existing equipment as a vessel for the anaerobic digestion process reduces the cost factor significantly. The tank is then covered with an air-tight insulated polymeric membrane which floats on the manure surface and is fixed around the rim of the tank. Mixing in the tank is provided only by the periodic addition of manure through the existing piping system, and temperature is allowed to fluctuate with the ambient climatic conditions, tempered by the mass of accumulated manure and the insulating properties of the surrounding earth, concrete and cover. Under these conditions in the Canadian climate, the manure temperature would fall into the 5-20 °C range (Cullimore et al., 1985).

Microbial species may be categorized by their preferred temperature range, the most common of which are psychrophilic (5-20 °C), mesophilic (25-40 °C) and thermophilic (45-60 °C). Species which thrive in one temperature range may survive when exposed to higher or lower temperatures; however, outside their preferred temperature range, their activity level is minimal (Stanier et al., 1970). Most commercial anaerobic digestion systems operate in the mesophilic or thermophilic ranges, and efforts to operate under psychrophilic conditions have shown mixed results. Though there have been some reports of success, most

systems produced relatively little methane and many were unable to operate under the loading conditions used for mesophilic systems (Kashyap et al., 2003; Lettinga et al., 2001). These studies may not have allowed sufficient time for acclimation, considering that complete acclimation for a dairy manure system was reported to take 1.5 years (Nozhevnikova et al., 1999).

Conceptually, however, if the manure in an ISPAD tank contained an appropriate psychrophilic microbial community, a slow, stable anaerobic digestion process needing no operator intervention could be expected to develop. A preliminary study determined that some methane production could be initiated in a laboratory ISPAD system by seeding with dairy manure, or by acclimation of the microbial community in the pig manure, suggesting that full-scale operations could be developed (Abou Nohra et al., 2003). However, the state of knowledge in the literature (Chapter 2) regarding psychrophilic anaerobic digestion is incomplete and frequently contradictory.

This research project was thus designed to determine whether ISPAD could be an effective treatment system for pig manure in Canada, and to develop an understanding of the process parameters required to design and optimize ISPAD systems. The work focused on the critical role of acclimation and its impact on the rates and relative importance of the component digestion processes, as well as the effect of the complete process on the composition of the manure.

1.2 Objectives

The objectives of this research project were to investigate and define, in the laboratory, the microbial processes occurring in a full-scale ISPAD

installation handling pig manure, their impact on the manure composition and the release of gaseous manure components to the environment. The work focused on the following goals:

i. Determine whether or not the microbial community occurring in manure acclimates to the operating conditions of ISPAD.
ii. Define the changes, due to microbial acclimation, in kinetics and relative importance of the major processes occurring in ISPAD and their impact on methane production.
iii. Define the processes, and acclimation thereof, occurring during the degradation of manure proteins in ISPAD, and their impact on TAN production.
iv. Determine the extent of ammonia volatilization from land-applied ISPAD manure, and the impact of this on the fertilizer value of the manure for crop production, compared to conventionally stored manure.

1.3 Hypotheses

The project was designed to test the following hypotheses:

i. The microbial community present in pig manure acclimates to the operating conditions of ISPAD within one or two years.
ii. The acclimated microbial community in ISPAD is capable of robust anaerobic digestion similar to a mesophilic system.
iii. Protein degradation in ISPAD is not as extensive as in mesophilic systems.

iv. Less ammonia is lost from ISPAD systems than from conventional uncovered storage systems during the storage and land application processes.

1.4 Scope

One full-scale ISPAD installation had been established when this research began in 2006. Experiments were carried out using samples of manure taken from this installation. The acclimation, methane production, and protein degradation experiments were carried out in laboratory microcosm bottles. The ammonia volatilization experiments were carried out in small wind tunnels to simulate land application. The results of these experiments using small samples under controlled conditions are assumed to represent processes occurring in the full-scale installation. In addition, results from this single installation are assumed to apply to other similar installations. Further research could be undertaken to verify the validity of these assumptions.

1.5 Organization of Thesis

This thesis consists of eight chapters which include the introduction, general literature review, four scientific articles, conclusions and references. Following the introduction in Chapter 1, Chapter 2 briefly reviews the history of anaerobic digestion and the essential features of the current biochemical understanding of the process. Following this, reports of the potential for in-storage anaerobic digestion of manure and of psychrophilic manure digestion trials are also reviewed. The importance of acclimation to these processes is

highlighted and methods used to characterize microbial communities and assess their performance are discussed. The chapter concludes by summarizing the state of knowledge pertaining to aspects of ISPAD indicating the areas to which a contribution was needed.

Chapter 3 describes the materials and methods used to analyze the composition of ISPAD manure, and to conduct 'Biochemical Methane Production' tests using ISPAD microbial biomass in addition to two control series. These results were analyzed and compared for evidence of microbial acclimation, and to estimate the effectiveness of the resulting acclimated anaerobic digestion process in terms of solids reduction and methane production.

In Chapter 4, the 'Specific Substrate Activity' protocol is described, along with the way these tests were used to investigate the kinetics of acclimated anaerobic digestion in ISPAD. Comparisons between the assessed ISPAD kinetics and the kinetics of two control materials were used to illustrate the changes in relative importance and rates of the intermediate processes occurring in ISPAD.

Chapter 5 describes how the substrate activity protocol used in Chapter 4 was modified to assess the protein degradation kinetics of the acclimated ISPAD process. The results of these assays were used to define the rates and limiting step in the protein degradation process occurring in ISPAD and to assess the conversion of nitrogen species in the ISPAD process.

In Chapter 6, the wind tunnel method for measurement of ammonia volatilization is described, along with the factors which were expected to have an impact on the volatilization of ammonia from land-applied ISPAD manure.

Comparison with measurements from traditionally stored manure was used to evaluate the reduction of volatilization from ISPAD manure. Statistical analyses were used to determine the importance of various manure and soil properties in producing this effect.

Chapter 7 presents a summary and the overall conclusions from the complete research project, including the contributions to knowledge and suggestions for further research. The complete list of references cited in the thesis chapters is presented in Chapter 8.

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Chapter 2. Literature Review

2.1 History of Anaerobic Digestion

While there has been anecdotal knowledge of flammable gases escaping from the sediments of stagnant waterways from time immemorial, the first recorded scientific studies of the phenomenon were completed in 1776 by Alessandro Volta at Lake Maggiore in Italy and in 1783 by Thomas Paine and George Washington in New Jersey, USA. A century later, the name 'methane' was accepted to describe this gas. Work by Bechamps and Tappeiner ascertained that it was produced by an anaerobic microbial process, which came to be known as 'methanogenesis' or 'anaerobic digestion'. It was understood that organic material, particularly cellulose from decaying plants, was the substrate consumed during anaerobic digestion. However, the study of methanogenesis from cellulose raised questions about the chemistry of the process, which took decades of research to resolve (Wolfe, 1993).

At the same time, systems were developed to apply anaerobic digestion to the problem of wastewater treatment, beginning with the first septic tanks in the 1880's. Improvements to these systems informed the theoretical work going on at the same time, and vice versa. The earliest systems focused on reduction of solids in the wastewater, by retaining it in an air-tight vessel, and the concurrent capture of methane for use as fuel. The addition of baffles or settling chambers improved digestion effectiveness, producing clearer effluent and illustrating the importance of sludge separation. Using some of the methane to heat the digestion tanks increased the efficiency further, and the existence of mesophilic (35 °C) and

thermophilic (55 °C) optimum temperatures for microbial activity was recognized. It was also discovered that, while the rate of gas production increased with temperature, the total gas production was not temperature dependant. Mixing of the microbial sludge caused by the influx of wastewater increased the process efficiency by enhancing contact between microbes and substrates, as did settling and recycling of sludge, which helped to maintain a larger microbial community (McCarty, 1981).

In the 1960's, bacterial cultures isolated from anaerobic digesters that were thought to contain a single species, were found to contain at least two. This opened the door to understanding that the anaerobic microbial community contained many populations that accomplished the degradation of organic matter and production of methane in a complex multi-step process containing several parallel pathways of activity. Following this, both the microbiological and biochemical understanding of the process progressed on several fronts: the culturing and identification of methanogenic species; the discovery and definition of the archaea to which the methanogens belong; and the identification of important enzymes and co-enzymes. The earlier reports of different possible stoichiometric relationships between organic substrate and methane production were explained by defining the intermediate processes leading to methane production from either acetate or hydrogen. The kinetics of these intermediate processes, in the mesophilic and thermophilic temperature ranges, and the variation of these with temperature, were described (McCarty, 1981; Wolfe, 1993).

2.2 Developments in the Late 20th Century

Technological developments drove new directions of inquiry in the 1980's and beyond. Molecular biological methods based on sequencing of DNA and RNA were used to identify microbial species in active anaerobic communities. As the metabolism of newly-identified species was determined, these analyses could be used to estimate the relative importance of the different degradation pathways in digestion under specific conditions (Collins et al., 2003; Leung and Topp, 2001; Tang et al., 2005).

From an engineering perspective, the increase in computer calculating power and availability was the most important development, as it allowed increasingly sophisticated mathematical models to be tested. These models began with a two-stage approximation of the anaerobic digestion process (Chen, 1983; Hill and Barth, 1977; Lavagno et al., 1983). The accuracy of these early models was soon increased by the change to four-stage approximations (Hill, 1985; Svendsen and Henry Blackburn, 1986), as well as evaluating different kinetic relationships for individual processes (Hill, 1987; Vavilin and Lokshina, 1996). Dynamic models were developed for generalized digestion of organic susbtrates (Moletta et al., 1986; Vavilin et al., 1994), as well as for specific substrates such as animal manures (Garcia-Ochoa et al., 1999; Parsons and Williams, 1987). Many of these authors, and others not mentioned in this text, came together at conferences, and co-operated in the synthesis and publication of the internationally-developed Anaerobic Digestion Model no. 1 which provides a

common theoretical base on which to build and evaluate further refinements (Batstone et al., 2002).

2.3 Understanding Anaerobic Digestion in the 21st Century

The Anaerobic Digestion Model no. 1 (ADM1) proposes a structured mathematical model of anaerobic digestion. This model is process-oriented, and thus uses terminology more familiar to the chemical engineer than the microbiologist, who might call the process flow chart presented by ADM1 a food web. The process begins with a-biotic disintegration of composite particulate organic material into complex carbohydrates, proteins and lipids. These compounds are then hydrolysed to simpler substrates: monosaccharides, amino acids and long-chain fatty acids. Acidogenesis converts these compounds to volatile fatty acids such as propionate, butyrate and valerate, along with hydrogen and carbon dioxide. Acetogenesis transforms these products, as well as a portion of the preceding substrates, to acetate, hydrogen and carbon dioxide. These three compounds can then be transformed to methane via the aceticlastic and hydrogenotrophic pathways. While other products and processes are known to occur as well, the model excludes those which are considered to have a minor role (Batstone et al., 2002).

The disintegration process as described is largely physico-chemical and non-microbial in nature, and may be represented by a first-order relationship. For complex organic matter such as manure, this is frequently the limiting step in anaerobic digestion. Hydrolysis is an enzyme-mediated extra-cellular microbial process that is also represented by a first-order relationship (Batstone et al., 2002;

Rittmann and McCarty, 2001). Acidogenesis and acetogenesis are metabolic microbial oxidation-reduction processes which result in cell growth and energy storage. The need for thermodynamically suitable electron acceptors mediates these processes, which are usually represented by Monod-type growth and substrate uptake equations. More precision may be obtained by including factors to account for cell lysis and process inhibition by pH, ammonia, or other intermediate products (Rittmann and McCarty, 2001). Activity of the hydrogen-using methanogens controls the partial pressure of hydrogen in the system, which in turn affects the pH and inhibition of intermediate processes. The aceticlastic methanogens are the slowest growing population within the community, so while most of the methane in a balanced system may be produced by this pathway, its development is often the limiting factor in anaerobic digester start-up; Homoacetogenesis, which converts hydrogen and carbon dioxide to acetate, links the two methanogenic pathways. While this process may be negligible under mesophilic conditions, it has great importance during acclimation to psychrophilic temperatures (Batstone et al., 2002; Kotsyurbenko, 2005).

Overall, the ADM1 provides a detailed description of the understanding of anaerobic digestion at the start of the 21st century. It may be used or adapted to describe novel and emerging digestion systems (Galí et al., 2009), such as In-Storage Psychrophilic Anaerobic Digestion (ISPAD) of swine manure, which was developed based on reports of spontaneous methane production from stored manure and trials of psychrophilic digestion conceived to reduce the cost of technology for agricultural applications.

2.4 Methanogenesis from Stored Manure

As methane is produced spontaneously from the organic sediments in natural water bodies (Nozhevnikova et al., 1997), it is not surprising to find similar production from artificial water bodies such as manure storage tanks and lagoons (Park et al., 2006; Safley and Westerman, 1988; Sharpe et al., 2002). This phenomenon has been investigated by several experimenters, who have used floating or inflated covers to capture the escaping biogas (Chandler et al., 1983; Safley and Westerman, 1989). The performance of these systems varied seasonally, with reduced but still measurable methane production occurring in the colder winter months (Safley and Westerman, 1992). It was also observed that methane production at low temperatures increased with time, and was initiated at progressively lower temperatures over a period of two years (Cullimore et al., 1985). Though methane production from manure lagoons was erratic and much lower than that from industrial-style digesters, these studies provided the impetus to investigate the possibility of developing ambient-temperature in-storage manure digesters (Abou Nohra et al., 2003; Safley and Westerman, 1990).

2.5 Psychrophilic Anaerobic Digestion of Pig Manure

Several types of purpose-built swine manure digesters were also tested at ambient temperatures. Conventional single-stage pilot scale digesters with intermittent mixing were operated at 25 °C and 22.5 °C for 100 and 80 days respectively; this study concluded that, with a controlled loading rate and solids retention time double that of a mesophilic digester, low temperature digestion was stable and produced methane at a rate close to the theoretical methane potential of

the manure (Stevens and Schulte, 1979). Sequencing batch reactors were seeded with granular anaerobic sludge from dairy processing wastewater treatment and operated at 20 °C; these digesters reached performance rates similar to mesophilic digesters, but demonstrated a lag phase prior to methane production; production then continued beyond the reaction period, suggesting that the 77 day start-up period was not sufficient for full acclimation of the microbial community (Massé et al., 1997). Two accumulation digesters operated at 20 °C developed methane production comparable to mesophilic systems after two years of operation, though the start-up process was difficult; increasing the loading rate too quickly caused methane production to stop (Wellinger and Kaufmann, 1982). Seeding with cow manure, which contains methanogens originating in the rumen, was proposed to accelerate the start-up procedure, and the use of temperature-adapted seed material developed methane production at 5 °C (Zeeman et al., 1988). When laboratory digesters were started up at 25 °C and reduced by one degree every two weeks to 10 °C, methane production was maintained; a steady increase in methane production over five months at 10 °C suggested that microbial acclimation was continuing during this operating period (Safley and Westerman, 1994). Detailed study of low-temperature digestion of both dairy and swine manure in Russia confirmed that while psychrophilic digestion was a feasible and effective system for manure treatment, the acclimation process was critical and required approximately 1.5 years at 6 °C (Nozhevnikova et al., 1999).

2.6 Acclimation in Psychrophilic Anaerobic Digestion

Each microbial species has an optimum temperature at which it uses substrate most efficiently and exhibits a maximum growth rate. These optima generally occur near 20, 35 or 55 °C, and are termed psychrophilic, mesophilic and thermophilic, respectively, though there are extremophiles whose preferences extend beyond either end of this range. While both psychrophilic and thermophilic organisms can often survive in a mesophilic environment, neither is present in the others' range (Stanier et al., 1970). This is why manures, which originate from livestock with body temperatures in the mesophilic range, contain mostly mesophilic species with which small psychrophilic and thermophilic populations can coexist (Panikov, 1995). Mesophilic species are active at psychrophilic temperatures, however, their affinity for substrate decreases with temperature. This appears to be due to increasing viscosity of cell membrane lipids, reducing the effectiveness of substrate transport during metabolism to a minimum temperature at which the membrane effectively solidifies. The cell membranes of psychrophilic organisms generally contain more unsaturated lipids, which retain fluidity at lower temperatures. Cell membrane lipid composition is resistant to change, so that adaptation of species to temperature is limited (Nedwell, 1999). The temperature acclimation process experienced by anaerobic digestion microbial communities is thus less a transformation of individual species, and more a competitive re-arrangement of the relations between, and relative importance of, different species within the community (Kotsyurbenko, 2005).

Evidence of acclimation by various populations within the microbial community may be seen in measures of methanogenic activity. Typically, when a mesophilic anaerobic community is incubated at a constant psychrophilic temperature with excess substrate, specific substrate uptake will increase not only when evaluated at the incubation temperature, but also at other temperatures in both the psychrophilic and mesophilic ranges. Frequently, a greater increase is seen across the whole psychrophilic range than in the original mesophilic range. For example, after four months incubation of a mesophilic microbial biomass at 20 °C, methanogenic activity increased by factors of seven and five at 11 and 22 °C, respectively (Kettunen and Rintala, 1997). Similarly, in nearly two years of operation at 18 °C, specific substrate uptake activities showed significant increases at 15 °C compared to 37 °C; these results were accompanied by sequential changes in microbial population densities, for both bacterial and archaeal species as measured using polymerase chain reaction-denaturing agent gel electrophoresis (Connaughton et al., 2006b). These results suggest that a psychrophilic population and a mesophilic population are both consuming the specific substrate, and that the psychrophilic population has grown more than the mesophilic one during the incubation. Thus while an acclimated anaerobic microbial population may appear to be a single population with an unusual relationship between temperature and substrate uptake, it may in fact contain co-existing mesophilic and psychrophilic species which share the same substrate (McKeown et al., 2009b; Nozhevnikova et al., 2003). Studies of psychrophilic digestion that found kinetic relationships different from the generally accepted

Monod formulation could be explained by the concurrent existence of two populations (Kettunen and Rintala, 1997; Lokshina et al., 2001).

2.7 Protein Degradation in Psychrophilic Anaerobic Digestion

Although most investigations of psychrophilic anaerobic digestion have focused on degradation of carbohydrates, or processing of intermediate products such as the volatile fatty acids, the ADM1 specifies that protein and lipid susbtrates be described individually. The small number of studies investigating the anaerobic degradation of proteins have reported contradictory findings, including inhibition of protein digestion in the presence of carbohydrates, acclimation of active biomass to protein, but not amino acids, and lack of acclimation at all (Breure et al., 1986; Perle et al., 1995; Sarada and Joseph, 1993). The complexity of protein structures, and the large number of component amino acids has made it difficult to estimate the stoichiometry of protein degrading anaerobic reactions (Ramsay and Pullammanappallil, 2001). The contribution of lipids has also been scantily covered, though it may be an important inhibitor of digestion (Neves et al., 2008; Ortega et al., 2008). The single available study on psychrophilic degradation of manure proteins reported methane production increased with time, and that the limiting factor in the process was availability of hydrogen in the intermediate stages (Parshina et al., 1993).

2.8 Characterization of Microbial Communities by Activity

While the species-identification methods of molecular biology provide fascinating and informative data about the identities of species within microbial

communities, measures of activity and performance are more appropriate for use in an engineering study, as they provide quantitative results which may be applied to design and optimization procedures. Two laboratory microcosm procedures are frequently used: the biochemical methane production assay, and the specific methanogenic or substrate uptake activity assay.

The biochemical methane production assay uses long-term incubation of batch cultures with periodic biogas sampling to provide a temporal profile of methane production from a known initial concentration of active biomass and organic substrate. When the methane production reaches a plateau, this is defined as the methane potential, B_0, of the supplied substrate. This assay is used to determine the potential for methane production from various feedstocks for anaerobic digestion, as well as to assess the toxicity of substrates to the active biomass. It also provides an overall assessment of substrate consumption and methane production (Chynoweth et al., 1993; Owen et al., 1979; Shelton and Tiedje, 1984). The specific activity assay is similar, except that monitoring is done for a shorter period of time, because the objective is to investigate the rates of production or uptake, and the substrates used are individual chemical compounds representing the intermediate stages of anaerobic digestion. Typical substrates used are glucose, acetate, propionate butyrate and hydrogen. Tests with each individual substrate and a known concentration of active biomass are run in parallel, to provide a profile of the relative activities of the major populations occurring in the anaerobic microbial community. Two variations of the procedure exist: methane production may be measured to evaluate methanogenic activity

from the specific substrate, or the concentration of supplied substrate may be monitored to determine the substrate uptake activity. A drawback of the first protocol is that it must assume that stages of methanogenesis beyond uptake of the specific substrate are not limiting (Coates et al., 1996; Colleran et al., 1992; Soto et al., 1993). In either case, however, the microbial community in the active biomass will acclimate to the conditions in the bottles during incubation, so that only measurements taken near the beginning of the assay are representative of the original community (Grady et al., 1996).

2.9 Directions for Research

While publication of the ADM1 model did not signal the end of theoretical research into the understanding of anaerobic digestion, it did usher in an era which has been characterized by applications of the model to specific cases, (Parker, 2005), and improvements to the model resulting from these applications (Galí et al., 2009; Lee et al., 2009; Ramirez et al., 2009). Thus while it may provide an excellent base from which to develop an ISPAD design and optimization approach, a number of important features of ISPAD do not conform to the assumptions made in the model. Psychrophilic digestion is known to have important levels of activity on the homoacetogenic pathway which vary with the extent of acclimation (Kotsyurbenko, 2005), though this pathway is excluded from ADM1 (Batstone et al., 2002). The model also confines operation to a single temperature range, with temperature variation in activity governed by an exponential Arhennius relationship. Psychrophilic digestion frequently does not fit these assumptions in that psychrophilic and mesophilic populations may be

active simultaneously(McKeown et al., 2009b; Nozhevnikova et al., 2003), and the resulting activity does not conform to the Arhennius assumption (Rebac et al., 1999). In addition, the model was conceived for industrial systems which use fixed volume vessels, controlled hydraulic retention times and uniform mixing processes. Anaerobic digestion in manure storage tanks and lagoons, however, has been described as a continuously-expanding process, where stratification and oxidation-reduction relationships result in non-uniform conditions occurring within the system (Hill, 1985).

Research on psychrophilic digestion during the last decade has also focused on specific substrates, such as brewery effluent (Connaughton et al., 2006a) or whey (McHugh et al., 2006), or particular reactor types, such as the expanded granular sludge-bed anaerobic filter (McKeown et al., 2009a) or the upflow anaerobic sludge blanket reactor (Akila and Chandra, 2007). The only available recent work on psychrophilic manure digestion has focused on the use of a sequencing batch reactor (Massé et al., 2007; Massé et al., 1997).

Though the successful production of methane from manure storage lagoons and experimental psychrophilic digesters supports the concept of In-Storage Psychrophilic Anaerobic Digestion for treatment of swine manure, the current state of knowledge in the literature is not complete enough to inform the theoretical design and optimization of such systems. In particular, the extent of acclimation occurring in ISPAD and the time required for this process to occur, must be determined. Following this, the kinetics of both carbohydrate and protein degradation by the acclimated community must be described (lipids form a small

part of manure and thus may be excluded at this time). The impact of these processes on the manure composition and production of methane for fuel will confirm the effectiveness of ISPAD as a treatment system. Finally, evaluation of the production and volatilization of ammonia nitrogen in the system and following land application will define the fertilizer value of the treated manure.

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Chapter 3. Acclimation of the Microbial Community

Connecting Statement

The literature reviewed in Chapter 2 indicates that microbial acclimation is the most important factor in the success of In-Storage Psychrophilic Anaerobic Digestion (ISPAD). The article which follows describes how the extent of microbial acclimation was evaluated, and the resulting methane production determined, for a three-year-old ISPAD installation.

This article was submitted to the Journal of Biomass and Bioenergy. The first contributing author, Susan M. King, designed the experiments, conducted the laboratory work, analyzed the data and wrote the article. The second contributing author, Dr. Suzelle Barrington, supervised, advised on the experimental design and methods of analysis, and revised the content of the article. The third author, Dr. Serge R. Guiot, supervised, advised on the experimental design and methods of analysis, and revised the content of the article.

Abstract

In-Storage Psychrophilic Anaerobic Digestion (ISPAD), achieved by covering a concrete manure storage tank with an airtight floating membrane, has been proposed as a manure treatment strategy adapted to the needs of Canadian pork producers. Previous laboratory work suggested that the microbial community in swine manure could develop a robust anaerobic digestion process given an acclimation time of at least 100 days at 35 °C. The objective of this study was to confirm that the microbial community in swine manure does acclimate to the psychrophilic operating conditions in ISPAD, resulting in significant anaerobic digestion of the manure in the tank. In the laboratory, manure from a three-year old full-scale pilot ISPAD facility located in St. Francois-Xavier, Quebec, Canada, was analyzed, along with fresh manure and manure from an uncovered storage tank. Analytical characterizations as well as biochemical methane production assays performed at the three temperatures of 8, 18 and 35 ^{o}C were used to quantify the performance of the microbial community. The results of this study indicate that a robust, acclimated microbial community is present in the pilot ISPAD installation. The ISPAD process reduced the organic matter content of the manure by 24% while releasing 63% of the potential methane in the manure, as opposed to the open storage tank where no measurable reduction in solids occurred, and only 15% of the potential methane was released.

KEYWORDS: psychrophilic anaerobic digestion, acclimation, swine manure, manure treatment.

3.1 Introduction

An increasing number of Canadian hogs, 33% in 1991 and 37% in 2001, are raised in regions of high livestock density (Beaulieu and Bedard, 2003) where, according to environmental regulations, manure production exceeds cropland nutrient requirements (De Kimpe and MacDonald, 1998; Gangbazo et al., 2005; Hofmann and Beaulieu, 2006a; Quebec, 2005). To continue operating within this constraint, many producers have improved their manure management practices (Le and Beaulieu, 2005). However, management alone may not be sufficient, and manure treatment strategies adapted to regional needs have yet to be developed (AAFC, 1998; Martinez et al., 2009).

In-Storage Psychrophilic Anaerobic Digestion (ISPAD) has been proposed as a treatment system suited to the needs of pork producers in Canada and other temperate regions (Abou Nohra et al., 2003). This process would operate under quite different conditions than conventional anaerobic digestion, as detailed in Table 3.1. ISPAD is expected to occur in manure storage tanks with an air-tight cover, once the anaerobic microbial community in the manure has acclimated to the ambient (psychrophilic) operating temperature.

Anaerobic digestion of swine manure at psychrophilic temperatures has been successful in several studies done at different scales. Experiments using four 2500-litre pilot scale reactors indicated that digestion at 22.5 °C was stable, with biogas production and solids reduction comparable to those in the mesophilic range, when the retention time was doubled (Stevens and Schulte, 1979). Two full-scale accumulation digesters, built below the floor of a piggery, reached net

biogas production rates comparable to mesophilic installations after 1.5 years of acclimation at 19-20 °C (Wellinger and Kaufmann, 1982). Laboratory digesters that were seeded with lagoon effluent successfully adapted to temperatures from 10-23 °C (Safley and Westerman, 1994). Seeded 25-liter laboratory-scale sequencing batch reactors operated at 20 °C exhibited methane production, COD removal and odour reduction comparable to mesophilic reports, after 250 days (Massé et al., 1997).

The acclimation process has been investigated in detail by incubating manure samples in biochemical methane production (BMP) assays to measure anaerobic digestion activity; Methane production was initiated at progressively lower temperatures (3-9 °C) when samples of pig manure from an un-heated earthen lagoon, taken at regular intervals over two years, were incubated (Cullimore et al., 1985). Pig manure samples incubated for 15 months at different temperatures between 6-20 °C showed continuously increasing rates of methanogenesis at each temperature over the study period (Nozhevnikova et al., 1999). Laboratory simulation of ISPAD start-up concluded that the microbial community would develop with several different seed materials, but swine manure alone with no seed could also develop a viable community, given an acclimation time of at least 100 days at 35 °C (Abou Nohra et al., 2003).

