DOCTOR OF PHILOSOPHY

Mitigating greenhouse gases emission from cattle slurry: An approach for small-medium scale farms

Bastami, Mohd Saufi

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MITIGATING GREENHOUSE GASES EMISSION FROM CATTLE SLURRY: AN APPROACH FOR SMALL-MEDIUM SCALE FARMS

A thesis submitted to Bangor University by

Mohd Saufi Bastami

In candidature for the degree

Philosophiae Doctor

2016

Supervisors: Professor Dave R. Chadwick and Professor Davey L. Jones

School of Environment, Natural Resources and Geography
Bangor University
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LL57 2UW
UK
SUMMARY

Slurry stores are an important source of both methane (CH$_4$) and ammonia (NH$_3$) emissions. Strategies to reduce these emissions include modifying the slurry environment through use of natural and biological additives. In this thesis, two such strategies were explored. The first three experimental chapters explored the potential to mitigate emissions by using inorganic acid (50% concentrated sulphuric acid H$_2$SO$_4$), fermentable carbohydrates at 10% w/v to reduce slurry pH, and biological additives (effective microorganisms - EM) at 5% v/v, to suppress methanogenesis. The addition of a carbohydrate source resulted in 'self-acidification' of cattle slurry from average pH 6.8 to pH's as low as pH 3.5 in laboratory and pot-scale experiments, under both warm and cool conditions. The reduced slurry pH inhibited both CH$_4$ and NH$_3$ emissions significantly (by between 72% and 84% for CH$_4$, and 57% and 92% for NH$_3$). The use of agriculture food-chain by-products as sources of available carbohydrates, such as brewery spent grains, successfully promoted self-acidification by producing large amounts of lactic acid and reducing the slurry pH to 4.0. As a result, the greenhouse gas (GHG) carbon dioxide equivalent (CO$_2$ eq.; CH$_4$+CO$_2$+N$_2$O) emission was inhibited by 86%. However, NH$_3$ emissions were not significantly inhibited. Meanwhile, methane oxidation by methanotrophs within slurry crusts was also explored, but bio-augmentation with EM showed no clear effect on this potential CH$_4$ sink. The application of self-acidified slurry to ryegrass in a pot experiment demonstrated significant NH$_3$ and CH$_4$ inhibition compared to the untreated slurry during the first 48 hours after application. However, plant health was severely affected by the acidic slurry treatment, resulting in plant death in most cases. The mechanism for the self-acidification of slurry was elucidated by a conducting a metagenomic analysis of the slurries immediately after carbohydrate addition, and at the end of the 30 day storage period. This linked the dominance of Lactobacillales in the self-acidified slurry to the high lactic acid production and reduced pH in the treatments that received the carbohydrate source. The metagenomic analysis also identified groups of hydrogenotrophic methanogens of the Order Methanobacteriales, Methanomicrobiales and Methanosarcinales, as the dominant methanogens in both treated and untreated slurries. The low CH$_4$ emission from the self-acidified slurry was associated with low abundance of methanogens. Whilst this study demonstrated the potential for self-acidification of slurry to reduce CH$_4$ production and emission, as well as reduction in NH$_3$ emissions from slurry stores and following land spreading, further research is necessary to test the strategy at a larger scale, and develop methods that minimize the negative effect of the acidified slurry on soil and plant health, e.g. via slurry injection or incorporation.
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**Statement 4:**
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<th>Description</th>
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Dedication

A dedication to my family and to those who seek knowledge...

“By Time, Surely man is in loss, Save those who believe and do good deeds, and enjoin on each other truth, and enjoin on each other patience”.

Surah ‘Asr, Verses 1-3
ACKNOWLEDGEMENTS

The completion of this thesis and the project associated with this study was achieved with help from many hands and institutions. I am extremely thankful to almighty ALLAH, the beneficent and merciful that helped me a lot in every moment of my life and making my dream come true. I offer my humblest thanks to The Holy Prophet “Hazrat Mohammad (SAW)”, who is forever a source of guidance and inspiration for humanity.

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<table>
<thead>
<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>AD</td>
<td>Anaerobic digesters</td>
</tr>
<tr>
<td>AFBP</td>
<td>Agricultural food chain by-products</td>
</tr>
<tr>
<td>AFEM</td>
<td>Actiferm effective microorganism</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AOB</td>
<td>Ammonia oxidising bacteria</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BES</td>
<td>2-bromoethanesulfonate</td>
</tr>
<tr>
<td>BOD</td>
<td>Biological oxygen demand</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSG</td>
<td>Brewery spent grains</td>
</tr>
<tr>
<td>C</td>
<td>Carbon</td>
</tr>
<tr>
<td>C₃H₆O₃</td>
<td>Lactic acid</td>
</tr>
<tr>
<td>Ca</td>
<td>Calcium</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium chloride</td>
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<tr>
<td>CFC</td>
<td>Chlorofluorocarbon</td>
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<tr>
<td>CH₄</td>
<td>Methane</td>
</tr>
<tr>
<td>CHCl₃</td>
<td>Chloroform</td>
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<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CO₂ eq.</td>
<td>Carbon dioxide equivalent</td>
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<tr>
<td>COD</td>
<td>Chemical oxygen demand</td>
</tr>
<tr>
<td>DCD</td>
<td>Dicyandiamide</td>
</tr>
<tr>
<td>DGGE</td>
<td>Denaturing gradient gel electrophoresis</td>
</tr>
<tr>
<td>DMPP</td>
<td>3, 4-dimethylpyrazole phosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EC</td>
<td>Electrical conductivity</td>
</tr>
<tr>
<td>EFs</td>
<td>Emission factor</td>
</tr>
<tr>
<td>EM</td>
<td>Effective microorganism</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
</tr>
<tr>
<td>FWt</td>
<td>Fresh weight</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>GHG</td>
<td>Greenhouse gases</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>GHG CO₂ eq.</td>
<td>Greenhouse gas CO₂ equivalent</td>
</tr>
<tr>
<td>GLM</td>
<td>General linear model</td>
</tr>
<tr>
<td>GWP</td>
<td>Global warming potential</td>
</tr>
<tr>
<td>H</td>
<td>Hydrogen</td>
</tr>
<tr>
<td>H₂</td>
<td>Dihydrogen</td>
</tr>
<tr>
<td>H₂O</td>
<td>Water</td>
</tr>
<tr>
<td>H₂S</td>
<td>Hydrogen sulphide</td>
</tr>
<tr>
<td>H₂SO₄</td>
<td>Sulphuric acid</td>
</tr>
<tr>
<td>H₃PO₄</td>
<td>Phosphoric acid</td>
</tr>
<tr>
<td>ha⁻¹</td>
<td>Per hectares(s)</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HNO₃</td>
<td>Nitric acid</td>
</tr>
<tr>
<td>IPCC</td>
<td>Intergovernmental Panel on Climate Change</td>
</tr>
<tr>
<td>K</td>
<td>Potassium</td>
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<tr>
<td>KCl</td>
<td>Potassium chloride</td>
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<tr>
<td>KNO₃</td>
<td>Potassium nitrate</td>
</tr>
<tr>
<td>L</td>
<td>Litre(s)</td>
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<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>mg</td>
<td>Milligrams</td>
</tr>
<tr>
<td>MgO</td>
<td>Magnesium oxide</td>
</tr>
<tr>
<td>mL</td>
<td>Millilitre(s)</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MOB</td>
<td>Methane-oxidizing bacteria</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>N</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>N₂</td>
<td>Nitrogen gas</td>
</tr>
<tr>
<td>N₂O</td>
<td>Nitrous oxide</td>
</tr>
<tr>
<td>Na</td>
<td>Sodium</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NBPT</td>
<td>N-(n-butyl) thiophosphoric triamide</td>
</tr>
<tr>
<td>NGS</td>
<td>Next generation sequencing</td>
</tr>
<tr>
<td>NH₂OH</td>
<td>Hydroxyl amine</td>
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<tr>
<td>NH₃</td>
<td>Ammonia</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>----------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>Ammonium</td>
</tr>
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<td>NH₄-N</td>
<td>Ammonium-N</td>
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<tr>
<td>NH₄Cl</td>
<td>Ammonium chloride</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NO₂</td>
<td>Nitrogen dioxide</td>
</tr>
<tr>
<td>NO₂⁻</td>
<td>Nitrite</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>Nitrate</td>
</tr>
<tr>
<td>NO₃-N</td>
<td>Nitrate-nitrogen</td>
</tr>
<tr>
<td>NOB</td>
<td>Nitrite-oxidizing bacteria</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
</tr>
<tr>
<td>OH</td>
<td>Hydroxide</td>
</tr>
<tr>
<td>OLR</td>
<td>Organic loading rate</td>
</tr>
<tr>
<td>OM</td>
<td>Organic matter</td>
</tr>
<tr>
<td>ORP</td>
<td>Oxidation redox potential</td>
</tr>
<tr>
<td>OTU</td>
<td>Operational taxonomic unit</td>
</tr>
<tr>
<td>P</td>
<td>Phosphorus</td>
</tr>
<tr>
<td>P₂O₅</td>
<td>Phosphate</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PKC</td>
<td>Palm kernel cake</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>S</td>
<td>Sulphur</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SO₃</td>
<td>Sulphur</td>
</tr>
<tr>
<td>SO₄</td>
<td>Sulphur trioxide</td>
</tr>
<tr>
<td>t</td>
<td>Tonne</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume to volume</td>
</tr>
<tr>
<td>v/w</td>
<td>Volume to weight</td>
</tr>
<tr>
<td>VFA</td>
<td>Volatile fatty acids</td>
</tr>
<tr>
<td>VS</td>
<td>Volatile solids</td>
</tr>
<tr>
<td>w/w</td>
<td>Weight to weight</td>
</tr>
<tr>
<td>WFPS</td>
<td>Water filled pore space</td>
</tr>
</tbody>
</table>
μL       Microliter
μg       Microgram
μM       Micromolar
μmol     Micromole
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Supplements

Paper 1: Published
Do Effective Micro-organisms Affect Greenhouse Gas Emissions from Slurry Crusts?

Paper 2: Submitted

Paper 3: Accepted, June 2016
CHAPTER 1

1 Introduction

1.1 General introduction

Livestock industries continue to gain pace in global food production as a major source of protein for human consumption (Devendra, 2006, 2007; Boland et al., 2013). Global consumption of meat, milk and eggs has increased gradually over the years, in parallel with the increase in per capita income especially in East and South East Asia (Devendra, 2007; Boland et al., 2013), human population growth and urbanization (Schwarzer et al., 2012). As a consequence, animal excreta has increased as well as the emissions of greenhouse gases (GHG). This is the case in the livestock industry where cattle farming are one of the major GHG contributors, arising from the animal house, manure store and during manure processing. Further GHG emission occurs following manure application to soil (Jungbluth et al., 2001; DeSutter and Ham, 2005; Amon et al., 2006; Berg and Pazsiczki, 2006; Lovanh et al., 2010; Chadwick et al., 2011), and deposition of dung and urine by grazing livestock (Saggar et al., 2004). Managing and reducing GHG from animal manure is important as they contribute to about 10% of global agriculture GHG emission (Owen and Silver, 2015). In this research context, cattle manure is responsible to over 33% of CH₄ livestock total manure emission to the atmosphere (FAO, 2016a).

All animal manures (cattle, chicken, sheep, goat and pig) are significant sources of GHG. The GHG associated with manure management are carbon dioxide (CO₂), methane (CH₄) and nitrous oxide (N₂O), and some traces of other gases (Danny Harvey, 1993; Clemens et al., 2006; Johnson et al., 2007). Recent updates by the Intergovernmental Panel on Climate Change (IPCC), as stated by Myhre et al., (2013), indicate that CH₄ and N₂O contribute 34 and 298 times the global warming potential (GWP) compared to CO₂ over 100 years’ time horizon. Therefore, improved manure management and further reduction of GHG emissions could deliver great benefits to the environment and economy.

Mitigating GHG is part of challenge to face the global climate change to ensure food security for future humankind (FAO, 2016b). Three general GHG mitigation
strategies have been suggested by Smith et al. (2007); (i) enhance removal of GHG, (ii) avoiding generation of GHG, (iii) reduce current emission of GHG, or a combination any of these approaches. The best practices to improve GHG mitigation are through better manure collection, storage facilities and anaerobic biogas production (Andeweg and Reisinger., 2014). Yet, other approaches are also practiced worldwide such as aerobic treatment lagoon of slurries, solid-liquid separation/filtration, use of nitrification inhibitors, acidification and cooling (Hristov et al., 2013; Petersen et al., 2013a; Andeweg and Reisinger, 2014). However, most of these approaches are impractical for small-scale farmers, such as those in Southeast Asian countries (Petersen et al., 2013a), resulting in the slow progress of manure management in terms of GHG mitigation practices. An alternative strategy to mitigate GHG from animal manures, particularly from slurry during storage is needed to fulfil the needs of cheap, practical and farmer-friendly practices. Such qualities are urgently needed to better suit manure management in the Malaysian cattle industry. – see Text box 1.

**Textbox 1: Malaysia cattle farming industry**

Integrated livestock farming systems involving crops are commonplace in the Malaysian cattle industry in comparison to intensive production system. These started over 30 years ago by smallholder or backyard paddy farmers in Kedah and Kelantan states after the paddy-harvesting period (Jalaludin and Halim, 2014). Nowadays, cattle farming integrated with palm oil production are the most favoured systems in Malaysia (Devendra, 2006, 2007). The Malaysian palm oil plantations cover over 3.2 million hectares, which is suitable for integration with cattle farming at a stocking rate at between 1.9 and 3.7 head ha\(^{-1}\) (Ayob and Hj Kabul, 2009). However, integrating cattle farming in palm oil plantation area is challenged by the topography of the area (hills, slopes) and uneven surfaces. In addition, many farms are owned by small enterprises, which further hinders the integration process.

Cattle farming is shifting from integrated systems to semi- and fully intensive systems to feedlots and special fattening farm as result of increasing meat demand, low beef meat self-sufficiency level (Malaysia beef meat self-sufficiency was at 29.5% in 2012) and insufficient or lack of grazing areas. The numbers of existing feedlot farms is unknown but it is estimated that 200 feedlots of various sizes existed in Malaysia in 1998 (Jalaludin and Halim, 2014). Theumber is likely to be much
larger now. Although larger feedlots are capable of producing 6000 to 7000 heads of cattle per year, this is unlikely to happen in Malaysia as most feedlots only hold about 10 to 200 heads at once. It is thought that < 10% of large ruminant farms in Malaysia have appropriate infrastructure and facilities (Mohamed et al., 2013). Thus, it is not surprising that >60% of the beef enterprise in Malaysia are small-medium sized entrepreneurs (Najim et al., 2015).

At present, manure management practices in cattle barns or feedlots are in either liquid (slurry) or solid forms. Cattle manure consists of faeces, urine and feed waste, which may contain mix ration concentrate or any agriculture by-products such as chopped oil palm frond, grasses, palm kernel cake (PKC), corn stem silage, essential oil by-product (citronella oil) or rice straw. The estimated production of manure in these systems is between 4.1 - 5.9 metric tons month⁻¹ 100 head⁻¹ (Najib et al., 2000). Meanwhile, slurry handling follows most slurry practices, in which liquid slurry is channel to multiple holding lagoons comprising of anaerobic and aerobic digestion ponds, before being released down to waterways. In certain areas of Malaysia, slurry from the holding ponds is pumped out and spread to cropland or grazing areas by gravity discharge or using a mechanical spreader following most practices in the United Kingdom (Chambers et al., 2001). The number of waste-ponds is increasing because of the transition from the conventional grazing farming system to intensive in-house production systems (Chung et al., 2013).

1.2 Aims and objectives

The aim of this thesis is to investigate alternative GHG mitigation approaches during manure (slurry) management (storage and land spreading), focussing on strategies applicable for small-scale and medium-scale farms. The objectives of the thesis are:

- To understand the available mitigation approaches and evaluate alternative CH₄ mitigation techniques by natural and biological additives using fermentable carbohydrate and effective microorganisms (EM).
- To evaluate the use of agriculture food chain by-products as an additive to slurry to mitigate GHG emissions.
- To examine further mitigation capability following slurry application to grassland.
- To determine EM’s influence on methane oxidation and N₂O emission in stored slurry crust.
To link changes in slurry microbial diversity profiles with the mechanism of GHG mitigation.

1.3 Thesis outline

The outline of this thesis is shown in Figure 1.1, which includes six experimental chapters.

Figure 1.1 Schematic representation of the thesis content and experimental chapters.

Mitigating Greenhouse Gases and Ammonia Emission from Cattle Slurry

Chapter 1 Introduction
Chapter 2: Literature Review

Chapter 3: Preliminary study: Effect of chemical and natural additives on methane emission.

Chapter 4: Addition of a carbohydrate substrate and biological additives of stored slurry

Chapter 5: Agriculture food chain by-products to supply carbohydrate to induce self-acidification during storage

Chapter 6: Slurry crust: methane sink or N₂O source?

Chapter 7: Effects of slurry treatment on biomass production, greenhouse gas and ammonia emission of following spreading: a pot study

Chapter 8: Microbial diversity and changes due to self-induced acidification

Chapter 9: General Discussion, Conclusion and suggestion for further work

Winter & summer
Chapter 1: General introduction.
This current chapter (Chapter 1) is an introduction to the subject area. It explains the importance of GHG emissions from management of livestock manures and discusses the research aims and objectives.

Chapter 2: Literature.
This chapter describes the general manure management practices, and the practices used by Malaysian farmers. It also describes the gases emitted during slurry storage. The importance of this chapter is to review the current major mitigation options practiced worldwide. The importance of EM and their potential benefits to the environment are also described as another potential mechanism for GHG mitigation. Mitigation potential for small-medium scale farm is discussed here especially for Malaysia or other developing countries.

Chapter 3: Mitigating methane emission from stored slurry using chemical and natural additives (a preliminary study).
The study is to observe the possible mitigation during slurry storage by measuring the percentage of CH₄ emission measured by a Micro-Oxymax respirometer. The study was carried at the small-scale (laboratory scale) using chemical, and biological additive and natural fermentable carbohydrate as the substrates. Slurry physiochemical changes were observed as potential indicators in determining the mitigation potential.

Chapter 4: Mitigating greenhouse gases and ammonia emissions from stored cattle slurry through carbohydrate substrates and biological additives.
The aim of this experiment was to further evaluate the effects of adding fermentable carbohydrate and EM to slurry during storage. Greenhouse gas fluxes and cumulative emissions during cold and warm environment (winter and summer) were measured periodically by measuring headspace concentration in static chambers over a 1 hour period. The results showed slurry acidification during the storage period and lower NH₃, CH₄ and GHG emissions.

Chapter 5: Ammonia and greenhouse gas emissions from slurry storage in response to the addition of agriculture food chain by-products.
In this chapter, six potential agriculture food chain by-products were used. Their sugar contents were measured and they were added to slurry to observe the acidification rate and GHG emissions inhibition. Brewery spent grains (BSG) were also used as additive in a larger-scale and longer term experiment.

Chapter 6: Slurry crust: Methane sink or nitrous oxide source?
Slurry crusts act can as primary barrier to reduce gaseous emissions from slurry, but they can also act as an oxidation medium and a suitable medium for N₂O emission by nitrification denitrification processes. In this chapter, the CH₄ oxidation potential and N₂O emission rates were evaluated and the effect of a biological additive (EM) was quantified.

Chapter 7: The effects of slurry modification on greenhouse gas and ammonia emissions following spreading: a pot study.

Applying slurry as an organic fertilizer increases soil fertility and should reduce the amount of N fertilizer needed. This chapter addresses GHG mitigation from soil following application of modified slurry to soil and its effects on grass growth.

Chapter 8: Do microbial diversity dynamics help explain the possible mechanism of self-induced acidification of slurry?

This chapter explained the potential mechanism responsible for methane mitigation via microbial characterization of treated slurries by ‘next generation sequencing’ and addresses the changes in microbial diversity in response to slurry acidification, storage period and environmental change.

Chapter 9: General discussion.

In the final chapter, key findings from all chapters are discussed from a broader perspective with highlights and conclusion. Possible areas for further work are also described. This chapter also lists all bibliographies used in this thesis.
CHAPTER 2

2 Literature

2.1 Literature search

A literature review was performed through the search of literature databases, searches engines and organizational websites. The websites of Government research organization and institutions’ were explored in the UK and Malaysia for additional related information. Because of the lack of published studies on greenhouse gas (GHG) emission from Malaysian cattle farms, the literature search was broadened to include other farming systems that were deemed appropriate to this study such as cattle-palm oil integration farming, manure composting, small ruminant and poultry manure managements, which were deemed appropriate to this study. The search was carried out using various related search terms in the key words, title, abstract and full text by established inclusion criteria for cattle farming, GHG emission, mitigation approaches and a pre-defined strategies and outcome key elements that are useful in the mitigation of GHG. Bibliographies of retrieved articles were checked for additional relevant references. Numbers of articles were identified and 191 articles were cited further for this research area.

2.1.1 The literature scope

Current Malaysian cattle farming systems are based on the integrated use of estate plantation areas especially on oil palm plantations, hence barn or animal houses are rarely used. However, the increase in meat demand during certain periods of the year (especially during Eid Fitr and Adha celebrations) has increased farmers’ interest to be involved with intensive production systems or in-house fattening. In addition, in-house and intensive beef production are also driven by other factors; i) biosecurity factor, ii) lack of grazing area due to urbanization, iii) evaporated ventilation enclosed house to overcome heat stress and, iv) access to automated technology suitable for handling slurry manure rather than solid manure. The slurry is later recycled as organic fertilizer to the sward or animal feed crops. Thus, the literature review focuses on GHG
mitigating during slurry management and is slightly extended into the general manure management wherever relevant.

2.2 Manure composition and decomposition

Generally, manure comprises of animal excreta consisting of inorganic and organic matters and often influenced by feeding practices and animal species (Jensen and Sommer, 2013). Ruminants are usually supplied with forage-based feedstuffs containing a high proportion of cellulose fibres. Thus, carbon (C), hydrogen (H), oxygen (O₂), nitrogen (N) and sulphur (S) are major constituents of organic matter in dry manure. The largest fractions of organic materials of manures are carbohydrates followed by protein, lipids, lignin and volatile fatty acids (VFAs). Lipids, proteins, carbohydrates, lignins and VFA contents in dairy cattle manure represent 6.9, 15.0, 62.5, 12.1 and 3.6% of volatile solids, respectively (Møller et al., 2004). The volatile fatty acids content is equivalent to about 8% of dry matter in cattle slurry (Sommer and Husted, 1995). Manures also contain microorganisms; mainly bacteria with typical numbers about 10¹⁰ bacteria g⁻¹ dry manure (Jensen and Sommer, 2013).

During manure storage, aerobic and anaerobic decomposition occurs. Aerobic decomposition by organoheterotrophic organism degrades organic matter (Jensen and Sommer, 2013). While aerobic decomposition occurs mostly on the slurry surface, anaerobic decomposition occurs in the absence of oxygen within the manure especially slurry. After all, slurry is usually anaerobic condition as oxygen diffusion into slurry is very slow, except for the crust when it develops (Nielsen et al., 2010; Jensen and Sommer, 2013). Decomposition starts with hydrolysis of organic polymers such as protein, cellulose, lignin or lipids which are transformed into long-chain fatty acids, glycerol, dissolved carbohydrate and amino acids (Figure 2.1). Acidogenesis takes place following the transformation of organic components to short chain VFAs, alcohols, amino acids, H and carbon dioxide (CO₂). The decomposition of any component containing N and S will produce H₂S and NH₃ as the end-products, usually detectable through unpleasant or foul odours. The third process that leads to methane (CH₄) emission is methanogenesis. This process is either involves two steps; i) the acetoclastic step (equation 1), which transforms acetic acid to CH₄ and CO₂ and ii) the hydrogenotrophic step (equation 2) where CO₂ and H₂ are combined to CH₄. As stated earlier, the end products of complete anaerobic biopolymer degradation are CH₄, CO₂, H₂S and ammonia (NH₃).
• Acetoclastic methanogenesis:

\[
\text{CH}_3\text{COO}^- + \text{H}_2\text{O} \rightarrow \text{CH}_4 + \text{HCO}_3^- (\Delta G^\circ = -76 \text{ kJ mol}^{-1})
\] - Equation 1

• Hydrogenotrophic methanogenesis:

\[
4\text{H}_2\text{O} + \text{HCO}_3^- + \text{H} \rightarrow \text{CH}_4 + 3\text{H}_2\text{O} (\Delta G^\circ = -130 \text{ kJ mol}^{-1})
\] - Equation 2

While anaerobic manure decomposition emits \(\text{CH}_4\), inorganic N that mainly comes in the form of ammonium (\(\text{NH}_4^+\)) may produce nitrous oxide (\(\text{N}_2\text{O}\)) as the result of \(\text{NH}_4^+\) and \(\text{NO}_2^-\) oxidation. Autotrophic microorganism (\(\text{Nitrobacteriaceae}\)) oxidised the total ammonical nitrogen (TAN) (\(\text{NH}_4^+ + \text{NH}_3\)) into nitrite (\(\text{NO}_2^-\)) and later to nitrate (\(\text{NO}_3^-\)). This is known as the nitrification process (Jensen and Sommer, 2013) and is an aerobic process. On the other hand, heterotrophic bacteria are involved in the denitrification or the stepwise conversion of \(\text{NO}_3^-\) via \(\text{NO}_2^-\), NO and \(\text{N}_2\text{O}\) to \(\text{N}_2\) (Figure 2.2) under anaerobic conditions. Nitrous oxide reductase enzyme may stimulate \(\text{N}_2\) emission via denitrifying bacteria (Firestone et al., 1980; Jensen and Sommer, 2013). Nitrification and denitrification often occur in manure management, where both processes increase the potential of production and emission of the potent climate gas, \(\text{N}_2\text{O}\).

![Diagram of degradation pathways and end-products of anaerobic decomposition of organic matter](image)

Figure 2.1 The degradation pathways and end-products of anaerobic decomposition of organic matter from animal manures through anaerobic decomposition. This consists of three main phases: hydrolysis, acidogenesis and methanogenesis. Source: Jensen and Sommer, (2013).
2.2.1 Cattle slurry characteristic

As reported earlier, slurry consists of animal excreta, which includes urine and faeces. The slurry is often diluted with washing water (barn cleaning activity) and rainfall water resulting in lower dry matter content with less than 15% moisture content. Either beef or dairy slurry in farms are most likely to have pH levels varying between pH 6.8 to 7.5 (Cooper and Cornforth, 1978; Paul and Beauchamp, 1989a). However, Sommer and Husted, (1995) reported that some slurry might reach 8.1 or even higher pH at 8.2 (Cooper and Cornforth, 1978). Slurries may have a lower nutrient content compared to solid manure due to their higher dry matter content, especially for beef cattle manure. Cattle slurry typically contains NH$_4^+$, phosphate (P$_2$O$_5$), potash (K$_2$O), sulphur (SO$_3$) and magnesium (MgO) 0.9, 1.2, 3.2, 29, 0.7 and 0.6 kg ton$^{-1}$ (Sommer and Husted, 1995; Defra, 2010a). These amount is twice to triple higher compared to pig slurry (Christensen and Sommer, 2013). Slurry temperature is claimed to be constant and does not self-heat during storage, as characterised by stable and equal temperature as the ambient surroundings (Petersen and Sommer, 2011; Jensen and Sommer, 2013) such as in Poland where the temperature were typically at about 6°C during the winter and 18°C during the summer (Skowron et al., 2013).

2.2.2 Slurry microbial diversity

While compost and digestate from biogas production have been extensively studied, change in microbial communities during cattle slurry storage remains somewhat underexplored. Very little is known about the microbial community and biodiversity of cattle slurry. van Vliet et al., (2006) conducted a semi-quantitative study using the denaturing gradient gel electrophoresis (DGGE) method to determine the bacterial community of cattle slurry. This technique of exploring microbial diversity only
classifies and groups microorganisms but does not identify individual species. Although some research had taken the initiative to identify the manure microbial diversity by DNA sequencing, these studies have only focussed on pig, poultry faeces and manure (Nodar et al., 1992; Whitehead and Cotta, 2001), while other studies focussed on compost (Ouwerkerk and Klieve, 2001; Liu et al., 2011). However, a recent study by Dhadse et al., (2012) reported a dominant microbial consortia for biogas production from cattle dung, which included Propionibacterium, Bacteroides, Peptostreptococcus and Clostridium.

GHG emissions from slurries are influenced by microbial activities, which are affected by the environment. Microbial shifts might take place due to environmental factors or changes as reported by Herrmann and Shann (1997) on municipal waste, which involved mesophilic and thermophilic organisms. However, changes in slurry microorganisms may not involve these bacteria groups as no rise in temperature is reported during slurry storage (Skowron et al., 2013). Similarly, pig slurry showed microbial changes during the first two weeks of storage which can be observed through the decreasing faecal indicators microbes, the Enterococci and Escherichia coli (Peu et al., 2006). Therefore, cattle slurry microbial biodiversity needs to be explored to better understand the microbial interactions within their environment.

2.3 Manure greenhouse gas emission

Greenhouse gas emissions, typically CH₄, N₂O, CO₂ and NH₃ from animal manure are unavoidable and are emitted from urine and faeces deposited in the animal houses (Bussink and Oenema, 1998; Pereira et al., 2010, 2012; Mathot et al., 2012), dairy parlours and collecting yards (Garnsworthy et al., 2012), solid manure heaps (Chadwick et al., 1999; Chadwick, 2005; Dong et al., 2011), slurry stores (Husted, 1993; Amon et al., 2006; Rodhe et al., 2009a) and from land receiving manure applications (Amon et al., 2006; Fangueiro et al., 2010b). Greenhouse gas emission from intensive cattle farming are shown in Figure 2.3. It is known that CH₄ and N₂O emission from manure management are estimated to represent around 18% - 20% of the total agriculture emission for most countries (Pattey et al., 2005; Chadwick et al., 2011; Sommer et al., 2013). Dairy cattle slurry in Canada emit as much as 1.301 and 0.068 (t CO₂ eq. head⁻¹ year⁻¹) of CH₄ and N₂O. However, the emission level is much lower from beef slurry (Pattey et al., 2005).
Studies on GHG emissions from livestock manures have focused on i) quantifying emissions from different sources (Husted, 1993; Williams, 1993; Ngwabie et al., 2009; Wiedemann et al., 2010; Tauseef et al., 2013), ii) biogas production, and iii) GHG mitigation strategies (Clemens et al., 2006; Cantrell et al., 2008; Chen et al., 2008; Martin, 2008; Chadwick et al., 2011; Petersen et al., 2013). Although CO₂ and other trace gases are also emitted from manure, the research interest has largely focused on CH₄ and N₂O emissions. The estimated world GHG emission from cattle manure (dairy and non-dairy) in 2005, 2010 and 2012 were at 156149, 161163 and 160025 gigagrams (Gg) of CO₂ equivalent (eq.), respectively (FAO, 2016a). These amounts are equivalent to about 46-47% of total GHG CO₂ eq. from manure management. Thus, it is important to reduce the amount of CH₄ and N₂O emissions from livestock production as it is regulated as part of the Kyoto Protocol (1997) under the United Nations Framework Convention on Climate Change (UNFCCC).
Figure 2.3 Schematic diagrams of common cattle slurry management and GHG pollution.
2.3.1 Carbon dioxide (CO$_2$)

Agriculture is not considered as net CO$_2$ contributor because it originates from plants which absorb CO$_2$ from the atmosphere. (Hilkiah Igoni et al., 2008). Carbon dioxide and CH$_4$ are the end products (Amon et al., 2006) of degraded organic compounds e.g. volatile acids, by acid producing bacteria through methanogenic processes during anaerobic respiration (Chadwick et al., 2011). However, CO$_2$ is reported to have an indirect effect on the emission mechanism of other gases, through crust formation from fibre particles carried by CO$_2$ and CH$_4$ bubbles to the slurry surface (Aguerre et al., 2012). Meanwhile, a study by Hafner et al., (2013) concluded that CO$_2$ emission influences NH$_3$ emission from manure by increasing the pH level at the surface.

2.3.2 Methane (CH$_4$)

Methane emission from manure evolves from the biodegradation of organic matter under anaerobic conditions. Complex biomass stepwise degradation involves hydrolysis, acidogenesis and acetogenesis by diverse microorganisms that mainly consist of the dominant group of facultative and obligate fermentative bacteria followed by methanogenesis (O’Flaherty et al., 2006; Barret et al., 2013; Lv et al., 2013). Biomethanation or methanogenesis are processes carried out by either two of the subdivided archaea (methanogen) groups. Acetotrophic archaea generate CH$_4$ by (i) acetate decarboxylation, in which acetate is cleaved with the carbonyl group and oxidised to CO$_2$. The acetate is then reduced to CH$_4$ by the methyl group (Whitman et al., 2006; Ferry, 2010, 2011). Or CH$_4$ is generated through (ii) the hydrogenotrophic pathway (also known as CO$_2$/H$_2$ reduction pathway), in which formate or H$_2$ is oxidised and CO$_2$ is reduced to CH$_4$ (Figure 2.4) (O’Flaherty et al., 2006; Ferry, 2010, 2011).
Figure 2.4 Anaerobic fermentation and methanogenesis in slurry.
1) Hydrolytic/fermentative bacteria; 2) obligate hydrogen producing bacteria; 3) homoacetogenic bacteria; 4a) acetoclastic methanogens; 4b) hydrogenophilic methanogens; 5) fatty acid synthesizing bacteria; dashed lines represent the action of sulphate reducing bacteria from a wide range of species.

2.3.3 Nitrous oxide

Nitrous oxide emission from manure is produced through nitrification and denitrification processes (Rahn et al., 1997; Maeda et al., 2010b). Ammonium (NH$_4^+$) is the main form of inorganic nitrogen (N) in manure (Chadwick et al., 2000; Defra, 2010a) and transformed to nitrate (NO$_3^-$) and a biological reduction of NO$_3^-$, resulting in N$_2$O gas production (Figure 2.5) (Bremner and Blackmer, 1978; Firestone et al., 1980; Maeda et al., 2010b; Chadwick et al., 2011). The presence of N$_2$O in the stratosphere will later be converted to nitrogen oxide (NO), contributing to the destruction of the ozone layer in the process (Portmann et al., 2012).

Nitrification process generates N$_2$O under aerobic conditions. It is an autotrophic process of converting NH$_4^+$ to NO$_2^-$ by microorganisms, mainly the Nitrosomonas species, then to NO$_3^-$ by Nitrobacter species (Dinçer and Kargi, 2000). On the other hand, denitrification process is the reduction process of NO$_3^-$ by microbial reaction (Dinçer and Kargi, 2000; Hayatsu et al., 2008). Stepwise reactions of NO$_3^-$ to N$_2$ are mainly carried out by heterotrophic, facultative anaerobic bacteria, some archaea and fungal species (Wrage et al., 2001; Hayatsu et al., 2008). In contrast to
nitrification, N\textsubscript{2}O emission is a regular intermediate product and has higher release if the pH level during the denitrification process is low (Wrage et al., 2001).

![Figure 2.5 Nitrogen cycling under nitrification and denitrification.](image)

1) Nitrogen fixation; 2) bacterial nitrification, archaeal nitrification and heterotrophic nitrification; 3) aerobic and anaerobic bacterial denitrification, nitrifier denitrification, fungal denitrification and archaeal denitrification; 4) and 5) co-denitrification (by fungi); 5) anammox and 6) N\textsubscript{2}O production during nitrification (ammonia oxidation). Source: Hayatsu et al., (2008)

### 2.3.4 Ammonia

Ammonia is a soluble and reactive gas but not considered as a direct GHG. But NH\textsubscript{3} emission followed by N deposition causes eutrophication and may acidify natural and semi natural ecosystems (Dise et al., 2011). Ammonia emission is caused by the ammonium (NH\textsubscript{4}\textsuperscript{+}) being exposed to the atmosphere, thus volatilisation of NH\textsubscript{3} occurs. Ammonia emission from manure especially slurry is major problem as its represents 80% of agricultural loses as reviewed by Fangueiro et al., (2015). More than 50% of applied N can be lost by NH\textsubscript{3} volatilisation following slurry application, especially during the first 24-48 hours after application (Maeda et al., 2010b). This significant loss strongly reduces the value of fertilizer in terms of N application.

### 2.4 Greenhouse gases mitigation strategies

Greenhouse gas mitigation strategies for manure management have been published worldwide e.g. (Mosier et al., 1998; Berg and Pazsiczki, 2006; Clemens et al., 2006; Kreuzer and Hindrichsen, 2006; Ndegwa et al., 2008; Sommer et al., 2009;
Chadwick et al., 2011; Kaparaju and Rintala, 2011; Massé et al., 2011; Borhan et al., 2012; Yusuf et al., 2012; Petersen et al., 2013a; Sommer and Christensen, 2013; Montes et al., 2013). The Food and Agriculture Organization of the United Nations (FAO, 2016a) reported that the total CH4 emission by animal manure for 2012 was estimated at 11002 Gg CH4, with cattle (beef, dairy and non-dairy) contributing 4075 Gg CH4 (FAO, 2016a). Mitigation strategies are believed able to reduce this amount in the future years. Below, I describe some of the GHG mitigation practices applicable to livestock slurry.