This body of research suggests that installing an air-tight polymeric cover over a swine-manure storage tank should develop a robust psychrophilic anaerobic digestion process equivalent to that of a mesophilic system. However, it

must be demonstrated that ISPAD does initiate the microbial acclimation process under ambient conditions.

The first objective of the present study was therefore to confirm that the microbial community in swine manure does acclimate to the psychrophilic operating conditions in ISPAD, resulting in significant anaerobic digestion of the manure. The second objective was to develop a procedure to monitor the progress of acclimation in an ISPAD manure microbial community. To accomplish these objectives, samples of manure from a three-year old full-scale ISPAD installation (X_f) were tested in the laboratory, along with two control materials: fresh manure (S_m) was used to evaluate the changes occurring in the ISPAD tank over time; manure from a standard uncovered storage tank (X_o) was used to evaluate the effect of residence in a storage tank separately from the treatment effect of the ISPAD cover. Organic matter fractionation and presence of intermediate products were evaluated by analysing the composition of the three subject materials. Biochemical methane production assays performed at three temperatures of 8, 18 and 35 °C, were used to evaluate methane production rates, time required for acclimation, and methane potential.

3.2 Materials and Methods

Experimental materials

In 2004, a full-scale swine manure ISPAD facility was established in St. Francois Xavier (St. F-X) in the central region of the Province of Quebec, Canada. This facility consisted of a circular concrete tank measuring 30 m in diameter by 3.66 m in depth, covered with an air-tight membrane (*GTI*,

Fredericton, NB, Canada). The tank received manure from the swine facility on a regular basis. Except for a depth of 0.3-0.6 m, the contents were removed for land-spreading twice yearly. The facility had been in operation for three years when this study started in 2007. As acclimation is reported to require 1 to 2 years (Connaughton et al., 2006b; Nozhevnikova et al., 1999), manure from the St. F-X tank (X_f) was expected to contain a mature ISPAD microbial population. The two controls, freshly produced manure (S_m) and one year old manure contained in an uncovered storage tank (X_o), were obtained from the swine research facility of the McGill University Macdonald Campus Experimental Swine Centre (Mac), located on the Island of Montreal, Quebec, Canada. As they are produced by hogs fed a standard corn and soybean based ration, the manures from the St. F-X and Mac operations were considered comparable in terms of solids and nutrients with significant differences only in water content (Conn et al., 2007).

All manure samples were collected in June 2007. Manure from the ISPAD tank (X_f) was collected through sampling ports in the tank cover, using a sludge-judge apparatus to collect a composite sample representing the average of depths and locations within the tank. The uncovered-tank samples (X_o) were collected from the side of the tank using a similar apparatus. The fresh manure samples (S_m) were obtained after thorough mixing from the Mac piggery pre-pit, which collects manure for no more than 3 days prior to its transfer to the storage tank. All collected manures were transported in sealed 500 ml Nalgene bottles, sieved at the lab to remove particles larger than 2 mm, transferred to glass bottles, flushed with N_2/CO_2 and stored at 4 °C until needed.

Characterization of experimental materials

Sub-samples of all three materials (X_f, X_o and S_m) were analyzed according to standard methods (APHA et al., 2005) to establish: solids (TS, VS, TSS, VSS, TDS and VDS), COD (total and soluble), pH, VFA (acetic, propionic and butyric acids), anions (NO_2^-, NO_3^-, PO_4^{-3}, Cl^-), cations (Na^+, NH_4^+, K^+), and ATP to measure the active microbial biomass.

Total solids were determined by drying at 103 °C overnight (*VWR*, *Sheldon Manufacturing*, model 1327F, OR, USA). Volatile solids were determined by incineration at 500 °C for two hours (*Barnstead Thermodyne,* model 48000, IA, USA). Suspended solids were separated from supernatant by centrifuging at 10000 rpm for 10 minutes at 4 °C. Chemical oxygen demand was measured using the potassium perchromate method and a spectrophotometer (*Hach Canada,* model DR 2800, Mississauga, ON). The pH of all samples was determined using a pH meter (*Corning,* model 450, NY, USA).

Volatile fatty acids were measured using a gas chromatograph (*Agilent*, model 6890, DE, USA) equipped with a flame ionization detector. Samples were centrifuged to remove solids. Sub-samples of supernatant were diluted 5-fold and 0.5 μL of each diluted sample was fortified at a ratio of 1:1 (V:V) using an internal standard of iso-butyric acid dissolved in 6 % formic acid. These were directly injected into a glass column of 1 mm x 2 mm *Carbopack C* (60 to 80 mesh) coated with 0.3 % *Carbowax 20M* and 0.1 % H_3PO_4. The column was held at 130 °C for 4 minutes and helium as carrier gas was injected at a rate of 20 ml

min^{-1}. The injector and the detector were both maintained at 200 °C. Quantification was made using iso-butyric acid as an internal standard.

Anion and cation analyses were carried out on diluted supernatant of centrifuged samples as well. Anions were analyzed by HPLC on a polymer-based chromatography column (250x41mm OD, *Hamilton,* model PRP-X100, NV, USA), on a high-performance liquid chromatograph (*TSP,* model P4000 & AS3000). Conductivity data were obtained by using a *Waters* Millipore detector, model 432. The parameters were: mobile phase 4.0 mM p-hydroxybenzoic acid, pH 8.5 with 2.5 % methanol, 100 µL injection, 1.8 ml/min flow rate at 40 °C. Cations were analyzed by HPLC on a *Hamilton* PRP-X200 cation resin-based chromatography column (250x41mm OD), on a high-performance liquid chromatograph (*TSP* model P4000 & AS3000). Conductivity data were obtained by using a *Waters* Millipore detector, model 432. The parameters used were: mobile phase 4 mM nitric acid with 30% methanol, 20 µL injection, and 1.8 ml/min flow rate at 40 °C.

The quantity of active microbial biomass in each manure sample was estimated using the *Luminultra* wastewater ATP kit (*Luminultra*, NB, Canada) and a *Sirius* Luminometer (V3.2, *Bethold Detection Systems*, TN, USA).

Biochemical methane production (BMP) assays

Two sets of BMP assays were performed: one using the ISPAD manure as biomass (X_f) and the other using the uncovered-tank manure as biomass (X_o). The substrate tested was fresh manure (S_m). Each triplicate set comprised three batches, one each at 8, 18 and 35 °C. Each batch included three bottle-filling

regimes: substrate alone, biomass alone and substrate and biomass together. Substrate alone and biomass alone served as controls for each batch of assays to separate the performance of manure-as-biomass from manure-as-substrate. For each bottle-filling regime, a fourth bottle was prepared and analyzed to establish the initial conditions: solids, COD, VFA, anions and cations. The experimental design is depicted in Table 3.2.

The tests were run based on established procedures (Owen et al., 1979; Shelton and Tiedje, 1984). For each batch, nine 160 ml bottles were prepared. Biomass was measured into six bottles, the quantity calculated to provide 10 g VSS/L. Defined media, sulphide solution and bicarbonate buffer were added to all the bottles in standard amounts. De-ionized water was added to all the bottles, the quantity calculated to provide 50.5 ml total liquid in each bottle after substrate addition. Bottles were capped, sealed and flushed with N_2/CO_2 gas to establish anaerobic conditions, and placed in a shaker operating at 100 rpm, in a dark, thermostatically controlled environment. After three or four days of adjustment to the test conditions, substrate was injected into three of the bottles containing biomass, and the three which contained no biomass, the quantity in both cases calculated to result in 4.5 g soluble COD/L in the bottles. Monitoring started at this point.

The biogas production of each bottle was monitored regularly until production ceased, following a schedule based on the assay temperature. For example, 35 °C assays were monitored every day for one week to establish the initial rate of methane production, then every 2 to 3 days for the next 10 weeks

until a near-plateau was reached, and then every 2 to 3 weeks for another 10 weeks. A similar pattern was used for the lower temperatures, with frequencies approximately halved for 18 °C and halved again for 8 °C, and the duration of each sampling frequency doubled for 18 °C and doubled again for 8 °C.

At each sampling time, the volume of biogas produced in the headspace of each serum bottle since the previous sampling was measured using the U-Tube method based on water displacement. The biogas composition (H_2, N_2+O_2, CH_4, CO_2) was measured by injecting 300 μL of the bottle head-space gas into a HP 6890 Series Gas Chromatograph System equipped with a flame ionization detector and a 11m x 3.2mm 60/80 mesh *Chromosorb* 102 column (*Supelco*, Bellafonte, PA). The carrier gas was argon. The column temperature was held at 50 °C for 3.9 minutes isocratic.

At the end of each assay, the bottle contents were analyzed for pH, solids, VFA, COD, anions and cations, as described previously.

Manipulation of biochemical methane production data

All gas measurements from the biochemical methane production assays were corrected to standard temperature and pressure (STP) of 0 °C and 1 atmosphere, according to the ideal gas law. The STP methane production of each bottle was normalized in terms of VS as m^3CH_4/kgVS. Table 3.2 indicates which data sets were used to evaluate each parameter described in detail below.

The specific methane production rate (*MP*) at a particular temperature is a property of the microbial community used as biomass, specific to the substrate used. The *MP* is defined as the steepest slope of the curve representing the

accumulation of methane over time, occurring during the initial period of a BMP assay. Using the initial period ensures, first, that activity reflects the original (extant) community, as the community acclimates to the bottle environment during the assay, and thus rates occurring later represent modified communities (Grady et al., 1996) and second, that substrate uptake is not limited or inhibited by depletion of any components of the substrate. The initial period considered in the present calculations represents one doubling time for the methanogen population, adjusted exponentially for temperature: 4 days at 35 °C, 14 days at 18 °C and 28 days at 8 °C (Rittmann and McCarty, 2001).

The time required for acclimation (*TA*) of each microbial community may be inferred from the same curves, when the steepest slope occurs after the initial period. In these cases, *TA* is defined as the time elapsed prior to the occurrence of the steepest slope. Since the methanogens are slowest to acclimate, the development of methane production is a good indicator of development of the community as a whole (Kotsyurbenko, 2005).

The ultimate methane potential (B_0) is a property of a substrate, independent of the biomass used. It represents the quantity of methane that can be released by digestion of the substrate under ideal conditions for an infinite time period. The value of B_0 for each of the study materials as a substrate was obtained from the 35 °C BMP assays. This was done by fitting the data sets using *Matlab* (®2008a, *the Mathworks*, Natick, MA) to a first-order curve of the form: $B = B_0 (1\text{-}e^{-kt})$ where t = time (*days*), B = methane released up to time t ($m^3\ CH_4 / kg\ FS$) and k = conversion constant ($days^{-1}$) (Chynoweth et al., 1993). Both B_0 and k are

estimated in the fitting process. The coefficient of determination, R^2, is used to evaluate the goodness of fit. Although 150 days long, these assays did not reach a true plateau because the slow hydrolysis of recalcitrant compounds in the manures continued releasing a small amount of methane. The total quantity of methane released in 150 days, B_{150}, was compared to B_0 obtained by curve fitting.

Statistical Analysis

All statistical analyses were performed using *SAS* 9.0 (®2009, *SAS Institute inc.*, Cary NC, USA). The characterization data were collected in triplicate, on sub-samples from the composite sample taken from each tank. The significance of differences between measured values was evaluated by the Student-Newman-Keuls method in a simple analysis of variance based on a completely randomized design. The biochemical methane production assays used a randomized complete block design, considering microbial community type (X_f, X_o and S_m) as the treatment factor, temperature (35, 18 and 8 °C) as the block factor, with specific methane production rate (*MP*), time required for acclimation (*TA*) and methane potential (B_0, B_{150}) as dependant variables. Treatments were assigned randomly to experimental units (bottles), and all treatment-block combinations were completed in triplicate. While the assays were completed in batches of nine bottles (three repetitions each of three regimes), the resulting data were used in within-set comparisons as well as between-set comparisons.

3.3 Results and Discussion

Characterization of experimental materials

The analytical results of analyses performed on the three study manures are presented in Table 3.3. Comparisons assume that both storage tank manures, X_f and X_o, developed by microbial digestion and volatilization from the source manure, S_m. The total solids (TS) data indicate that X_f is 19% less concentrated than S_m, reflecting the inclusion of wash water in X_f, while X_o is 52% less concentrated than S_m, due to both wash water and precipitation. For both S_m and X_o, volatile solids (VS) represent 72% of TS, while X_f shows a reduction of VS to 66% of TS. To eliminate the effect of dilution, all the remaining data in Table 3.3 are presented in terms of *VS*.

Values of COD were lower in both X_o and X_f, as compared to S_m, which supports the above assumption, as COD may be reduced by anaerobic processes in X_f and by anaerobic, aerobic and volatilization processes in X_o. The profile of VFA that is present in S_m is different from the profile in X_o, while the total VFA are respectively 243.0 and 182.5 mg / g VS. In contrast, there are effectively no VFA detected in X_f. This further supports the assumption that both X_o and X_f developed by microbial and chemical processes from manures equivalent to S_m, and suggests that, in X_f, VFA are consumed as quickly as they are produced.

The anion and cation data indicate higher chloride (Cl^-) in S_m and X_o, explained by the use of municipally treated water at Mac (S_m and X_o), and untreated well water in St.F-X (X_f). Sulphate (SO_4^{-2}) is only present in X_f, which could also be due to the water source. Phosphate (PO_4^{-3}) is lower in both storage

tanks (X_o and X_f), due to settling, which is a known phenomenon. Sodium and potassium (Na^+ and K^+) vary between the three samples, due to a combination of water, feed and ionization.

In many anaerobic digestion systems, the active microbial biomass is distinct from the influent VSS, and thus can be quantified separately. For example, packed bed processes develop an attached microbial slime, fluidized bed reactors develop granules of active biomass and anaerobic contact reactors develop a microbial biomass layer through sludge recycling. In a manure storage tank, however, the active community develops over time from the community introduced with and forming a small part of the manure. The size of the microbial population in relation to the total concentration of VSS in the manure can be estimated by measuring adenosine tri-phosphate (ATP), present only in viable microbial cells (Horiuchi et al., 2003; Hwang and Hansen, 1997). Because microbial species contain varying concentrations of ATP, the conversion of ATP data to % VSS is an estimate based on empirical averages, 2-6 μmol ATP / g cells, dry weight (Jay et al., 2005). Preliminary ATP analysis of the study materials indicates that the three active microbial communities are similar in size and account for less than 2% of the VSS. As the remaining 98% of VSS represents the organic matter available as substrate for the digestion process, VSS is equated to organic substrate in this study.

Intermediate products

Concentrations of volatile solids and volatile fatty acids are presented in Table 3.4. These intermediate products represent the sequential conversion of

VSS through several stages to form methane, with each conversion performed by a specific population within each anaerobic microbial community (S_m, X_o and X_f). To compare these data from different processes on a basis referring to the initial fresh manure fed to the tank, characteristics of both digested materials, X_f and X_o, are presented in terms of the VS of fresh manure, VS_i . The conversion uses the fixed solids (FS or ash) as reference, as variations in FS for manures from different sources are insignificant when a standard diet is used (Conn et al., 2007; Kerr et al., 2006). Thus characteristics of X_f and X_o measured as g/kg VS are multiplied by the ratio VS/FS for the appropriate sample then multiplied by the FS/VS ratio of fresh manure, to finally be presented in terms of g/kg VS_i.

The initial substrate consumed by anaerobic digestion overall is the volatile (organic) solids (VS) fraction of the manure. The ratio of volatile to initial volatile solids ($VS:VS_i$) for fresh manure (S_m), at 1.00, and uncovered tank manure (X_o), at 1.05, are statistically similar, while the manure from the ISPAD tank (X_f), at 0.76, is significantly lower than both of these. These data indicate that VS_i in the fresh manure fed to each tank is not being consumed by the X_o community, while 24% of VS_i has been consumed by the X_f community.

The pool of VS includes volatile suspended solids (VSS), the substrate for hydrolysis, and volatile dissolved solids (VDS), the product of hydrolysis and substrate for acidogenesis and acetogenesis. The fresh manure contains 0.80 $gVSS/gVS_i$, whereas both the uncovered tank and the ISPAD tank contain 0.71 $gVSS/gVS_i$. This indicates that hydrolysis is occurring at the same rate in both tanks. However, the uncovered tank accumulates the products of hydrolysis, with

0.34 gVDS/gVS_i, higher than fresh manure at 0.20 gVDS/gVS_i, implying that acidogenesis and acetogenesis are not occurring at rates sufficient to keep the process in balance. In contrast, the ISPAD tank contains only 0.04 gVDS/gVS_i, suggesting that acidogenesis and acetogenesis consume the VDS to balance hydrolysis in the ISPAD community.

The data on volatile fatty acids provides similar results. The uncovered tank manure shows an accumulation of propionic acid along with a moderate decrease in both acetic and butyric acids, as compared to the fresh manure. Thus methanogenesis consuming these products has not developed to the required level for the community to be balanced. The ISPAD manure shows only traces of acetic acid and no measurable concentration of either propionic or butyric acids, indicating strong methanogenesis from these intermediate products.

These results suggest that the ISPAD microbial community (X_f) is acclimated to its operating conditions, with balanced hydrolysis, acidogenesis, acetogenesis and methanogenesis (Kotsyurbenko, 2005). In the uncovered tank community (X_o) hydrolysis is equivalent to ISPAD but the corresponding acidogens, acetogens and methanogens are not fully developed, resulting in an unbalanced community.

Methane production trends during BMP assays

The development of methane production, with fresh swine manure (S_m) as substrate, during the course of the BMP assays at 8, 18 and 35 °C, is illustrated in Figure 3.1 for the microbial community in fresh manure (S_m), Figure 3.2 for the microbial community in uncovered-tank manure (X_o) and Figure 3.3 for the

microbial community in ISPAD manure (X_f). Note that slight decreases seen in some of these cumulative curves reflect the compounding of experimental error when values form the control bottles were subtracted from the experimental data.

As shown in Figure 3.1, the microbial community in fresh manure produced methane at a very low rate during the initial period at all three assay temperatures. This indicates that there is a viable anaerobic microbial community in fresh manure, which is minimally active at both psychrophilic and mesophilic temperatures. At 35 °C and 18 °C, the rate increased to a maximum, the steepest slope, in 15 days and 60 days, respectively, demonstrating acclimation to the BMP bottle conditions during these assays. However, in nearly 500 days at 8 °C, an exponential growth phase was not reached, and methane was produced at a minimal rate only, which suggests that acclimation to this temperature requires much more time, or other conditions to encourage the process.

In contrast, the uncovered-tank community illustrated in Figure 3.2 developed its maximum methane production rate within the initial period of the assays at both 35 °C and 18 °C, indicating that this community had acclimated in the tank environment to the range of 18-35 °C. At 8 °C, however, the initial low methane production rate was maintained for over 200 days, increasing around the 300-day mark to reach another steady stage for the rest of the assay. This demonstrates that acclimation to the 8 °C conditions occurred in the X_o BMP bottles during the first 300 days. This suggests that a partial acclimation must have occurred within the X_o tank environment, which allowed further acclimation

to occur once the sample was placed in ideal anaerobic conditions in the BMP bottles.

Figure 3.3 shows that, for the ISPAD community, the maximum methane production rate occurred immediately at all three temperatures, confirming that this community is fully acclimated to all temperatures in the range of 8-35 °C. This corroborates the results from the X_o BMP assays, demonstrating that the ideal anaerobic conditions occurring in the ISPAD covered tank, as well as within the BMP assay bottles, allows the facultative community in fresh manure to acclimate to these conditions, developing a robust psychro-active anaerobic digestion process.

Methane production rates (MP)

The rates of methane production developed during the initial period of these assays, for each microbial community at each assay temperature, are presented in Figure 3.4. All three microbial communities had measurable methane production rates at all three assay temperatures, increasing with temperature, being highest at 35 °C. This confirms that these communities are mesophilic overall, consisting of a combination of meso-tolerant psychrophilic populations and psychro-tolerant mesophilic populations. Though both tanks had experienced long-term exposure to mostly psychrophilic conditions, a wholly psychrophilic community had not developed in either tank. Rather, the original mixed community acclimated to the variable conditions occurring in the tanks, retaining the potential for activity in the psychrophilic and mesophilic ranges.

At each temperature, methane production was highest for the ISPAD community, moderate for the uncovered-tank community and lowest for the fresh manure. Values for X_f were from 3-10 times higher than those for X_o, which were between 3-10 times the values for S_m. The ATP analysis mentioned previously indicated that the X_o and S_m communities were roughly the same size, while the X_f community was approximately 50% larger, so the differences in methane production can be ascribed only slightly to differences in community size. Rather, it may be inferred that differences in community structure are the dominant factor.

The ISPAD tank community shows a definitely non-linear relationship between methane production rate and temperature, while the fresh manure and the uncovered-tank communities show a relatively linear relationship. Neither community type (S_m, X_o, X_f) nor temperature alone were found to significantly affect the relationship between methane production rate and temperature ($P = 0.19$ and $P = 0.16$, respectively), but the interaction between these two factors was significant ($P < 0.001$). Accordingly, the three communities react differently to temperature, indicating that the original community has not simply grown, but has also acclimated metabolically to the tank environments.

Ultimate methane potential (B_0)

Materials used as a substrate for anaerobic digestion are characterized by their ultimate methane potential, B_0. Table 3.5 presents B_0 from first-order curve fitting, as well as B_{150} from the assay data, for all three experimental manures. Figure 3.5 illustrates the self-fermentation data used to determine these values. Data from the fresh manure, S_m, exhibited a significant lag phase prior to reaching

first-order (exponential) growth. Using the data points from the beginning of the first-order region to the end of the data set gave a good fit between the experimental and calculated curves, with an R^2 of 0.99, and equal values for B_0 and B_{150}, of 0.27 m^3 CH_4/kg VS_i. For the uncovered-tank manure, X_o, the entire data set was used, as it followed a first-order form. A good fit, with R^2 of 0.99, was obtained with correspondence between B_0 and B_{150}, at 0.22 and 0.23 m^3 CH_4/kg VS_i. For the ISPAD manure, X_f, the data appeared to follow a first-order form, but the curve-fitting showed that the initial maximum rate region entered a prolonged and flattened transition phase, and did not reach a clearly defined asymptote. As a result a poor first-order fit, with R^2 of 0.87, was obtained. At 0.08 and 0.10 m^3 CH_4/kg FS, B_0 underestimated B_{150} by 20%. This may be due to the fact that the X_f manure had undergone significant digestion in the tank, so that the remaining material available for digestion in the BMP assay was limited by the hydrolysis process. For this reason, B_{150} was a better estimator of ultimate methane potential for X_f, and equivalent to B_0 for both X_o and S_m. Overall B_{150} gave a good estimate of B_0 for all three manures without the need to assume first-order behaviour.

Ultimate methane potential, B_0, and its indicator, B_{150}, are expected to be constant for a specific material (Hashimoto et al., 1980), and thus all three materials, as samples of swine manure, should present similar results. However, B_{150} for the uncovered tank manure, at 0.23 m^3 CH_4/kg VS_i, is lower than B_{150} for fresh manure, at 0.27 m^3 CH_4/kg VS_i. The ISPAD manure is even lower, at 0.10 m^3 CH_4/kg VS_i. This means that anaerobic digestion in the uncovered storage tank

has released 15% of the manure's potential methane, while more robust digestion in the ISPAD covered tank has released 63% of its potential methane. It is difficult to compare the ISPAD methane release values to other reports due to the variety of units and measures used. However, a review of swine manure anaerobic digestion reports that methane production by mesophilic systems ranges from 0.26 to 0.35 m^3CH_4/kg VS, and that values of B_0 for swine manure range from 0.32 to 0.48 m^3CH_4/kg VS (Chynoweth et al., 1998). These data suggest an average methane release of approximately 75% of B_0 which compares favourably to the 63% of B_0 released by ISPAD.

Time for acclimation (TA)

The BMP assays indicate the time required for acclimation at three constant temperatures. However, in practice, the temperature of a Canadian manure storage tank varies with ambient air temperature through the seasons, remaining 4^oC warmer, 60 cm below the surface, than the average air temperature (Park et al., 2006). This means that acclimation to temperature occurs as a continuous process which cannot be estimated precisely from these single-temperature assays. However, the results may be used to provide the boundary conditions if the three assay temperatures are used to represent the seasons, 35 °C for summer, 18 °C for spring and fall, and 8 °C for winter. Accordingly, a newly covered tank containing fresh manure only will acclimate to summer temperatures within 30 days, to spring and fall temperatures within 100 days and to winter temperatures in more than 500 days. A newly covered tank which has held manure for the preceding year will acclimate to summer temperatures within a

week, to spring and fall temperatures within a month and to winter temperatures in the first year.

Monitoring protocol

The parameters demonstrating evidence of microbial acclimation in this study could be used to monitor the acclimation process in other ISPAD installations. The simplest measure is the reduction in VS, a standard analytical procedure that could be repeated on initial and monthly samples from the new installation. The ultimate methane potential, B_0, could be evaluated seasonally using BMP assays performed at 35 °C, lasting 75 days. Figure 3.5 shows that a 75-day incubation would give a good approximation of B_{150}, and would be complete within the three-month repeat cycle. Incubations of 15 days or less, at temperatures lower than 35 °C could also be done seasonally to evaluate the initial methane production rates. Combining these three procedures would give a clear picture of the development of acclimation in a new ISPAD microbial community. A meaningful comparison could be made by reporting all values based on the initial manure VS (VS_i). Reporting the quantity of methane released from the system in relative terms based on the ultimate methane potential of the original manure is also more meaningful than an absolute value, allowing installations with different manure compositions to be compared.

Further investigation

This study has demonstrated that a swine manure microbial community can acclimate to psychrophilic ISPAD operating conditions, resulting in significant anaerobic digestion of manure. However, to develop an ISPAD design

model, further studies are needed to define the kinetic parameters of the ISPAD process stages, describe the variation of these parameters with temperature, and to confirm that the results presented here may be generalized to other ISPAD installations.

A microbial community may be considered acclimated to its environmental conditions when initial substrate is being consumed, and intermediate substrates are not accumulating (Kotsyurbenko, 2005). However, measuring only the concentration of these substrates combines information about production and consumption which does not completely define the behaviour of each population. Using specific activity tests with individual pure substrates representing each stage in the digestion process would permit the kinetics of each population to be defined (Colleran et al., 1992). These data could then be used in modeling and simulation of the community as a whole, allowing the design and operation of ISPAD systems to be optimized (Vavilin et al., 1998). Specific activity tests using H_2/CO_2 would provide more information on the progress of acclimation, as the H_2 methanogenesis pathway is expected to dominate during low-temperature acclimation, with the acetate pathway providing 95% of methanogenesis once acclimation is complete (Kotsyurbenko, 2005). The data obtained from the uncovered storage tank supports this hypothesis in a qualitative sense, as methane is produced in the system while volatile fatty acids accumulate, suggesting that H_2 is the major source of methane in this partially acclimated system.

Because the operating temperature of an ISPAD system varies with the seasons, unlike the controlled constant-temperature processes used in industry, it is important to have an accurate representation of the relationship between methane production rate and temperature for an ISPAD model. The present study, using three temperatures, indicates that this relationship has a different form for acclimated and non-acclimated communities, agreeing with the conclusions of other studies (Kettunen and Rintala, 1997; Lokshina et al., 2001). However, these studies do not agree on the appropriate mathematical description of this relationship. Further investigation using more than three temperatures would help to define this more clearly.

The effectiveness of digestion in ISPAD was determined by laboratory analysis of small samples from the system. These values could be used with more confidence to evaluate the system's performance if their accuracy were confirmed by conducting a carbon mass balance on a whole ISPAD system. In addition, all of the study results were based on data from a single ISPAD location, as no others were in operation when the study began. These results could be generalized from the specific site to the process in general if other installations were monitored using the protocol developed in the preceding section. This would be an important step in developing the design model needed to bring the ISPAD system into common use.

3.4 Conclusion

This study has confirmed that in-storage psychrophilic anaerobic digestion (ISPAD) of swine manure is feasible and effective, and develops by microbial

acclimation of the microbial community in the manure. When an ISPAD air-tight cover is installed on an existing manure storage tank, the acclimation process should be complete within one year. This process may be monitored using standard laboratory analysis of periodic manure samples from the tank. Future laboratory studies are required to investigate the kinetics of the microbial community responsible for digestion, while field studies could monitor the acclimation process occurring in two new installations, as well as confirming the solids reduction and methane release by mass balance. These data could be combined to create an ISPAD design and operation model. The treatment effects, including ammonia release, phosphorus separation and odour reduction could also be evaluated.

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3.7 Tables

Table 3.1: Features of ISPAD compared to conventional anaerobic digestion systems

	Conventional Anaerobic systems	**ISPAD**
Temperature	Constant (20, 35 or 55 °C)	Variable with seasons (0 to 25 °C)
Pressure	Ambient or higher	Ambient
Volume	Constant	Continuously expanding
Mixing	Completely mixed or plug flow	No mixing applied
Loading rate	Constant	Decreasing over time
Retention time	5 to 40 days	200 to 365 days

Table 3.2: Experimental design

Designation			Bottle contents		Initial conditions		Variables evaluated		
							Microbial community		*Substrate*
Set	Batch	Bottle name	Biomass	Substrate	COD *(g / L)*	VSS *(g / L)*	Lag time	Initial slope	Plateau
X_f									
	35°C	S_m	-	S_m	4.5	VSS(S_m)	*TA(S_m-35)*	*MP(S_m-35:S_m)*	*B_0(S_m)*
		X_f	X_f	-	COD(X_f)	10	*TA(X_f-35)*		*B_0(X_f)*
		$X_f + S_m$	X_f	S_m	4.5 + COD(X_f)	10 + VSS(S_m)		*MP(X_f-35:S_m)*	
	18°C	S_m	-	S_m	4.5	VSS(S_m)	*TA(S_m-18)*	*MP(S_m-18:S_m)*	
		X_f	X_f	-	COD(X_f)	10	*TA(X_f-18)*		
		$X_f + S_m$	X_f	S_m	4.5 + COD(X_f)	10 + VSS(S_m)		*MP(X_f-18:S_m)*	
	8°C	S_m	-	S_m	4.5	VSS(S_m)	*TA(S_m-8)*	*MP(S_m-8:S_m)*	
		X_f	X_f	-	COD(X_f)	10	*TA(X_f-8)*		
		$X_f + S_m$	X_f	S_m	4.5 + COD(X_f)	10 + VSS(S_m)		*MP(X_f-8:S_m)*	
X_o									
	35°C	S_m	-	S_m	4.5	VSS(Sm)	*TA(S_m-35)*	*MP(S_m-35:S_m)*	*B_0(S_m)*
		X_o	X_o	-	COD(X_o)	10	*TA(X_o-35)*		*B_0(X_o)*
		$X_o + S_m$	X_o	S_m	4.5 + COD(X_o)	10 + VSS(S_m)		*MP(X_o-35:S_m)*	
	18°C	S_m	-	S_m	4.5	VSS(S_m)	*TA(S_m-18)*	*MP(S_m-18:S_m)*	
		X_o	X_o	-	COD(X_o)	10	*TA(X_o-18)*		
		$X_o + S_m$	X_o	S_m	4.5 + COD(X_o)	10 + VSS(S_m)		*MP(X_o-18:S_m)*	
	8°C	S_m	-	S_m	4.5	VSS(S_m)	*TA(S_m-8)*	*MP(S_m-8:S_m)*	
		X_o	X_o	-	COD(X_o)	10	*TA(X_o-8)*		
		$X_o + S_m$	X_o	S_m	4.5 + COD(X_o)	10 + VSS(S_m)		*MP(X_o-8:S_m)*	

Notes: S_m = fresh manure

X_f = ISPAD manure

X_o = uncovered tank manure

COD(biomass) = COD in quantity of biomass containing specifies VSS

VSS(substrate) = VSS in quantity of substrate containing specified COD

Table 3.3: Characteristics of experimental manures

			Fresh manure S_m		Uncovered tank X_o		ISPAD tank X_f	
			average	standard deviation	average	standard deviation	average	standard deviation
Solids								
	TS	*g/L*	48.01	0.26	22.71	0.18	38.71	0.35
	VS	*g/L*	34.34	0.22	16.45	0.14	25.40	0.15
	FS	*g/L*	13.67	0.04	6.25	0.05	13.31	0.20
	VSS	*g/L*	27.38	1.22	11.17	0.57	24.01	0.19
	VDS	*g/L*	6.96	0.72	5.29	0.35	1.38	0.17
pH								
	pH	*pH*	6.90	0.03	7.33	0.04	7.46	0.05
COD								
	total	*g/gVS*	2.43	n/a	2.07	0.05	1.99	0.10
	soluble	*g/gVS*	0.88	n/a	0.67	0.01	0.08	0.02
VFA								
	acetic	*mg/gVS*	142.04	13.30	29.66	0.87	0.33	0.15
	propionic	*mg/gVS*	60.31	2.93	152.83	4.11	0.00	0.00
	butyric	*mg/gVS*	40.69	1.70	0.00	0.00	0.00	0.00
Anions								
	Cl^-	*mg/g VS*	33.81	0.54	40.69	0.11	21.33	0.18
	NO_2^-	*mg/g VS*	2.93	0.04	0.00	0.00	0.04	0.03
	NO_3^-	*mg/g VS*	0.00	0.00	0.00	0.00	0.00	0.00
	PO_4^{3-}	*mg/g VS*	15.61	0.24	6.88	0.20	6.94	0.05
	SO_4^{2-}	*mg/g VS*	0.00	0.00	0.00	0.00	17.38	0.29
Cations								
	Na^+	*mg/g VS*	19.19	0.23	24.05	0.32	13.43	0.29
	NH_4^+	*mg/g VS*	108.88	0.23	111.37	0.65	79.16	0.19
	K^+	*mg/g VS*	70.23	0.38	99.82	0.45	34.51	0.24
ATP								
	ATP	*μg/g VSS*	12.0	n/a	10.7	n/a	16.7	n/a
	Active	*% VSS*	0.4 – 1.2	n/a	0.3 – 1.0	n/a	0.5 – 1.6	n/a

Table 3.4: Intermediate products of anaerobic digestion in experimental manures

			Fresh manure, S_m		Uncovered tank manure, X_0		ISPAD manure, X_f	
			average	standard deviation	average	standard deviation	average	standard deviation
Solids								
	VS/FS	*kg / kg*	2.51	0.01	2.63	0.01	1.91	0.02
	VS/VS_i	*kg / kg* VS_i	1.00	0.004	1.05	0.004	0.76	0.008
	VSS / VS_i	*kg / kg* VS_i	0.80	0.04	0.71	0.04	0.71	0.016
	VDS/VS_i	*kg / kg* VS_i	0.20	0.04	0.34	0.04	0.04	0.008
VFA								
	Acetic	*g / kg* VS_i	28.4	2.66	6.2	0.18	0.05	0.02
	Propionic	*g / kg* VS_i	12.1	0.59	32.0	0.86	0.00	0.00
	Butyric	*g / kg* VS_i	8.2	0.34	0.0	0.00	0.00	0.00

Table 3.5: Methane potential of experimental manures

		Fresh manure, S_m		Uncovered tank manure, X_0		ISPAD manure, X_f	
		average	standard deviation	average	standard deviation	average	standard deviation
B_{150}	$m^3\ CH_4/kg\ VS_i$	0.27	0.01	0.23	0.004	0.10	0.01
		average	R^2	average	R^2	average	R^2
B_0	$m^3\ CH_4/kg\ VS_i$	0.27	0.99	0.22	0.99	0.08	0.87

Notes: B_{150} is the total methane released during anaerobic incubation in a BMP assay at 35°C for 150 days.

B_0 is the maximum possible methane release from the same manure, calculated by fitting a first-order equation to the data from the same BMP assay.

3.8 Figures

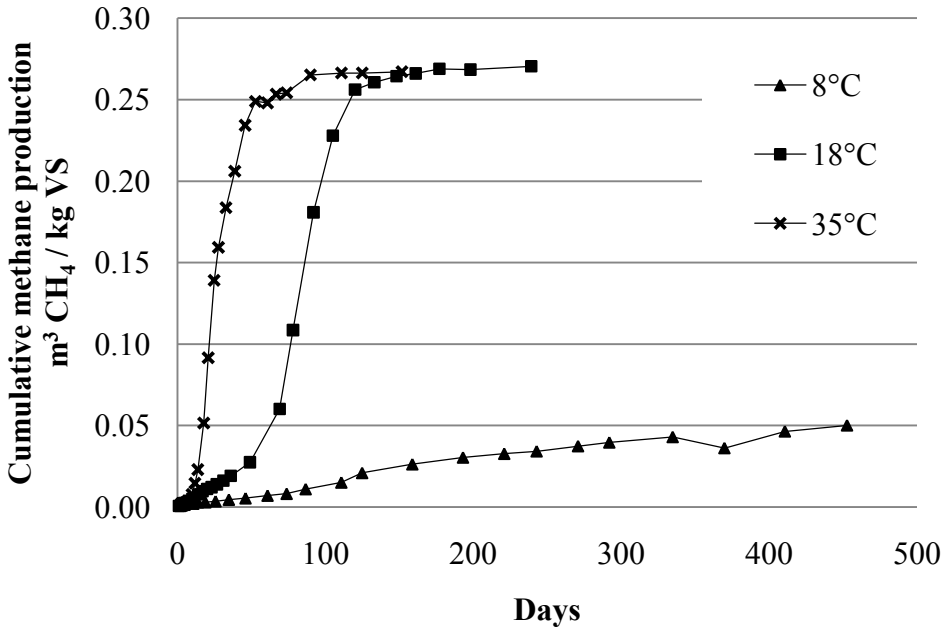

Figure 3.1: Development of methane production by the microbial community in fresh manure (Sm) during BMP incubation with fresh manure as substrate.

Notes: Data points represent the average of three replicates (bottles). Standard deviations are less than 10 % for all data points.

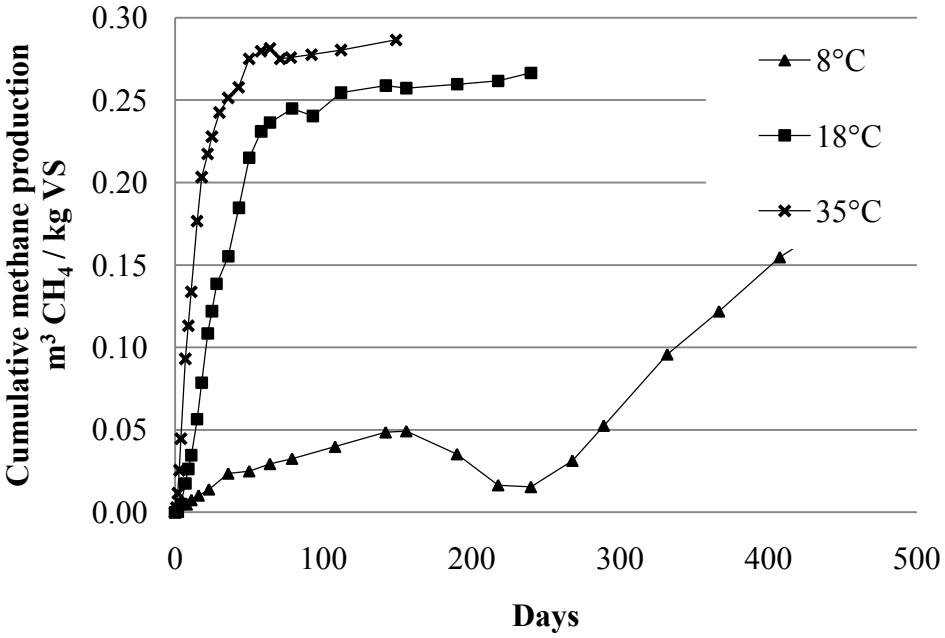

Figure 3.2: Development of methane production by the microbial community in manure from an uncovered storage tank (X_o) during BMP incubation with fresh manure as substrate.

Notes: Data points represent methane production in X_o+S_m bottles, subtracting control data from X_o bottles.
Data points represent the average of three replicates.
Standard deviations are less than 10 % for all data points.

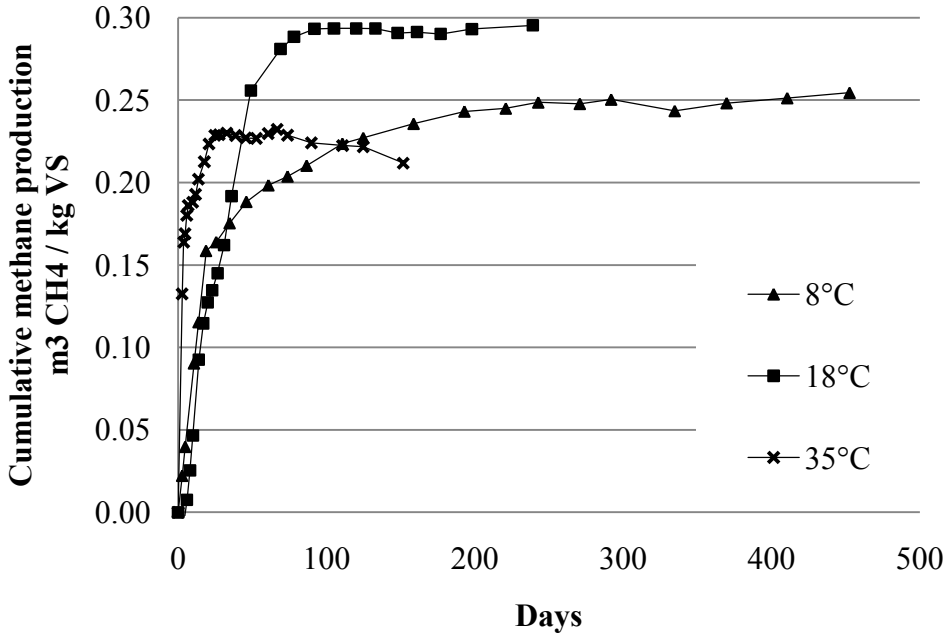

Figure 3.3: Development of methane production by the microbial community in manure from an ISPAD covered storage tank (X_f) during BMP incubation with fresh manure as substrate.

Notes: Data points represent methane production in X_f+S_m bottles, subtracting control data from X_f bottles.
Data points represent the average of three replicates.
Standard deviations are less than 10 % for all data points.

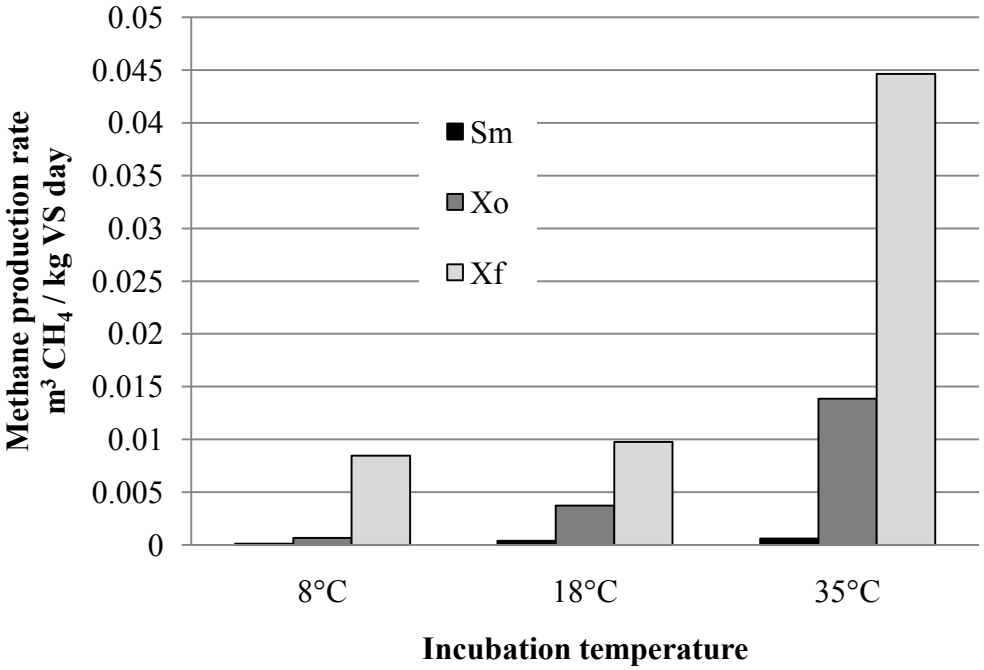

Figure 3.4: Methane production rates during the initial period of BMP incubation, by the microbial communities in fresh manure (S_m), uncovered tank manure (X_o) and ISPAD tank manure (X_f), with fresh manure as substrate.

Notes: Data points represent the average of three replicates (bottles). Standard deviations are less than 10 % for all data points.

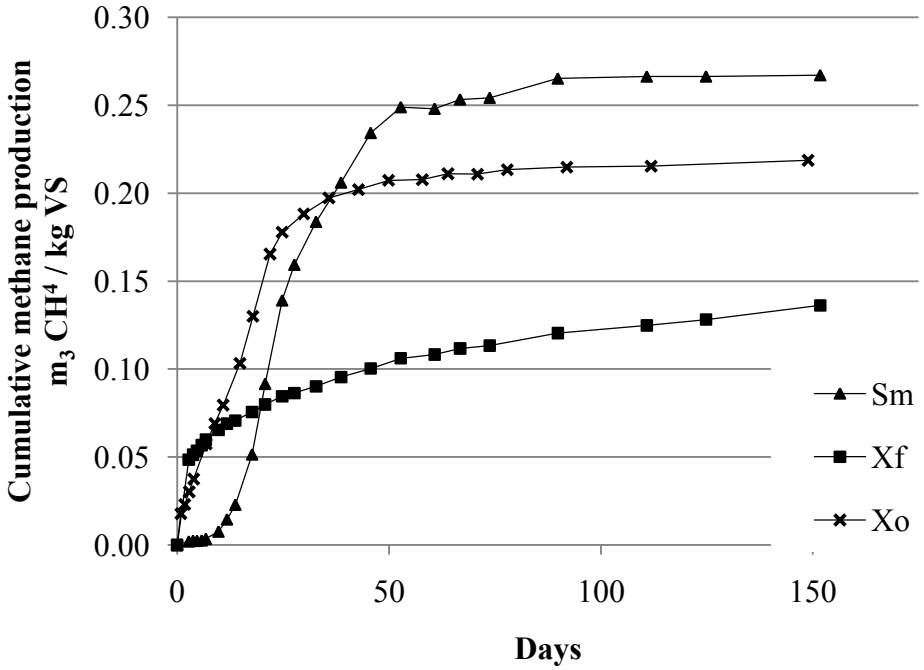

Figure 3.5: Cumulative methane production during self-fermentation BMP incubation at 35°C, with no added substrate, for fresh manure (S_m), uncovered tank manure (X_o) and ISPAD tank manure (X_f).

Notes: Data points represent the average of three replicates (bottles). Standard deviations are less than 10 % for all data points.

Chapter 4. Acclimated Microbial Kinetics

Connecting Statement

In Chapter 3, it was demonstrated that the microbial community in an ISPAD installation was acclimated to its operating conditions. This implied that the kinetics describing the stages in the ISPAD process would be different than for a typical mesophilic community. The article which follows describes how the kinetics of the main processes in ISPAD were evaluated in the laboratory, and how the results illustrate the acclimated nature of the ISPAD microbial community.

This article was submitted to the Journal of Water Research. The first contributing author, Susan M. King, designed the experiments, conducted the laboratory work, analysed the data and wrote the article. The second contributing author, Dr. Suzelle Barrington, supervised, advised on the experimental design and methods of analysis, and revised the content of the article. The third contributing author, Dr. Serge R.Guiot, supervised, advised on the experimental design and methods of analysis, and revised the content of the article. The fourth contributing author, Pierre Courvoisier, wrote the computer program for estimating kinetic coefficients and revised the related content of the article.

Abstract

In-storage psychrophilic anaerobic digestion (ISPAD) is a manure treatment system achieved by covering a concrete manure storage tank with an airtight membrane. Microbial acclimation and the long retention time of the treatment result in robust methane production during the treatment process. This suggests that ISPAD productivity could be improved to equal that of mesophilic systems if the operating parameters were optimized using a kinetic model. The objective of the present study was to obtain data for modeling by evaluating the kinetic parameters that describe the main populations in an acclimated ISPAD microbial community, and defining the variation of these parameters with temperature. This characterization was done using specific activity tests at three incubation temperatures (8, 18, and 35 °C) on five substrates (glucose, acetate, propionate, butyrate and H_2/CO_2) using three swine manure inocula (freshly produced, from an uncovered storage tank and from a three-year-old ISPAD system). Extant substrate activities were determined analytically for each case, and intrinsic kinetic parameters for glucose uptake were estimated by grid search fitting to the Monod model. The results demonstrate that the acclimated ISPAD microbial community exhibits different kinetics than the mesophilic communities currently modeled in the literature, and suggest that active psychrophilic and mesophilic microbial populations co-exist within the community. This will require development of an ISPAD-specific model.

Keywords: Psychrophilic anaerobic digestion, specific substrate uptake, manure treatment, microbial kinetics.

4.1 Introduction

In-storage psychrophilic anaerobic digestion (ISPAD) is a combined manure treatment and storage system especially well suited for livestock operations with up to 300 animal units of 1000 kg in Canada and other temperate regions (Abou Nohra et al., 2003). This un-mixed, fed-batch process occurs in concrete manure storage tanks with an air-tight floating cover when the anaerobic microbial community in the manure has acclimated to the ambient (psychrophilic) operating conditions. An ISPAD system can reduce manure solids by 24 % and release 63 % of the potential methane in the manure, compared to 50 % and 75 %, respectively, for an average mesophilic manure digester (King et al., 2010a). To optimize ISPAD performance, the process variables could be analyzed using a model. The Anaerobic Digestion Model no.1 (ADM1), developed by the International Water Association (Batstone et al., 2002), was successfully used to represent the decomposition of manure and other agricultural wastes under mesophilic conditions (Galí et al., 2009), and could be used to analyze ISPAD if the major assumptions of ADM1 are shown to apply.

The ADM1 assumes that substrate uptake parameters are available for each microbial population in the anaerobic community and that each population is mesophilic (Batstone et al., 2002). It is also assumed that microbial acidogenesis, acetogenesis and methanogenesis processes may be represented by Monod-type substrate uptake kinetics, and that the kinetic parameters describing substrate uptake by each population vary with temperature according to the Arrhenius relationship (Batstone et al., 2002).

The substrate uptake behaviour of anaerobic microbial communities is often assessed using specific activity assays, providing a profile of the populations forming the anaerobic community (Colleran et al., 1992). Substrates are selected which represent the major stages of anaerobic digestion: glucose is used as the model substrate for acidogenesis; propionate and butyrate are used for acetogenesis; acetate is used for acetoclastic methanogenesis; and H_2/CO_2 for hydrogenotrophic methanogenesis and homo-acetogenesis (Soto et al., 1993). Microbial communities acclimated to psychrophilic conditions are characterized by increased substrate uptake activity at lower temperatures, as compared to non-acclimated communities, but the increases are not uniform across substrates and temperatures (Kotsyurbenko, 2005). For example, after cultivation of granular sludge for 306 days at 10 °C, activities on acetate, propionate and butyrate increased by factors of 3.65, 1.45 and 4.15 respectively at 10 °C, and 2.44, 1.20 and 2.61 at 30 °C (Rebac et al., 1999). In another study using granular sludge, operating at 15 °C for 625 days, activities on acetate and butyrate increased at both 15 and 37 °C, while propionate activity remained low, and H_2/CO_2 activity increased continually throughout the experimental period (Connaughton et al., 2006b). Propionate activity appears to be the most sensitive to temperature change and the slowest to acclimate (Arbeli et al., 2006; McHugh et al., 2004), while the highest acclimated VFA activity is reported for butyrate (Parshina et al., 2000).

When a mesophilic anaerobic microbial community acclimates to psychrophilic conditions, such as those occurring in ISPAD, for at least a year, the component populations generally exhibit maximum substrate uptake at 35 °C,

which is taken to mean that they are still mesophilic (Connaughton et al., 2006b; Rebac et al., 1995). At the same time, greater activity increases at low temperatures indicate that these communities are psychro-active (McHugh et al., 2006). Occasionally a fully psychrophilic microbial population with temperature optimum near 15 °C is found within the community (Akila and Chandra, 2007; McKeown et al., 2009a). However, psychrophilic and mesophilic populations consuming a single substrate may also coexist in a single community (McKeown et al., 2009b; Nozhevnikova et al., 2003), in which case the substrate uptake data may exhibit a mesophilic optimum on a bi-modal curve with an inflection point at the psychrophilic optimum temperature, as it is in fact composed of two superposed uptake curves (Panikov, 1995).

Experimental substrate uptake data may be fitted mathematically to an assumed equation form, such as Monod, to estimate kinetic parameters such as the Monod half-saturation constant K_s (Goudar et al., 1999; Robinson and Tiedje, 1983). For example, non-linear regression was used to calculate kinetic parameters from substrate uptake data for acclimated psychro-active acetoclastic methanogens assuming a Michaelis-Menten type of relationship (Rebac et al., 1999). The same study noted that a key difficulty in assessing and comparing kinetic parameters from substrate uptake data is the evaluation of the size of the active microbial population involved (Rebac et al., 1999). Note that the Michaelis-Menten relationship has the same form as the Monod, though the former is usually used to describe enzyme catalyzed reactions, while the latter describes microbial substrate uptake and growth.

The goodness of fit is used to compare the suitability of different relationship forms to the process represented by the data (Bhunia and Ghangrekar, 2008). Using this approach, a study of aceticlastic methanogenesis data found that both the Monod and Haldane models were accurate at 30 °C, while the Haldane model produced a better fit between 6 and 22 °C (Lokshina et al., 2001). A similar study concluded that the Haldane model was preferred at 22 °C while at 11 °C both Haldane and a non-competitive model fit the data equally well, proposing that differences may be attributed to the role of and representation of inhibition in each model (Kettunen and Rintala, 1997).

The relationship between the maximum substrate uptake kinetic parameter, q_{max}, and temperature is usually described using the exponential Arrhenius equation (Rittmann and McCarty, 2001). Accordingly, in a study using granular sludge adapted to 10 °C for 235 days, the temperature dependence of acetate conversion was well described by an Arrhenius model; however propionate, butyrate and mixed VFA activities were better described by a square-root formulation (Rebac et al., 1995). Conversely, when digested sewage sludge was adapted to 20 °C for 4 months, the temperature dependence of the resulting methane production from acetate was poorly described by both the Arrhenius and Haldane equation forms (Kettunen and Rintala, 1997).

These results indicate that the substrate uptake kinetics of an ISPAD community cannot be estimated from known data, and illustrate that some of the ADM1 assumptions may not apply to acclimated anaerobic communities. The kinetics of ISPAD must be defined in the laboratory, and the ADM1 assumptions

tested, to determine whether the ADM1 could be used to optimize the ISPAD process. Therefore the objective of the present study was to measure the substrate uptake activities of the main microbial populations in an ISPAD microbial community, and the variation of these with temperature and substrate concentration. To accomplish this, samples of pig manure from a three-year old ISPAD installation (X_f), from a similar uncovered storage tank (X_o), and freshly produced manure (X_m) were evaluated using specific substrate activity tests, an established laboratory procedure. Assays were performed at three temperatures, 8, 18 and 35 °C, using glucose, acetate, propionate, butyrate and H_2/CO_2 as substrates. These data were analyzed to determine extant substrate uptake activity and optimal temperature for each population. Intrinsic activity was estimated by curve fitting, testing the fit of the Monod substrate uptake model and the accuracy of the Arrhenius temperature variation assumption.