2.4.1 Aerobic treatment lagoon

Increasing soluble oxygen (O2) in slurry and treatment lagoon is a possible approach to minimise the activity of anaerobic organisms such as methanogens (Fetzer et al., 1993; Zitomer and Shrout, 1998; Clemens et al., 2006; Chae et al., 2010; Jiang et al., 2011). The approach relies on the passive diffusion of O2 to surface layers, or the oxygen produced by phytoplankton during photosynthesis. Another possible approach is by enhancing the rate of O2 diffusion into the slurry treatment pond by aeration (Amon et al., 2006; Molodovskaya et al., 2008; Jiang et al., 2011). This can significantly reduce CH4 production. As a result, more aerobic conditions exist which reduces the production of CH4. Techniques such as aeration by bubbling, passive aeration on oxidation pond or air blowing over lagoon surfaces maybe able to increase the oxygen content of effluent and increase aerobic bacteria activity. Increasing the activity of aerobic bacteria will increase lagoon treatment potential (Bhagat and Proctor, 1969). Bhagat and Proctor (1969) reported a significant decrease in chemical oxygen demand (COD) between 84-88%, while the biological oxygen demand (BOD) was significantly reduced to 88-93% in the dairy lagoon pond. Yet, the reduction in BOD and COD may differ in pig slurry (lower reduction rate) as pig slurry dry matter content was usually less than 2% (Zhang et al., 2006).

The recommended optimum depth for passive diffusion of waste pond is 1.0-1.5 m, in order to minimise the anaerobic conditions in the bottom layers (Amoatey and Bani, 2011). In contrast, N2O emissions on undisturbed slurry lagoon are reported to be low or negligible (Rodhe et al., 2009a). Therefore, the production of N2O is likely to increase with aeration or oxygenation at the slurry lagoon surface (Molodovskaya et al., 2008). Moreover, due to the maintenance and start up equipment cost that will
offset some of the CH$_4$ mitigation (as CO$_2$ eq.), this approach may not be suitable (Amon et al., 2006).

2.4.2 Anaerobic digestion

Slurry manure handling usually ends up at serial treatment lagoons with static or anaerobic lagoon where organic matters is trapped and digested. In those anaerobic storages, microorganisms degrade organic matter and produce CO$_2$, CH$_4$ and some trace gases (Clemens et al., 2006). As CH$_4$ is continuously produced from manure for more than 14 weeks (Kreuzer and Hindrichsen, 2006), capturing CH$_4$ emitted by slurry cover is a useful way to mitigate CH$_4$ emissions. The technique has the potential to be adopted by many livestock farms if more cost-effective systems are developed. In addition, by completely covering the manure prior to anaerobic digestion ensures that neither CH$_4$, NH$_3$ nor N$_2$O can be emitted (Clemens et al., 2006). This is the principle in biogas production, which is practiced in anaerobic digesters (AD). Anaerobic digesters for biogas capture are useful for CH$_4$ production and capable of reducing up to 80% of global warming potential (GWP) (Turnbull and Kamthunzi, 2008). New CH$_4$ concentration of biogas produced is estimated to range between 60 to 80% (Massé et al., 2011).

Although biogas production from livestock manures can be increased by adopting optimal temperatures (Clemens et al., 2006; Siddique et al., 2014) or co-digestion with crops (Comino et al., 2010) or food wastes (Li et al., 2010), positive CH$_4$ losses from manure stores are still reportedly large (DeSutter and Ham, 2005). In addition, large scale biogas generation, especially for biogas-generated electricity, may require high capital costs and large numbers of animals to ensure it runs efficiently (Heinonen-Tanski et al., 2006; Martin, 2008). From the economic perspective, the technique does not seem attractive or viable compared to the capital investment required for small farms. Some farms tend to simply use the gases for local heating purposes, internal-combustion engines (Hilkiah Igoni et al., 2008) or even just for flaring, resulting in CO$_2$ release. With the high capital investment to start (Liang et al., 2008; Yiridoe et al., 2009; Massé et al., 2011) and over 25 years payback for the period, AD is not favourable by small farm practitioners (Yiridoe et al., 2009). In a previous study in Malaysia, Liang et al., (2008) summarised the challenges of adopting wide scale AD in Malaysia and other Asian countries. The challenges are; i) high capital
is required for infrastructure modification and AD equipment on farms, ii) insufficient electricity generation to cater for daily farm usage, iii) lack of government support for funding and policy support, and iv) inadequate knowledge of waste treatment, carbon cycle and GHG emission, especially by small farmers. Thus, an anaerobic digestion as mitigation option may not be a simple digestion and fermentation process. Chen et al., (2008) described the factors dissuading anaerobic digestion process through inhibition and toxicity.

I. Anaerobic digestion limiting factor

Anaerobic digestion for CH$_4$ production requires controlled parameters, which often need to be monitored to achieve the optimum CH$_4$ emission level. These parameters are; optimum pH level, temperature, salt, ionic strength, sulphide, NH$_3$, sulphide$^-$ or heavy metal ions and organics content where they may act as inhibitors or toxic substances when accumulated in large amounts and suppress the methanogen activity (Rajeshwari et al., 2000; Chen et al., 2008; Suryawanshi et al., 2013). It is also important to control the anaerobic condition with optimum liquid velocity and organic loading rate (OLR). Rajeshwari (2000) suggested the optimal OLR of 24 kg COD m$^{-3}$ day$^{-1}$ and 3 m h$^{-1}$ of liquid velocity. However, digester temperature need to reach the optimum mesophilic or thermophilic methanogen conditions with an oxidation-reduction potential (ORP) value less than -175 mV (Gerardi, 2007; Borhan et al., 2012). The limiting factors such as biomass retention, start-up cost, operation and maintenance requirements remain the major constraints in capturing CH$_4$ through AD (Rajeshwari et al., 2000). In addition, the volume of slurry produced depends heavily on the number of animals and water usage, which may not able to cater to the digesters’ capacity.

2.4.3 Separation or filtration

The use of bedding material such as straw or chipped wood to increase dry matter in the manure is an important ingredient apart from the forages. Reduction of dry matter in slurry manure through liquid separation, sludge or solid sedimentation may reduce CH$_4$ although it may increase NH$_3$ (Amon et al., 2006) and N$_2$O emissions (Amon et al., 2006; Fangueiro et al., 2010a). Separate storage of the liquid and solid
fractions results in CH₄ reduction by over 41% (Amon et al., 2006). Despite its ability to reduce CH₄ emission, removal of bedding material from the manure can increase N₂O and NH₃ emissions by 126.5% and 86.4%, respectively. In contrast, storage of solid fraction indicates lower GHG emission compared to liquid and raw slurry storage (Dinuccio et al., 2011). Lower GHG emission was also observed on soil application (Dinuccio et al., 2011). Recently, a review by Petersen et al., (2013) concluded that this mitigation method was not always successful in reducing GHG emission. Therefore, liquid and solid manure separation as GHG mitigation requires further exploration and can be applied only if other GHG reduction measures are adopted on liquid fraction during storage and soil application.

2.4.4 Methane oxidation

The amount of methane gas from anaerobic ponds can be reduced through oxidation by methane-oxidizing bacteria (MOB) (Duan, 2012; Pratt et al., 2012; Duan et al., 2013). Methane oxidation can be encouraged by retaining surface crusts on anaerobic ponds or slurry (Petersen et al., 2005; Duan et al., 2013). Crusts are often formed naturally or are promoted by the addition of fibrous material such as wood chips and straw on the slurry surface (Duan et al., 2013). Crust characteristics are; porous and floating on top of liquid slurry, contain organic materials, rich in inorganic N with neutral to alkaline pH to provide a conducive environment and medium for methanotrophic bacteria to grow. Methane oxidation by MOB is also achievable by biofiltration process through a porous media supporting the methanothrophic bacteria, as reported by Pratt et al., (2012). This study used volcanic soil as the bio-filter containing Type II methanotrophs (Methylocystis sp.-related). It showed significant CH₄ oxidation by up to 16 g m⁻³ hr⁻¹ (greater than landfill soil CH₄ oxidation) (Pratt et al., 2012).

2.4.5 Inhibitors

I. Nitrification inhibitors

Manure application to grassland or crop field also causes GHG emission, especially N₂O. Monteny et al., (2006) and Pereira et al., (2010) reported the use of nitrification inhibitors and demonstrated a significant effectiveness in inhibiting N₂O emissions from fertiliser and animal slurries. Between 32 and 48% of N₂O inhibition was recorded on slurry and slurry fraction when they were treated with
inhibitor during slurry-soil application (Pereira et al., 2010b). Their research has focussed on selectively few inhibitors, e.g. nitrapyrin, dicyandiamide (DCD) and 3, 4-dimethylpyrazole phosphate (DMPP) (Petersen et al., 2013a). Another type of additive is urease inhibitor, e.g. N-(n-butyl) thiophosphoric triamide (NBPT), which significantly reduces NH₃ volatilisation by 58% from urea-based fertilizer and poultry manure (Abalos et al., 2012; Jones et al., 2013). NBPT application slows down NH₄⁺ hydrolysis and lowers N emission to the atmosphere. It has also been shown to reduce N₂O and NO emissions by over 86 and 88%, respectively (Abalos et al., 2012).

II. Methane inhibitors

Few inhibitors have specifically shown effectiveness in suppressing CH₄ formation (Anderson et al., 2006; Chen et al., 2008; Božic et al., 2009). Lignin based compounds with aldehyde groups or chemicals substances may be toxic to methanogens, (Chen et al., 2008). These inhibitors either block the function of the corrinoids enzyme and inhibit methyl-coenzyme M reductase (Chidthaisong and Conrad, 2000), or partially inhibit acetate-dependent sulphate reduction. However, these studies only focussed on the rumination in bovine to enhance energy production and increase feed conversion instead of CH₄ formation (Chae et al., 2010; Xu et al., 2010). In 2010, a study on AD sludge using chloroform (CHCl₃) and 2-bromoethanesulfonate (BES) was able to suppress methanogenesis process during the digestion period (Xu et al., 2010), but it is unclear whether this may also affect the microorganism involved in other biodegradation processes. Therefore, their effectiveness to reduce CH₄ emission from slurry waste storage is doubtful due to negative environmental effects and the co-existence of other microorganisms.

2.4.6 Frequent slurry removal

Another simple practise that might be useful in reducing CH₄ emission is through frequent slurry removal (Haeussermann et al., 2006; Masse et al., 2008; Wood et al., 2014). Removing slurry will also remove the CH₄ producing microorganisms (methanogens) and prolong the lag phase of CH₄ production and emission from newly added slurry in the store (Masse et al., 2008). Wood (2014) showed that complete
removal with cleaning may further reduce CH$_4$ by 40% compared to non-completed slurry removal. A significant reduction might be observed if complete slurry removal is practised during warmer conditions such as through the summer period (Masse et al., 2008). The frequent slurry removal process may, however, result in increases in NH$_3$ and N$_2$O loss. However, even though the amount of NH$_3$ and N$_2$O emitted increased significantly, the total GHG CO$_2$ eq. was still much less compared to the emission level in non-removal of slurry from the tank (Wood et al., 2014). Although this practice may be useful, it requires additional machinery and increased labour, which would need to be considered by farmers.

# 2.4.7 Acidification

Manure acidification is a possible approach to mitigate CH$_4$ and NH$_3$ emission (Clemens et al., 2002; Berg et al., 2006b; Kai et al., 2008; Petersen et al., 2012). Average CH$_4$ reduction was reported between 67 to 87% (Petersen et al., 2012), and 70-90% on NH$_3$ emission (Stevens et al., 1992; Clemens et al., 2002; Berg et al., 2006b). Although slurry acidification has been developed and tested for over 30 years, the numbers of countries applying this practice is still very small (Fangueiro et al., 2015b). Acidification can be applied to slurry or separated slurry fractions. The whole acidification process may ability delay nitrification therefore; N$_2$O emission during field application may be further suppressed (Sommer et al., 2013). Acidification may be promoted by using soluble carbohydrate such as glucose or sucrose (Clemens et al., 2002), lactose and maltose (Nykanen et al., 2010), or chemical substances examples; concentrated sulphuric acid (H$_2$SO$_4$), lactic acid (C$_3$H$_6$O$_3$) nitric acid (HNO$_3$) and hydrochloric acid (HCl) (Stevens et al., 1992; Berg et al., 2006b; Kai et al., 2008; Ottosen et al., 2009; Moset et al., 2012; Petersen et al., 2012; David. Fangueiro et al., 2013). In addition, Fangueiro et al., (2010a) concluded the combination solid-liquid separation with acidification is an efficient strategy to reduce N$_2$O and CO$_2$ emission after soil application.

# 2.4.8 Others

As reported by some researchers, a number of environmental factors affect the level of CH$_4$ emission. As increasing CH$_4$ emission is related to higher temperature (Clemens et al., 2006; Monteny et al., 2006; Sommer et al., 2009; Pereira et al., 2012),
emissions may be suppressed through inhibiting methanogen activities by lowering the slurry temperature, i.e. slurry cooling (Petersen et al., 2013a). Ammonia emission is also slowed down by cooling slurry (Groenestein et al., 2011). Some other possible mitigation approaches include high dilution of slurry (Møller et al., 2004) and inhibit anaerobic condition through various inhibitory substances during biodegradation (Chen et al., 2008). Interestingly, Hindrichsen et al., (2005) and Kreuzer and Hindrichsen, (2006) suggested supplying a balanced protein diet to the animal in order to indirectly mitigate GHG emission from slurry stores (Petersen et al., 2013).

2.5 Slurry-soil application

Slurry-soil or swards/crops application may improve soil fertility and increase crops yield. However, this practice results in dispersing malodours smell and GHG emission (Lovanh et al., 2010). Slurry can be applied to the soil by surface by broadcasting, splash plate or trail hose methods (Rubæk et al., 1996; Lovanh et al., 2010). These traditional practices are practiced in most countries. Either modifying the application method by injection, trailing-shoe, time of application or modifying the slurry characteristic are the next generation approaches to reduce the NH\textsubscript{3} loss while simultaneously likely to increase the available N in the soil (Wulf et al., 2001b; Misselbrook et al., 2002; Bourdin et al., 2014). Yet this approach (application method) definitely increases management costs by as much $GBP 31 ha\textsuperscript{-1} (Frost et al., 2006). Slurry spreading also results in an immediate burst of CH\textsubscript{4} emission (Chadwick and Pain, 1997; Rodhe et al., 2006; Fangueiro et al., 2008b) and might be lowered by being acidified to pH <5.5 (Fangueiro et al., 2015b). It is also known that acidification reduces the level of NH\textsubscript{3} emission (Stevens et al., 1992; Fangueiro et al., 2010b, 2015a).

Meanwhile, N\textsubscript{2}O which is 298 times more potent than CO\textsubscript{2} in term of global warming potential (GWP) (Myhre et al., 2013) compared to CO\textsubscript{2}, is emitted following slurry application (Rodhe et al., 2006) and reported to be higher if it is actively incorporated (injected into the soil) (Wulf et al., 2001). Although the use of nitrification inhibitors (DCD, DMPP, nitrapyrin) can reduce N\textsubscript{2}O emissions, this approach is not currently thought to be economical, in addition nitrapyrin use is associated with hazard, health and safety issues (Zerulla et al., 2001; Alexeeff et al., 2015).
2.6 Effectives microorganisms (EM)

Effective microorganisms (EM) are now widely used throughout the world. Kyan et al., (1999) described the multiple use of EMs in agriculture, including as additions to organic fertilizers, mushroom cultivation and in orchard crops, paddy fields, animal production and management, aquaculture and environmental management (van Vliet et al., 2006; Zhou et al., 2009; Zakaria et al., 2010). Effective microorganisms are also reported to improve the conversion of solid municipal waste during the composting period to a product that can be utilised as a soil fertilizer (Freitag and Meihoef, 2000; Kale and Kumar Anthappan, 2012). Furthermore, the amount of organic matter and minerals including N, phosphate and potassium contents of waste are reported to increase following a two week incubation period (Kale and Kumar Anthappan, 2012). Higa and Parr, (1994) suggested that the mode of EM action is related to microbial diversity that interacts with the ecosystems. Greater diversity causes higher interaction and produces a more stable ecosystem. The application of EM to soil is thought to act as an inoculant to increase the microbial diversity and population, and subsequently inhibit parasite infection on the crops. The statement is strengthened by Sigstad et al., (2013) when he suggested that EM works, (i) by competing with harmful microorganisms and, (ii) producing beneficial substances that promote healthier environment such as enzymes, organic acids, amino acids, hormones and antioxidants.

The presence of five bacterial groups (Cóndor Golec et al., 2007) in EM is responsible for the synergistic and non-harmful microbial activities. The microbes involved are the photosynthetic bacteria and yeast which synthesise antimicrobial and others substances. Another microbe is the lactic acid producing bacteria which is capable of accelerating the breakdown and decomposition processes of organic matter such as lignin and cellulose. Lactic acid produced by the acid producing bacteria suppresses harmful microorganisms in the environment. Fungi’s presence in the EM is important for rapid decomposition of organic matter to produce alcohol, esters and antimicrobial substances. Antimicrobials are also produced by Actinomycetes, a microorganism that is grouped as an intermediate between bacteria and fungi. However, Zimmermann and Kamukuenjandje, (2008) suggested that EM’s effectiveness is best shown when the natural microbial community is disrupted. However, there is lack of consistent scientific evidence of the benefits of EM (Carozzi et al., 2013). There are needs to explicitly state the microorganism responsible for the
observed effects (Higa and Parr, 1994), illustrate the interaction of each microorganisms and their ‘cause and effect’ to the observed subject environment (observe plant, water, municipals waste, animal) (Carozzi et al., 2013). Non-reliable information (non-scientific claims) could be easily distributed, but it is the clear sales, commercialization and marketing promotion that will garner consumers’ satisfaction and trust (Carozzi et al., 2013). Many countries have introduced local EMs and EM products, which are claimed as comparable to the EMs produced by others. Yet, EM’s potential to mitigate GHG emissions during slurry storage needs to be proven as a possible mitigation strategy to reduce GHG emission at low cost while producing significant result.