4.2 Materials and Methods

Experimental manures

In 2004, a full-scale swine manure ISPAD facility was established in St. Francois Xavier (St. F-X) Quebec, Canada. This facility consisted of a circular concrete tank measuring 30 m in diameter by 3.66 m in depth, covered with an air-tight membrane (GTI, Fredericton, NB, Canada). The tank received manure from the swine facility on a regular basis. Except for a depth of 0.3-0.6 m, the contents were removed for land-spreading twice yearly. The facility had been in operation for three years when this study started in 2007. Manure from this facility (X_f) was used to represent ISPAD in this study. The two controls, freshly

produced manure (X_m) and one year old manure contained in an uncovered storage tank (X_o), were obtained from the swine research facility of the McGill University Macdonald Campus Experimental Centre (Mac), located on the Island of Montreal, Quebec, Canada. The manures from the St. F-X and Mac operations were considered comparable in terms of solids and nutrients with significant differences only in water content, as they are produced by hogs fed a standard corn and soybean based ration (Conn et al., 2007). Samples of each manure were collected in June 2007 as described previously (King et al., 2010a).

Manure characterization

Sub-samples of all three manures (X_f, X_o and X_m) were analyzed according to standard methods (APHA et al., 2005) to establish: solids, chemical oxygen demand (total and soluble) and pH. Total solids (TS) were determined by drying whole samples at 103 °C overnight in an oven (*VWR*, Sheldon Manufacturing, model 1327F, OR, USA). Volatile solids (VS) were determined by incineration of dried samples at 500 °C for two hours in a muffle furnace (*Barnstead Thermodyne*, model 48000, IA, USA). Suspended solids were separated from supernatant by centrifuging at 10000 rpm for 10 minutes at 4 °C. These samples were dried and incinerated as described above to determine volatile suspended solids (VSS) and volatile dissolved solids (VDS). Chemical oxygen demand (COD) was measured using the potassium perchromate method and a spectrophotometer (*Hach*, model DR 2800, CO, USA). The pH of all samples was determined using a pH meter (*Corning*, model 450, NY, USA).

The quantity of active microbial biomass in each manure sample was estimated using the *Luminultra* wastewater ATP kit (*Luminultra*, NB, Canada) and a luminometer (*Sirius*, model V3.2, Bethold Detection Systems, TN, USA).

Specific substrate activity tests

Three sets of substrate activity tests (SAT) were performed (Guiot et al., 1995): one using the microbial community contained in the St. F-X manure as active biomass (X_f); one using the community in the uncovered tank manure (X_o), and the third using the community in fresh manure (X_m). Each set comprised three batches, one each at 8, 18 and 35 °C. Each batch included five individual substrate assays: glucose, acetate, propionate and butyrate were the liquid substrates and H_2/CO_2 was the gaseous substrate used. All combinations were run in triplicate.

For each batch, twelve 120 ml bottles (for liquid substrates) and three 60 ml bottles (for gaseous substrate) were prepared. Manure, with its facultative biomass, was measured before being added to all the bottles, the quantity calculated to provide 5 g VSS l^{-1} for liquids and 2 g VSS l^{-1} for the gaseous substrate. Phosphate buffer was added to each bottle, to bring to 20 ml the volume of liquid in each bottle. Bottles were capped, sealed and flushed with N_2/CO_2 gas (80 % / 20%) to establish anaerobic conditions, and placed in a shaker (*New Brunswick Scientific*, Edison, NJ, USA) operating at 100 rpm (400 rpm for gaseous substrates), in a thermostatically controlled environment, in the dark. After three or four days of adjustment to the test conditions, liquid substrate was injected through the cap of each 120 ml bottle, and the 60 ml bottles were flushed

and pressurized to 140 kPa with H_2/CO_2. The first sample was immediately taken, either 0.5 ml of liquid or 100 µl of gas. Liquid samples were centrifuged to remove solids. Sub-samples of supernatant were taken for glucose analysis, and others were diluted 5-fold for VFA analysis. Gaseous samples were analysed immediately by gas chromatograph. Sampling was repeated at regular intervals during each assay period. At the end of each assay, the bottle contents were analyzed to determine solids and pH.

Analytical methods

Glucose was measured using high pressure liquid chromatography (*Waters Chromatography Division*, Milford, MA, USA) equipped with an injector (model 717+), photodiode array detector (model 2996), pump (model 600), refractive index detector (model 2414). The column used for the separation was ICSep IC ION-300 column (*Transgenomics*, San Jose, CA, USA) of 300 mm × 7.8 mm id and an ion guard GC-801 column (*Transgenomics*). The mobile phase consisted of 0.035 N H_2SO_4 at a pH of 4, flowing at a rate of 0.4 ml min^{-1}. The measurements were conducted using a wavelength of 210 nm. Analysis was carried out at 35 °C.

Acetic, propionic and butyric acid concentrations were measured using a gas chromatograph (*Agilent*, model 6890, Wilmington, DE, USA) equipped with a flame ionization detector of 0.2 µl. The samples were fortified at a ratio of 1:1 (by volume) using an internal standard of *iso*-butyric acid dissolved in 6 % formic acid. These were directly injected into a glass column of 1 mm × 2 mm *Carbopack C* (60 to 80 mesh) coated with 0.3 % *Carbowax* 20M and 0.1 %

H_3PO_4. The column was held at 130 °C for 4 minutes and helium as carrier gas was injected at a rate of 20 ml min^{-1}. The injector and the detector were both maintained at 200 °C.

To quantify hydrogen consumption, biogas composition (H_2, N_2+O_2, CH_4, CO_2) was measured using a gas chromatograph (*Hewlett Packard*, 6890 Series, Wilmington, DE, USA) equipped with a thermal conductivity detector and a 900 mm × 3mm 60/80 mesh *Chromosorb* 102 column (*Supelco*, Bellefonte, PA, USA).

Data Manipulation and Computations

All gas measurements were corrected to standard temperature and pressure of 0 °C and 101.3 kPa, according to the ideal gas law. For each assay, substrate concentration was plotted versus time. The extant substrate uptake rate was then defined as the maximum slope of the substrate concentration versus time curve, within the initial period for each assay bottle. This substrate uptake rate was then divided by the VSS concentration of the appropriate bottle, to give the extant activity, q_{ext}, of each population within each community. In cases where the curve had a suitable form, the entire curve was fitted to a Monod-type equation to extract intrinsic kinetic parameters (K_s, Y and q_{max}) for each population.

Initial period definition

Several authors mention that microbial growth during a specific activity assay is to be avoided, suggesting that activity measurements be restricted to the first few hours of the assay (Coates et al., 1996; Colleran et al., 1992; Soto et al., 1993). Others have suggested that activity measured during the first hours of an

assay be called *extant*, while activities during the growth phase of an assay be termed *intrinsic* (Grady et al., 1996; Kovarova-Kovar and Egli, 1998). In this study, the initial assay period used to assess extant activities was defined as equal to one doubling time for each specific population under examination, at the temperature of the assay.

The doubling time values for 35 °C were determined based on ADM1 model parameters (Batstone et al., 2002). The appropriate values for 18 and 8 °C were extrapolated using the common Q10 assumption, that reaction rate roughly doubles for a temperature increase of 10 °C. This has been shown to be a reliable estimate for many microbial processes, including anaerobic digestion, in the temperature range of 5-40 °C (Loehr, 1984). This approach assumes that the rate of a reaction r_2 at temperature T_2 may be determined from a known reaction rate r_1 at temperature T_1, as follows:

$$r_2 = r_1 * 1.072^{(T2-T1)} \qquad (1)$$

The ADM1 documentation lists maximum substrate uptake rate, q_{max}, and microbial yield, Y, for each population at 35 °C, which are related to the maximum growth rate, μ_{max35}, by the relationship:

$$\mu_{max35} = q_{max} \times Y \qquad (2)$$

The doubling time at 35 °C, d_{35}, may then be calculated from:

$$d_{35} = \ln(2) / \mu_{max35} \qquad (3)$$

The growth rates at 18 and 8 °C, respectively μ_{max18} and μ_{max18}, are then calculated using Equation (1), and corresponding doubling times d_{18} and d_8 from Equation (3). The values of q_{max} and Y from the ADM1 documentation and the

calculated growth rates and doubling times for each population are presented in Table 4.1.

Estimation of kinetic parameters

Kinetic parameters were estimated by fitting the substrate uptake data to the Monod-type equations for population growth and substrate uptake (McCarty and Rittmann, 2001):

$$x = x_i + Y (S_i - S) \qquad (4)$$

$$dS/dt = (q_{max} \times S \times x) / (K_s + S) \qquad (5)$$

In these equations, x is the concentration of active microbial biomass in g l^{-1}, Y is the yield of microbial biomass from the substrate in g biomass (g substrate)$^{-1}$, S is the concentration of substrate in g l^{-1}, q_{max} is the maximum substrate uptake rate in g substrate (g biomass day)$^{-1}$ (equivalent to k_m in the ADM1 documentation) and K_s is the half-saturation constant in g substrate l^{-1}. The subscript "i" indicates the initial value of a parameter.

The method of fitting was developed using the means of the triplicate glucose consumption data for the uncovered tank biomass, X_o, which had a clearly defined Monod-type form at each assay temperature. The ADM1 literature values of K_s, Y and q_{max} for glucose-consumers were used with the 35 °C data to find a starting value for x_i. Values for all four variables were then optimized using the grid search method, minimizing the sum of squares error between the calculated value and the experimental data. This method finds local minima but can be trapped in a local minimum and miss the global minimum. However, as fitted values were expected to be in the same order of magnitude of the ADM1

values, this was assumed to be acceptable. The first iteration step size was 15 % of the initial parameter value. For each subsequent iteration the step size was halved. The iterative process was stopped when the change in sum of squares error (SSE) from the previous iteration was less than 5 % of the previous SSE value. A maximum of seven iterations were performed, at which point the step size was 0.12 % of the initial value.

Because the data contained a small number of points, the algorithm tended to come up with extreme values that fitted the data well, but did not reduce the SSE by more than 5 %. These values were also unrealistic in terms of the shape of the curve and the difference from the starting literature values. To control this behaviour, the grid search method was modified to keep the values of the parameters within reasonable boundaries. Boundary values for q_{max}, Y and K_s were selected from those used by the ADM1 model (Batstone et al., 2002), while x_i was kept within one order of magnitude from the starting value. The optimized parameter values for the 35 °C data were then used as starting values for the 18 and 8 °C data sets to optimize K_s and q_{max} for each temperature, with the value of Y and the ratio of x_i to VSS kept constant throughout. Once the method was perfected, it was applied with the same algorithms, starting points, step sizes and assumptions to the glucose consumption data sets for the fresh manure and the ISPAD tank communities. For each optimization, the starting and boundary values for the optimized parameters are presented in Table 4.2.

Statistical Analysis

All statistical analyses were performed using SAS 9.0 (2009, SAS Institute inc., Cary NC, USA).The characterization data were collected in triplicate, on sub-samples from the composite sample taken from each tank. Significance of differences between these measured values was evaluated by the Student-Newman-Keuls method in a simple analysis of variance based on a completely randomized design. The specific substrate activity assays used a randomized complete block design, considering microbial community type (X_f, X_o and X_m) as the treatment factor, temperature (35, 18 and 8 °C) as the block factor, with substrate uptake rate (q_{ext}) as the dependant variable. Treatments were assigned randomly to experimental units (bottles), and all treatment-block combinations were completed in triplicate.

4.3 Results and Discussion

Characterization of experimental manures

Table 4.3 lists the characteristics of the three manures used as sources of active biomass in the specific activity assays. The fresh manure, X_m, has the highest concentration of total solids (TS), while X_f and X_o are respectively 19 % and 52 % less concentrated than X_m. This is due to the fact that wash water is periodically flushed into both storage tanks, diluting both X_f and X_o, while the uncovered tank containing X_o is further diluted by accumulated rain and snow. For both X_m and X_o, volatile solids (VS) represent 72 % of TS, while X_f shows a reduction of VS to 66 % of TS. This is the result of anaerobic digestion consuming VS in the ISPAD tank (King et al., 2010a). The data subdividing VS

into suspended and dissolved fractions, VSS and VDS, provide further information about processes occurring in the storage tanks. The substrate for anaerobic digestion, VDS, represents 20 % of VS in fresh manure, X_m. In the uncovered tank manure, X_o, this has increased to 32 % indicating that hydrolysis is breaking down VSS to VDS, but anaerobic digestion is not consuming the resulting VDS. In the ISPAD manure, X_f, VDS is conversely reduced to 5 % of VS, which itself has also decreased as mentioned above, indicating active anaerobic digestion in the tank. Soluble COD, which also represents substrate, is steady at 32 % of total COD in both X_m and X_o, and reduced to 4 % in X_f, confirming these trends. The pH of all three manures is similar and thus does not need to be considered a variable in the analysis of substrate uptake data. However, the presence of soluble substrate (VDS) in the manure for X_m and X_o may interfere with the SAT procedure that endeavours to control precisely the availability of substrate.

The ATP analysis results indicate that the active microbial communities in the three manures are similar in size and account for less than 2 % of the VSS. However, this data may only be considered preliminary, because repeated assays with different dilutions of manure (data not shown) did not produce consistent results, suggesting the presence of inhibition in the assay. In terms of the substrate activity assays (SAT) conducted using these materials as sources of active biomass, this similarity in community size means that activity results for the three communities, presented in terms of VSS, may be directly compared.

Typical substrate activity test (SAT) results

There were three main types of response observed in the substrate uptake data from the SAT assays. They may be classified as exponential, linear and accumulation, though a few of the data-sets display patterns of intermediate forms. Table 4.4 lists the response type for each biomass-substrate-temperature combination. Samples of each response type are illustrated in Figures 4.1 to 4.4.

Figure 4.1 illustrates the glucose activity of the uncovered tank biomass, X_o, at all three temperatures, 8, 18 and 35 °C. Each curve is of the exponential form associated with the Monod model of microbial activity, with a low-slope initial uptake rate increasing in an exponential fashion representing microbial growth to a steeply sloping final uptake rate. The shape of the curve is stretched out in time as the assay temperature decreases, which confirms that the activity of the biomass decreases with temperature. The shallow slope at the beginning of each curve represents the extant activity of the glucose-consuming acidogen population, while the exponential phase which follows illustrates the response of the fastest-growing members of the population to the ideal conditions of the assay bottle. This part of the data may be used to extract intrinsic kinetic parameters representing this portion of the population.

Figure 4.2 illustrates butyrate activity for the ISPAD tank biomass, X_f, at all three temperatures. The nearly linear form of these data-sets suggests a robust butyrate-consuming acetogen population that consumes the butyrate quickly without significant growth. The rate of butyrate consumption decreases toward the end of the assay as the low remaining concentration becomes limiting. This

type of data-set can be used to evaluate the initial, extant activity rate, but does not illustrate the growth pattern required to allow for estimation of intrinsic kinetic parameters.

There were a few instances of a combined linear-exponential response, in which the data-set began with a strong steady slope that did not level off as the substrate became limiting, but demonstrated a mild growth phase near the end of the assay. This response type is illustrated in Figure 4.3 for propionate consumption by the ISPAD biomass, X_f, at 35 °C. As with the linear activity response, this data-set provides a good estimation of the extant propionate-consuming acetogen activity, but may not have sufficient exponential characteristics to allow the corresponding intrinsic parameters to be determined.

The two accumulation response types are illustrated in Figure 4.4. The fresh manure biomass, X_m, with acetate as substrate shows an overall upward trend at 18 °C, indicating accumulation of the substrate, while the corresponding 35 °C dataset shows initial accumulation, followed by population growth and consumption of the substrate. Because acetate is an intermediate substrate in the anaerobic digestion process, the net accumulation may include a modest rate of consumption by the methanogens that is not visible in these assays; however, because the microbial community occurs in manure, it cannot easily be separated from the manure organic matter which is the source of acetate production. This suggests that the SAT method, which was developed for granular microbial communities, should be modified for analysis of those associated with manure. Specifically, the ratio of active biomass to substrate concentration must be

established by a more accurate measure than VSS, which in this study contained less that 2 % active biomass, and means of increasing this ratio must be developed.

Substrate uptake activities

The extant substrate uptake activities, q_{ext}, for all substrate-biomass combinations are presented in Table 4.5. The progress of acclimation from fresh manure, X_m, through the uncovered tank, X_o, to the ISPAD tank, X_f, is demonstrated by the values in this table. The fresh manure community, X_m, showing measureable activity on glucose and H_2/CO_2 only, does not contain a well developed anaerobic community. The community in X_o, being active also on butyrate at all assay temperatures, and on acetate and propionate at 35 °C, is semi-acclimated to ambient conditions, as butyrate activity is developed preferentially, followed by the development of acetate and then propionate activity later in the acclimation process (Parshina et al., 2000). In the ISPAD tank, X_f, which is active on all substrates tested and at all assay temperatures, is a fully acclimated balanced anaerobic microbial community (Kotsyurbenko, 2005). As reported by other researchers, the variations between communities, substrates and temperatures are not uniform, and the values tend to support the mesophilic optimum assumption, with maximum activities at 35 °C. The H_2/CO_2 activity of X_o, which increases with decreasing temperature, is the exception. In this case, the test procedure may have been a limiting factor, or it may be a further indication of acclimation occurring in the uncovered tank: While homoacetogens were reported to have a larger role in low-temperature digestion than in the mesophilic

process (Vavilin et al., 2000), it has been proposed more recently that homoacetogen activity may be highest during the acclimation process, and reduced in a fully acclimated psychro-active community (Kotsyurbenko, 2005). These results confirm the conclusions of a previous study, which reported on the basis of methane production that X_m contained a minimally active anaerobic community, while X_o was semi-acclimated compared to the fully acclimated ISPAD community, X_f (King et al., 2010a).

The other issue evident in Table 4.5 is the magnitude and variety of standard deviations among the assays. This can be understood as a compound error, because substrate concentration, time elapsed and VSS concentration are all measured factors exposed to experimental error, which combine to determine each specific substrate activity. Thus the errors, in some cases, may be additive and, in others, may be subtractive, resulting in the wide variety of deviations reported. Despite this variation, the data illustrate trends that have been observed by other researchers, which strengthen their validity. In fact, substrate uptake activities are known to be highly variable, as many related parameters used by ADM1 report up to 300% variability (Batstone et al., 2002). For the modeling of ISPAD, the elaboration of a modified substrate activity test protocol for manure microbial communities could improve the accuracy of the resulting kinetic parameters.

Kinetic parameters and temperature

Glucose consumption was used for the fitting exercise, since the Monod equations may only be fitted to data sets with an exponential form. Table 4.6

presents the resulting fitted kinetic parameters q_{max}, Y and K_s, for glucose consumption by each of the three studied microbial communities, as well as the population size estimate, x_i. The population sizes, representing 0.03-0.05 % VSS, are much smaller than those from the preliminary ATP analysis (Table 4.3), which is to be expected since only a small number of the microbial species present are expected to be glucose consumers. However, because the values obtained are similar for the three communities, this confirms that the comparison of activities and kinetic parameters for the different communities studied, based on VSS, is a valid approach.

For X_o and X_m, the maximum substrate uptake rate, q_{max}, at 35 °C remains equal to the literature value presented in Table 4.2, at 39.12 g substrate (g biomass day)$^{-1}$, while X_f exhibits a 33 % increase to 51.84 g substrate (g biomass day)$^{-1}$. This increase in the processing efficiency of the glucose-consuming population within X_f illustrates acclimation to the substrate. There is further evidence at 18 and 8 °C, where X_f shows increases of 220 % and 90 % respectively in comparison to the values calculated for X_m at these temperatures. These greater increases at lower temperatures indicate acclimation to the psychrophilic operating temperature of ISPAD, as reported by other researchers (Connaughton et al., 2006b; Rebac et al., 1999).

The q_{max} values for X_o, while equal to X_m at 35 °C, show modest increases of 20 and 44 % at 18 and 8 °C, respectively. This suggests again that X_o contains a partially acclimated population. In addition, as illustrated in Figure 4.5, the values of q_{max} for X_o and X_m describe an exponential Arrhenius-type curve with

temperature, indicating that the population in X_o remains primarily mesophilic. In contrast, for X_f the relationship between q_{max} and temperature appears to be curved in the reverse direction. Assuming that the q_{max} values for X_m represent an average mesophilic population, these 'mesophilic' values were subtracted from the q_{max} values of X_f to produce the 'psychrophilic' values, both illustrated in Figure 4.6, which represent the effect of the temperature acclimation process on the ISPAD microbial community. With the clear peak near 18 °C, these 'psychrophilic' q_{max} values for X_f may illustrate the development of a true psychrophilic population coexisting with the original mesophilic population. This concept is corroborated by recent reports of similar results (McKeown et al., 2009b; Nozhevnikova et al., 2003). Assessing q_{max} at other intermediate temperatures could further define the relationship. The coexistence of two populations such as these performing the same function with different kinetics would have to be explicitly included in an ISPAD process model. One approach to modeling microbial diversity that could be appropriate, using multiple microbial populations for each substrate, has recently been applied to the ADM1(Ramirez et al., 2009).

The microbial yield exhibits a similar pattern, with Y for X_o and X_m identical at 0.128 g biomass/g substrate, while the Y value for X_f is 35 % lower. This indicates a change in cellular metabolic processing, as the glucose consuming population in X_f produces less microbial biomass from each gram of substrate consumed, while concurrently consuming more substrate. The excess consumed substrate, then, is converted to intermediate products in the anaerobic

digestion process, VFA. This contributes eventually to the increase in methane production previously reported for the ISPAD biomass (King et al., 2010a). Because Y is expected to resist change for any given species (Nedwell, 1999), this effect may represent a change in the predominance of species making up the glucose-consuming population as it acclimates to the ISPAD operating conditions. Follow-up molecular biology investigation of the ISPAD microbial community is planned to illuminate this possibility.

The apparent half-saturation constant, K_s, indicates the concentration at which the population is able to process the substrate at half its maximum rate, which represents the affinity of the microbial population for the substrate. The implication is that, at concentrations lower than K_s, processing is slow and inefficient, and may result in accumulation. Continuing the pattern seen with q_{max} and Y, the K_s values obtained for X_m remain similar to the original literature values representing a standard mesophilic population. The values of K_s representing X_f are 90 - 96 % lower, showing that the glucose consuming population in X_f is able to efficiently utilize substrate even at concentrations a full order of magnitude lower than the standard mesophilic population. This indicates that the ISPAD microbial community in X_f is acclimated not only to temperature and substrate, but also to the low substrate concentrations which occur in the lightly-fed ISPAD tank. The uncovered tank community in X_o, with a low K_s comparable to that of X_f at 35 °C, illustrates the beginning of the acclimation process, while the higher values similar to X_m at 18 and 8 °C indicate that the community in X_o remains only semi-acclimated and primarily mesophilic.

4.4 Conclusions

Analysis of the substrate uptake kinetics, defined in the laboratory, of an ISPAD microbial community and two control communities, to test the major assumptions of the ADM1 model, revealed that:

i. While glucose uptake data for microbial communities contained in manure was well defined using the established Substrate Activity Test (SAT) protocol, data for intermediate substrate/products was less conclusive due to the high manure substrate content, and low biomass concentration. For future modeling work using manure, the SAT protocol needs to be modified to extract the uptake data for intermediate substrate/products.

ii. Extant substrate uptake activities of the ISPAD populations appear to have mesophilic optima, but the intrinsic kinetic parameters for glucose suggest that in fact robust psychrophilic and mesophilic populations coexist within the community. This should be further investigated to allow inclusion of both populations in a future process model.

iii. The Monod representation of substrate uptake appeared accurate for glucose uptake, but could not be verified for volatile fatty acids (VFA). For modeling, further investigations are required in conjunction with the development of a modified SAT protocol, to permit this confirmation.

iv. Although valid between maximum substrate uptake and temperature for the non-acclimated control communities, the Arrhenius relationship did

not apply to the fitted kinetic parameters for glucose uptake by the ISPAD community. However, more experimental data may define separate Arrhenius curves, useful for future modeling, for the apparent co-existing psychrophilic and mesophilic populations.

The ADM1 model cannot be used to simulate the acclimated microbial community in an ISPAD manure tank, because its main assumptions, investigated in this study, do not apply to the acclimated ISPAD microbial community. However, several interesting avenues for further research have been identified, which could lead to development of a satisfactory ISPAD process model. Future work should evaluate other modeling approaches and develop protocols for substrate uptake parameter estimation that are better suited to manure microbial communities.