2.7 Mitigation strategy for livestock manure management in Malaysia

Reducing the release of GHG, especially in the intensive cattle farming is becoming very important. Instead of solid manure, the GHG mitigation interest is now slurry storage because of the increase of in-house cattle farming and the received benefits of slurry recycling to fertilise crops. Some of the prevention methods mentioned earlier which is useful in reducing GHG but tend to be expensive or not practical in terms of a Malaysian farmer’s herd size, even these technologies may be effective. Thus, practical and cheaper preventive approaches suitable for the Malaysian cattle farms (small-medium scale herd size) are needed. Below, I summarise two possible methods, which may be applied to slurry management in Malaysia.

As recommended by Nykanen et al. (2010), carbohydrate additives (e.g., sucrose, milled wheat, and potato starch) may favour lactic acid bacteria growth in stored slurry and modify the slurry pH and should decrease the concentration of bacteria producing malodorous metabolites. As stated earlier, the capability of Lactobacillus plantarum to utilise the soluble carbohydrates and dramatically reduces the pH level will result in decreasing NH₃ volatilisation and odour (McCrory and Hobbs, 2001; Petersen et al., 2012). An EM is widely used in many aspects and it is deemed as a move towards a better environment, although Amon et al., (2005) indicated that the effect of EM use in manure management may have a small effect. Effective microorganisms were successfully studied in wastewater and kitchen waste (Sekaran et al., 2005). Sekeran et al., (2005) suggested that EM converted the waste to become better quality compost, leading to detoxification and decontamination of landfill and
environment without foul smells (free of odours). It has also been reported that EM can improve the conversion of solid municipal waste during the composting period to a product that can be utilised as soil fertilizer (Freitag and Meihoefer, 2000; Kale and Kumar Anthappan, 2012). In addition, Namsivayam et al., (2011) found decreasing sludge volume after employing the EM. These results suggested the advantages of EM to be incorporated into the cattle slurry sector to improve the GHG inhibition or suppress methanogen growth.
CHAPTER 3

3 Mitigating methane emission from stored slurry using chemical and natural additives (a preliminary study).

Abstract

The primary animal waste management strategy in intensive dairy and beef cattle farming is typically slurry storage, which is an important source of GHG emission that contributes to climate change. Biogas production from livestock slurries was initially introduced as a mitigation approach; however, start-up infrastructure and maintenance costs as well as efficiency remain major issues, which must be addressed. This Chapter presents a preliminary study to assess an alternative strategy, i.e. to reduce methane (CH₄) emissions from slurry stores through the use of additives. Methane emissions during storage were monitored using a closed respiratory meter (Micro-Oxymax). A significant reduction in slurry pH (pH 4.0) was achieved through the addition of hydrochloric acid (HCl), and this significantly reduced CH₄ production. However, as a result of 10% (w/v) glucose addition, slurry pH was also decreased to pH <4.0 and this also resulted in significantly reduced CH₄ emissions. In contrast, slurry amendment by various concentrations of effective microorganisms (EM) resulted in only a small reduction in CH₄ emissions for a short period (three days). ‘Self-induced’ acidification in both fresh and aged slurry was found to reduce CH₄ emissions during storage. The mechanism for the ‘self-acidification’ is unclear; glucose addition had an inconsistent effect on acetic acid production, but significantly increased butyric acid production. Total VFA production was higher in ‘self-acidified’ slurries, but it is unclear if this was the cause of the acidification.

Key words: methane, mitigation, slurry, additives, effective microorganisms
3.1 Introduction

Typical intensive beef production systems (in barn/house/buildings or feedlots) require less area than conventional systems, with quick and efficient production before animals are sent for slaughter. There is a high probability of increasing cattle numbers reared in intensive farms over the next decade (Chung et al., 2013) to fulfil consumer demand for meat and milk products. Therefore, manure management has become more important from both agronomic and environmental perspectives. In intensive farming, farmers often choose liquid manure management systems rather than solid manure handling, because it offers easier handling with less need for heavy machinery. Slurry handling is also important to the dairy industry. Currently, only a few farms are practicing slurry management in Malaysia compared to the UK; however, the numbers have been increasing following reports in changes of cattle production systems from grazing to feedlot systems (Chung et al., 2013). Slurry stores are a significant source of CH₄ emissions, but strategies are being explored and implemented to reduce these emissions, e.g. via production of biogas, which also (primarily) generates renewable energy. However, two important factors need to be addressed when planning and building biogas biofuel facilities; i.e. i) capital and maintenance costs; and ii) the number of livestock needed to make the system economically viable and productive. These factors can be significant barriers to small-medium farmers, especially in Malaysia’s cattle farming systems. Inefficient and impractical biogas production approaches for the small-medium farms size means that there is a need for alternative approaches to reduce CH₄ and other GHG emissions from slurry stores.

A possible approach to mitigate CH₄ emission is the application of additives during storage. The uses of additives are not limited to chemicals (Petersen et al., 2013a), such as acids (Stevens et al., 1992; Berg et al., 2006b; Kai et al., 2008; Ottosen et al., 2009; Moset et al., 2012), but also include inhibitors (Anderson et al., 2006; Chen et al., 2008; Božic et al., 2009), base precipitating salts such soluble magnesium or calcium (McCrory and Hobbs, 2001), and sodium or potassium (Zupan and Grilc, 2007). However, salts have only been effective in inhibiting CH₄ when they are added at very high concentrations, e.g. magnesium (1000 mg L⁻¹) calcium and potassium (2500 mg L⁻¹) and sodium (3500 mg L⁻¹), respectively. Other substrates containing labile carbon have shown inhibitory effects on methanogenesis, e.g. sucrose, glucose, and fructose (Clemens et al., 2002), lactose and maltose (Nykanen et al., 2010).
Slurry acidification to pH 5-6, either by addition of acids or organic substances, has been shown to lead to noticeable reductions in CH₄ emissions from stored slurry (Clemens et al., 2002; Berg and Pazsiczki, 2006; Berg et al., 2006b). Sulphuric acid (H₂SO₄) has been used commercially in Denmark to acidify pig slurry to pH 6.0 (Kai et al., 2008; Sørensen and Eriksen, 2009). It is mainly used to reduce NH₃ volatilisation. The challenge of using H₂SO₄ is the formation and increased emission of hydrogen sulphide gas (H₂S) (Dai and Blanes-Vidal, 2013; Wang et al., 2014) and the health and safety implications of handling large volumes of concentrated acids on farms. Storing slurry at acidic pH levels can reduce CH₄ emission by between 67 and 87%. The use of lactic acid (C₃H₆O₃) has shown higher CH₄ inhibition than nitric acid (Berg et al., 2006b).

In comparison, acidification by organic substances requires anaerobic fermentation by living microorganisms within the slurry over a prolonged period. It is not an instantaneous acidification process (Clemens et al., 2002), and causes an increase in dry matter content (Nykanen et al., 2010).

The use of microbial substances or organisms such as effective microorganism (EM) either as a mixture or single culture, e.g. *Lactobacillus plantarum*, (Nykanen et al., 2010) or *Lactococcus lactis* (Takahashi, 2013) is another possible approach to mitigate CH₄ by a different mechanism. However, the effectiveness of a single culture or species remains unconvincing (Carozzi et al., 2013), as slurry contains many multiple dominant microorganisms responsible for bio-degradation, which later contribute to CH₄ emission. Amon et al., (2005) added effective microorganisms (EM) to slurry in the absence of other additives at 1% m⁻³ slurry and observed 1.8% increase of CH₄ emissions. Unfortunately, the study did not explore whether higher or lower emissions could be achieved if EM was supplied with simple carbohydrate as studied by Huang et al., (2006). The addition of carbohydrates could facilitate multiplication in the EM numbers to overcome the dominant microbes in the slurry, with the use of a monosaccharide as a fast degrading energy source, rather than the hydrolysis of large particulate organic substrate. The use of fermentable carbohydrate additives during slurry storage may promote organic compound production through metabolism by anaerobic microbes (Nykanen et al., 2010).

The aim of this study was to provide an initial evaluation of the potential of additives to mitigate CH₄ emission. Specifically, the experiment was designed to, 1) determine the effect of slurry pH on CH₄ emissions, 2) compare acidification and CH₄
inhibition by ‘forced’ (addition of strong acid) and ‘induced-acidification’ (anaerobic fermentation), 3) compare acidification with amendment of effective microorganisms (EM), and 4) evaluate the effect of these amendments on CH\textsubscript{4} inhibition from both fresh and aged slurry.

3.2 Materials and method

3.2.1 Cattle slurry origin and characterization

Cattle slurry was obtained from a commercial beef farm, located close to the Henfaes Research Centre, Abergwyngregyn, United Kingdom (UK), and stored in a sealed container and refrigerated before use at <4°C. Slurries were classified as aged cattle slurry (AS), collected from an area of the lagoon that was not frequently mixed. Fresh cattle slurry (FS) was obtained from the reception pit within 24-48 hours after daily cleaning took place. (All experiments in this thesis are subjected to FS cattle slurry unless otherwise stated). Briefly, the farm housed 80 head of mainly male Limousine crossbred cattle with an average weight 450 kg and aged around 20 months old. The cattle were fed with total mixed ration (TMR) feeds on average 11% of body weight. The feed consisted of whole crop barley, rolled barley, straw, grass silage, and brewery by-product (Trafford Gold, Manchester UK.) (3.0, 5.8, 1.3, 75.0, 15.0%), with addition of less than 0.002% mineral and trace element for the animal growth requirement. Slurries taken from the farm were then mixed using a wooden stick and slurry subsamples was collected into 50 mL plastic centrifuge tubes (Falcon) and kept at 4°C until analysis. Cattle slurry subsamples were then used to characterize the pH, oxidation redox potential (ORP), dry matter (DM), volatile solid (VS), volatile fatty acid content (VFA), total carbon (C), total nitrogen (N), extractable nitrate-N (NO\textsubscript{3}-N) and ammonium-N (NH\textsubscript{4}-N) content. Details of these analytical methods are described below

I. Slurry dry matter (DM) and volatile solids (VS) content

Slurry dry matter was determined by drying slurries samples at 80°C (24-48 hr) before further drying at 105°C to constant weight (24-36 hr), while volatile solids content was measured on dried samples as loss on ignition at 450°C for 16 hr in muffle furnace Carbolite CWF 1200 (Carbolite Ltd, UK).

II. Slurry pH and oxidation redox potential (ORP)
Slurries pH and ORP determination were measured using a probe connected to a Hanna pH meter model HI 991003 (Hanna Instrument, USA).

III. Ammonium-N (NH₄-N) and nitrate-N (NO₃-N) determination

Slurry for NH₄-N and NO₃-N determination was measured after extraction by 2 M KCl. 2-4 grams (g) of slurry sample were weighed into 50 mL centrifuges tubes. Two molar (2 M) KCl was added at 1:5 ratio (following method described by Chadwick et al., 2000) and continuously shaken on a rotary shaker (SM30 Edmund Bühler GmbH) for 1 hr at 250 rpm. Samples were then centrifuged (Eppendorf centrifuge 5810, UK) at 4000 rpm for 10 minutes and the supernatant kept at -20°C prior to defrosting and analysis for NH₄-N and NO₃-N.

a) Ammonium-N (NH₄-N) determination

Ammonium-N determination was carried out using protocol as described as Mulvaney, (1996). Determination was carried out using a 96 well microtiter plate. Prior to incubation at 30°C, 6% Na₂EDTA, Na-Salicylate-nitroprusside and hypochlorite solution was added (15, 60 and 30 mL). Na-Salicylate-nitroprusside solution consists of 7.8% (w/v) Na-Salicylate and 0.125% (w/v) Na-nitroprusside, while hypochlorite solution (pH 13) contains 2.96% (w/v) NaOH, 9.96% K₂HPO₄ (w/v) and 10% (v/v) Na-hypochlorite. Following 30 minutes incubation, absorbance readings were measured using a microplate reader Biotek Power Wave XS at wavelength 667nm and analysed by Gen 5 software Biotech (Instruments, Inc., USA).

b) Slurry nitrate-N analysis

The method of Miranda et al. (2001) was used to determine nitrate-N. Extracted samples from slurry and vanadium III chloride (VCl₃) and Greiss reagent were brought to room temperature prior to use. Reagents are, Vanadium III chloride, which consists of 400 mg VCl₃ in 50 mL 1M HCl, Greiss reagent consists of 0.1 (%) N-(1-Naphthyl) ethylenediamine dihydrochloride (NEDD) and sulphanilamide (SULF) 0.4g in 5% HCl. Vanadium III chloride reagent was kept in a dark for maximum usage 2 weeks. Briefly, 100 µL samples were placed in a microtiter plate and reacted with 100 µL VCl₃, 50 µL NEDD and
50 µL SULF. Reaction absorbance was measured by microplate reader Biotek Power Wave XS at 540 nm and analysed by Gen 5 software Biotech (Instruments, Inc., USA).

**IV. Slurry volatile fatty acid analysis**

Slurries samples for VFA determination were processed immediately or kept at 4°C. Slurry was homogenized with 1:1 (v/v) distilled water if necessary. Briefly, 15 mL of slurry were centrifuged at 4000 rpm (Eppendorf centrifuge 5810, UK) at 15°C for 30 minutes. Slurry supernatants were transferred into clean tubes and sediments were discarded. Slurry supernatant was then processed as mention by Playne (1985) and Paul and Beauchamp (1989). Briefly, 400 µL metaphosphoric acid (HPO₃) was added into 2 mL samples to acidify and precipitate protein (Playne, 1985). Samples were centrifuged again at 10000 rpm (Eppendorf centrifuge 5810, UK) at 15°C for 20 minutes, after 30 minutes incubation at 4°C. Supernatants were transferred into GC vial and 2-ethyl butyric acid 300 mM internal standard was added at 1:100 ratio during experiment. Samples were then injected into a gas chromatograph fitted with a Free Fatty Acid Phase (FFAP) column (25 m x 0.32 mm x 0.5 µm) (Agilent J&W GC column). Half a microliter (0.5 µL) of the VFA sample was injected by the auto sampler (Varian CP 8400) directly into a Varian 3380 gas chromatograph equipped with a flame ionization detector with split ratio 1:10. A glass liner of glass wool (Phenomenex, USA) 4 mm x 78.5 mm x 6.3 mm fitted at the injector was used to trap dirt or contaminants accumulating as a result of direct injection of manure supernatant. The carrier gas used was nitrogen (N₂) and the column flow rate was 1.4 mL min⁻¹. The GC machine was setup with the temperature of the injection head at 250°C, column oven temperature of 80°C (0.2 s) ramped at 20°C min⁻¹ to 170°C (3.2 min), and then ramped at 65°C min⁻¹ to 240°C (4.5 min). Total time for analysis each sample was less than 14 minutes. The slurry VFA’s peaks obtained was identified and quantified using a set of VFA reference standards.
V. **Total carbon (C) and nitrogen (N)**

The total C and N of fresh slurry samples were measured by TruSpec® CN analyzer (Leco Corp, St Joseph, MI).

### 3.2.2 Effect of additives on methane emission

#### I. **Chemical acidification by hydrochloric acid (HCl)**

The relative effect of CH$_4$ emissions on diluted fresh slurries (1:1 ratio, slurry: distilled water – v/v) from slurry acidification was observed. Slurry was diluted to simulate the average cattle slurry DM content at 6-9%. Three hundred (300) mL of slurry was acidified to pH 4.0 ±0.2 using concentrated HCl (50%), (Sigma-Aldrich Inc., Gillingham, UK) and the emission of CH$_4$ was measured using Micro-Oxymax respiratory meter (Columbus Instrument, Inc. Ohio, USA). A 300 mL of acidified slurry and non-acidified slurry (Ctrl) were placed in 500 mL glass bottles (Schott) with five replicates in each groups and randomly fitted to a Micro-Oxymax respiratory meter. The process was operated in the standard aerobic selection mode at room temperature (20-23°C). Briefly, the vessel was capped with a lid attached to the Micro-Oxymax through a tube. The Micro-Oxymax purged the channel (bottle) headspace before measuring the headspace gas. The machine then analysed the CH$_4$ concentration level in the headspace and compared it to a reference channel within the machine. The system was setup to alternately record CH4 headspace concentration level in all channels continuously for eleven days. Slurry pH levels and ORP were immediately recorded prior to connecting to Micro-Oxymax respiratory meter. The pH levels were observed daily using pH meter electrode probe by opening the vessel lid during non-reading points.

#### II. **Biological additives (EM) effect**

Activated effective microorganisms (Actiferm EM®; later term as AFEM) used during this study were provided by Effective Micro-organism® Limited, Exeter UK. Methane emission and the effects of different concentration of AFEM added to slurries were observed by adding 5, 10, 20, 30% (v/v) AFEM (termed as EM5, EM10, EM20 and EM30 respectively) into 100 mL distilled water and mixed into 200 g of cattle slurries. The mixture was then thoroughly mixed and incubated in the Micro-Oxymax system, as above, to determine the effect of AFEM addition.
on CH₄ emissions. Due to the Micro-Oxymax system limitation, each treatment will only have three replicates. Slurry pH levels and ORP were immediately recorded prior to connecting to Micro-Oxymax respiratory meter and daily using pH meter electrode probe by opening the vessel lid during non-reading points.

III. Carbohydrate induced self-acidification (glucose)

Slurry amendment by simple carbohydrate (glucose) was observed by adding D-glucose (Sigma-Aldrich Inc., Gillingham, UK) at concentration between 0-25% (w/w) (G0, G5, G10, G15, G20 and G25) in 200 g cattle slurry in 5 replicates. Methane emissions were not monitored during this observation due to equipment failure, but the effect of glucose addition on slurry pH and ORP was quantified.