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4.7 Tables

Table 4.1: Initial periods for determination of extant substrate activity from SAT assays

	q_{max} [a,b]	Y [a,c]	**Initial period** *(hours)*		
			35 °C	18 °C	8 °C
Glucose	30	0.10	6	18	36
Butyrate	20	0.06	14	45	91
Propionate	13	0.04	32	104	209
Acetate	8	0.05	42	136	272
H_2	35	0.06	8	26	52

[a](Batstone et. al. 2002)

[b]Maximum substrate uptake rate *(COD $COD^{-1}d^{-1}$)*

[c]Microbial yield *(COD COD^{-1})*

Table 4.2: Initial parameter estimates and boundary values for glucose consumption at 35 °C

	Minimum	Starting value	Maximum
q_{max} [a,b]	38.5	39.8	165.9
Y [a,c]	0.008	0.075	0.128
K_s [a,d]	0.022	0.469	0.591
x_i [e]	0.1	1.0	10.0

[a](Batstone et. al. 2002)

[b]Maximum substrate uptake rate *(g glucose (g biomass)$^{-1}$ day^{-1})*

[c]Microbial yield *(g biomass (g glucose)$^{-1}$)*

[d]Half-saturation constant *(g glucose l^{-1})*

[e]Biomass concentration (*% VSS)*

Table 4.3: Characteristics of experimental manures

	Fresh manure, X_m	**Uncovered tank, X_o**	**ISPAD tank, X_f**
TS *(g l^{-1})*	48.0 (0.3)	22.7 (0.2)	38.7 (0.4)
VS *(g l^{-1})*	34.3 (0.2)	16.5 (0.1)	25.4 (0.2)
FS *(g l^{-1})*	13.7 (0.1)	6.3 (0.1)	13.3 (0.2)
VSS *(g l^{-1})*	27.4 (1.2)	11.2 (0.6)	24.0 (0.2)
VDS *(g l^{-1})*	7.0 (0.7)	5.3 (0.4)	1.4 (0.2)
pH	6.9 (0.1)	7.3 (0.1)	7.5 (0.1)
Total COD *(g (g VS) $^{-1}$)*	2.4 (nd)	2.1 (0.1)	2.0 (0.1)
Soluble COD *(g (g VS) $^{-1}$)*	0.9 (nd)	0.7 (0.01)	0.1 (0.02)
ATP *(μg (g VSS) $^{-1}$)*	12.0	10.7	16.7
Active biomass *(% VSS)*	0.4 – 1.2	0.3 – 1.0	0.5 – 1.6

(standard deviation in brackets)

(nd) = not determined

Table 4.4: Form of substrate uptake response curve for each SAT assay

		Fresh manure, X_m	Uncovered tank, X_o	ISPAD tank, X_f
Glucose				
	35 °C	E	E	E
	18 °C	E	E	E
	8 °C	L/E	E	E
Butyrate				
	35 °C	A/E	L	L
	18 °C	A	L	L
	8 °C	A	L	L
Propionate				
	35 °C	A/E	L	L/E
	18 °C	A	A	E
	8 °C	A	A	L
Acetate				
	35 °C	A/E	L/E	L
	18 °C	A	A	L
	8 °C	A	A	L
H_2/CO_2				
	35 °C	E	E	L
	18 °C	E	E	L/E
	8 °C	E	L	E

E = exponential

L = linear

A = accumulating

Table 4.5: Extant substrate activities from SAT assays

		Fresh manure, X_m	Uncovered tank, X_o	ISPAD tank, X_f
Glucose				
	35°C	55 (68)	307 (243)	128 (100)
	18°C	36 (23)	-	87 (12)
	8°C	12 (1)	10(3)	7 (7)
Butyrate				
	35°C	-	18 (29)	204 (37)
	18°C	-	81 (1)	105 (23)
	8°C	-	12 (8)	10 (2)
Propionate				
	35°C	-	24 (35)	32 (10)
	18°C	-	-	7 (9)
	8°C	-	-	12 (5)
Acetate				
	35°C	-	83 (254)	316 (135)
	18°C	-	-	28 (9)
	8°C	-	-	47 (10)
H_2/CO_2				
	35°C	14 (7)	-	131 (225)
	18°C	-	8 (1)	59 (24)
	8°C	-	52 (5)	1 (0.3)

Activities in *(mg (g VSS)$^{-1}$ day^{-1})*

(standard deviation in brackets)

"-" = no measurable activity

Table 4.6: Fitted kinetic parameters, biomass concentration and error for glucose consumption

		Fresh manure, X_m	Uncovered tank, X_o	ISPAD tank, X_f
q_{max} [a]				
	35°C	39.12	39.12	51.84
	18°C	10.01	12.02	31.92
	8°C	3.10	4.47	5.90
Y [b]				
	35°C	0.128	0.128	0.082
	18°C	0.128	0.128	0.082
	8°C	0.128	0.128	0.082
K_s [c]				
	35°C	0.238	0.022	0.022
	18°C	0.776	0.574	0.029
	8°C	0.679	0.469	0.029
x_i [d]				
	35°C	7.16	5.26	7.41
	18°C	7.16	5.26	7.41
	8°C	7.16	5.26	7.41
e^2 [e]				
	35°C	0.021	0.433	0.076
	18°C	0.036	0.192	0.003
	8°C	0.013	0.049	0.003

[a]Maximum substrate uptake rate *(g glucose (g biomass)$^{-1}$ day^{-1})*

[b]Microbial yield *(g biomass (g glucose)$^{-1}$)*

[c]Half-saturation constant *(g glucose l^{-1})*

[d]Biomass concentration *(mg biomass l^{-1})*

[e]Sum-of-squares error *(g glucose l^{-1})^2*

4.8 Figures

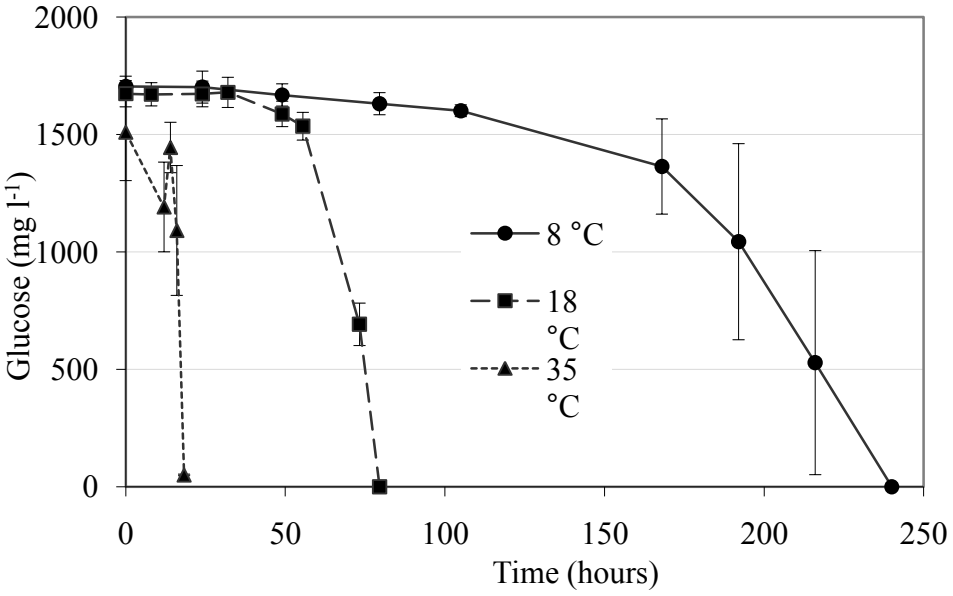

Figure 4.1: Exponential glucose uptake by uncovered tank biomass, X_o.

Notes: Data points represent the average of three replicates (bottles)
Error bars represent +/- one standard deviation

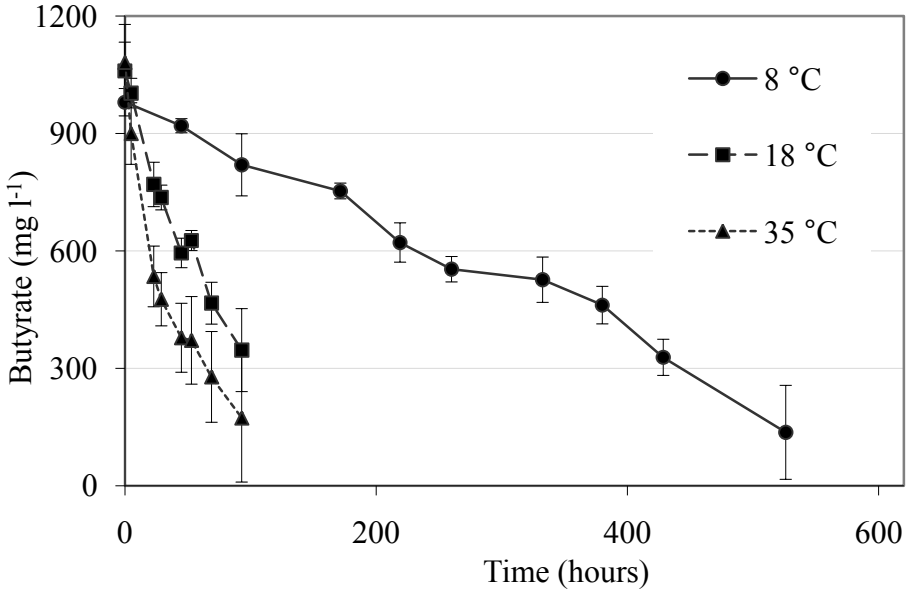

Figure 4.2: Linear butyrate uptake by ISPAD tank biomass, X_f.

Notes: Data points represent the average of three replicates (bottles)
Error bars represent +/- one standard deviation

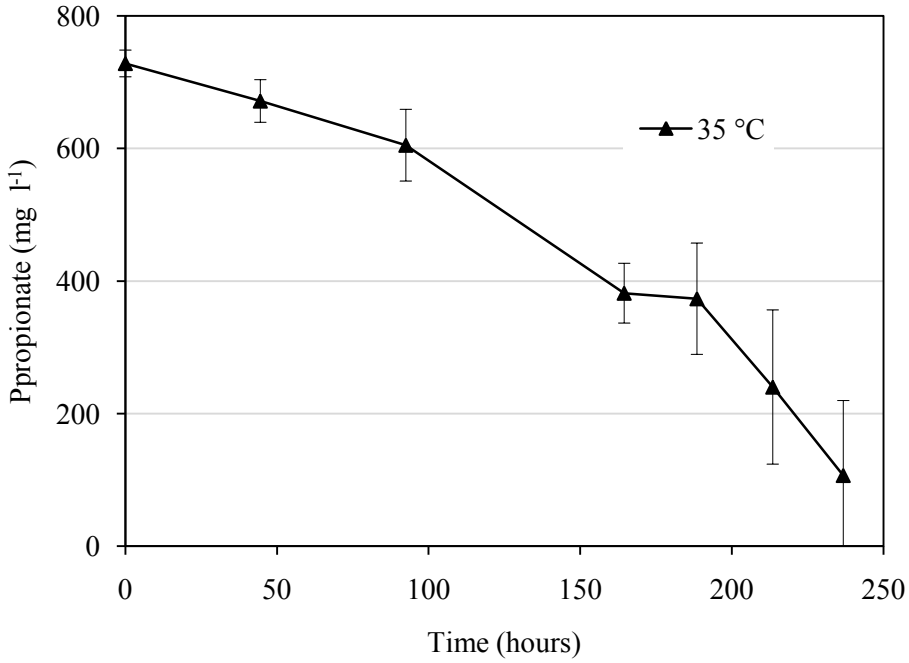

Figure 4.3: Linear-exponential propionate uptake by ISPAD tank biomass, X_f.

Notes: Data points represent the average of three replicates (bottles)
Error bars represent +/- one standard deviation

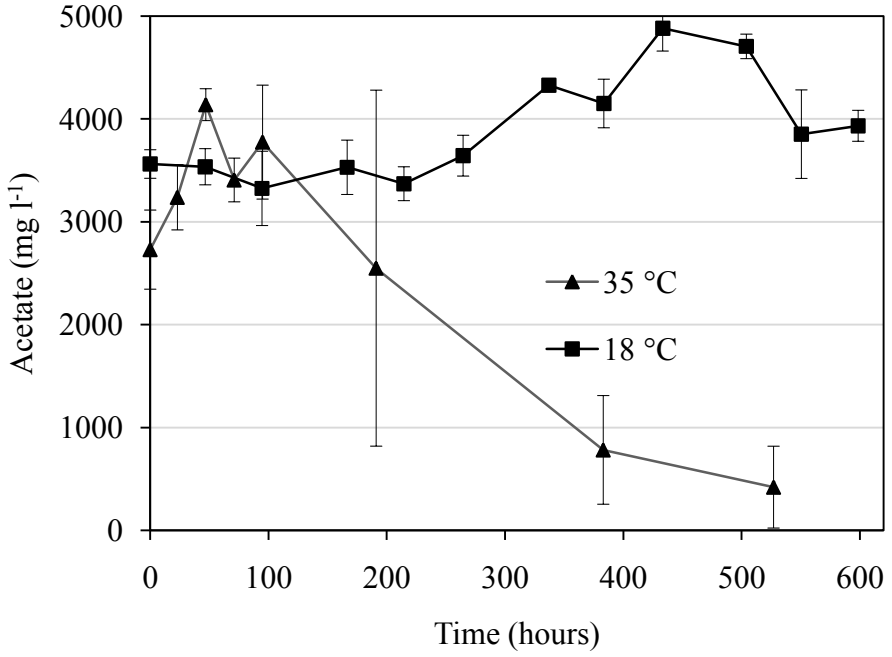

Figure 4.4: Accumulating and accumulating-linear acetate uptake by fresh manure biomass, X_m.

Notes: Data points represent the average of three replicates (bottles)
Error bars represent +/- one standard deviation

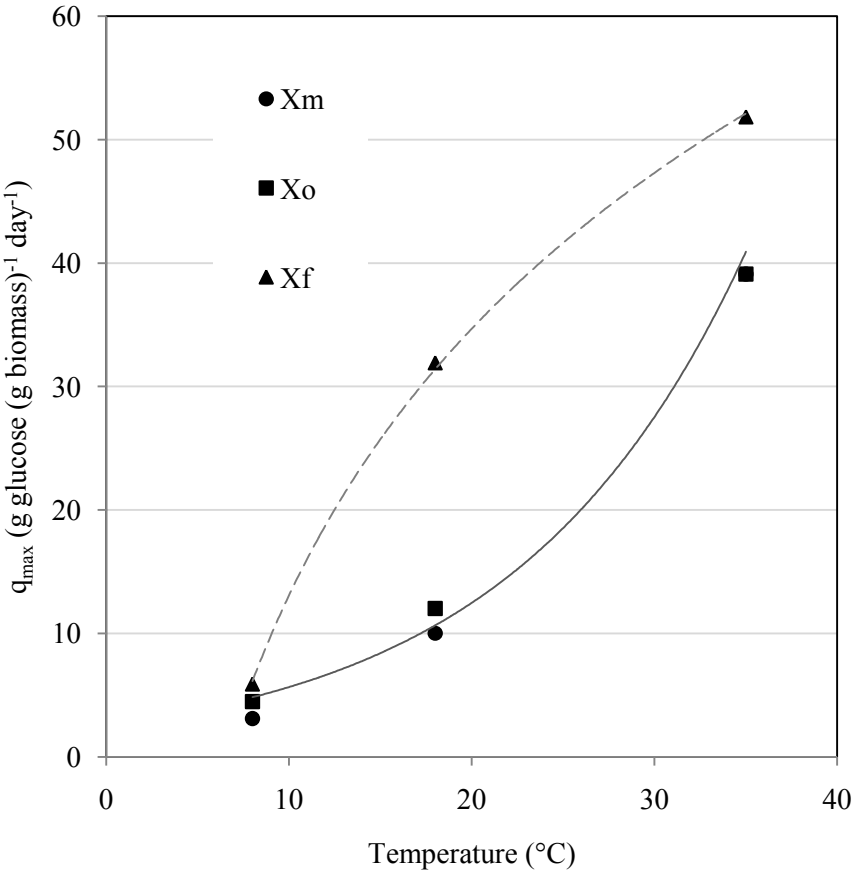

Figure 4.5: Maximum glucose uptake rates, q_{max}, for fresh manure biomass, X_m, uncovered tank biomass, X_o, and ISPAD tank biomass, X_f.

Notes: Data points represent the average of three replicates (bottles)
Data are derived by curve fitting
See table 4.6 for error data for the fitting process

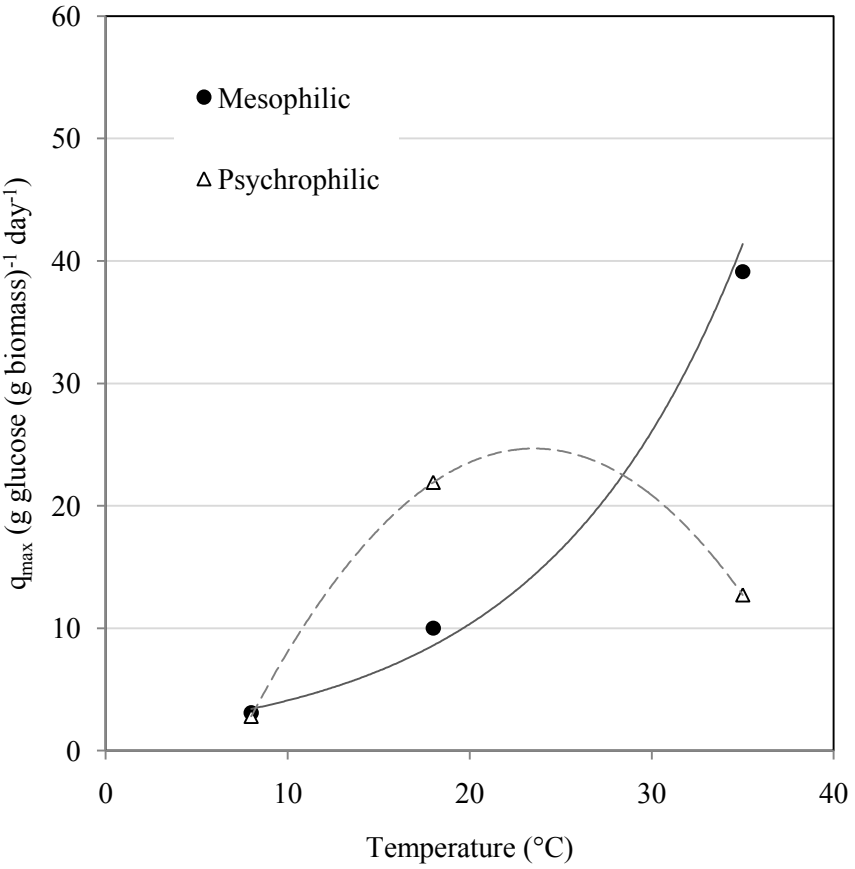

Figure 4.6: Maximum glucose uptake rates, q_{max}, for possible coexisting mesophilic and psychrophilic populations within the ISPAD tank biomass, X_f.

Notes: Data points represent the average of three replicates (bottles)
Data are derived by curve fitting
See table 4.6 for error data for the fitting process

Chapter 5. Protein Degradation Kinetics

Connecting Statement

In Chapter 4, the temperature-acclimated nature of the ISPAD microbial community was illustrated by the results of a kinetic study focusing on the carbohydrate degradation pathway. The article which follows extends this line of inquiry to the protein degradation pathway, describing the laboratory protocol that was developed for this purpose, and the results of the study, which indicate that the microbial populations that degrade proteins in ISPAD remain mesophilic in nature.

This article will be submitted to Journal of Bioresource Technology. The first contributing author, Ms. Susan M. King, designed the experiments, conducted the laboratory work, analysed the data and wrote the article. The second contributing author, Dr. Suzelle Barrington, supervised, advised on the experimental design and methods of analysis, and revised the content of the article. The third contributing author, Dr. Serge R.Guiot, supervised, advised on the experimental design and methods of analysis, and revised the content of the article.

Abstract

In-Storage Psychrophilic Anaerobic Digestion of swine manure develops by acclimation of its microbial community, producing methane from the organic matter, and ammonia from the portion which is protein. The ammonia content of the treated manure is an important component of its fertilizer value. To estimate this value for treated manures, the protein degradation pathway must be understood. This laboratory study using manure from a 5-year-old full-scale ISPAD installation investigated the rates of key processes in this pathway, their variation with temperature and the occurrence of interaction between these and other components of the anaerobic digestion process. A specific proteolytic activity assay was developed for this purpose, based on the specific substrate uptake activity assay commonly used to study anaerobic digestion processes. These assays were performed over the range of 8 to 35 °C. The results indicated that the microbial protein degrading population remained mesophilic, exhibiting low degradation rates in the psychrophilic temperature range; however combined with the long retention time of the ISPAD process, approximately 64% manure proteins were effectively degraded. The produced ammonia-N was conserved in the ISPAD tank. This should be available for crops when the treated manure is applied to cropland, providing an economic benefit to producers using this technology.

Keywords: protein degradation; psychrophilic anaerobic digestion; pig manure; albumin; lysine;

5.1 Introduction

In-storage psychrophilic anaerobic digestion (ISPAD) is a manure treatment strategy developed to meet the needs of Canadian pork producers (Abou Nohra et al., 2003). This process occurs in manure storage tanks with air-tight covers through acclimation of the anaerobic microbial community to the ambient operating conditions (King et al., 2010a). Anaerobic digestion consumes both carbohydrates and proteins in the manure. While the kinetics of carbohydrate metabolism as the primary source of methanogenesis in ISPAD have been established (King et al., 2010c), it is not clear how the degradation of proteins to amino acids, ammonia and methane, are affected by ISPAD conditions.

Anaerobic digestion of complex wastewaters has been modeled by defining separate pathways for the degradation of carbohydrates, proteins and lipids; these contribute to common pools of volatile fatty acids, acetic acid and hydrogen, which lead to methanogenesis by the aceticlastic and hydrogen-using pathways (Batstone et al., 2002). Proteins are first hydrolysed to amino acids, which are then transformed to volatile fatty acids (VFA), hydrogen and ammonium (Tang et al., 2005). A variety of microbial species in the genus *Clostridia* perform these transformations of amino acids through numerous reactions, some singly and some in the paired Strickland form (Barker, 1981). At least thirty of these reactions have been identified, and to define the overall degradation stoichiometry for a single protein requires making assumptions about appropriate pathways that are difficult to fully verify (Ramsay and Pullammanappallil, 2001). When the anaerobic community includes active

methanogenesis, the syntrophic (non-Strickland) amino acid degradation pathway has been reported to dominate (Tang et al., 2005). Due to the large and varying number of intermediate products produced during anaerobic digestion of protein substrates, many studies of protein degradation have examined methane production as an indicator of changes in the complete process (Ortega et al., 2008; Parshina et al., 1993; Perle et al., 1995). Others follow ammonium production to monitor the protein pathway up to the acetogenesis and methanogenesis stages that are common to all substrate components (Maharaj and Elefsiniotis, 2001; Sarada and Joseph, 1993).

A study using protein-acclimated biomass and the single-compound substrates gelatine, glucose and lactose, concluded that protein degradation increased with increasing dilution of the substrate, and decreased with increasing carbohydrate concentration; this was interpreted as preferential degradation of carbohydrates first and proteins second (Breure et al., 1986). Degradation of proteins in primary municipal wastewater sludge decreased with temperature and increased with increasing hydraulic retention time, suggesting that acclimation to the protein substrate was occurring; this process appeared to be inhibited by the addition of starch-rich potato processing waste (Maharaj and Elefsiniotis, 2001). Using a simulated dairy effluent as substrate, the rate of protein degradation increased with acclimation of the microbial population to the protein substrate, though no acclimation effect was detected for amino acid degradation (Perle et al., 1995). In laboratory incubation of tomato-processing wastewater, twice as much protein was degraded in batch assays of 100 days as in semi-continuous assays

with hydraulic retention times of 8 to 32 days, suggesting again that acclimation to substrate is a factor in the anaerobic degradation of proteins (Sarada and Joseph, 1993). For simulated restaurant waste, methane production was equivalent for a protein-rich food waste and a similar carbohydrate-rich waste, though there was greater accumulation of volatile fatty acids during the high-protein digestion, suggesting poor acclimation of the microbial biomass to the substrate (Neves et al., 2008). Using sludge from a pig-manure storage tank as active biomass and four pure proteins as substrates in incubations at four temperatures, the rate of protein degradation decreased with decreasing temperature and increased with acclimation time; the process appeared to be limited by competition for H_2 between methanogens and homo-acetogens (Parshina et al., 1993). During laboratory acidogenesis from gelatine, the temperature effect was well described by an Arrhenius-type equation, though the variation was less than 10 % over the range 20-55 °C (Yu and Fang, 2003).

The anaerobic degradation of proteins is thus affected by microbial acclimation, process temperature and the presence of other substrates and intermediate products. As all of these factors are present in the ISPAD system, the net impact of ISPAD on the degradation of manure proteins cannot be inferred from the current literature. It is important to predict, however, because this degradation directly affects the fertiliser value of the treated manure, which is based on the ammonia-N, phosphorus (P) and potassium (K) content of the manure. When manure land-application rates are based on the phosphorus content of the manure, as in Quebec, mineral nitrogen must also be applied to meet the

crops' needs. Thus an increase in the ammonia-N content of ISPAD-treated manure would reduce the need for mineral nitrogen, providing an economic benefit to the producer. For this reason, it is essential for a future ISPAD process model, based on the results of this and other studies, to accurately predict the protein and ammonia-N content of ISPAD-treated manure.

The objectives of the present study were therefore: to determine how protein degradation proceeds in ISPAD; how this varies with temperature; and to investigate the occurrence of preferential or inhibitory relationships between the protein and carbohydrate fractions of the manure. The degradation of protein by the acclimated microbial biomass from an established ISPAD installation was examined in the laboratory by incubating samples of this biomass using a procedure based on specific methanogenic activity assays, with several substrates: fresh pig manure, albumin, lysine and glucose. The variations of these processes with temperature were assessed by repeating the assays at three incubation temperatures, 35, 18 and 8 °C. Protein-carbohydrate interactions were investigated in a series of assays using albumin combined with glucose.

5.2 Materials and Methods

Experimental materials

In 2004, a full-scale swine manure ISPAD facility was established in St. Francois Xavier (St. F-X) in the central region of the Province of Quebec, Canada. This facility consisted of a circular concrete tank measuring 30 m in diameter by 3.66 m in depth, covered with an air-tight membrane (*GTI*, Fredericton, NB, Canada). The tank received manure from the swine facility on a

regular basis. Except for a depth of 0.3-0.6 m, the contents were removed for land-spreading twice yearly. The facility had been in operation for five years when this study started in 2009. Manure from this facility was used to represent ISPAD (X_f), as the source of active microbial biomass. Freshly produced manure (S_m) for use as substrate was obtained from the swine research facility of the McGill University Macdonald Campus Experimental Centre (Mac), located on the Island of Montreal, Quebec, Canada. As they are produced by hogs fed a standard corn and soybean based ration, the manures from the St. F-X and Mac operations were considered comparable in terms of solids and nutrients (Conn et al., 2007). All manure samples were collected in June 2009 as previously described (King et al., 2010a).

Characterization of experimental manures

Samples of both manures (X_f and S_m) were analyzed according to standard methods (APHA et al., 2005) to establish solids, pH, and total Kjeldahl nitrogen (TKN). Total solids (TS) were determined by drying whole samples at 103 °C overnight in an oven (*VWR*, Sheldon Manufacturing, model 1327F, OR, USA). Volatile solids (VS) were determined by incineration of dried samples at 500 °C for two hours in a muffle furnace (*Barnstead Thermodyne*, model 48000, IA, USA). Suspended solids were separated from supernatant by centrifuging at 10000 rpm for 10 minutes at 4 °C. These samples were subsequently dried and incinerated as described above to determine total suspended solids (TSS) and volatile suspended solids (VSS). The pH of all samples was measured using a pH meter (*Corning*, model 450, NY, USA). Total Kjeldahl Nitrogen (TKN) was

determined by digesting the samples with sulphuric acid and 50 % hydrogen peroxide at 500 °C for 15 minutes (Digesdahl® Digestion Apparatus, *Hach Canada*, Mississauga, ON); the samples were diluted with de-ionized water, the pH adjusted to 13 with NaOH, and the NH_3-N content measured using an ammonia sensitive probe (*Orion*, model BCN, Boston, MA, USA) connected to a pH meter (*Corning*, model 450, NY, USA). Other properties of the manures were calculated from analysis of the initial conditions of the appropriate specific proteolytic activity bottles, as described in section 2.3.

One set of substrate activity tests (SAT) was performed at 35 °C to define the methanogenic activity profile of the ISPAD microbial community contained in the St. F-X manure (X_f), as previously described (King et al., 2010c). Four liquid and one gaseous substrate were used: glucose, acetate, propionate, butyrate and H_2/CO_2.

Specific proteolytic activity (SPA) assays

To evaluate the microbial activity in each stage of the protein degradation pathway, a specific proteolytic activity (SPA) protocol was adapted from the SAT procedure used above. Substrates were selected to represent the main stages, and thus the activity of the main populations in the process. Water soluble bovine serum albumin (*Sigma Aldrich*, item A4503, Oakville, ON, Canada) was used to represent proteins and their hydrolysis. Lysine (*Sigma Aldrich*, item L5501, Oakville, ON, Canada) was selected as a representative amino acid, present and essential in pig feed (Harmon et al., 1972), which may be degraded by non-Strickland single-substrate reactions (Ramsay and Pullammanappallil, 2001).

Glucose was retained to represent the degradation of carbohydrates for the purpose of comparison, and used in combination with albumin to investigate inhibition of protein degradation by carbohydrates. A series of assays with fresh pig manure as substrate were performed to follow protein degradation in the real ISPAD case, and to allow correlation of the pure substrates to those of the real system. In addition, a set of control assays with no added substrate were used to evaluate the component of protein degradation occurring from the ISPAD manure organic matter. All assays were performed at three temperatures, 8, 18 and 35 °C, to evaluate the impact of temperature on each studied stage.

While the SAT protocol monitored the concentration of the specific substrate throughout each assay, methods were not available to monitor albumin or lysine directly. However, ammonia is produced during anaerobic digestion only from protein degradation via amino acids. Thus to follow the protein degradation pathway, the SPA protocol monitored the concentrations of ammonium ion, NH_4^+, in all the assays. Volatile fatty acids (VFA) were monitored to illustrate effects on the acidogenesis and acetogenesis processes, including possible inhibition relationships. Glucose concentration was monitored in all the glucose-containing assays. The pH of each assay bottle solution was also measured at each sampling time.

The SPA assays were performed in triplicate 120 ml serum bottles. To provide 5 g VSS/l, 13.6 ml of manure from the ISPAD tank, with its facultative biomass, was added to each bottle. Phosphate buffer was added to bring the volume of liquid in each bottle to 20 ml. Bottles were capped, sealed and flushed

with N_2/CO_2 gas (80 % / 20 %) to establish anaerobic conditions, and placed in a thermostatically controlled shaker (*New Brunswick Scientific*, Edison NJ USA) operating at 100 rpm, in a darkened environment. Glucose, albumin and lysine were each dissolved in de-ionized water to produce liquid substrates, with concentrations of 25 g/l. After two hours of adjustment to the test conditions, 2.0 ml of each appropriate liquid substrate, or 2.4 ml of fresh manure, was injected through the cap of each 120 ml bottle, except for the control bottles. The first samples, 0.5 ml of liquid, were immediately taken and centrifuged to remove solids. Sub-samples of supernatant were diluted 2-fold for glucose analysis, 5-fold for VFA analysis and 20-fold for NH_4^+ analysis. The pH of each sample was determined using paper chromatography. Sampling was repeated periodically for the duration of the test. The length of the assays was determined as the estimated doubling time of methanogens at each temperature, adjusted exponentially for temperature: 4 days at 35 ^oC, 14 days at 18 ^oC and 28 days at 8 ^oC (King et al., 2010a). At the end of each assay, the bottle contents were analyzed to determine solids and TKN. An extra set of three bottles was prepared without substrate addition and used to analyze initial solids and TKN.

Analytical methods - SAT and SPA

Glucose was measured using a high pressure liquid chromatograph (*Waters Chromatography Division*, Milford, MA, USA) equipped with an injector (model 717+), photodiode array detector (model 2996), pump (model 600), refractive index detector (model 2414). The column used for the separation was ICSep IC ION-300 column (*Transgenomics*, San Jose, CA, USA) of 300 mm ×

7.8 mm id and an ion guard GC-801 column (*Transgenomics*). The mobile phase consisted of 0.035 N H_2SO_4 at a pH of 4, flowing at a rate of 0.4 ml min^{-1}. The measurements were conducted using a wavelength of 210 nm. Analysis was carried out at 35 °C.

For VFA analysis, acetic, propionic and butyric acid concentrations were measured using a gas chromatograph (*Agilent*, model 6890, Wilmington, DE, USA) equipped with a flame ionization detector of 0.2 µl. The samples were fortified at a ratio of 1:1 (by volume) using an internal standard of *iso*-butyric acid dissolved in 6 % formic acid. These were directly injected into a glass column of 1 mm × 2 mm *Carbopack C* (60 to 80 mesh) coated with 0.3 % *Carbowax* 20M and 0.1 % H_3PO_4. The column was held at 130 °C for 4 minutes and helium as carrier gas was injected at a rate of 20 ml min^{-1}. The injector and the detector were both maintained at 200 °C.

To quantify hydrogen consumption, biogas composition (H_2, N_2+O_2, CH_4, CO_2) was measured using a gas chromatograph (*Hewlett Packard*, 6890 Series, Wilmington, DE, USA) equipped with a thermal conductivity detector and a 900 mm × 3mm 60/80 mesh Chromosorb 102 column (*Supelco*, Bellefonte, PA, USA).

To quantify NH_4^+, cations were analyzed by HPLC on a Hamilton PRP-X200 cation resin-based chromatography column (250x41mm OD), on a high-performance liquid chromatograph (TSP model P4000 & AS3000). Conductivity data were obtained by using a Waters Millipore detector model 432. The

parameters used were: mobile phase 4mM nitric acid with 30% methanol, 20 µl injection, pH=3, and 1.8 ml/min flow rate at 40^{o} C.

Data interpretation

The time-series data from the specific proteolytic assays were interpreted by plotting the average of triplicates versus time. Peaks and troughs composed of single points were assumed to be results of experimental error, due to the multi-step nature of the data collection process, which included sampling and initial dilution for processing, further sampling, pH adjustment and dilution during processing, and reliance on the calibration of detectors for chromatography. Trends, increasing or decreasing, were identified where they spanned three or more data points. The rates of increase or decrease of these trends were estimated by fitting linear equations to the trend region of the plot, and reporting the slope of this line.

Statistical analysis

All statistical analyses were performed using SAS 9.0 (®2009, *SAS Institute inc.*, Cary NC, USA).The characterization data were collected in triplicate, on sub-samples from the composite sample taken from each tank. Significance of differences between these measured values was evaluated by the Student-Newman-Keuls method in a simple analysis of variance based on a completely randomized design. The specific substrate activity assays used a completely randomized design, considering substrate (glucose, butyrate, propionate, acetate, H_2/CO_2) as the treatment factor and substrate uptake rate as the dependant variable. Treatments were assigned randomly to experimental units

(bottles), and all treatment-block combinations were completed in triplicate. The specific proteolytic assays used a randomized complete block design, with substrate (none, glucose, glucose and albumin, albumin, lysine, pig manure) as the treatment factor, temperature (35, 18 or 8 °C) as the block factor and ammonia-N production rate as the dependant variable.

5.3 Results and Discussion

Characteristics of experimental manures

The characteristics of the fresh manure, S_m, and the manure from the ISPAD tank, X_f, are presented in Table 5.1. Pig manures from all types of operations in several countries have consistently similar composition with the exception of water content (Conn et al., 2007; Moore et al., 2005; Sánchez and González, 2005). Differences between the fresh and ISPAD manure characteristics may therefore be considered to represent the result of processes occurring in the ISPAD tank (King et al., 2010a). This assumption allows the initial volatile solids content, VS_i, of the ISPAD manure to be calculated from its fixed solids content, based on the ratio of volatile to fixed solids of the fresh manure (VS/FS). The characteristics of the manures are normalized to a VS_i basis in the lower section of Table 5.1. This calculation indicates that approximately 43 % of the volatile manure solids fed to the ISPAD tank were consumed by the anaerobic digestion process in the tank. The reduced concentrations of acetate, propionate and butyrate in the ISPAD manure, as well as the resulting increase in pH, confirm that the process is well acclimated to its operating conditions (Kotsyurbenko, 2005); butyrate, which is not measurable in the ISPAD manure,

tends to be consumed most rapidly in psychrophilic digestion, while propionate, which has the highest concentration, tends to be consumed much more slowly (Arbeli et al., 2006; Parshina et al., 2000).

Total Kjedahl nitrogen (TKN) includes the organic nitrogen and ammonia-ammonium nitrogen components of the manure, while total ammonia nitrogen (TAN) represents only the ammonia and ammonium portions. Thus, the increased TAN/TKN ratio of the ISPAD manure indicates that 65 % of the organic-N, representing manure proteins, were broken down to form ammonia-N in the digestion process, while the consistent TKN/VS_i ratio demonstrates that total nitrogen was conserved. This means that the TAN is not being lost as NH_3 in the biogas released from the tank.

Specific activity profile of ISPAD manure microbial community

The specific substrate uptake activity profile of the ISPAD microbial community is presented in Table 5.2. The first column lists the activities measured in the usual units of *mg substrate g* VSS^{-1} $hour^{-1}$; however, because the microbial biomass is contained within the manure organic matter (VSS), the values must not be considered absolute. For this reason, the second column normalizes the activities on the basis of acetate consumption as follows: normalized substrate activity (*mg substrate (mg acetate)*$^{-1}$) = specific substrate activity (*mg substrate g* VSS^{-1} $hour^{-1}$) / specific acetate activity (*mg acetate g* VSS^{-1} $hour^{-1}$). As a comparison, the third column lists activities reported in a large literature survey of mesophilic anaerobic digesters (Batstone et al., 2002), also in the same normalized form. Comparing the normalized ISPAD and mesophilic

profiles illustrates the temperature-acclimated nature of the ISPAD community; while the glucose uptake rates are similar, the propionate activity of ISPAD is reduced by 83 % compared to the mesophilic value, the butyrate activity is reduced by 24 % and the hydrogen activity by 30 %. Similarly, temperature acclimated communities have been reported by other researchers to have robust butyrate activity (Connaughton et al., 2006b) and minimal propionate activity (Arbeli et al., 2006). While hydrogen activity has been reported to increase during the acclimation process, once the community has stabilized, the this activity is lower than for mesophilic communities (Kotsyurbenko, 2005).

Specific proteolytic activity – Nitrogen balance

During the anaerobic digestion process, proteins are broken down to amino acids, which then degrade to volatile fatty acids, releasing ammonium ions which form an equilibrium with gaseous ammonia, NH_3, in the solution. While all the ammonia-N generated must originate from proteins, a primary concern with the use of ammonia-N concentration as a measure of protein degradation is its potential for volatilization into the bottle headspace biogas. To determine if NH_3 was volatilizing during the specific proteolytic activity assays, total Kjeldahl nitrogen, TKN, which includes both organic-N and ammonia-N, was measured at the beginning and end of each assay, for each bottle (data not shown). For the control bottles containing no added substrate, and the bottles fed glucose or albumin, separately or together, the TKN was conserved within ±10 % of the original value, which approximates the accuracy of the measurements. For the bottles containing fresh manure or lysine as substrate, there were no measurable

losses at 8 or 18 °C, but at 35 °C approximately 20 % of the TKN was lost from the bottle solution as NH_3. Thus, for these two assays, fresh manure and lysine at 35°C, a correction factor was applied to the measured rates of ammonia-N production by increasing the final concentration if TKN by 20 %.

Protein degradation by the ISPAD biomass

When albumin, a model protein, was fed to the ISPAD microbial biomass in the specific proteolytic assays, a similar pattern of degradation occurred at each assay temperature, as shown in Figures 5.1 a, b and c. In each case, NH_4^+ concentration increased steadily during most of the assay, levelling off toward the end, indicating that the quantity of albumin and length of assay were appropriately matched. At the same time, concentrations of acetate and propionate increased to a plateau concentration, and in some cases began to decrease near the end of the assay. There was no measurable concentration of butyrate in any of the samples. The immediate and steady production of NH_4^+ in each assay indicates that the ISPAD biomass degrades proteins effectively, and thus is acclimated to this substrate at all the assay temperatures. However, the transient accumulation of volatile fatty acids at all temperatures suggests that the ISPAD biomass may not be completely acclimated to the concentration of protein available in albumin. This is to be expected, as readily available proteins would have been absorbed in the animals' digestive tract, so that proteins excreted in manure are likely to be those that are still bound up in complex organic matter. The manure proteins are released by the disintegration of this organic matter, which is slow and may be the limiting step in this part of the ISPAD process.

The initial rate of production for NH_4^+, acetate and propionate for each assay was determined from the first 4 to 6 sampling points; these values are presented in Figure 5.2 a. In each case, an exponential Arrhenius-type trendline describes the relationship between production rate and temperature, indicating that the protein-degrading microbial population remains mesophilic, unlike the ISPAD glucose degraders examined in a previous study, which demonstrated the development of a co-existing psychrophilic population with maximum activity near 18°C (King et al., 2010c).

Interactions between protein and glucose degradation by ISPAD biomass

The specific proteolytic assay bottles fed albumin and glucose together exhibited the same pattern of degradation as the albumin-only assays, though about 20 % more acetate and propionate accumulated in the albumin-glucose bottles. Figures 5.2 a and b summarize the initial production rates for acetate, propionate and NH_4^+ for these assays. At each temperature, the rate of NH_4^+ production was similar for both substrate types. This indicates that glucose did not inhibit the degradation of albumin. The increased rates of VFA production in the glucose-albumin assays reflect the concurrent degradation of albumin and glucose.

In the same way, the pattern of glucose consumption in the assays fed only glucose was similar to that of the assays fed glucose and albumin together. This indicates that the presence of protein did not inhibit the degradation of glucose. An interesting observation, however, is that the production rate of acetate from both glucose and glucose-albumin, shown in Figures 5.2 b and c, was much

higher at 18 °C compared to albumin alone, resulting in a trendline which curves in the opposite direction from the Arrhenius-type relationship illustrated for albumin. This reinforces the evidence of a psychrophilic glucose-consuming population in the ISPAD microbial community, as mentioned in the preceding section (King et al., 2010c).

Degradation of lysine by ISPAD biomass

As lysine is an important amino acid used to enrich pig feed, it was expected to be present in pig manure and thus actively digested in ISPAD; however, as illustrated in Figures 5.3 a, b and c, the assays fed lysine demonstrated a significant lag time prior to active degradation, with sharp increases in NH_4^+ and acetate concentration after 60 and 200 hours respectively at 35 and 18 °C, and no activity in 550 hours at 8 °C. In the 35 °C assay only, butyrate production also began after 60 hours, corresponding to the degradation pathway proposed by previous studies; lysine is expected to degrade via a non-Strickland reaction, requiring no other amino acids and producing acetate and butyrate but no propionate, (Batstone et al., 2002; Ramsay and Pullammanappallil, 2001). These assay results suggest that either the ISPAD biomass is not acclimated to lysine as a substrate, possibly because enriched lysine may be entirely assimilated by the animals and not be present in manure as was assumed, or that some other inhibiting factor, such as lack of H_2 (Parshina et al., 1993), prevented the degradation of lysine when supplied with no other substrates. There was slow consumption of propionate during the lag phase at 35 °C and both propionate and acetate at 18 °C, which could have provided the

amount of H_2 necessary to allow the degradation of lysine. Thus in the real case of fresh manure containing many substrates and intermediate products, lysine, if present, could be consumed without any lag.

Degradation of manure proteins by ISPAD biomass

The specific proteolytic assay control bottles, containing ISPAD manure as biomass only with no added substrate (data not shown), showed no evidence of active protein degradation. The concentration of NH_4^+ remained constant throughout each assay, and the concentrations of acetate and propionate gradually decreased, except for the 35 °C assay where there was a slight production of acetate. This indicates that some non-protein manure substrate was degrading at 35 °C, at a rate or concentration to which the active biomass was not perfectly acclimated. This is reasonable as the ISPAD biomass has been shown to be acclimated to its operating conditions which include psychrophilic temperatures and low substrate concentrations (King et al., 2010a; King et al., 2010c). As indicated in Table 5.1, 43 % of the original manure organic matter had previously been degraded in the ISPAD tank, and within this, 64% of the manure protein had been degraded to ammonia-N. The remaining organics, including protein, are likely to be recalcitrant or slowly degradable and thus these results are expected.

The SPA bottles which were fed fresh manure, as illustrated in Figures 5.4 a, b and c, also show no measurable NH_4^+ production, though degradation of non-protein substrates is evident from fluctuating concentrations of acetate and propionate, in contrast with the steady decreases in the control bottles. The lack of measurable NH_4^+ production suggests that manure proteins are bound within the

manure organic matter and are not readily available for digestion, as the assays with albumin demonstrate that available protein will be quickly degraded. As mentioned in section 3.3 above, approximately 20 % of the fresh manure TKN was volatilized as NH_3 from the 35 °C assay bottles, which, in combination with the constant NH_4^+ concentration in these bottles means that about 20 % of the manure protein was in fact degraded in this assay. Other factors such as ionic saturation may have driven the NH_3/NH_4^+ equilibrium to release more NH_3 than the other assays (Hafner and Bisogni Jr, 2009). Thus it appears that disintegration of organic matter to release proteins is the limiting step in the anaerobic digestion of manure proteins in ISPAD.

5.4 Conclusions

The anaerobic microbial community which developed during 5 years of In-Storage Psychrophilic Anaerobic Digestion (ISPAD) of swine manure was assessed in the laboratory to describe its protein degradation characteristics. These analyses revealed that:

i. In contrast to previously assessed carbohydrate degrading populations which develop a robust psychrophilic component, the protein and amino acid degrading populations within the acclimated ISPAD community remained mesophilic; the relationship between the protein degradation rate and temperature appeared to follow an Arrhenius-type exponential curve in the studied temperature range of 8 to 35 °C.

ii. Unlike other anaerobic digestion systems which have been studied, there was no evidence of interaction or inhibition between the protein and carbohydrate degradation processes in ISPAD.

iii. The limiting step in degradation of proteins by the ISPAD microbial community appeared to be the disintegration of manure organic matter.

iv. The long residence time of manure in the ISPAD tank compensated for the slow degradation rate, resulting in 65% of manure proteins transformed to ammonia-N which was conserved in the manure with no loss of gaseous NH_3 during the residence period.

Manure treated by ISPAD should therefore have a high plant-available ammonia-N content, comparable to manure from a mesophilic digester in which protein is degraded at a higher rate; the ISPAD ammonia-N content should be much higher than in manure from a short-residence psychrophilic digester, which would undergo minimal protein degradation due to the low rate of the process; the ISPAD ammonia-N content should also be much higher than manure from an industry-standard uncovered storage tank, which has a long residence time but loses important amounts of the ammonia-N produced to volatilisation. Using ISPAD-treated manure as fertiliser could then reduce the need for mineral nitrogen fertilizer, if the rich ammonia-N content is not lost to volatilization following its application to cropland.

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5.7 Tables

Table 5.1: Characteristics of experimental manures

	Fresh manure, S_m	**ISPAD tank, X_f**
TS ($g\ l^{-1}$)	73.04 (0.56)	15.33 (0.15)
VS ($g\ l^{-1}$)	52.92 (0.48)	9.17 (0.07)
VSS ($g\ l^{-1}$)	42.25 (0.74)	7.37 (0.23)
FS ($g\ l^{-1}$)	20.13 (0.10)	6.16 (0.08)
pH	6.90 (0.03)	7.34 (0.04)
TKN ($g\ l^{-1}$)	6.36 (0.32)	1.95 (0.06)
TAN ($g\ l^{-1}$)	1.69 (0.70)	1.44 (0.07)
Acetate ($g\ l^{-1}$)	4.58 (0.78)	0.13 (0.04)
Propionate ($g\ l^{-1}$)	1.65 (0.28)	0.26 (0.03)
Butyrate ($g\ l^{-1}$)	0.12 (0.07)	0.00 (0.00)
VS/TS	2.62	1.49
Initial VS ($g\ l^{-1}$)	-	16.20
VS consumed (%)	-	43
Acetate ($mg\ (gVSi)^{-1}$)	86.48	7.74
Propionate ($mg\ (gVSi)^{-1}$)	31.10	16.03
Butyrate ($mg\ (gVSi)^{-1}$)	2.25	0.00
TKN / VSi	0.12	0.12
TAN / TKN	0.26	0.74
Protein degraded (%)	-	64

(standard deviation in brackets)

Table 5.2: Specific substrate uptake activity of ISPAD microbial community, X_f, and mesophilic activity from literature

	Activity, X_f *mg (g VSS)$^{-1}$ hour^{-1}*	**Normalized activity, X_f** *mg (mg acetate)$^{-1}$*	**Mesophilic activity**[a] *mg (mg acetate)$^{-1}$*
Glucose	9.49	4.02	3.77
Acetate	2.36	1.00	1.00
Propionate	0.48	0.20	1.16
Butyrate	2.62	1.11	1.47
Hydrogen	0.83	0.35	0.59

[a](Batstone et al., 2002)

5.8 Figures

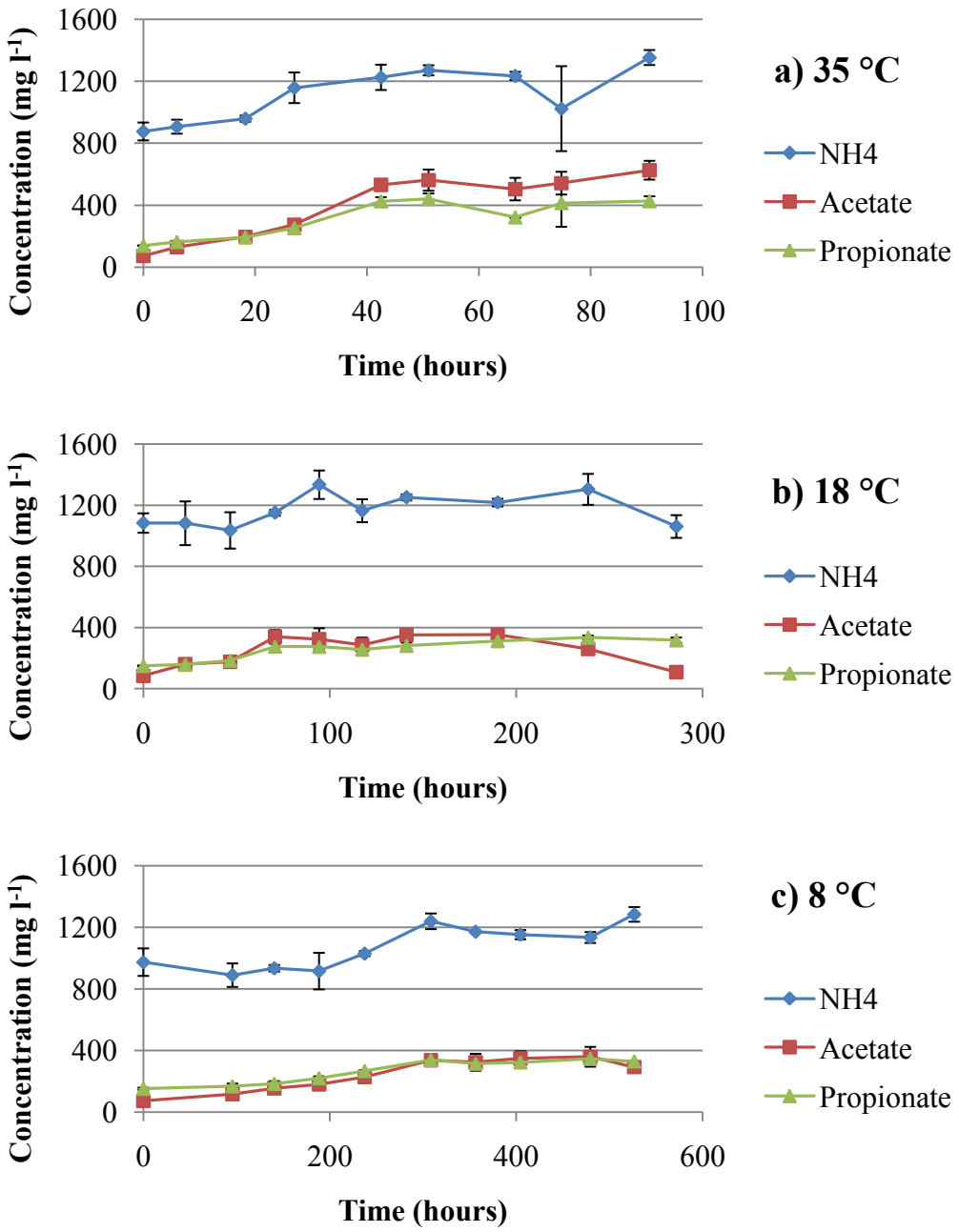

Figure 5.1: Concentrations of NH_4^+, acetate and propionate during specific proteolytic assays with albumin as substrate at: a) 35 °C, b) 18 °C and c) 8 °C.

Notes: Data points represent the average of three replicates.

Error bars represent +/- one standard deviation.

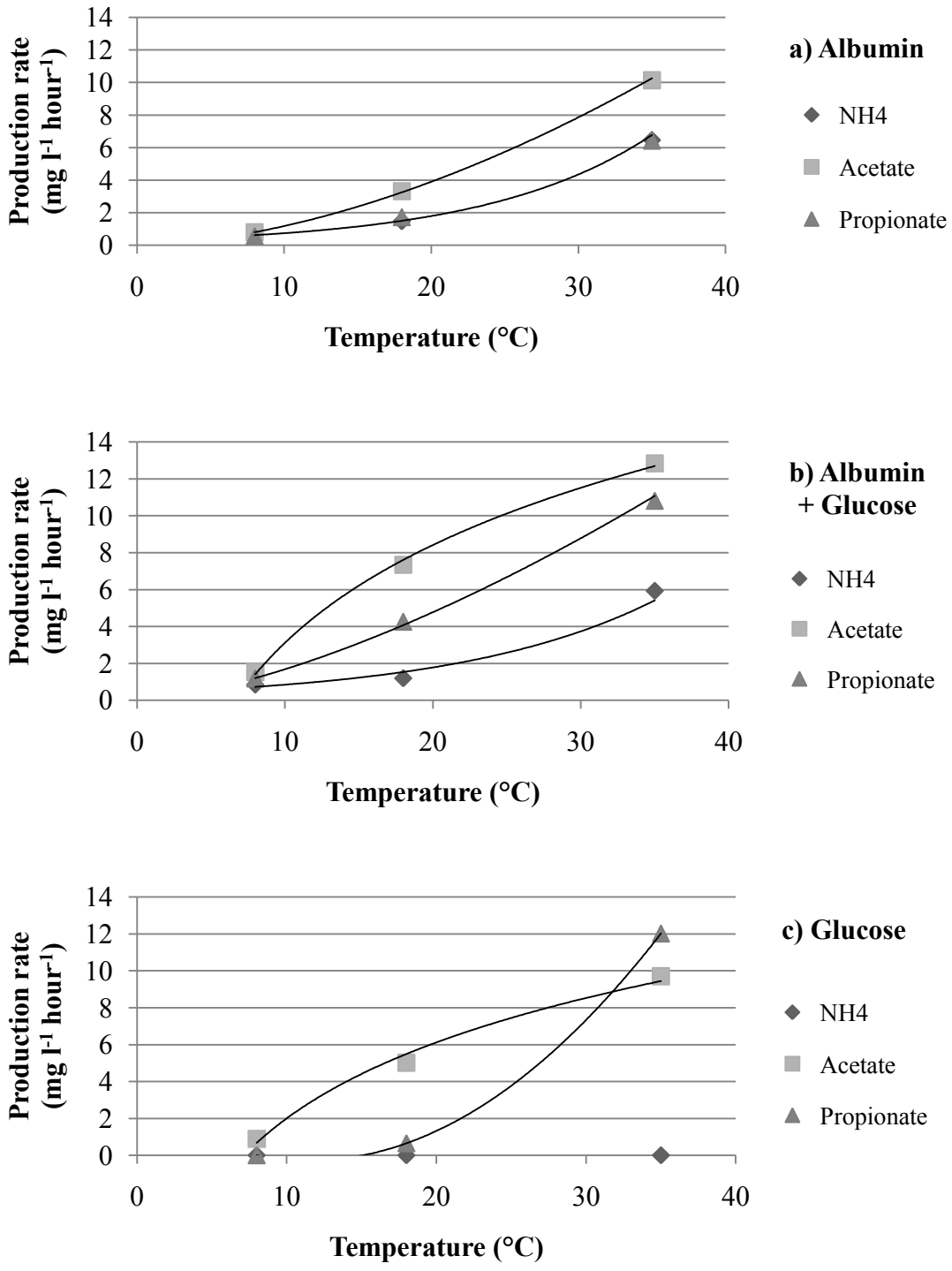

Figure 5.2: Initial production rates of NH_4^+, acetate and propionate during specific proteolytic assays at 8, 18 and 35 °C with substrates: a) albumin, b) albumin and glucose and c) glucose.