3.2.3 Induced acidification on fresh and aged slurry: Biological effect of glucose and effective microorganism addition.

Fresh and aged slurries were obtained from the commercial farm as described previously. Four treatments were established for each age of slurry using 150 mL of diluted slurry (1:1 v/v, slurry: distilled water) for each. These treatments were: (1) an untreated slurry (Ctrl); (2) a mixture of slurry with 5% (v/w) Actiferm EM (EM); (3) a mixture of slurry with 10% (w/w) glucose (0.56 M) (Sugar) and (4) a mixture of slurry with 10% (w/w) glucose (0.56 M) and 5% (v/w) Actiferm EM, (Sugar + EM). Table 3.1 summarises these eight treatments (i.e. including both the fresh and aged slurry). There were five replicates of each treatment. The slurry treatments were mixed and assigned in a complete randomized block design on the Micro-Oxymax machine, after which the headspace CH₄ concentrations were measured and recorded daily for 10 days. The treatments for fresh slurry (FS) were known as FGEM, FG, FEM, F Ctrl, and for aged slurry (AS) as AGEM, AG, AEM, and A Ctrl.
Table 3.1 An overview of treatments in two manipulation experiments with storage of fresh and aged cattle slurry.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fresh Slurry</th>
<th>Aged Slurry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slurry + Glucose 10% + AFEM 5%</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Slurry + Glucose 10%</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Slurry + AFEM 5%</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Slurry (Control untreated)</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Note: AFEM, Actiferm activated effective microorganism

3.2.4 Statistical analysis

The effect of slurry amendment by HCl was analysed by t-test while slurry amended with glucose or AFEM additive was analysed by two-way ANOVA by Minitab 16 statistical software, (Minitab ltd. Coventry, UK). Later, the effects of AFEM and glucose additives on slurry acidification during storage period were analysed using general linear model (GLM) over the incubation period followed by Tukey’s post-hoc test, with $P<0.05$ used to signify statistical significance. Analyses of both fresh and aged slurry on the effect of combination of AFEM and glucose were carried out separately using the statistical analyses mentioned above.

3.3 Result
3.3.1 Slurry characterization

Slurry characteristics are summarized in Table 3.2. The data shows FS had a higher DM and VS content, but a lower C/N ratio and total VFA content. Although significant differences were observed for % DM and % VS DM$^{-1}$, no significant differences were observed for other parameters (% C, % N, ORP, pH, NH$_4$-N and total VFA content).
Table 3.2 Physicochemical properties of fresh and aged slurry.

<table>
<thead>
<tr>
<th>Slurry physiochemical properties</th>
<th>Fresh Slurry (FS)</th>
<th>Aged Slurry (AG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry Matter (% FWt)</td>
<td>10.7 ±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.85 ±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Volatile solids (% DM&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>81.4 ±0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>77.2 ±0.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Oxidation redox potential (mV)</td>
<td>-231 ±7.5</td>
<td>-282 ±13.0</td>
</tr>
<tr>
<td>Nitrate-N (g N kg&lt;sup&gt;-1&lt;/sup&gt; FWt)</td>
<td>0.00</td>
<td>0.07 ±0.02</td>
</tr>
<tr>
<td>Ammonium-N (g N kg&lt;sup&gt;-1&lt;/sup&gt; FWt)</td>
<td>1.03 ±0.07</td>
<td>1.30 ±0.01</td>
</tr>
<tr>
<td>pH</td>
<td>7.48 ±0.1</td>
<td>7.2 ±0.25</td>
</tr>
<tr>
<td>Total C (g kg&lt;sup&gt;-1&lt;/sup&gt; FWt)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>49.3 ±0.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>60.3 ±0.99&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total N (g kg&lt;sup&gt;-1&lt;/sup&gt; FWt)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>4.5 ±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.3 ±0.10&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C:N&lt;sup&gt;*&lt;/sup&gt;</td>
<td>10.71</td>
<td>9.55</td>
</tr>
<tr>
<td>Volatile fatty acids content (g L&lt;sup&gt;-1&lt;/sup&gt;)&lt;sup&gt;**&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetic acid</td>
<td>2.27 ±0.41</td>
<td>3.40 ±0.46</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>0.64 ±0.13</td>
<td>0.87 ±0.07</td>
</tr>
<tr>
<td>Iso-butyric acid</td>
<td>0.09 ±0.02</td>
<td>0.45 ±0.03</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>0.36 ±0.07</td>
<td>0.49 ±0.08</td>
</tr>
<tr>
<td>Iso-valeric acid</td>
<td>0.15 ±0.03</td>
<td>0.53 ±0.53</td>
</tr>
<tr>
<td>Valeric acid</td>
<td>0.05 ±0.01</td>
<td>0.06 ±0.01</td>
</tr>
<tr>
<td>Total (g L&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>4.63 ±0.64</td>
<td>5.79 ±0.68</td>
</tr>
</tbody>
</table>

Means between columns with no common superscript differ significantly (P ≤0.05). Data represent mean ± SEM (n=5). <sup>*</sup> n=3; <sup>**</sup>, n=4.
3.3.2 Effect of additives on slurry pH and CH₄ emission

I. HCl amendment

Slurry acidification using concentrated HCl caused slurry pH to decrease from 7.4 to 4.1 and lead to significant lower CH₄ headspace concentration \((P<0.05)\) compared to non-acidified slurry during the 11 days storage (Figure 3.1). The pH of acidified slurry varied from 4.1 - 5.3, while in non-acidified slurry, the pH fluctuated between 7.1 and 7.7 with no significant differences \((P>0.05)\). During the incubation period, non-acidified slurry produced CH₄ headspace concentration that ranged from 0.04 to 0.22% CH₄ reaching the highest emission levels on day 5. However, the headspace concentration remained constant after day 8 at 0.13 ± 0.01% CH₄. This headspace concentration was significantly higher than the acidified slurry, which had a low CH₄ headspace concentration at 0.04% throughout the experiment. The pH of the acidified slurry treatment then increased 0.2 - 0.3 units every 2 days and reached its highest point at pH 5.5 on day 10, while the CH₄ emission rate remained unchanged.

The acidified slurry had lower individual and total VFA content \((P<0.05)\) compared to untreated slurry (Table 3.3). Total VFA content of the acidified slurry was over 53% lower than non-acidified slurry.

![Figure 3.1 Cattle slurry pH (a) and CH₄ headspace concentration (b) during the 11 day incubation. Values represent means \((n=5)\), vertical bars indicate the standard error of the mean \((±SEM)\).](image)
Table 3.3 Volatile fatty acid (VFA) content in HCl-acidified and non-acidified slurry.

<table>
<thead>
<tr>
<th>Volatile fatty acids types</th>
<th>HCl-acidified slurry (g L⁻¹)</th>
<th>Non-acidified slurry (g L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>1.06 ±0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.98 ±0.41&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>0.37 ±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.07 ±0.34&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Iso-butyric acid</td>
<td>0.05 ±0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.11 ±0.03&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>0.25 ±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.47 ±0.12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Iso-valeric acid</td>
<td>0.07 ±0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.15 ±0.03&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Valeric acid</td>
<td>0.03 ±0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.06 ±0.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total (g L⁻¹)</td>
<td>1.83 ±0.09</td>
<td>3.84 ±0.94</td>
</tr>
</tbody>
</table>

Means within the same row with different letters are significantly different (P≤0.05). Values represent means ±SEM, n=5.

II. Carbohydrate induced acidification

Glucose addition induced organic acid production resulting in an acidification of the slurry, while the pH of the untreated slurry remains unchanged (Table 3.4; Appendix 3.1). The addition of glucose into slurry immediately changed the slurry pH, and the dynamics of slurry pH during the incubation period provide an indication of acidification process. A uniform pH decrease was measured in all treated slurries, (i.e. at all glucose addition levels) during the experimental period, with differences between treatments. Statistical analysis further revealed that there was a significant difference (P<0.05) between treatments showed by the different rates of acidification activity.

The microorganism consumed simple sugar immediately, and as a result the slurry pH decreased rapidly, at about ±1.0 unit in all treatments (G5, G10, G15, G20, G25) on day 1 (P<0.05). The lowest pH was recorded at pH 4.3 in G5 and G10 on day 11. Addition of glucose higher than 10% w/w did not lead to faster acidification; this is clearly seen for the slurry amended with 25% D-glucose (G25). At the end of day 11, G10 had a significantly lower pH level (P<0.05) compared to G25 and untreated slurry. Although glucose at 5 and 15% (G5 and G25) concentration showed similar trends for pH, the 10% addition (G10) caused faster acidification of cattle slurry with a higher coefficient determination (R²) (Appendix 3.1). This was indicated by a polynomial equation fitted to the data.
The G10 treatment had an acidification rate at -0.5338 pH units day\(^{-1}\) (\(R^2=0.9419\)) compared to G5 (-0.4811, \(R^2=0.928\)) and G15 (-0.5428, \(R^2=0.9292\)).

III. Biological additives (AFEM) effect

An immediate pH decrease was observed for slurries amended with AFEM as shown in Figure 3.2. Significantly lower pH levels were recorded at the initial starting point 6.85 and 6.90 (\(P<0.05\)) on slurries treated with 20 and 30% EM\(^\text{®}\) (EM20, EM30). Overall, no significant changes of slurry pH or ORP were observed during the 10 day incubation (Figure 3.2a, b) following AFEM amendment. Significant differences in CH\(_4\) headspace concentration were only recorded during first 3 days (\(P<0.05\)) for treatments EM20 and EM30.
Table 3.4 Effect of different glucose concentrations on slurry acidification during the 11 day incubation.

<table>
<thead>
<tr>
<th>Glucose level</th>
<th>Slurry pH (Incubation day)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>7</td>
<td>9</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>G0</td>
<td>7.45 ±0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.07 ±0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.47 ±0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.60 ±0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.95 ±0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.81 ±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.07 ±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>G5</td>
<td>6.60 ±0.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.57 ±0.24&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>4.97 ±0.11&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.00 ±0.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.43 ±0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.32 ±0.05&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.28 ±0.05&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>G10</td>
<td>6.26 ±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.04 ±0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.84 ±0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.49 ±0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.44 ±0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.42 ±0.05&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.29 ±0.04&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>G15</td>
<td>6.33 ±0.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.04 ±0.24&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.90 ±0.11&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.59 ±0.16&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>4.49 ±0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.52 ±0.05&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>4.40 ±0.05&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>G20</td>
<td>6.26 ±0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.31 ±0.04&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>4.98 ±0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.50 ±0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.54 ±0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.46 ±0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.36 ±0.01&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>G25</td>
<td>6.44 ±0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.65 ±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.32 ±0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.74 ±0.02&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>4.71 ±0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.67 ±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.65 ±0.01&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

G0, glucose 0%; G5, glucose 5%; G10, glucose 10%; G15, glucose 15%; G20, glucose 20%; G25, glucose 25% (w/w). Means with different letters within same column are significantly different (P≤0.05). Data represent mean ±SEM (n=3).
Figure 3.2 Effect of various concentration of AFEM on slurry pH (a), Oxidation redox potential (ORP), (b) and percentage methane concentration in the headspace (c). Values represent means (n=3), vertical bars indicate the standard error of the mean (±SEM).
3.3.3 Induced acidification on fresh and aged slurry: Biological effect of glucose and effective microorganism addition.

Observation of the effects of glucose and AFEM amendment on the slurry characteristics of FS and AS was carried out at room temperature (20°C ±1.0). Table 3.5 shows that the additives resulted in higher percentage DM and VS in slurries. Amendment with glucose at 10% (w/w) caused a significant pH drop (P<0.05) in both FS and AS (Figure 3.3a, b). The slurry pH decreased sharply in the first 4 days and then gradually after this point in both slurries and reached the lowest point at day 10, at 3.6 and 3.9 (AS and FS). Meanwhile, slurries with AFEM without glucose (FEM and AEM) addition showed no significant differences (P>0.05) in pH, although the pH was slightly lower than untreated slurry for both slurries (FS and AS). The pH of the aged slurry of non-glucose treatments (AEM and A Ctrl) was between 6.4 to 7.0, 6.6 to 7.3, while the FS pH was at pH 6.8 to 7.2, 6.8 to 7.4 (FEM, F Ctrl), respectively, during the experiments.

Methane headspace concentrations were expected to decrease as a result of acidification. As can be seen in Figure 3.3c and d, the CH₄ concentration was lower (P<0.05) than for the untreated slurries, for both FS and AS. Significantly lower CH₄ concentrations (P<0.05) were seen on day 3 (FGEM, FG) and day 2 (AGEM, AG), where they remained lower than 0.05% CH₄ concentration. Their concentrations were unchanged throughout the rest of the incubation period.

The VFA content from both slurries (FS and AS) were analysed at the end of the incubation period. An obvious difference was observed when slurries were amended with glucose (Table 3.6). The acetic acid content was significantly lower in FS treated with glucose; however, they were higher in AS. There was a similar butyric acid content in both slurries, which was significantly higher (P<0.05) in slurries with the glucose addition for both FS and AS. Other individual VFAs components were small and may not have been significant in causing acidification. In contrast, any slurries treated with AFEM did not show any significant changes in the VFA content.
Table 3.5 Characteristics of fresh and aged slurries after the 10 day incubation.

<table>
<thead>
<tr>
<th>Slurries Type</th>
<th>Treatment</th>
<th>Fresh Slurry</th>
<th>Aged Slurry</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FGEM</td>
<td>FG</td>
<td>FEM</td>
</tr>
<tr>
<td>Dry Matter (% FWt)</td>
<td>13.6 ±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.9 ±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.3 ±0.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Volatile solids (% DM⁻¹)</td>
<td>85.3 ±1.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>85.7 ±1.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>75.0 ±2.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ammonium-N (g N kg⁻¹ FWt)*</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
</tr>
<tr>
<td>Nitrate-N (g N kg⁻¹ FWt)</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Dry Matter (% FWt)</td>
<td>14.3 ±0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.0 ±1.86&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.3 ±0.29&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Volatile solids (% DM⁻¹)</td>
<td>88.7 ±1.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>74.1 ±1.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>67.1 ±1.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ammonium-N (g N kg⁻¹ FWt)*</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
</tr>
<tr>
<td>Nitrate-N (g N kg⁻¹ FWt)</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Means within the same row with different letters are significantly different (P≤0.05). Values represent means ±SEM, n=5. FGEM, Fresh Slurry + Glucose 10% + AFEM 5%; FG, Fresh Slurry + Glucose 10%; FEM, Fresh Slurry + AFEM 5%; F (Ctrl), Fresh Slurry – Control; AGEM, Fresh Slurry + Glucose 10% + AFEM 5%; AG, Aged Slurry + Glucose 10%; AEM, Aged Slurry + AFEM 5%; A Ctrl, Aged Slurry – Control. Values represent means ±SEM, n=5. * n.d: not determined.
Figure 3.3 Effect of additives on fresh slurry pH (a) and CH₄ emission (c), and aged slurry pH (b) and CH₄ emission (d) during the 10 day incubation. Vertical bars indicate the standard error of the mean (±SEM). Values represent means ±SEM (n=5).

Note: (a), Fresh slurry pH; (b), Aged slurry pH; (c), Fresh slurry CH₄ % emission; (d), Aged slurry CH₄ % emission.
Table 3.6 Effect of additives on the volatile fatty acid content of fresh and aged slurries after the 10 day storage period.

<table>
<thead>
<tr>
<th>Volatile Fatty acid type (g L(^{-1}))</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GEM</td>
</tr>
<tr>
<td><strong>Fresh Slurries</strong></td>
<td></td>
</tr>
<tr>
<td>Acetic acid</td>
<td>0.54 ±0.014(^a)</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>1.00 ±0.020(^a)</td>
</tr>
<tr>
<td>Iso-butyric acid</td>
<td>0.03 ±0.001(^a)</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>2.66 ±0.072(^a)</td>
</tr>
<tr>
<td>Iso-valeric acid</td>
<td>0.05 ±0.001(^a)</td>
</tr>
<tr>
<td>Valeric acid</td>
<td>0.02 ±0.002(^a)</td>
</tr>
<tr>
<td><strong>Total VFA</strong></td>
<td>4.30 ±0.110</td>
</tr>
<tr>
<td><strong>Aged Slurries</strong></td>
<td></td>
</tr>
<tr>
<td>Acetic acid</td>
<td>1.06 ±0.087(^a)</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>0.39 ±0.011(^a)</td>
</tr>
<tr>
<td>Iso-butyric acid</td>
<td>0.05 ±0.003(^b)</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>1.56 ±0.071(^a)</td>
</tr>
<tr>
<td>Iso-valeric acid</td>
<td>0.11 ±0.014(^a)</td>
</tr>
<tr>
<td>Valeric acid</td>
<td>0.04 ±0.003(^a)</td>
</tr>
<tr>
<td><strong>Total VFA</strong></td>
<td>3.21 ±0.189</td>
</tr>
</tbody>
</table>

Means within the same row with different letters are significantly different (\(P\leq0.05\)). Values represent means ±SEM, \(n=5\).

3.4 Discussion

3.4.1 Slurry characteristic

In this chapter, both fresh (FS) and aged slurry (AS) was taken from the same commercial beef farm in Abergwyngregyn, Wales. Although the slurries’ DM content was at the high end of the typical UK cattle’s slurry (average 6%; Defra, 2010), the percentage (%) of DM was close to that reported by other authors (Table 3.7). Thicker slurries result from barn management (e.g. as a result of scraping rather than washing concrete floors), high moisture loss, diet provided to the animals, and excluding clean water entering the slurry lagoon. Thicker (higher % DM) slurries have a lower moisture content and maximize the quantity of VS in a given volume, thus possible longer storage (holding) time. The practical disadvantage of thicker slurry is that it may require a more powerful pump during slurry transfer, especially in field applications (Weir, 2009). Thicker slurry also promotes faster crust formation (Petersen et al., 2005;
Aguerre et al., 2012) which can reduce CH$_4$ and NH$_3$ losses, but can also lead to nitrification of NH$_4^+$ and the potential for N$_2$O production and emission (Dustan, 2002) in the slurry crust. In comparison, thinner slurry (lower % DM) infiltrates soils easier; thus, the CH$_4$ emission is immediately suppressed during slurry-soil application (Chadwick et al., 2011).

Slurry typically has low or zero NO$_3^-$ content because conditions are too anaerobic for nitrification to take place (Chadwick et al., 2011; Skiba et al., 2012). The NO$_3^-$ content of the slurries used in these experiments were negligible or beyond detection limit. The VFA content of the aged slurry was high, indicating accumulation of VFA from microbial fermentation and biodegradation activity. The normal range for slurry VFA content is between 1.21 - 17.0 g L$^{-1}$ (Cooper and Cornforth, 1978; Paul and Beauchamp, 1989a; Kirchmann and Lundvall, 1993). I was unable to report on Malaysian typical slurry characteristic, as there do not appear to be any records available.
Table 3.7 Characteristics of the cattle slurries used compared with published values.

<table>
<thead>
<tr>
<th>Slurry type</th>
<th>pH</th>
<th>Dry matter (%)</th>
<th>Total N (g kg(^{-1}) FWT)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh cattle slurry (beef &amp; dairy)</td>
<td>7.5</td>
<td>10.7</td>
<td>4.5</td>
<td>Our study</td>
</tr>
<tr>
<td>Mix aged slurry (beef &amp; dairy)</td>
<td>7.2</td>
<td>6.9</td>
<td>6.3</td>
<td>Our study</td>
</tr>
<tr>
<td>Fresh dairy slurry</td>
<td>6.5</td>
<td>7.8-8</td>
<td>3.1</td>
<td>Berg et al., 2006</td>
</tr>
<tr>
<td>Fresh cattle slurry</td>
<td>7.1</td>
<td>7.5</td>
<td>3.6</td>
<td>Dinuccio et al., 2008</td>
</tr>
<tr>
<td>Aged slurry</td>
<td>8.1</td>
<td>9.6</td>
<td>4.3</td>
<td>Pereira et al., 2010</td>
</tr>
<tr>
<td>Aged slurry</td>
<td>7.4</td>
<td>5.6</td>
<td>3.1</td>
<td>Petersen et al., 2012</td>
</tr>
<tr>
<td>Beef cattle slurry</td>
<td>6.7</td>
<td>9.2</td>
<td>3.7</td>
<td>Wulf et al., 2001a</td>
</tr>
<tr>
<td>Cattle slurry</td>
<td>7.4</td>
<td>3.3</td>
<td>2.2</td>
<td>Clemens et al., 2006</td>
</tr>
<tr>
<td>Cattle slurry</td>
<td>6.5</td>
<td>5.8</td>
<td>1.1</td>
<td>Fangueiro et al., 2008</td>
</tr>
<tr>
<td>Cattle slurry</td>
<td>NS</td>
<td>2.0-9.0</td>
<td>3.5-4.0</td>
<td>Defra, 2010</td>
</tr>
<tr>
<td>Cattle slurry</td>
<td>7.8-8.1</td>
<td>2-11.4</td>
<td>NS</td>
<td>Sommer and Husted, 1995</td>
</tr>
<tr>
<td>Cattle slurry</td>
<td>7.5-7.7</td>
<td>5.9</td>
<td>4.1-5.2</td>
<td>Sommer et al., 1993</td>
</tr>
<tr>
<td>Cattle slurry</td>
<td>7.03</td>
<td>4.7-5</td>
<td>2.7</td>
<td>Sørensen and Eriksen, 2009</td>
</tr>
<tr>
<td>Cattle slurry</td>
<td>7.1-7.8</td>
<td>8.2-11.3</td>
<td>4.1-6.3</td>
<td>Stevens et al., 1992</td>
</tr>
<tr>
<td>Cattle slurry</td>
<td>7.5-8.6</td>
<td>3.5-10.2</td>
<td>1.5-3.5</td>
<td>Stevens et al., 1989</td>
</tr>
<tr>
<td>Dairy slurry</td>
<td>6.9</td>
<td>9.16</td>
<td>3.7</td>
<td>Clemens and Huschka, 2001</td>
</tr>
<tr>
<td>Dairy slurry</td>
<td>7.1-7.8</td>
<td>5.7-9.2</td>
<td>3.3-4.0</td>
<td>Amon et al., 2006</td>
</tr>
<tr>
<td>Dairy slurry</td>
<td>7.2</td>
<td>2.1-6.3</td>
<td>NS</td>
<td>Chadwick and Pain, 1997</td>
</tr>
<tr>
<td>Dairy slurry</td>
<td>NS</td>
<td>2.3-7.7</td>
<td>4.2</td>
<td>Christensen and Sommer, 2013</td>
</tr>
<tr>
<td>Dairy slurry</td>
<td>6.78</td>
<td>4.1-9.2</td>
<td>2.1-3.9</td>
<td>Massé et al., 2003</td>
</tr>
<tr>
<td>Dairy slurry</td>
<td>6.4-7.6</td>
<td>7.1-10.4</td>
<td>2.7-4.1</td>
<td>Masse et al., 2008</td>
</tr>
<tr>
<td>Dairy slurry</td>
<td>7.1-8.4</td>
<td>4.3-6.8</td>
<td>3.7</td>
<td>Pain et al., 1990</td>
</tr>
<tr>
<td>Dairy slurry</td>
<td>NS</td>
<td>9.8</td>
<td>NS</td>
<td>Patni and Juli, 1985</td>
</tr>
<tr>
<td>Dairy slurry</td>
<td>7</td>
<td>9.9</td>
<td>3.7</td>
<td>Petersen et al., 2012</td>
</tr>
<tr>
<td>Dairy slurry</td>
<td>6.9</td>
<td>7.9-8.5</td>
<td>4.0-4.9</td>
<td>Rodhe et al., 2009</td>
</tr>
<tr>
<td>Fermented beef slurry (aged)</td>
<td>6.8</td>
<td>5.1</td>
<td>3.2</td>
<td>Wulf et al., 2001a</td>
</tr>
<tr>
<td>Unfermented cattle slurry</td>
<td>7.6</td>
<td>8.1</td>
<td>4.3</td>
<td>Wulf et al., 2001b</td>
</tr>
<tr>
<td>1 months old dairy slurry</td>
<td>NS</td>
<td>11.8</td>
<td>4.0</td>
<td>Fangueiro et al., 2010</td>
</tr>
</tbody>
</table>

*NS: not stated.
3.4.2 Effect of additives on slurry pH and methane emission

Slurry acidification was studied as a GHG mitigation strategy. According to Stevens et al. (1989), slurry acidification was introduced in 1938 by Salter and Schollenberger, and later by Pain (1987) and Thomson (1987), as a strategy to reduce NH$_3$ volatilisation. Later Berg et al. (2006) showed that slurry acidification also significantly reduced CH$_4$ emission during storage. Our findings in this chapter corroborate results from previous studies, and indicate the potential for mitigating CH$_4$ emissions by acidification during slurry storage. These findings are in line with Petersen et al., (2012) and promise a long-term mitigation approach (Petersen et al., 2012). However, there is concern that HCl-acidification could be temporary, as a gradual increase in pH is observed, which may result in CH$_4$ emission again when the pH returns to the optimal level for methanogenesis. During this short incubation period (11 days) after the initial decrease in slurry pH following HCl addition, there was a significant pH increase from 4.0 to 5.3 ($P<0.05$), although it did not return to the initial pH value. Similarly, Petersen (2012) also observed a gradual pH increase during a 3 months storage experiment, thus acidification by HCl might require periodic addition of acid to be successful.

The choice of correct chemical is important, as use of H$_2$SO$_4$ leads to higher hydrogen sulphide (e.g. as H$_2$S) gas production, emission, and a higher sulphur content for the slurry (Sørensen and Eriksen, 2009; Dai and Blanes-Vidal, 2013; Wang et al., 2014), while nitric acid (HNO$_3$) leads to significant N$_2$O production (Berg et al., 2006b). Greater volume of acid would be needed if a slurry were acidified by weak acid such as lactic acid, (pKa 3.86), (Berg et al., 2006b). The uses of EM is not to act as acidifying agent; however, EM is a natural biological additive contains non-pathogenic and naturally occurring microbes to compete with microbes within slurry (Freitag and Meihoefer, 2000). The pH of the AFEM used was in the range pH 3.9-4.1, such that the higher doses used (e.g. EM30) would probably have disrupted the slurry buffer stability and thus decreased the slurry pH to a small degree. As a result, there was lower CH$_4$ production observed during first 6 days of incubation (Figure 3.2). The increasing trend of CH$_4$ emission in all treatments is unknown, as slurry pH remained unchanged in all treatments. However, the low ORP level (below -300 mV) indicates a suitable environment for methanogenic microbes (Mah and Smith, 1981) thus, we suggest the oxygen (O$_2$) ingress into the slurry during mixing resulted in inhibition of
methanogenesis, inducing a new lag phase in CH₄ production and emission during the initial storage period.

Slurry acidification represents an additional management cost in livestock production, but it also returns the benefits to the farmers in terms of higher N availability (assuming NH₃ emissions are reduced during slurry storage and during slurry spreading) if applied to crops (Sørensen and Eriksen, 2009). It also promotes an improved in-house climate if used in slurry stores under buildings. According to Donham (1977), as reviewed by Petersen et al., (2012), acidification may harm animals and threaten human health in closed buildings or barns. However, acidification causes lower biodegradation activity; thus, organic matter retention might be higher compared to non-acidified slurry (Table 3.5) as in the report by Sørensen and Eriksen, (2009).

### 3.4.3 Fresh and aged slurry preliminary observation

Both FS and AS had an anaerobic environment with ORP values below -200 mV, and thus bacterial fermentation could be carried out by facultative anaerobic or obligate anaerobe bacteria. The addition of glucose would have promoted anaerobic respiration; as a result, organic and inorganic by-products were produced. It is a form of oxidation-reduction of organic compounds by microbes to obtain energy for their consumption involving organic compounds, CO₂, CH₄, S₂, and H molecules (Muller, 2001; Gerardi, 2003). The end-product of anaerobic fermentation depends on the fermentation pathway involved, which is related to the of microbes types (Muller, 2001; Gerardi, 2003). Lactate, acetate, butyrate, propionate, organic acid and alcohol are major products obtained from the process. In this preliminary study on FS and AS amended with a simple carbohydrate, the slurry pH decreased to ca. pH 4.0 because of organic acids accumulation during the 10 days incubation. Subsequent to the initial pH drop (after glucose addition), CH₄ production was reduced during the entire experimental period. This result is similar to the HCl-acidification in the earlier experiment. The result suggests that ‘self-induced’ acidification during slurry storage can be used to mitigate CH₄ emission, rather than forced-acidification using concentrated acid (Wulf et al., 2001a; Berg and Pazsiczki, 2006; Berg et al., 2006b). This may also be a more cost-effective approach.

The use of the Micro-Oxymax system in this study was useful to compare the relative CH₄ emission concentration between treatments continuously at a laboratory scale of up to 2 litres volume. However, the system could not be configured to provide
data to calculate actual CH$_4$ fluxes, thus an alternative methodology was required to generate such data, and a manual headspace sampling and determination by gas chromatography was used in subsequent experiments.

Following induced-acidification by anaerobic fermentation, the total VFA observed in both slurries (FS and AS) was higher than without glucose addition, but changes of the different slurry VFA profiles were not clear except for the greater production of butyric acid. It is doubtful that changes in the total VFA content were responsible for the ‘self-acidification’ mechanism. This is because the VFA concentration observed in the ‘self-acidified’ slurry treatments (AG and FG) were not high enough compared to untreated slurry from the HCl acidified experiment. The mechanism of ‘self-acidification’ is explored in following Chapters. The use of fermentable carbohydrate source to promote ‘self-acidification’ resulted in a high percentage of DM and VS in slurries (Appendix 3.2).

In this study, there was no difference in CH$_4$ production between fresh and aged slurry, nor any difference in CH$_4$ inhibition between fresh and aged slurry. In contrast, previous studies (Sommer et al., 2007; Petersen et al., 2012) have shown that fresh slurry had lower CH$_4$ emission at early slurry production due to a microbial lag phase, unless slurry was inoculated with fermented slurry (Sommer et al., 2007).

The fresh slurry acidification technique is a practical approach to reduce NH$_3$ loss and inhibit CH$_4$ emission (Sørensen and Eriksen, 2009; Petersen et al., 2012). This study demonstrated that ‘self-acidification’ through anaerobic fermentation using fermentable carbohydrate could reduce slurry pH to pH values lower than chemical-acidified slurries in commercial farms in Denmark (Sørensen and Eriksen, 2009) e.g. to pH values of 3.9 and 3.6 for FS and AS on G10 treatment, respectively. According to Sommer, (2007) and Petersen, (2012) mitigation should start as early as possible, before the fresh slurry is mix with the aged slurry, which further acts as a methanogen inoculum.
3.5 Conclusion

The aim of this study was to provide a preliminary indication of the potential for chemical acidification and the addition of biological additives to reduce CH₄ emissions. Slurry acidification by 50% (v/w) HCl immediately caused inhibition of CH₄ production, which remained low if the slurry pH remained <5.5. The addition of glucose as a labile carbohydrate promoted ‘self-acidification’ and increased the slurry acidity to pH <5.0 in less than 7 days, and this was observed in both fresh and aged slurries. Consequently, CH₄ emission reduced dramatically. However, it was not clear if ‘self-acidification’ in both slurry types was caused by VFA production. There was no evidence of an effect of effective microorganisms in inhibiting CH₄ emission from slurry. Since the labile carbohydrate addition to slurry resulted in ‘self-acidification’ and a subsequent reduction in CH₄ production, a deeper exploration is now required to determine a true flux and the efficacy in inhibition CH₄ emission and perhaps other GHGs during the storage period, and the mechanism of this acidification process.
3.6 Appendix

Appendix 3.1: Effect of various concentration of glucose on fresh cattle slurry during 11 days storing period

Values represent means (n=4), vertical bars indicate the standard error of the mean (±SEM).

\[
y = 0.031x^2 - 0.5338x + 6.5915
\]

\[
R^2 = 0.9419
\]
Appendix 3.2: Slurry characteristics at the end of the different incubations

<table>
<thead>
<tr>
<th>Additives observation</th>
<th>Dry matter (%)</th>
<th>Volatile solid (% VS DM)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemical additives</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCl 50 %</td>
<td>8.0 ±0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>81.6 ±0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.3 ±0.14&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ctrl</td>
<td>7.1 ±0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>79.3 ±0.17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.2 ±0.13&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>&lt;sup&gt;n&lt;/sup&gt;</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>&lt;sup&gt;P value&lt;/sup&gt;</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Biological additives in fresh slurry

|                       |                |                          |          |
| F GEM                 | 12.2 ±0.16<sup>a</sup> | 91.0 ±0.52<sup>a</sup> | 4.0 ±0.1<sup>a</sup> |
| F G                   | 12.5 ±0.13<sup>a</sup> | 90.0 ±0.60<sup>a</sup> | 3.9 ±0.02<sup>a</sup> |
| F EM                  | 6.9 ±0.10<sup>b</sup> | 82.7 ±0.28<sup>b</sup> | 6.7 ±0.04<sup>b</sup> |
| F Ctrl                | 7.2 ±0.21<sup>b</sup> | 81.6 ±0.61<sup>b</sup> | 6.7 ±0.03<sup>b</sup> |
| <sup>n</sup>          | 5              | 5                        | 5        |
| <sup>P value</sup>    | < 0.001        | < 0.001                  | < 0.001  |

Biological additives in aged slurry

|                       |                |                          |          |
| A GEM                 | 13.6 ±0.15<sup>a</sup> | 85.2 ±0.31<sup>a</sup> | 0.06<sup>a</sup> |
| A G                   | 13.9 ±0.10<sup>a</sup> | 85.1 ±0.34<sup>a</sup> | 1.86<sup>a</sup> |
| A EM                  | 8.3 ±0.43<sup>b</sup> | 72.2 ±1.10<sup>b</sup> | 0.29<sup>b</sup> |
| A Ctrl                | 8.0 ±0.24<sup>b</sup> | 71.8 ±0.12<sup>b</sup> | 0.17<sup>b</sup> |
| <sup>n</sup>          | 5              | 5                        | 5        |
| <sup>P value</sup>    | < 0.001        | < 0.001                  | < 0.001  |

Biological additives (AFEM)

|                       |                |                          |          |
| EM 0                  | 6.9 ±0.18      | 79.6 ±0.80               | 7.4 ±0.18 |
| EM 5                  | 7.1 ±0.59      | 80.6 ±0.59               | 7.1 ±0.20 |
| EM 10                 | 7.0 ±0.45      | 79.2 ±0.45               | 7.0 ±0.18 |
| EM 20                 | 7.4 ±0.44      | 78.6 ±0.44               | 6.6 ±0.26 |
| EM 30                 | 7.7 ±0.24      | 79.6 ±0.24               | 6.6 ±0.07 |
| <sup>n</sup>          | 4              | 4                        | 4        |
| <sup>P value</sup>    | 0.072          | 0.183                    | 0.063    |

<sup>ab</sup> Means within the same column with different letters are significantly different (<i>P</i> ≤0.05). F, Fresh slurry; A, Aged slurry; HCl, acid hydrochloric; EM, effective microorganism AFEM; GEM, glucose 10 % + AFEM 5%; G, glucose 10 %; EM, AFEM 5 %; Ctrl, control untreated.
CHAPTER 4

4 Mitigating greenhouse gases and ammonia emissions from stored cattle slurry through carbohydrate substrate and biological additives

Abstract

Greenhouse gases (GHG) and ammonia (NH$_3$) emission from cattle slurry during storage occurs in any season, albeit at different rates. GHG and NH$_3$ emission inhibition were examined during slurry storage for 120 days (winter) and 60 days (summer) following the addition of brewing sugar (10% $w/w$), as a fermentable carbohydrate, and effective micro-organisms (EM) (5% $v/w$). Addition of brewing sugar decreased the slurry pH to <4.5 within 30 days during winter condition, and 14 days in summer condition. This ‘self-acidification’ led to a significant inhibition of net GHG emission (19%) and average NH$_3$ loss (57%) during winter, and 77% and 92% during the summer. Bio-augmentation with a commercial effective microorganism culture (EM) had an inconsistent effect on GHG and NH$_3$ emission during slurry storage.

Keywords: Manure management, greenhouse gas, ammonia, brewing sugar, acidification, effective microorganisms
4.1 Introduction

Livestock greenhouse gas (GHG) emissions are responsible for between 7 and 18% of global anthropogenic GHG emissions (Hristov et al., 2013). Thus, there is a significant challenge for the livestock industry to produce healthy livestock products for consumption, reduce nuisance odours and manage GHG emissions. Moreover, animal excreta are responsible for ca. 80% of ammonia (NH₃) losses in the EU (Nieder and Benbi, 2008). Despite only 13% NH₃ were emitted from cattle manure during storage (Misselbrook et al., 2006), this emission can be significantly higher if the surface area per m³ manure during storage is increased (Bussink and Oenema, 1998). Moreover, rates of emission are also influenced by the complex interaction between other biological, chemical and physical factors (Dinuccio et al., 2008). Ammonia loss from manure represents an agronomic loss to the farmer, as it reduces the nitrogen (N) content of the manure that is important for crop growth (Ma et al., 2010). In addition, NH₃ volatilisation followed by N deposition results in eutrophication of natural and semi natural ecosystems, soil acidification, forest dieback and also has implications for human health through particulate formation (Pyatt, 2003; Dise et al., 2011). Nitrogen deposition also results in subsequent nitrous oxide (N₂O) emission from soil (Clemens et al., 2006). Livestock manures are a significant source of GHG especially methane (CH₄) and NH₃, thus, an optimal strategy to mitigate CH₄ losses is needed immediately after slurry is generated, i.e. at the start of the manure management chain (Chadwick et al., 2011).