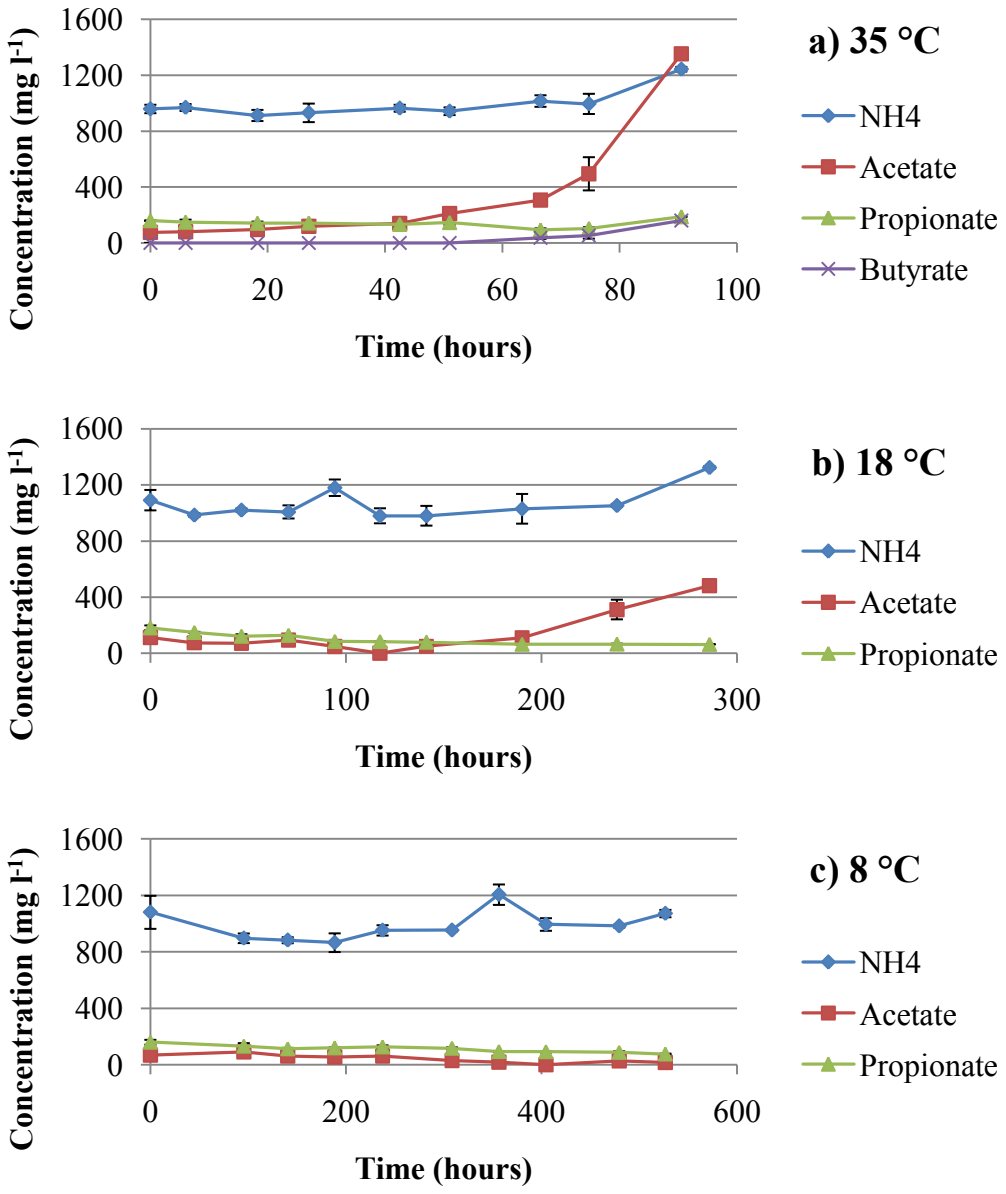

Figure 5.3: Concentrations of NH_4^+, acetate, butyrate and propionate during specific proteolytic assays with lysine as substrate at: a) 35 °C, b) 18 °C and c) 8 °C.

Notes: Data points represent the average of three replicates.
Error bars represent +/- one standard deviation.

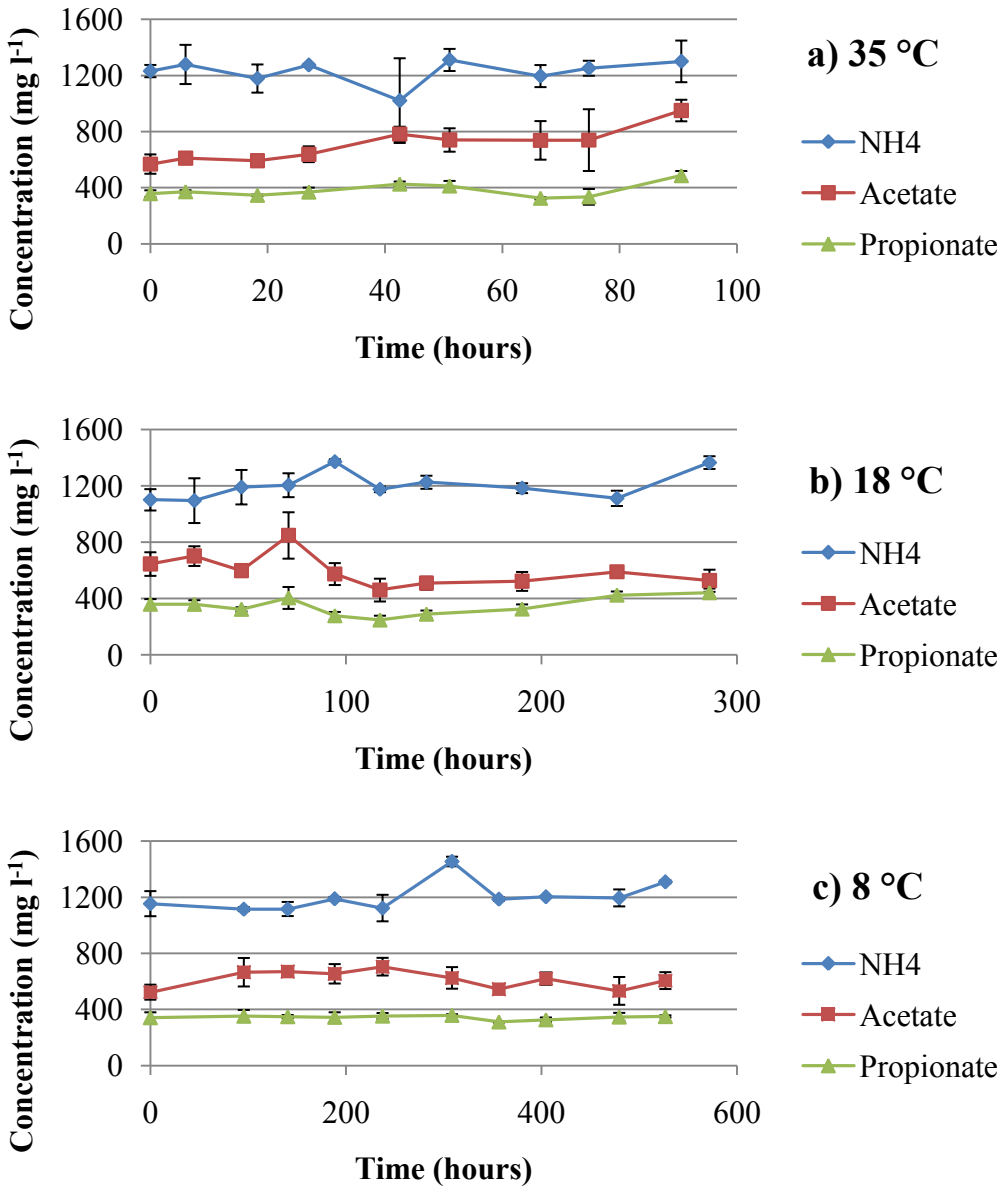

Figure 5.4: Concentrations of NH_4^+, acetate and propionate during specific proteolytic assays with fresh manure as substrate at: a) 35 °C, b) 18 °C and c) 8 °C.

Notes: Data points represent the average of three replicates.
Error bars represent +/- one standard deviation.

Chapter 6. Nitrogen Conservation

Connecting Statement

In Chapter 5, it was demonstrated that protein degradation in ISPAD is carried out by a mesophilic microbial community, and thus the rate at which the process occurs is much lower than for a comparable mesophilic digestion system. However, in combination with the long retention time of manure in the ISPAD system, this results in significant degradation of manure proteins to plant-available ammonia/ammonium nitrogen. The article which follows describes the wind tunnel method used for simulated manure land application, which was used to assess the extent of NH_3 volatilization from ISPAD manure compared to conventionally stored manure, and the resulting improved N-fertilizer value of the ISPAD manure.

This article will be submitted to the Journal of Applied and Environmental Soil Science, special issue on 'Biosolids Soil Application: Agronomic and Environmental Implications'. The first contributing author, Ms. Susan M. King, designed the experiments, supervised the laboratory work, analysed the data and wrote the article. The second contributing author, Mr. Michael Schwalb, conducted the laboratory work and contributed to the Methods section of the article. The third contributing author, Dr. Suzelle Barrington, supervised, advised on the experimental design and methods of analysis, and revised the content of the article. The fourth contributing author, Dr. Joann Whalen, advised on the experimental design and methods of analysis, and revised the content of the article.

Abstract

During In-Storage Psychrophilic Anaerobic Digestion (ISPAD) of swine manure, proteins are broken down to form plant-available ammonia nitrogen which is conserved during the treatment and storage process. This means that prior to land application, the treated manure has a higher nitrogen fertilizer value than that from standard storage tanks. However, if it is lost by volatilization following land application, then this advantage may be reduced or reversed. This study evaluated the ammonia volatilization and resulting nitrogen fertilizer value of both ISPAD and conventionally stored pig manure following land application to different soils. This was done by simulating land application using the wind tunnel technique and five soils varying in properties (clean sand, Ste Rosalie clay, Upland sandy loam, St Bernard loam and Ormstown silt). The amount of ammonia nitrogen volatilized during the wind tunnel tests varied with both manure and soil type. Over all soil types, the ISPAD manure consistently volatilized less ammonia than the conventionally stored manure, averaging 53% of this. Within each manure type, the amount of ammonia volatilized was highest for clean sand and lowest for the clay soil. As a result, the nitrogen fertilizer value of ISPAD manure is up to 21 % higher than that of conventionally stored manure for an equivalent phosphorus value, depending on the conditions of application.

Keywords: psychrophilic anaerobic digestion, NH_3 volatilization, manure application, swine, soil

6.1 Introduction

In-storage psychrophilic anaerobic digestion (ISPAD) occurs in manure storage tanks with an air-tight cover when its anaerobic microbial community acclimates to ambient conditions (King et al., 2010c). This system, developed for Canadian pork producers, effectively reduces the volatile solids content of the treated manure while releasing up to 63% of the manure's potential methane (King et al., 2010a). In addition, the microbial community actively breaks down manure proteins in the ISPAD tank without losing ammonia (NH_3) to the atmosphere, increasing the proportion of plant-available total ammonia nitrogen (TAN) in the manure (King et al., 2010b). However, the conserved TAN may be lost to the atmosphere when the treated manure is subsequently applied to cropland. This could result in a net loss of TAN, affecting the fertilizer value of the ISPAD manure.

The quantity of NH_3-N lost through volatilization following land application of pig manure has been shown to depend on several factors, some of which are independent of manure storage and treatment, such as pig diet (Paschold et al., 2008), incorporation of manure into soil (Rochette et al., 2001), timing of application (Gordon, 2001) and weather conditions following application (Huijsmans et al., 2003; Misselbrook et al., 2005; Mkhabela et al., 2009). However, when the diet manipulation involved reducing protein intake, the resulting manure contained less TAN , and the volatilization of NH_3 following land application was also reduced (Velthof et al., 2005). This was corroborated by a study examining a database of field measurements, which concluded that pig

manure with higher TAN released more ammonia to the atmosphere than pig manure with a lower TAN, though the percentage released remained constant (Huijsmans et al., 2003).

Several characteristics of the land-applied manure, as well as interactions of these with properties of the receiving soil, have been shown to influence NH_3 volatilisation. The speciation of TAN into NH_3-N and NH_4^+-N depends on the pH of the manure slurry (Avnimelech and Laher, 1977). This relationship is regulated by the equilibrium constant, K_N, and Henry's constant, K_H, both of which vary exponentially with temperature (Sommer et al., 2003). The presence of other ionized species in the manure also influences this equilibrium, and may be accounted for using an activity factor (Hafner and Bisogni Jr, 2009). The manure pH is also influenced by the buffering capacity of the manure (Avnimelech and Laher, 1977; Husted et al., 1991), which can be approximated by its alkalinity (Vlek and Stumpe, 1978). The ammonia speciation equilibrium is further affected by the buffered cation exchange capacity (CEC) of the receiving soil (O'Toole et al., 1985). Manure TAN may be removed from the liquid equilibrium process by precipitation when the soil contains sufficient cations, particularly Ca^{2+} and Mg^{2+} which enhance production of calcite and struvite (Sommer et al., 2003). In conjunction with these chemical equilibrium considerations, ammonia volatilisation decreases as the manure liquid fraction infiltrates the soil; infiltration depends on both manure and soil physical properties, increasing with decreasing manure dry matter content (Misselbrook et al., 2005), and with increasing soil water potential (Sommer et al., 2006).

While anaerobic digestion of manure may influence many of the above-mentioned physical and chemical properties, it has also been evaluated as a separate effect. When pig manure treated by mesophilic anaerobic digestion was compared to freshly produced pig manure, NH_3 volatilization after application to grassland was not affected (Pain et al., 1990). Similarly, in a comparison of anaerobically digested and anaerobically stored pig manure, the percentage of total manure N volatilized remained constant, though the soil pH and soil NH_4^+-N content affected volatilization following application to bare soil (Chantigny et al., 2004). However, in comparison to raw manure, NH_3 volatilisation was 22 % less for anaerobically digested manure applied to a bare Le Bras silty clay loam (Chantigny et al., 2009).

Pig manures from all types of operations in several countries have consistently similar nutrient content in terms of the ratios of total N, phosphorus (P) and potassium (K) content (Conn et al., 2007; Moore et al., 2005; Sánchez and González, 2005). However, the availability to plants of each nutrient may be affected by many factors and must be assessed on a case-by-case basis for best nutrient management practices (Schröder, 2005). Once manure has been applied to arable land, soil testing has indicated that raw and digested manures supply similar quantities of plant-available TAN and P (Loria and Sawyer, 2005). From plant growth studies, it has been reported that anaerobically digested manure is equivalent to or better than freshly produced manure as an N source for wheat (Dahlberg et al., 1988), corn (Loria et al., 2007) and timothy (Chantigny et al., 2007). During anaerobic digestion, some of the organic N compounds are broken

down to release TAN (Field et al., 1984; King et al., 2010b), which results in a stabilisation of organic matter in the digested manure (Marcato et al., 2009). These effects, combined with the reduction in the dry matter content and carbon/nitrogen ratio of digested manure from the anaerobic processes, enhance infiltration of the manure liquid fraction into the soil and reduce both TAN immobilisation and NH_3 volatilisation, resulting in higher levels of plant-available TAN in soils amended with digested manures (Gutser et al., 2005).

Due to the number of factors which affect NH_3 volatilization, the impact of ISPAD on the ultimate N fertilizer value of land-applied manure cannot be inferred from the current literature. If the ammonia nitrogen released from manure proteins during the ISPAD process is conserved following land application of the treated manure, then the ISPAD manure would have a higher nitrogen fertilizer value than un-treated manure stored in standard uncovered tanks. If this hypothesis is correct, then, as mineral N is often added to top-up the N:P ratio of manure for crop fertilization, less mineral N would be required for crops fertilized with ISPAD-treated manure. This could result in significant cost savings for pork producers using this technology.

The objectives of the present study were to compare the extent of NH_3 volatilization following application of ISPAD-treated manure and manure from a standard storage tank to soils with different properties, and to test the hypothesis stated above. This was done by simulating manure land application of both manures using the wind tunnel technique, monitoring NH_3 release with boric acid

traps. Five different soils were used to evaluate the impact of the soil type on the release of NH_3.

6.2 Materials and Methods

Experimental manures

In 2004, a full-scale swine manure ISPAD facility was established in St. Francois Xavier, Quebec, Canada. This facility consisted of a circular concrete tank, 30 m in diameter and 3.66 m deep, covered with an air-tight membrane (*GTI*, Fredericton, NB, Canada). The tank received manure from the swine facility on a regular basis. Except for a depth of 0.3-0.6 m, the contents were removed for land-spreading twice yearly. The facility had been in operation for five years when this study started in 2009. Manure from this facility was used to represent ISPAD. One year old conventionally-stored manure contained in an uncovered storage tank, was obtained from the swine research facility of the McGill University Macdonald Campus Experimental Centre located on the Island of Montreal, Quebec, Canada. As they are produced by hogs fed a standard corn and soybean based ration, these two manures were considered comparable in terms of solids and nutrients (Conn et al., 2007). All manure samples were collected in March 2010 as previously described (King et al., 2010a).

Characterization of experimental manures

Samples of both manures were analyzed according to standard methods (APHA et al., 2005) to establish solids, pH, total Kjeldahl nitrogen (TKN), total ammonia nitrogen (TAN), phosphorus (P) and potassium (K) content. Total solids (TS) were determined by drying whole samples at 103 ^{o}C overnight (*VWR*,

Sheldon Manufacturing, model 1327F, OR, USA). Volatile solids (VS) were determined by incineration of dried samples at 500 ^{o}C for two hours (*Barnstead Thermodyne*, model 48000, IA, USA). The pH of all samples was determined using a pH meter (*Corning*, model 450, NY, USA). The TKN, P and K were determined by digesting samples of each manure with sulphuric acid and 50 % hydrogen peroxide at 500 °C for 15 minutes (*Hach Canada*, Digesdahl model 23130-20, Mississauga, ON). Sub-samples of digestate were used to quantify P and K colorimetrically at a pH of 7, using a spectrophotometer (*Hach*, model DR 5000, Loveland CO, USA). For TKN, the pH of sub-samples was adjusted to 13 using NaOH, and the NH_3-N content was measured with an NH_3-sensitive electrode (*Orion*, Boston MA, USA) connected to a pH meter (*Corning*, model 450, NY, USA). Total ammonia nitrogen (TAN) was measured in the same way using undigested samples, after adjusting the pH to 13.

Experimental soils

Five soils were selected to evaluate the impact of soil properties on ammonia volatilization following land application of the experimental manures. Washed sand was purchased in 18 kg bags (*S. Boudrias inc.*, Laval, QC, Canada). Topsoil from the Sainte Rosalie clay, Upland sandy loam and St Bernard loam soil series were collected from different cultivated fields on the MacDonald Campus of McGill University in Ste-Anne-de-Bellevue, QC, Canada. Topsoil from the Ormstown silt soil series was collected in the Suroit region southwest of Montreal. These four soils were collected manually in 20 litre buckets, in August 2009. Buckets were stored uncovered for 6 months indoors to dry. All the dried

soil samples were crushed or ground, then passed through a 6mm sieve to become homogenized and free from organic debris.

Characterization of experimental soils

Each of the five experimental soils was analyzed to determine the following properties: pH, buffer pH, organic matter, mineral content (Ca, P, Al, K, Mg) and cation exchange capacity (CEC) were evaluated by a commercial soil-test facility; soil texture (particle size distribution), gravimetric water-holding capacity, total ammonia nitrogen (TAN) and total Kjeldahl nitrogen (TKN) were evaluated in the research laboratory.

The pH was measured by suspending the soil in an equal volume of distilled water, letting it sit for 30 minutes and then using a pH electrode to measure the pH (CEAE, 2003c). The organic content of the experimental soils was determined by first drying the samples in an oven at 150 ^{o}C for 16 hours and then by heating the samples in a muffle furnace at 375 ^{o} C for 16 hours (CEAE, 2003b). Minerals and trace elements were measured by first extracting them with a Mehlich III solution and then by using a plasma emission spectrometer (CEAE, 2003a). The CEC was determined by saturating the samples with NH_4^+ (using an ammonium acetate solution at pH 7) and then by treating the samples with NaCl; the CEC was quantified based on the amount of NH_4^+ liberated (CRAAQ, 2003).

The soil particle size distribution was determined using the hydrometer method (Sheldrick and Wang, 1993). The gravimetric moisture holding capacity was measured by soaking previously dried sub-samples of each soil in distilled water for 24 hours, draining off the excess water by gravity, under cover to

prevent evaporation, and finally drying at 103 °C for 24 hours to determine the final moisture content. The TAN and TKN of the soils were analyzed using the same method as the experimental manures.

Wind tunnel installation

Five small wind tunnels designed for manure spreading simulations (Choinière et al., 2007) were installed in the Bioresource Engineering workshop on the Macdonald campus of McGill University. As illustrated in Figure 6.1, each tunnel sat on a soil pan measuring 1.5 m long x 0.1 m wide x 0.05 m deep. The tunnels were 2.0 m in length, with an inlet diffuser 0.3 m long, and an outlet reducer 0.15 m long. The five tunnels were connected in parallel. Fresh air was blown through the tunnels using 50 mm tubing to maintain a consistent air speed of 0.3 m/s. Exhaust air was routed through 75 mm tubing to the exterior of the building. To monitor NH_3 volatilization inside the tunnels, air was drawn constantly at a rate of 6 l min^{-1} from inside each tunnel through 3 mm I.D. tubing (*Laurentian Valve & Fittings ltd.*, Saint-Laurent, QC, Canada) by a 0.5 kW vacuum pump (*Gast*, model 0823, *Wainbee ltd.*, Pointe-Claire, QC, Canada). Each air stream passed through a flow meter (*Rate-Master*, model RMA-21-BV, *ITM*, Ste-Anne-de-Bellevue, QC, Canada) and then through an NH_3 trap consisting of 250 ml of 0.32 M HBO_3 indicator solution in a sealed 500ml flask (Miles et al., 2008). The tubes from each tunnel converged in a plenum prior to passing through a liquid trap (500 ml flask) and the vacuum pump. The sampled air was then exhausted outside.

Land-application simulation

Land application simulations for both the ISPAD and the conventionally stored manure were done in combination with each of the five soil types, with every manure-soil combination repeated in triplicate, resulting in 30 simulations. Each land-application simulation used all five wind tunnels. For each simulation, 7.5 l of prepared soil was spread in each soil pan. Water was added to each soil sample to bring it to 25% of the gravimetric water holding capacity previously established. Manure samples were prepared by weight, and were quickly applied to the soil in the pans by hand. The manure application rate was equivalent to 115 kg TKN ha^{-1}. The tunnels were quickly placed on the pans and airflow was started.

Volatilized NH_3 sampling was done after 2, 4, 6, 8, 24 and 47 hours. At each sampling time for each tunnel, the acid trap was removed and a fresh one installed. The sampling air flow rate and the wind speed inside each tunnel were noted, as was the ambient temperature. The removed acid traps were returned to the lab and chilled to 4 °C. Each trap was subsequently analyzed for NH_4^+-N content by titration with 0.1 M HCl (Miles et al., 2008). The trap NH_4^+-N content was then scaled up to represent NH_3 volatilization in the tunnel based on the ratio of measured sampling and the tunnel airflows, and the time elapsed since the previous sample.

Statistical analysis

All statistical analyses were performed using SAS 9.2 (®2010, *SAS Institute inc.*, Cary NC, USA). The manure and soil characterization data were

collected in triplicate or quintuplicate from the composite samples collected from manure tanks and fields. The significance of differences between measured values was evaluated by the Student-Newman-Keuls method in a simple analysis of variance based on a completely randomized design. The NH_3 volatilization wind tunnel tests used a randomized complete block design, considering manure type as the treatment factor and soil type as the block factor. The dependant variable was NH_3 volatilization. Treatments were assigned randomly to experimental units (wind tunnels) and all treatments-block combinations were completed in triplicate.

6.3 Results and Discussion

Manure characteristics

The measured characteristics of the experimental manures are presented in Table 6.1. While the concentrations of both total and volatile solids were statistically similar for both manures, due in part to the variability of these values, the ratio of volatile to total solids was significantly different ($P = 0.0059$), reflecting the consumption of organic matter by the anaerobic digestion processes occurring in the ISPAD tank. The pH of both manures was similar, as was the total nitrogen content (TKN); however the total ammonia/ammonium nitrogen content (TAN) and the ratio of TAN:TKN were significantly different, with $P = 0.0096$ and $P = 0.0407$ respectively. This ratio, at 0.69 and 0.49, respectively for the ISPAD and conventional manures, indicates greater breakdown of manure proteins to TAN, confirming the results of a previous study on protein degradation and nitrogen conservation in ISPAD (King et al., 2010b). The total

phosphorus and potassium content were also similar. This combination of similarities and differences allowed comparisons of the wind tunnel results based on only the significantly different characteristics: for the selected application rate of 115 kg TKN ha^{-1} the quantities of manure used were nearly identical, resulting in equal moisture content of the soil-manure mixture; the manure solids added, affecting the rate of manure infiltration into the soil, were equivalent; the most important measured difference between the manures was TAN content, which most authors report does not impact volatilization and should result in similar ammonia loss from each tunnel on a % TAN basis (Chantigny et al., 2004; Huijsmans et al., 2003).

Soil characteristics

Table 6.2 presents the assessed characteristics of the experimental soils used in this study. The soils, all topsoil from the indicated series, were selected to represent as much variety in properties as possible, though there is no significant difference in pH between the five soils. The Student-Newman-Keuls test was used to group the soils for each property, illustrating the variety achieved in the selection process: in terms of organic matter, the clean sand, Upland sandy loam and Ste Rosalie clay were each significantly different from each other, and from the other two soils which were grouped together. A similar grouping, with clean sand and Ste Rosalie clay each distinct and the other three together, held for gravitational water holding (H_2O) capacity. For cation saturation, the groupings were quite different in that the Upland sandy loam and Ormstown silt series were grouped together, while the Ormstown silt, Ste Rosalie clay and Saint Bernard

loam series were in a second group, and the clean sand again was distinct. The five soils were all significantly different from each other in terms of all particle size distribution and CEC. The TKN values were highly variable, due to the non-uniform nature of soils and the fact that the organic nitrogen represented by the TKN is present only in the organic portion of the soils. Though initial and final soil and soil-manure samples were analyzed for TKN to do a mass balance on nitrogen in the tunnels, the high variability of these values rendered the calculations inconclusive. While the variability of the soil TAN content was high, the values were extremely small, confirming that it was appropriate to assume that all NH_3 volatilized during the wind tunnel tests originated in the applied manure.

Ammonia volatilization from land application

The wind tunnel simulations of manure land-application were performed at a uniform temperature of 21 °C, with the air-flow through the tunnels maintained close to 0.3 m s^{-1} to ensure that these variables did not impact the volatilization in the tunnels. The progress of NH_3 volatilization during these tests is summarized in Figures 6.2 a and b. These figures present volatilization on the basis of % TAN, though the statistical analysis was done on this basis as well as using the absolute values in mg NH_3-N. This analysis revealed that the effect of both manure type and soil type were significant on both bases, $P < 0.003$ for mg N and $P < 0.0001$ for % TAN. There was no significant interaction between the factors in either case. The significance of the manure effect using the mg N values confirms that the results were not limited by NH_3-saturation of the air flowing through the wind tunnels, which would have resulted in equal values for the

tunnels exhibiting higher volatilization rates. This means that discussion of the remaining results on the basis of % TAN is appropriate.

As mentioned in section 6.1, the literature suggests that volatilization should be consistent in terms of % TAN, with other factors held constant, meaning that there should not be a significant effect of manure type, though there should be an important effect of soil type. As most properties of the manures were similar, the manure type effect seen in the current study can be attributed to the anaerobic digestion treatment process. A study using more than two types of manure, based on a wider characterization such as electrical conductivity and buffering capacity, could be used to determine which characteristic resulting from anaerobic digestion is the controlling factor for ammonia volatilization.

The volatilization results based on soil type were also grouped using the Student-Newman-Keuls method, indicating that, for each manure type, volatilization from the Upland sandy loam, St Bernard loam and Ormstown silt series were statistically similar while the Ste Rosalie clay and the clean sand were each significantly different. In each case, volatilization was highest for the clean sand, intermediate for the three similar soils, and lowest for the Ste Rosalie clay series. Returning to the analysis of soil properties presented in Table 6.2, the same grouping was seen for gravimetric water holding capacity. Exploratory plots of volatilized ammonia versus each soil property individually also revealed patterns suggesting linear relationships for water holding capacity, CEC and cation saturation. These relationships were then examined statistically using a two-factorial rather than a blocked experimental design, which allowed the qualitative

values for soil type to be replaced with quantitative values for individual soil properties. A variety of combinations based on the properties identified above revealed a significant effect of moisture holding capacity with $P < 0.0001$, and the same for CEC, but only when considered separately. Again, the use of only two levels of manure type limited the scope of analysis available. However, these results provide an interesting lead to be followed in future research, which could perhaps extract a numerical equation relating the appropriate manure and soil characteristics to ammonia volatilization.