Mitigating GHG emissions by separating the solid and liquid fractions of cattle slurry may be an option but may result in subsequent losses of N₂O and total manure N loss from the separated solid fraction (Smith et al., 2014). In addition, it requires additional infrastructure, management and maintenance costs to farming businesses (Moller et al., 2002). Thus, this approach maybe not favoured by typical small-scale farmers. As slurries are usually stored in reception pits, above and below-ground constructed tanks or lagoons before being either further processed or applied to land. There is opportunity to introduce mitigation strategies for both GHG and NH₃ emissions during this phase, or earlier in the livestock building where the animal urine and faeces are produced.

Slurry acidification has the potential to reduce NH₃ volatilisation and hence retain N in the form of NH₄⁺ (Sørensen and Eriksen, 2009; Petersen et al., 2012). Acids that are generally used in slurry acidification include sulphuric acid (H₂SO₄) and nitric
(HNO₃) acid. They have been shown to reduce NH₃ loss by up to 70% from stores below livestock buildings when slurry pH was reduced to pH <6.0 (Stevens et al., 1989; Berg et al., 2006b). Slurry acidification also has been reported to reduce CH₄ emission from stored slurry (Clemens et al., 2002; Berg and Pazsiczki, 2006; Berg et al., 2006b). However, there are health and safety implications of managing large quantities of concentrated acids on farms. In addition, correct choice of acid is important otherwise, it is not economical, and could cause undesirable environmental effects and increases N₂O losses (Vandré and Clemens, 1996; Berg et al., 2006b). Recent studies have investigated the effects of slurry acidification on emission of both CH₄ and NH₃ (Petersen et al., 2012; Wang et al., 2014). This is an important finding, as a concurrent decrease of both gases would help to justify the costs of implementation.

Induced ‘self-acidification’ by simple carbohydrate addition into slurry was reported by Clemens et al., (2002) and Nykänen et al., (2010). The fermentable carbohydrate source acts as a rapidly metabolised carbon source for the microbes in the slurry. As a result of anaerobic respiration, an acidic environment is created by production of organic acids, which increases the acidity of the environment to a pH <5.0 (Monilola et al., 2013). The use of biological additives (other than acids) during slurry storage is not well explored. Slurry supplied with effective microorganism (EM) additives was carried earlier by Amon et al. (2005) and found that the EM addition to cattle slurries reduced the NH₃ and N₂O emissions, but slightly increased CH₄ emission, although there were no significant differences. The reduction in NH₃ and N₂O emissions was not observed with pig slurries supplied with EM (Amon et al., 2005). Yet, the emission pattern indicated the potential of EM as additives to reduce NH₃ and GHG emissions from stored slurry, although environmental factors may also influence the emission during the storage period.

The aim of this investigation was to explore further the effect of natural additives as a mitigation approach to reduce CH₄ and N₂O emission, and NH₃ volatilisation, during slurry storage. The hypothesis tested was that natural additives promote biologically induced changes to the slurry environment, thus inhibiting key processes such as methanogenesis and ammonia volatilisation. The experiment was design to investigate the effect of adding EMs to slurry, with and without fermentable carbohydrate supplementation, at a small scale (10 kg) during winter and summer climates.
4.2 Materials and methods

4.2.1 Experimental design

The studies were carried in two different seasons (summer and winter) at Henfaes Research Centre (at Bangor University farm) located at Abergwyngregyn Gwynedd, UK (53° 23' 91.29'' N, 4° 01' 91.09’ W). The winter slurry storage study was carried on the 9th December 2013 for 120 days, while the summer study started on the 27th July 2014 for 60 days. Fresh beef slurry was obtained from a commercial farm nearby, and any coarse material such as uneaten grass/hay or straw was screened out prior to use in the experiments. Ten kilograms of slurry was added to 16 litre plastic storage vessels, and four treatments were established. The four treatments were: (1) an untreated slurry (Ctrl); (2) a mixture of slurry with 5% (v/w) Actiferm EM (EM); (3) a mixture of slurry with 10% (w/w) glucose (0.56 M) (Sugar) and (4) a mixture of slurry with 10% (w/w) glucose (0.56 M) and 5% (v/w) Actiferm EM, (Sugar + EM). The ratio of brewing sugar added to the slurry used in this study was based on the optimum ratio needed to induced ‘self-acidification’ that was identified in Chapter 3. The brewing sugar was dextrose, a fermentable carbohydrate as carbon (C) source. The activated EM was provided by Effective Micro-organism Limited, Exeter, UK. Slurry treatments were placed under cover, in a shed, and left open to the atmosphere between gaseous emission sampling.

The physiochemical slurry compositions after the treatments were established and by the end of the storage periods were characterised using the methods described in Chapter 3. During slurry sampling at the end of storage period, any formed crust was removed and the slurry was properly mixed (sediment at the bottom and slurry was mixed). Meanwhile, the lactic acid (C$_3$H$_6$O$_3$) content was determined by using a D-/L-Lactic Acid (D-/L-Lactate) rapid assay kit (Megazyme, Co. Wicklow, Ireland).

4.2.2 Slurry sampling strategy

Periodic sampling was carried out to measure the slurry pH, oxidation redox potential (ORP) and slurry temperature by a portable pH/ORP/Temp device with electrodes (model HI 991003, Hanna Instrument, USA). Sampling was carried out on days 1, 3, 5, 7, 14, 21, 30, 60, 90 and 120. Data logger Ibuttons (Ibuttonlink, Whitewater, USA) were used to record temperature of the environments every 4 hours.

I. Ammonia (NH$_3$) volatilisation
Relative NH₃ volatilisation was determined using a 0.02 M orthophosphoric acid (H₃PO₄) trap placed in a non-ventilated environment (Misselbrook and Powell, 2005) at the same time when the GHG measurements were made. In this method, all volatilised NH₃ will be trapped into the acid trap and create an equilibrium between the acid trap and chamber headspace. Following the one-hour ‘incubation’, ammonium-N (NH₄-N) concentration in the H₃PO₄ acid was analysed using the NH₄-N determination method as described earlier in Chapter 3.

II. Greenhouse gases sampling

Greenhouse gas fluxes were sampled from the container headspace after fitting a gas tight lid with butyl rubber septa. Each vessel had a headspace above the slurry treatment of ca. 3.5 litres. Headspace gas samples were taken immediately (T0), after 30 (T30) and 60 minutes (T60). Gas samples were placed in 20 mL pre-evacuated gas vials and analysed within 1 week on a Perkin Elmer Clarus 580 GC. The GC was equipped with identical megabore capillary Q PLOT columns, and fitted with a flame ionization detector, with methanizer to detect both CH₄ and CO₂, and an ‘electron capture detector to trace N₂O. The net gas fluxes were calculated based on the increase in gas concentration between the T0 and T60 samples over the 1 hour period, headspace volume and slurry weight. Gas accumulation linearity was check prior to GHG fluxes calculation. Cumulative emissions for the 120 days and 60 days periods were also calculated by interpolating the measurements using the trapezoidal rule, based on fluxes obtained.

4.2.3 Statistical analysis

The effect of EM and carbohydrate additives on slurry pH, ORP, VFA, lactic acid and the physiochemical properties were analysed using general linear model (GLM) by Minitab 16 statistical software, (Minitab Ltd. Coventry, UK) followed by Tukey's as post-hoc test, where statistical significance was determined at P<0.05.

Differences in GHG and NH₃ fluxes between treatments in each experiment were carried using corrected P values (by multiple transformation, where necessary) and associated loss of degrees of freedom when variances found to be unequal (Levene’s test; P>0.05). For these variables, reported arithmetic mean and standard
error were from untransformed values, whereas $P$ values reflect statistical analysis of transformed data.

4.3 Results

4.3.1 Winter observation

I. Physiochemical characteristic

Adding brewing sugar increased slurry DM content. The highest dry matter (DM) content was found in S Ctrl at the end of storage period, even though the finding was not statistically significant from the DM of other treatments (Table 4.1). Total carbon (C) was significantly greater ($P<0.05$) in both brewing sugar treatments (SGEM and SG) at the start and end of the storage periods. The NH$_4$-N content was also found to be greater in the SGEM and SG. Slurries supplied with brewing sugar resulted in a thin, lighter coloured and softer surface crust (Appendix 4.1). There was large moisture loss from SEM and S Ctrl (26% and 28% respectively) compared to SGEM and SG treatments, which recorded less than 20% of moisture loss.

II. Slurry temperature, pH and ORP dynamics

The ambient air and slurry temperatures fluctuated throughout the experimental periods, following the ambient air temperature, between 3.7 to 15.0°C (Figure 4.1a). Meanwhile, slurry pH changed significantly ($P<0.001$) during the winter incubation period for those slurries with carbohydrate addition. Both treatments (SGEM and SG) showed a continuous decrease in pH from the start of the incubation (Figure 4.1b). The lowest pH recorded was on day 90 at pH 4.1. Meanwhile the SEM and S Ctrl treatments resulted in a slight increase in pH by the end of the incubation, pH 7.4 (day 120).

In addition, the glucose treatments (SGEM and SG) resulted in significantly higher ($P<0.05$) ORP level from -231.6 mV at day 0 to 46.8 mV at day 120, compared to the SEM and S Ctrl treatments where the ORP remained lower at -287.6 and -315.6 mV at day 120 (Figure 4.1c).
**Table 4.1 Comparison of cattle slurry characteristics at the start and end of the winter storage period.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SGEM</th>
<th>SG</th>
<th>SEM</th>
<th>S Ctrl</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Start experiment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry matter (% FWt)</td>
<td>13.8 ±0.4a</td>
<td>18.4 ±1.3b</td>
<td>13.0 ±2.2a</td>
<td>10.7 ±0.02a</td>
</tr>
<tr>
<td>Volatile solids (% DM⁻¹)</td>
<td>88.6 ±1.0a</td>
<td>90.3 ±0.8ab</td>
<td>83.3 ±2.1ab</td>
<td>81.4 ±0.3b</td>
</tr>
<tr>
<td>Oxidation redox potential (mV)</td>
<td>-224 ±10.5</td>
<td>-247.7 ±11.3</td>
<td>-239.4 ±9.0</td>
<td>-231.6 ±7.5</td>
</tr>
<tr>
<td>Ammonium-N (g N kg⁻¹ FWt)</td>
<td>0.89 ±0.05ab</td>
<td>0.78 ±0.08b</td>
<td>0.98 ±0.05a</td>
<td>0.88 ±0.05ab</td>
</tr>
<tr>
<td>pH</td>
<td>7.2 ±0.1a</td>
<td>7.2 ±0.1ab</td>
<td>7.0 ±0.1b</td>
<td>7.5 ±0.1ab</td>
</tr>
<tr>
<td>Total C (g C kg⁻¹ FWt)</td>
<td>64.4 ±1.53b</td>
<td>85.2 ±3.36a</td>
<td>40.0 ±0.58c</td>
<td>43.4 ±2.07c</td>
</tr>
<tr>
<td>Total N (g N kg⁻¹ FWt)</td>
<td>5.0 ±0.15abc</td>
<td>4.6 ±0.06c</td>
<td>4.9 ±0.13c</td>
<td>5.3 ±0.20ab</td>
</tr>
<tr>
<td><strong>End of storage (120 days)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry matter (% FWt)</td>
<td>15.2 ±2.33</td>
<td>13.0 ±2.70</td>
<td>13.2 ±2.61</td>
<td>16.0 ±2.52</td>
</tr>
<tr>
<td>Volatile solids (% DM⁻¹)</td>
<td>83.6 ±2.26</td>
<td>81.9 ±2.61</td>
<td>81.1 ±2.08</td>
<td>83.5 ±2.31</td>
</tr>
<tr>
<td>Oxidation redox potential (mV)</td>
<td>52.8 ±14.0a</td>
<td>46.8 ±18.8a</td>
<td>-315.6 ±17.6b</td>
<td>-287.6 ±9.7b</td>
</tr>
<tr>
<td>Ammonium-N (g N kg⁻¹ FWt)</td>
<td>1.59 ±0.13a</td>
<td>1.77 ±0.07a</td>
<td>1.44 ±0.22a</td>
<td>1.18 ±0.18a</td>
</tr>
<tr>
<td>pH</td>
<td>4.2 ±0.14a</td>
<td>4.2 ±0.04a</td>
<td>7.4 ±0.24a</td>
<td>7.4 ±0.14a</td>
</tr>
<tr>
<td>Total C (g C kg⁻¹ FWt)</td>
<td>98.2 ±1.68a</td>
<td>116.7 ±10.65a</td>
<td>41.3 ±1.51b</td>
<td>41.7 ±1.23b</td>
</tr>
<tr>
<td>Total N (g N kg⁻¹ FWt)</td>
<td>6.6 ±0.21a</td>
<td>7.2 ±0.65a</td>
<td>4.6 ±0.22b</td>
<td>4.8 ±0.36b</td>
</tr>
</tbody>
</table>

Means with different letters within the same row are significantly different \( (P<0.05) \). Data represent mean ±SEM \( (n=5) \).

#Note: Initial slurry dry matter content seem inconsistent, it was expected to be higher in SGEM and SG, this is possibly due to sampling error.
Figure 4.1 Slurry temperature (a), pH (b) and ORP (c) dynamics during the winter storage period. Values represent means (n=5), vertical bars indicate the standard error of the mean (±SEM).
III. Ammonia volatilisation

Figure 4.2 shows the relative NH₃ fluxes during the winter incubation period. The highest NH₃ fluxes were seen in the first 4 days, with 364.8 and 302.1 µg NH₃ m⁻² hr⁻¹, from the SGEM and SG treatments, and 322.1 and 314.0 µg NH₃ m⁻² hr⁻¹, from the SEM and S Ctrl treatments. Ammonia fluxes decreased immediately at day 7 and remained low for the rest of the storing period, (between 0.0 to 0.7 µg NH₃ m⁻² hr⁻¹). The cumulative NH₃ emissions during the 120 days winter storage period for the SGEM, SG, SEM, and S Ctrl treatments were 61.0, 60.4, 139.7 and 153.4 mg NH₃ m⁻² respectively, with the cumulative NH₃ emissions from the two glucose treatments being significantly lower than those from the S Ctrl and SEM treatments ($P<0.05$).

![Figure 4.2 Slurry ammonia volatilisation observed during winter storage.](image)

(a) Ammonia flux; (b) Cumulative emission. Values represent means ($n=5$), vertical bars indicate the standard error of the mean (±SEM).
IV. Greenhouse gas emission

Figure 4.3 illustrates the dynamics of GHG (CH₄, CO₂ and N₂O) emissions during the 120 days winter storage period. It is clear that CH₄ fluxes were greater from the SEM and S Ctrl treatments, while CH₄ emissions from the SGEM and SG treatments remained lower until day 120. However, significant differences ($P<0.05$) were only observed from day 30 until the end of slurry storage period. Treatments with brewed sugar resulted in small fluxes within 0.0 and 2.6 µg CH₄ kg⁻¹ VS hr⁻¹ range, while the SEM and S Ctrl treatments resulted higher fluxes ranging from 31.1 to 39.8 µg CH₄ kg⁻¹ VS hr⁻¹. The cumulative CH₄ flux was significantly highest ($P<0.05$) from S Ctrl (11.63 g CH₄ kg⁻¹ VS) followed by SEM (10.17 g CH₄ kg⁻¹ VS), SG (0.32 g CH₄ kg⁻¹ VS) and SGEM (0.22 g CH₄ kg⁻¹ VS), respectively.

Meanwhile, CO₂ fluxes fluctuated during the study. There were significant lower CO₂ emissions ($P<0.05$) from the brewing sugar added treatment at day 14 until the end of the storage period, resulting in lower cumulative emissions than S Ctrl and SEM. Nitrous oxide fluxes were negligible from all treatments for the first 60 days, and small but significant ($P<0.05$) emissions recorded from S Ctrl and SEM on day 90 and day 120. The cumulative N₂O emissions were 39.7, 50.1, 62.2 and 180.8 mg N₂O kg⁻¹ VS for SGEM, SG, SEM and S Ctrl, respectively.

V. Volatile fatty acids content

Table 4.2 summarises the effect of the slurry treatments on the slurry volatile fatty acids (VFA) content. There was no significant difference in VFA content at the start of the incubation, but at the end of incubation, significant differences ($P<0.05$) were observed for some of the VFA types (except for acetic and propionic acids). The slurries with glucose addition had significantly lower total VFA contents at end of the storage period compared to without glucose addition. Total VFA contents were 5.4 (SGEM), 4.7 (SG), 6.2 (SEM) and 6.0 g L⁻¹ (S Ctrl), respectively.
Figure 4.3 Dynamics of GHG fluxes and cumulative emissions from slurry treatments during the winter storage period.

(a) CH₄ flux; (b) CH₄ cumulative; (c) CO₂ flux; (d) CO₂ cumulative; (e) N₂O Flux; (f) N₂O cumulative. Values represent means (n=5), vertical bars indicates the standard error of the mean (±SEM). Note different y-axis scales.
Table 4.2 Volatile fatty acid content of slurries at the start and end of the winter storage period.

<table>
<thead>
<tr>
<th>Volatile fatty acid type (g L(^{-1}))</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SGEM</td>
</tr>
<tr>
<td><strong>Start experiment</strong></td>
<td></td>
</tr>
<tr>
<td>Acetic acid</td>
<td>3.32 ±0.05</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>0.80 ±0.02</td>
</tr>
<tr>
<td>Iso-butyric acid</td>
<td>0.09 ±0.00</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>0.39 ±0.02</td>
</tr>
<tr>
<td>Iso-valeric acid</td>
<td>0.17 ±0.01</td>
</tr>
<tr>
<td>Valeric acid</td>
<td>0.03 ±0.00</td>
</tr>
<tr>
<td>Total VFA</td>
<td>4.81 ±0.04</td>
</tr>
<tr>
<td><strong>End of storage (120 days)</strong></td>
<td></td>
</tr>
<tr>
<td>Acetic acid</td>
<td>3.01 ±0.20</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>1.50 ±0.13</td>
</tr>
<tr>
<td>Iso-butyric acid</td>
<td>0.10 ±0.00(a)</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>0.60 ±0.03(a)</td>
</tr>
<tr>
<td>Iso-valeric acid</td>
<td>0.10 ±0.01(a)</td>
</tr>
<tr>
<td>Valeric acid</td>
<td>0.00 ±0.00(a)</td>
</tr>
<tr>
<td>Total VFA</td>
<td>5.42 ±0.37(ab)</td>
</tr>
</tbody>
</table>

Means within different letters within each row are significantly different \((P<0.05)\). Values represent means ±SEM, \(n=5\).
4.3.2 Summer observation

I. Physiochemical characteristic

Slurry physiochemical characteristic at the start and end of the 60 days summer storage experiment are summarised in Table 4.3. Slurries with glucose added treatments had significantly \((P<0.05)\) higher percentage DM at the start and at the end of the experiment. There was large moisture loss from SEM and S Ctrl (26% and 31% respectively) compared to SGEM and SG treatments, which recorded at average 21% moisture loss.