Table 6.3 summarizes the % TAN volatilized in 47 hours for each manure-soil combination with averages by manure and soil type. The ISPAD manure was found to lose 47 % less TAN than the conventionally-stored manure. The highest quantity of NH_3 was volatilized from the clean sand, while the lowest was from the Ste Rosalie clay series, at 33 % of this. Three intermediate soils lost approximately 54 % of the NH_3 lost from the clean sand.

Fertilizer value of ISPAD manure applied to land/crops

Because the Quebec government has stringent requirements for nutrient management planning for the province's livestock and crop producers (MDDEP, 2005), they have also published detailed methods for calculating the fertilizer value of manures (CRAAQ, 2003). Based on extensive study of the current literature, this document recommends the following equation for calculating the nitrogen fertilizer value ($N_{available}$) of manure when the appropriate data are available:

$$N_{available} = (TAN + (N_{organic} \times CEFO)) \times CV^{-1} \times CA^{-1} \times CP \quad (1)$$

where:

$N_{organic} = TKN - TAN$ (2)

and:

CEFO = organic fraction efficiency factor based on C:N ratio

CV = volatilization factor based on land-application method

CA = availability factor based on application date (spring or fall)

CP = previous application factor based on years of manure applied

Using Equations (1) and (2), $N_{available}$ was calculated for the two experimental manures, for several possible cases: Case 1 included direct manure injection to a clay soil supporting row crops in spring; Case 2 covered spray application incorporated in 48 hours, to a sandy loam soil in row crops in summer; Case 3 represents spray application, unincorporated, on a sandy loam soil hayfield in autumn. These combinations were selected to illustrate the range of possible factors. In all cases it was assumed for simplicity that no manure had been previously applied to these fields, thus CP = 1. For both manures, the C:N ratio was estimated at 6:1 based on the VS and TKN values (Table 6.1), despite the loss of carbon as methane from the ISPAD-treated manure. For the ISPAD manure, the values for CV were modified using the ratio of NH_3 volatilized from ISPAD as compared to conventionally-stored manure (0.53:1) presented in Table 6.3, so that:

$CV_{ISPAD} = 1 + ((CV - 1) \times 0.53)$ (3)

Noting that CV is a dividing factor with values between 1 and 1.8, equation (3) reduces the volatilization losses to 53 % of the expected losses for

standard manures. The resulting nitrogen fertilizer values are summarized in Table 6.4. These results demonstrate that in Case 1, representing the ideal manure land-application conditions, the ISPAD manure has a modest advantage of 6 % over the conventionally-stored manure; this increases to 18 % under the average conditions of Case 2, and to 21 % under the least controlled application conditions of Case 3. From these calculations it can be proposed that, for equivalent phosphorus content, manure from the ISPAD tank can provide up to 21 % more plant-available N than equivalent manure from a conventional uncovered storage tank, thus reducing the mineral nitrogen required for top-up by an equivalent amount.

6.4 Conclusions:

Pig manure from a five-year-old In-Storage Psychrophilic Anaerobic Digestion system and similar manure from a conventional uncovered storage tank were tested for ammonia volatilization by wind tunnel simulations of land application on five different soils. These comparisons revealed that:

i. When ISPAD manure and conventionally stored manure were applied to arable soils, the % TAN volatilized from ISPAD manure was less than that from conventionally stored manure, averaging 53% , on every soil type tested, which indicates that a property of the manure other than TAN content is controlling volatilization.

ii. The most important soil parameters influencing volatilisation of ammonia nitrogen from the land applied manure appear to be gravimetric water holding capacity and CEC.

iii. Pig manure treated and stored in an ISPAD covered tank contains 40 % more ammonia nitrogen than manure with similar TKN stored in a standard uncovered tank. Less of this plant-available TAN is volatilized during land application, resulting in a nitrogen-fertilizer value up to 21 % higher, with equivalent phosphorus content, than for conventionally stored manure.

The results of this study demonstrate that the fertilizer value of ISPAD-treated manure cannot be evaluated using the same assumptions as are used for conventionally stored manure. Further research could pinpoint the most important parameters influencing this effect, to develop guidelines that would include ISPAD manure in nutrient management planning.

6.5 Acknowledgements

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6.7 Tables

Table 6.1: Characteristics of experimental manures

	Uncovered tank manure	ISPAD tank manure
TS ($g\ l^{-1}$)	37.68 (1.93)	33.46 (4.12)
VS ($g\ l^{-1}$)	27.68 (1.75)	22.95 (3.24)
VS:TS	0.73 (0.01)	0.69 (0.01)
pH	7.54 (0.01)	7.60 (0.05)
TAN ($g\ l^{-1}$)	1.31 (0.06)	1.63 (0.08)
TKN ($g\ l^{-1}$)	2.66 (0.20)	2.35 (0.30)
TAN:TKN	0.49 (0.08)	0.69 (0.12)
P ($g\ l^{-1}$)	2.29 (0.49)	2.82 (0.48)
K ($g\ l^{-1}$)	1.42 (0.39)	1.04 (0.16)

(standard deviation in brackets)

Table 6.2: Characteristics of experimental soils

	Clean sand	**Ste Rosalie clay**	**Upland sandy loam**	**St Bernard loam**	**Ormstown silt**
Organic matter (*%*)	0.1 (0.0)	6.2 (0.5)	5.0 (0.3)	6.5 (0.4)	3.9 (0.2)
H_2O capacity (*%*)	14.3 (0.0)	32.8 (1.6)	24.8 (1.7)	25.4 (1.5)	23.8 (2.5)
Particle size (*%*):					
Sand	82.9 (0.9)	7.4 (1.7)	71.2 (1.8)	38.6 (1.5)	1.6 (3.4)
Silt	0.5 (0.9)	25.7 (1.5)	6.8 (1.7)	31.3 (1.9)	62.2 (1.3)
Clay	16.6 (0.9)	66.9 (2.2)	22.0 (0.1)	30.1 (0.4)	36.3 (2.2)
pH	6.3 (0.1)	6.8 (0.1)	6.5 (0.1)	6.9 (0.3)	6.5 (0.9)
CEC (*meq/100g*)	2.0 (0.5)	36.8 (2.8)	18.3 (1.0)	23.3 (2.2)	20.6 (1.1)
Cation saturation (*%*)	26.0 (5.9)	92.0 (2.6)	73.0 (3.5)	90.6 (8.8)	81.8 (17.3)
TKN ($g\ kg^{-1}$)	2.4 (0.6)	5.4 (0.8)	4.4 (0.2)	3.5 (0.9)	3.7 (1.3)
TAN ($mg\ kg^{-1}$)	3.0 (2.0)	3.0 (0.4)	60.0(10.0)	50.0(30.0)	8.0 (3.0)

(standard deviation in brackets)

Table 6.3: Total ammonia volatilized (% TAN) in 47 hours from experimental manures applied to different soils in wind tunnel simulations

	Uncovered tank manure	ISPAD tank manure	Average
Clean sand	61.4 (2.4)	36.6 (3.5)	49.6
Upland Sandy Loam	38.2 (12.9)	16.4 (3.3)	27.3
Ormstown Silt	34.3 (7.9)	18.7 (8.0)	26.5
St Bernard Loam	33.0 (4.7)	24.7 (5.9)	28.8
Ste Rosalie Clay	25.0 (5.4)	7.8 (2.6)	16.4
Average	38.6	20.8	

(standard deviation in brackets)

Table 6.4: Nitrogen fertilizer value of experimental manures for three land-application scenarios described in Section 3.4 (for similar phosphorus and potassium fertilizer values)

	Uncovered tank manure	**ISPAD tank manure**
Case 1 – Ideal		
$N_{available}$ ($g\ l^{-1}$)	1.78	1.88
Case 2 - Average		
$N_{available}$ ($g\ l^{-1}$)	1.25	1.48
Case 3 - Uncontrolled		
$N_{available}$ ($g\ l^{-1}$)	0.80	0.97

6.8 Figures

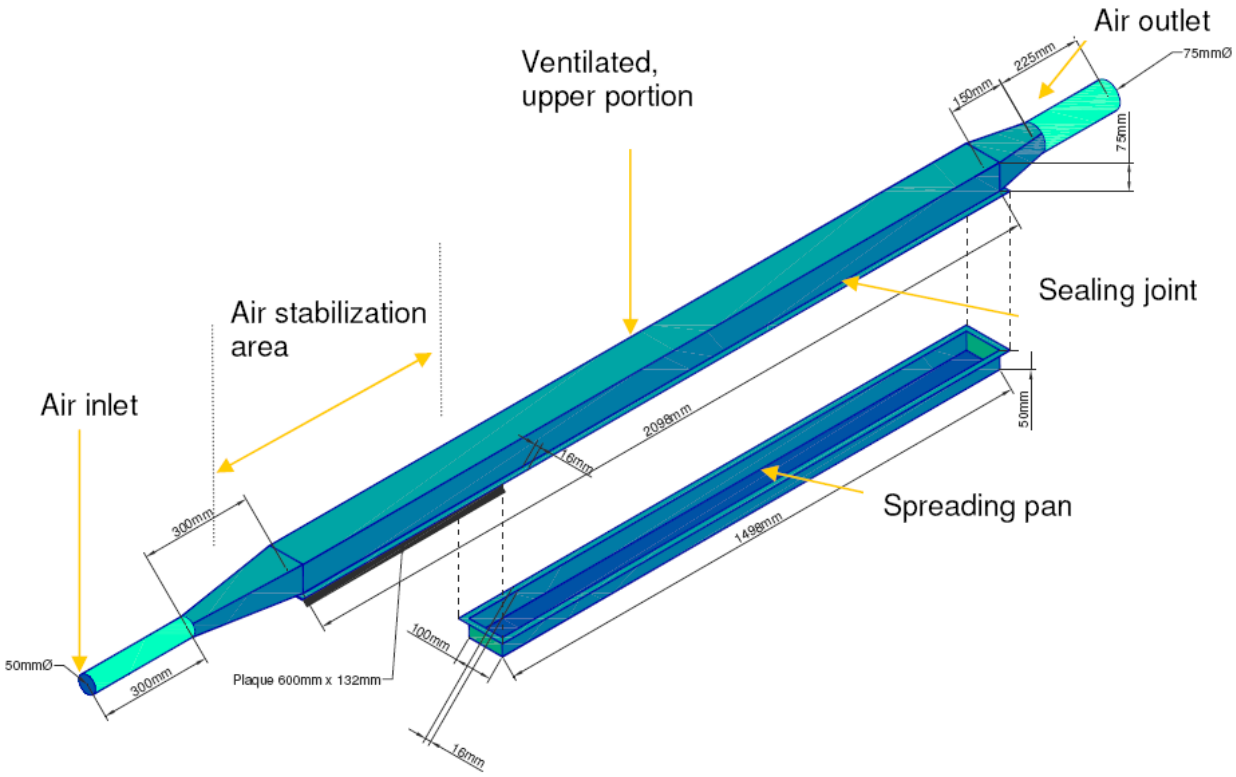

Figure 6.1: Wind tunnels used for manure land application simulations

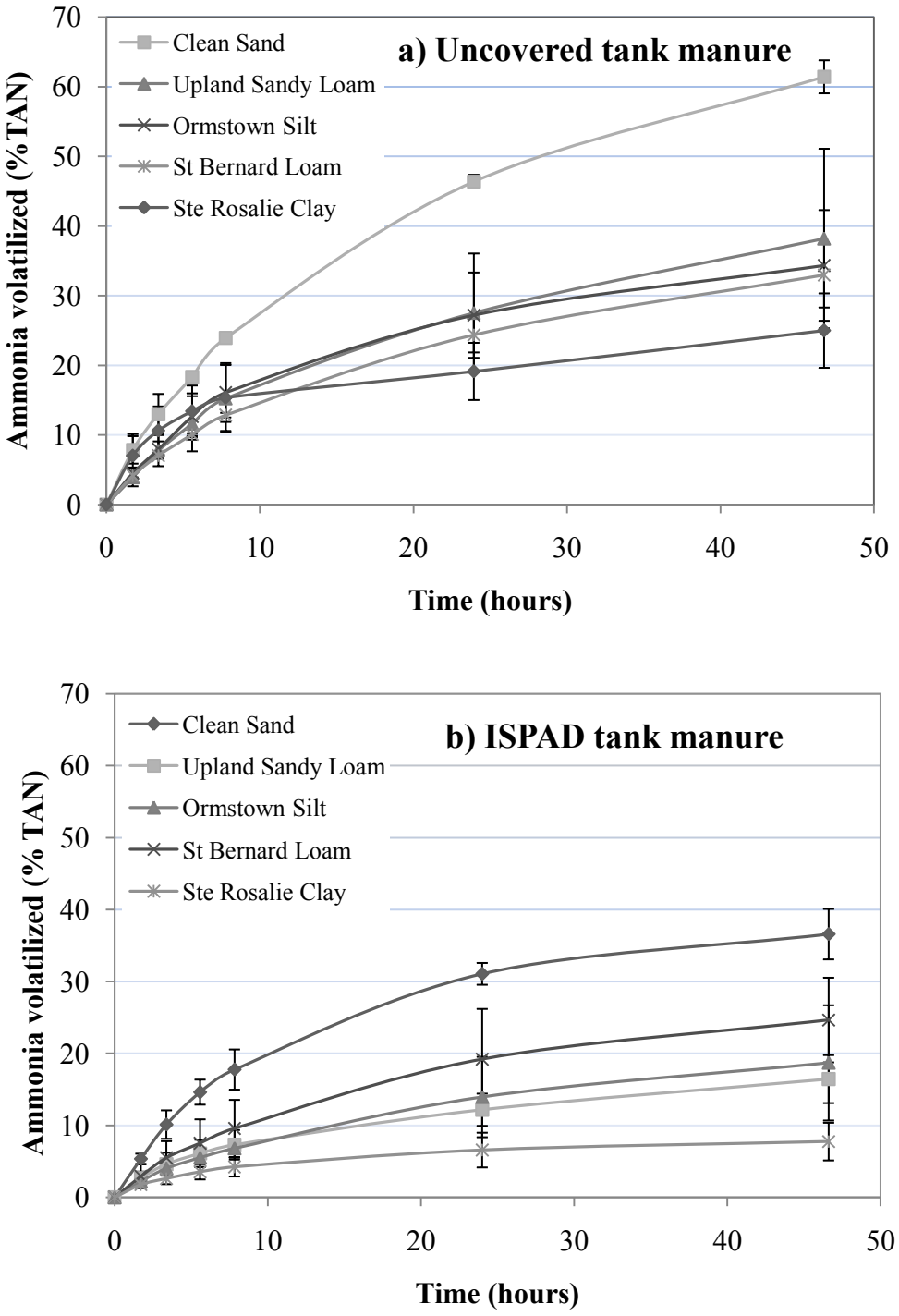

Figure 6.2: Cumulative NH_3 volatilized during land application simulations

Notes: Data points represent the average of three replicates.
Error bars represent +/- one standard deviation.

Chapter 7. Summary and Conclusions

7.1 Summary

This research project was designed to address four critical issues affecting the potential for success of In-Storage Psychrophilic Anaerobic Digestion (ISPAD): (i) the anaerobic microbial community in the manure must be shown to acclimate to the ambient (psychrophilic) operating temperature; (ii) the acclimated microbial kinetics and the resulting production of methane must be defined; (iii) the kinetics of protein degradation and their effect on nitrogen speciation must be determined; and (iv) the effect of ISPAD treatment on the volatilization of NH_3 following land application of the treated manure, and its impact on fertilizer value must be assessed. These issues were investigated sequentially by performing a variety of laboratory analyses, incubations and simulations on samples of manure taken from a full-scale ISPAD installation treating swine manure. Assays were also run using fresh manure and manure from a conventional uncovered storage tank to allow comparisons to be made.

Stage 1: Microbial Acclimation

The first series of experiments used biochemical methane production (BMP) assays to investigate the occurrence of microbial acclimation. In these assays, known quantities of active biomass and organic substrate were incubated under controlled conditions. Methane production was measured periodically over the long incubation, producing a temporal profile which illustrated both the production rate and cumulative amount of methane produced. Assays were performed at three temperatures: 8, 18 and 35 °C; using active biomass from three sources: fresh manure, conventionally stored manure

and ISPAD manure; with fresh manure as substrate or with no substrate for endogenous control. In addition, standard laboratory methods were used to analyse the composition of the three experimental manures.

The composition study revealed evidence of prior digestion in the ISPAD tank through reduced volatile solids, and of microbial acclimation by the absence of intermediate substrates such as volatile fatty acids. The initial rates of methane production in the BMP assays demonstrated robust microbial activity in the ISPAD biomass at all temperatures, which indicated acclimation to the studied temperature range, in comparison to minimal activity of the fresh manure biomass and modest activity in the conventionally stored manure biomass. The final cumulative quantity of methane produced from endogenous digestion demonstrated that manure in the ISPAD tank had undergone extensive digestion prior to the incubation, having released 63 % of its potential methane in contrast to 15 % released from the conventional storage tank. The time elapsed prior to maximum methane production during the BMP assays provided an estimator for the acclimation time that might be required for new ISPAD installations, suggesting that an existing storage tank converted to ISPAD could acclimate fully in the first year of operation.

Stage 2: Process Kinetics

Because the first stage of research demonstrated that the microbial community in ISPAD was acclimated to its psychrophilic operating conditions, the second stage proceeded to investigate the kinetics of the resulting acclimated digestion process. This was done using specific substrate activity (SAT) assays, again comparing the active biomass of ISPAD with the biomass from fresh and conventionally stored manure. In

these assays, as in the BMP's, known quantities of active biomass and substrate were incubated under controlled conditions. The substrates used were individual compounds selected to demonstrate the activity of specific stages in the overall digestion process. Concentrations of the substrate were monitored during the assay to illustrate the temporal progression of substrate uptake by the appropriate microbial population. Glucose was used to represent acidogenesis, propionate and butyrate were both substrates for acetogenesis, acetate drove acetoclastic methanogenesis, and hydrogen was the substrate for both homoacetogenesis and hydrogen-using methanogenesis. These assays were also performed at 8, 18 and 35 °C.

The initial rate of substrate uptake in each assay was defined as the extant activity for that substrate. Comparing these rates across the set of assays revealed strong activity on all substrates at all temperatures for the ISPAD biomass, which, in comparison to minimal activity for the fresh manure biomass and modest activity for the conventionally stored manure biomass confirmed the acclimated nature of the ISPAD microbial community. Mathematical curve fitting of a Monod-type substrate uptake equation was performed on the glucose uptake datasets, to estimate three kinetic parameters: maximum substrate uptake rate, q_{max}, microbial yield, Y, and half-saturation constant, K_s. Comparing these values for the three microbial communities, the q_{max} results showed greater activity with a peak near 18 °C for the ISPAD community, suggesting that robust psychrophilic and mesophilic glucose-consuming populations coexist in ISPAD. The Y results indicate increased efficiency of glucose consumption in ISPAD, and the decrease in K_s for ISPAD demonstrates increased substrate affinity. These results reinforce the classification of the ISPAD biomass as acclimated, and illustrate differences in kinetic

values that would prevent use of models designed for mesophilic digestion to represent the ISPAD process, without important modifications.

Stage 3: Protein Degradation

Since the results of stage two demonstrated acclimated kinetics for the carbohydrate degradation pathway, it was essential to investigate the protein degradation pathway as well, as it was less well documented in the literature, and has an important effect on the ultimate nitrogen fertilizer value of ISPAD treated manure. To accomplish this, the SAT protocol described in the preceding section was modified to examine the major stages in protein degradation. Incubations in these specific proteolytic assays (SPA) were done as for the SAT's, using only the active biomass in ISPAD manure, at the same temperatures, with appropriate substrates: albumin to represent protein de-amination and acidogenesis from amino acids, lysine for acidogenesis from this specific amino acid, glucose alone and in combination with albumin to investigate interactions between carbohydrate and protein degradation mentioned in the literature, and fresh manure for protein disintegration, hydrolysis and acidogenesis. The rates of degradation in these assays were evaluated by measuring, over the course of the assay, the concentration of NH_4^+, which is released by acetogenesis of amino acids, producing a temporal profile of activity on the protein degradation pathway.

As the ISPAD manure samples used in this stage of research were collected two years after those used in the first two stages, a composition analysis and set of SAT assays were performed to characterize the active biomass in this sample, and to compare it to the previous sample. These data revealed extensive prior digestion through decreased volatile solids content, as for the previous sample. The activity profile from the SAT's

was compared to values from the literature for mesophilic communities, and again illustrated an acclimated nature, with strong activity on all substrates but relatively lower propionate, and moderately lower butyrate and hydrogen activity for equal acetate and glucose activities. In addition, the composition data indicated that while the total nitrogen in ISPAD treated manure was conserved during the process, 65 % of the initial organic nitrogen, composed of proteins, had been transformed in the digestion process to ammonia/ammonium nitrogen (TAN).

The results of the SPA assays were compared on the basis of response patterns and activity levels, as volatile fatty acid concentrations were measured along with NH_4^+. These comparisons revealed that the ISPAD biomass actively degraded albumin at all temperatures, though the variation of rates with temperature indicated that this population was mesophilic in nature. Lysine was degraded only after a lag period, suggesting either non-acclimation, or a lack of another essential compound such as H_2 in the single substrate assay. Degradation of proteins in fresh manure was minimal, indicating that disintegration of complex organic matter could be the limiting step on this pathway. Comparing assays fed albumin and glucose together and separately did not expose any evidence of inhibition between these substrates. As the overall digestion effectiveness for protein degradation mentioned above was 65 %, it was postulated that the long retention time of the manure in the ISPAD tank compensated for the slow activity of the mesophilic protein-degrading populations to provide effective degradation over the treatment period.

Stage 4: Nitrogen conservation

The results of stage three indicated that manure nitrogen was conserved during ISPAD treatment, and that this should contain a high level of plant available TAN. Therefore stage four of the research was designed to determine whether this TAN would be volatilized as NH_3 following land application, and estimate the impact on N fertilizer value of the treated manure. This was done by simulating land application of ISPAD manure as well as conventionally stored manure, using small wind tunnels. To ensure that results would be true for most soil types, topsoil from five soil series with different properties were used. Ammonia volatilized from the manure in the tunnels was monitored, to provide a 46 hour profile of volatilization from each manure-soil combination.

The extent of NH_3 volatilization was affected both by manure and soil type. As many of the manure parameters were similar, this suggested an effect of ISPAD treatment that was not evident on the measured parameters. It was, however, a beneficial effect, resulting in the ISPAD manure losing on average over all the soil types only 53 % of the % TAN lost by the conventionally stored manure. This value was used to modify Quebec government guidelines for estimating the fertilizer value of land applied manures. Assessing three theoretical cases in this way demonstrated that manure treated by ISPAD could contribute 21 % more plant available TAN for an equivalent P concentration, compared to conventionally stored manure.

7.2 Conclusions

The most important conclusion of this research project is that In-Storage Psychrophilic Anaerobic Digestion, which develops a robust and effective anaerobic digestion process due to microbial acclimation, is a feasible and effective system for the treatment of liquid swine manure in the Canadian climate.

Four major conclusions about the anaerobic microbial process occurring in ISPAD may be drawn from the research summarized in the preceding sections:

- The manure microbial community present in a full-scale ISPAD installation did acclimate to the psychrophilic conditions in the tank.
- The kinetics of intermediate stages in the ISPAD process changed significantly due to microbial acclimation, showing the development of co-existing psychrophilic and mesophilic glucose-consumers.
- The protein degradation pathway in the ISPAD process, while active, remained mesophilic.
- An aspect of the ISPAD process that should be identified changes the kinetics of NH_3 volatilization of the treated manure when applied to cropland.

In addition, the effectiveness of manure treatment by ISPAD may be estimated by the results obtained in this study:

- 24 to 43 % reduction of manure volatile solids
- 63 % of manure potential methane released for use as fuel
- 47 % less ammonia volatilized following land application
- 21 % more plant available N supplied to crops

For the design and optimization of future ISPAD installations, the following points may be made:

- Studies of other ISPAD installations must be undertaken to confirm that the results of this study can be generalized to future installations.
- The acclimated kinetics of stages in the ISPAD process, and the co-existence of psychrophilic and mesophilic populations sharing a single substrate require that an ISPAD-specific model be developed to optimize the process.
- The protein degradation process in ISPAD must be included in such a model due to its importance in determining the fertilizer value of the treated manure.
- While an ISPAD process model would predict the TAN content of treated manure, a land application model incorporating the effect of both ISPAD and soil type could also be developed using the output parameters of the process model.

7.3 Contributions to Knowledge

The primary goal of this study was to evaluate the feasibility and effectiveness of In-Storage Psychrophilic Anaerobic Digestion for the treatment of liquid swine manure in the Canadian climate. The study was specifically designed to contribute to knowledge, focusing on gaps and controversies described in Chapter 2, and summarized in section 2.9. While there have been some contributions on the issues from other researchers

during the course of the project, the contributions to knowledge made in this study may be summarized as follows:

i. Evidence for microbial acclimation was found using several different measures, including biochemical methane production rates and total; substrate uptake activity rates and fitted kinetic parameters; chemical composition of treated manure and relative rates of substrate uptake from standard mesophilic activity tests using acetate activity as a base. These provided quantitative assessment of the time required for and extent of acclimation in an ISPAD installation, and may be referred to by researchers investigating other acclimated anaerobic processes. This has contributed both data and methods for monitoring the progress of acclimation.

ii. Assessing the kinetics of the acclimated ISPAD microbial community provided evidence of co-existing psychrophilic and mesophilic populations sharing a single substrate, which may explain the results of previous studies in the literature that reported unusual relationships between temperature and substrate uptake. While a very small number of other researchers have mentioned this possibility, these results provide concrete evidence to support the multi-population hypothesis, and may help to make this view more generally accepted.

iii. Describing the kinetics of both carbohydrate and protein degradation illustrated the important point that in acclimated communities, each population develops in a different way, some remaining mesophilic and others developing co-existing psychrophilic populations. This demonstrates that there is not a single

temperature acclimation factor that could be applied to all the pathways in psychrophilic anaerobic digestion. Rather, each pathway must be evaluated individually and modified accordingly. These results provide important guidance for the development of future models of psychrophilic digestion, which, to the author's knowledge, have not been studied since the publication of the ADM1.

iv. Though studies of anaerobic protein degradation in general are scarce and somewhat dated, investigation of protein degradation during psychrophilic digestion of manure has only been reported in a single article in the literature, published in Russian, of which the study author graciously provided an English translation for reference in the current research project. Thus the current study, taking a different experimental approach, provided new information about the kinetics of psychrophilic degradation of protein, for manure in particular, though they may be generalized to other substrates in the future.

v. Reports in the literature regarding the volatilization of ammonia from digested manures have used varying measures and methods, producing conflicting results that cannot then be generalized to other applications. This study demonstrated that the volatilization of ammonia from land applied ISPAD manure was consistently and significantly less than for conventional manure over several soil types. An approach for applying this result to the calculation of manure fertilizer value was also proposed.

7.4 Directions for Further Research

From the conclusions listed above, the further research on ISPAD may take three directions:

- Studies of other ISPAD installations, including development of the ISPAD microbial community following start-up and performance of the system both during and after acclimation, must be undertaken to confirm that the results of this study can be applied to future installations.
- An ISPAD process model could be developed, including the variation of kinetics during acclimation, the effect of multiple co-existing microbial populations sharing a substrate and the important role of protein degradation.
- An additional land application model could be developed, following investigation of the manure property(ies) that affect NH_3 volatilization, and the ISPAD process that controls this, linking the output of the process model to calculations of fertilizer value following application to cropland.

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