Table 4.3 Comparison of cattle slurry characteristics at the start and end of the summer storage period.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SGEM</th>
<th>SG</th>
<th>SEM</th>
<th>S Ctrl</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Start experiment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry matter (% FWt)</td>
<td>11.5 ±0.74a</td>
<td>12.4 ±0.42a</td>
<td>9.3 ±0.36b</td>
<td>8.9 ±0.25b</td>
</tr>
<tr>
<td>Volatile solids (% DM⁻¹)</td>
<td>93.5 ±6.08a</td>
<td>85.5 ±0.29ab</td>
<td>78.8 ±0.49b</td>
<td>78.7 ±0.31b</td>
</tr>
<tr>
<td>Oxidation redox potential (mV)</td>
<td>-250.8 ±6.9</td>
<td>-245.8 ±3.7</td>
<td>-259.8 ±2.8</td>
<td>-258 ±4.3</td>
</tr>
<tr>
<td>Ammonium-N (g N kg⁻¹ FWt)</td>
<td>0.67 ±0.04</td>
<td>0.67 ±0.02</td>
<td>0.67 ±0.03</td>
<td>0.67 ±0.02</td>
</tr>
<tr>
<td>pH</td>
<td>6.5 ±0.06</td>
<td>6.5 ±0.03</td>
<td>6.5 ±0.04</td>
<td>6.5 ±0.02</td>
</tr>
<tr>
<td>Total C (g C kg⁻¹ FWt)</td>
<td>45.0 ±3.97</td>
<td>49.3 ±5.39</td>
<td>35.5 ±2.50</td>
<td>42.1 ±6.50</td>
</tr>
<tr>
<td>Total N (g N kg⁻¹ FWt)</td>
<td>5.1 ±0.06</td>
<td>4.9 ±0.25</td>
<td>6.0 ±0.23</td>
<td>5.5 ±0.49</td>
</tr>
<tr>
<td><strong>End of storage (60 days)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry matter (% FWt)</td>
<td>19.5 ±3.61a</td>
<td>13.5 ±0.53a</td>
<td>10.1 ±1.39b</td>
<td>11.5 ±0.99b</td>
</tr>
<tr>
<td>Volatile solids (% DM⁻¹)</td>
<td>91.3 ±1.59a</td>
<td>85.1 ±1.65ab</td>
<td>72.0 ±1.87b</td>
<td>73.7 ±0.52ab</td>
</tr>
<tr>
<td>Oxidation redox potential (mV)</td>
<td>-35.2 ±19.6a</td>
<td>-5.8 ±22.3a</td>
<td>-366.8 ±12.2b</td>
<td>-338 ±20.3b</td>
</tr>
<tr>
<td>Ammonium-N (g N kg⁻¹ FWt)</td>
<td>1.12 ±0.05a</td>
<td>1.13 ±0.02a</td>
<td>1.09 ±0.11ab</td>
<td>0.8 ±0.09b</td>
</tr>
<tr>
<td>pH</td>
<td>4.2 ±0.03a</td>
<td>4.2 ±0.03a</td>
<td>8.4 ±0.09b</td>
<td>8.5 ±0.07b</td>
</tr>
<tr>
<td>Total C (g C kg⁻¹ FWt)</td>
<td>66.6 ±4.60a</td>
<td>61.7 ±3.14a</td>
<td>36.3 ±5.98b</td>
<td>39.8 ±5.69b</td>
</tr>
<tr>
<td>Total N (g N kg⁻¹ FWt)</td>
<td>4.4 ±0.32a</td>
<td>4.4 ±0.27a</td>
<td>4.4 ±0.50b</td>
<td>4.4 ±0.22b</td>
</tr>
</tbody>
</table>

Means with the same letter within same row are not significantly different. Data represent mean ±SEM \((n=5)\).

#Note: Total C from SGEM and SG at day 0 indicates lower proportion of C although after the addition of carbohydrates, this is possibly due to sampling error.
II. Slurry temperature, pH and ORP dynamics

Figure 4.4 shows the ambient air temperature, slurry temperature, pH and ORP levels during the summer storage period. The slurry temperatures ranged between 12.7°C and 22.8°C. Meanwhile, the slurry pH with glucose supplementation decreased significantly ($P<0.05$); the pH of the slurry declined immediately after glucose addition and continued to decrease to the lowest pH, of 3.5, on day 21. In contrast, pH of SEM and S Ctrl treatments gradually increased from a starting value of 6.5 to 8.4 (SEM) and 8.5 (S Ctrl) by day 60.

Similarly, the slurry ORP level increased for the SGEM and SG treatments, but decreased in the non-glucose treatments, SEM and S Ctrl. The ORP of the SGEM and SG treatment slurries increased from -246 and -251 mV to -35 and -5 mV at day 60, while the SEM and S Ctrl slurries decreased from -260 and -258 mV to -367 and -338 mV.

III. Ammonia volatilisation

Relative NH$_3$ emission measurements during the summer storage period are shown in Figure 4.5. There were lower emissions from the glucose added treatments (SGEM and SG) than from the SEM and S Ctrl slurries. However, significant differences ($P<0.05$) in NH$_3$ fluxes between the two sets of treatments were observed only after 14 days incubation (Figure 4.5). Cumulative NH$_3$ emissions were greatest ($P<0.05$) from S Ctrl and SEM at 96.8 and 86.9 mg NH$_3$ m$^{-2}$, compared to SGEM and SG, at 19.8 and 21.4 mg NH$_3$ m$^{-2}$.
Figure 4.4 Slurry temperature (a), pH (b) and ORP (c) dynamics during the summer storage period. Values represent means (n=5), vertical bars indicate the standard error of the mean (±SEM).
Figure 4.5 Relative ammonia emissions from slurry treatments during the summer storage period. (a) Ammonia fluxes (b) Cumulative emission. Values represent means ($n=5$), vertical bars indicate the standard error of the mean (±SEM).
IV. Greenhouse gas emission

Figure 4.6 shows the dynamics of GHG (CH\textsubscript{4}, CO\textsubscript{2} and N\textsubscript{2}O) emissions during the 60 day summer storage experiment. Lower CH\textsubscript{4} fluxes were recorded from those slurries with fermentable carbohydrate addition, SG and SGEM, during the entire 60 days storage period (with the exception on day 4 for SGEM). However, the significant difference in CH\textsubscript{4} fluxes between the SGEM and SG treatments, and SEM did not occur until after day 21 \((P<0.05)\) although CH\textsubscript{4} fluxes from S Ctrl and SEM were numerically higher. The cumulative CH\textsubscript{4} emission was significantly highest \((P<0.05)\) from SEM, followed by S Ctrl and lowest from SG. The cumulative CH\textsubscript{4} emission from S Ctrl, SEM, SG and SGEM were 2.0, 3.3, 0.5 and 0.3 g CH\textsubscript{4} kg\textsuperscript{-1} VS.

There were no significant differences \((P>0.05)\) in CO\textsubscript{2} fluxes from the different slurry treatments, with cumulative CO\textsubscript{2} emissions ranging between 0.9 and 1.0 g CO\textsubscript{2} kg\textsuperscript{-1} VS. Meanwhile, N\textsubscript{2}O emissions from all treatments were very small, with no significant difference \((P>0.05)\) between treatments. The cumulative N\textsubscript{2}O emissions were lower than 242 (SG) µg N\textsubscript{2}O kg\textsuperscript{-1} VS, with apparent negative emissions (below zero) observed for the SEM and SGEM treatments.
Figure 4.6 Dynamics of GHG fluxes and cumulative emissions during the summer storage period. (a) CH₄ flux; (b) CH₄ cumulative; (c) CO₂ flux; (d) CO₂ cumulative; (e) N₂O flux; (f) N₂O cumulative. Values represent means (n=5), vertical bars indicate the standard error of the mean (±SEM). Note different y-axis scales.
V. Volatile fatty acids content

Table 4.4 summarises the effect of the slurry treatments on slurry VFA content during the summer storage period. Significant differences ($P<0.05$) were observed between the start and end of the storage period for most VFA types and total VFA content. The slurries with glucose addition (SGEM and SG) had significantly ($P<0.05$) greater content of acetic, propionic, butyric acid, and subsequently the total VFA content, at the end of the storage period. Total VFA contents were 6.1, 6.9, 1.1 and 0.4 g L$^{-1}$ (SGEM, SG, EM, S Ctrl), respectively.

Table 4.4 Volatile fatty acid content of slurries at the start and end of the 60 day summer storage experiment.

<table>
<thead>
<tr>
<th>Volatile Fatty acid type (g L$^{-1}$ FWt)</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SGEM</td>
</tr>
<tr>
<td><strong>Start experiment</strong></td>
<td></td>
</tr>
<tr>
<td>Acetic acid</td>
<td>0.88 ±0.180</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>0.33 ±0.067</td>
</tr>
<tr>
<td>Iso-butyric acid</td>
<td>0.00 ±0.002</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>0.09 ±0.025</td>
</tr>
<tr>
<td>Iso-valeric acid</td>
<td>0.02 ±0.008</td>
</tr>
<tr>
<td>Valeric acid</td>
<td>0.00 ±0.002</td>
</tr>
<tr>
<td><strong>Total VFA</strong></td>
<td>1.32 ±0.284</td>
</tr>
<tr>
<td><strong>End of storage (60 days)</strong></td>
<td></td>
</tr>
<tr>
<td>Acetic acid</td>
<td>2.51 ±0.159$^a$</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>1.40 ±0.077$^a$</td>
</tr>
<tr>
<td>Iso-butyric acid</td>
<td>0.01 ±0.006</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>2.10 ±0.406$^a$</td>
</tr>
<tr>
<td>Iso-valeric acid</td>
<td>0.04 ±0.004$^{bc}$</td>
</tr>
<tr>
<td>Valeric acid</td>
<td>0.04 ±0.002$^{ab}$</td>
</tr>
<tr>
<td><strong>Total VFA</strong></td>
<td>6.10 ±0.653$^a$</td>
</tr>
</tbody>
</table>

Means with different letters within same row are significantly different ($P<0.05$). Data represent mean ±SEM (n=5).
4.3.3 Lactic acid content

The total lactic acid concentration of the different slurry treatments in both storage periods (winter and summer) was significantly \((P<0.05)\) greater in the glucose treated slurries, SGEM and SG (Table 4.5). At the end of the winter storage period, lactic acid concentrations of the SGEM and SG treatments were 4.20 and 4.28 g L\(^{-1}\) FWt, whilst concentrations were greater after the summer storage period, 5.02 and 5.1 g L\(^{-1}\) FWt respectively.

Table 4.5 Total lactic acid content in slurries (g L\(^{-1}\) FWt.) at the end of the summer and winter storage periods. Data presented on a fresh weight basis.

<table>
<thead>
<tr>
<th>Season</th>
<th>Lactic acid (C(_3)H(_6)O(_3)) concentration (g L(^{-1}) FWt)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SGEM</td>
</tr>
<tr>
<td>Winter</td>
<td>4.2 ±0.05(^a)</td>
</tr>
<tr>
<td>Summer</td>
<td>5.02 ±0.11(^a)</td>
</tr>
</tbody>
</table>

Means with different letters within the same row are significantly different \((P<0.01)\). Data represent mean ±SEM \((n=5)\).

4.3.4 Total greenhouse gases emissions - CO\(_2\) equivalent

The GHG emission during summer and winter storage were expressed as net GHG emissions CO\(_2\) equivalent (eq.) following the IPCC (2013) guidance (Myhre et al., 2013) (Table 4.6), where the global warming potential for N\(_2\)O and CH\(_4\) (over 100 years) are 298 and 34, respectively. Cumulative GHG emissions during the winter storage period were greatest from S Ctrl at 615.7 g CO\(_2\) eq. kg\(^{-1}\) VS, while during summer storage the greatest cumulative emission was from the SEM treatment, at 112 g CO\(_2\) eq. kg\(^{-1}\) VS. Lowest emissions were observed from ‘self-acidified’ slurries treatments in both climates. This reduced emission was equated to an inhibition in GHG (CO\(_2\) eq.) of ca. 85% and 84% (SGEM, SG) during the winter, and 82% (SGEM) and 72% (SG) during the summer, compared with emissions from the unamended (Ctrl) slurry.
Table 4.6 Total GHG emission, expressed as CO₂ eq. from winter and summer storage periods.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CH₄</th>
<th>CO₂</th>
<th>N₂O</th>
<th>Total emission (CO₂ eq.)</th>
<th>Percentage GHG Reduction compare to control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Winter climate observation (120 days)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SGEM</td>
<td>0.22</td>
<td>70.5</td>
<td>0.040</td>
<td>90.0</td>
<td>85.4</td>
</tr>
<tr>
<td>SG</td>
<td>0.33</td>
<td>74.9</td>
<td>0.050</td>
<td>101.0</td>
<td>83.6</td>
</tr>
<tr>
<td>SEM</td>
<td>10.17</td>
<td>129.6</td>
<td>0.062</td>
<td>494.1</td>
<td>19.8</td>
</tr>
<tr>
<td>S Ctrl</td>
<td>11.63</td>
<td>166.2</td>
<td>0.181</td>
<td>615.7</td>
<td>0.0</td>
</tr>
<tr>
<td>Summer climate observation (60 days)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SGEM</td>
<td>0.52</td>
<td>0.86</td>
<td>0.000</td>
<td>18.50</td>
<td>72.6</td>
</tr>
<tr>
<td>SG</td>
<td>0.33</td>
<td>1.01</td>
<td>0.000</td>
<td>12.18</td>
<td>81.9</td>
</tr>
<tr>
<td>SEM</td>
<td>3.27</td>
<td>0.90</td>
<td>0.000</td>
<td>112.02</td>
<td>-66.1</td>
</tr>
<tr>
<td>S Ctrl</td>
<td>1.96</td>
<td>0.90</td>
<td>0.000</td>
<td>67.43</td>
<td>0.0</td>
</tr>
</tbody>
</table>

The percentage reductions were calculated based on emission from non-amended treatment (S Ctrl) in each season. GHG emissions were expressed as CO₂ eq. using the GWPs as stated by IPCC (2013), i.e. CH₄=34, N₂O=298 (Myhre et al., 2013).

4.4 Discussion

The slurries used in the two experiments had different starting characteristics, even though they came from the same commercial farm. Although direct comparisons between the two experiments are not applicable, the relative differences between treatments in both experiments can be explored (in addition, both studies were conducted at different storage period). At the end of the storage, there is slight increase of slurry pH on non-acidified slurries (SEM, S Ctrl) on both (winter and summer) studies. The increased of slurries pH is found similar to other studies (Clemens et al., 2002; Petersen et al., 2012; Wang et al., 2014). The higher pH recorded during summer observation (pH 8.5) were similar to the latter finding. These increment was associated with the slurry VFA/(NH₄⁺+NH₃) ratio (Sommer and Husted, 1995). However, Paul and Beauchamp, 1989, suggested that the microbial activity uptake an acid from the medium (removes H⁺ ion from the medium) during neutral pH, or possibly by the result of oxidized VFA thus releases hydroxyl ion (OH⁻) to the medium.

In this study, both experiments consistently demonstrated the effects of slurry additives on the slurry environment and on emissions of GHGs and NH₃ emissions during storage. Results showed that emissions were affected by the ‘self-acidification’
during anaerobic fermentation, and suggest that production of large amounts of organic acids reduced the slurry pH. Slurries amended with the 10% brewing sugar had significantly ($P<0.05$) lower pH than the other treatments, as a result of organic acid production. However, the total VFA content was not the main reason for this pH decrease; our measurements suggest that the total lactic acid (Table 4.5) content of the glucose treated slurries was probably responsible for this acidification. Hence, we hypothesize that the addition of 10% brewing sugar to slurry promotes the homolactic or heterolactic acid fermentation process (Rogers et al., 2013) in both temperature environments, subsequently decreasing the slurry pH. Livestock slurry is known to contain indigenous lactic acid producing bacteria capable of degrading carbohydrate rapidly (Nykanen et al., 2010). This ‘self-acidification’ offers the potential to replace current methods of acidifying slurry on the farm (Kai et al., 2008), i.e. those that use concentrated mineral acids, which represent a risk to human health (Fangueiro et al., 2015).

### 4.4.1 Ammonia volatilisation

As has been noted, the slurry’s pH gradually decreased as a result of ‘self-acidification’ by organic acid production, specifically lactic acid production. The acidification subsequently resulted in inhibition of NH$_3$ loss in both temperature climates (Figure 4.2b and Figure 4.5b). Hence, NH$_3$ loss from the ‘self-acidified’ treatments (SGEM and SG) were significantly reduced during summer storage representing ca. 77.2% and 75.4% compared to the emissions from the untreated slurry, and reductions of ca. 58% and 56% during the 60 days winter storage period. The percentage inhibition of cumulative NH$_3$ emission recorded in these experiments were similar to finding by Blanes-Vidal et al., (2012) but lower than Petersen et al., (2013) who reported 95% inhibition and Wang et al., (2014) recorded up to 81% with H$_2$SO$_4$ acidification at pH below 5.5.

Cumulative NH$_3$ losses were comparatively greater in the summer (SEM and S Ctrl), between 86.9 and 96.8 mg NH$_3$ m$^{-2}$ (Figure 4.2b) during a 60 day storage period, than the from the 120 day winter storage period (132.3 to 140.0 mg NH$_3$ m$^{-2}$, Figure 4.5b), respectively. This can be partially explained by the higher ambient summer temperature and higher slurry pH of the SEM and S Ctrl slurries in the summer storage resulted in differences in the air-water equilibrium. It is known that Henry’s law constant referring to gas solubility especially at the surface layer where the atmosphere gas and
liquid pressure change with the changes of temperature. In addition, the presence of maggots and disappearance of crust during summer storage may have influenced the NH$_3$ fluxes (Appendix 4.2) through continuous disturbance of the slurry crust. Slurry crusts formed on the surface during the winter storage and acted as a passive barrier to air flow, slowing the NH$_3$ transfer rate from the slurry surface to the air (Sommer et al., 1993).

4.4.2 Greenhouse gas emission

Methane fluxes were highly suppressed in the acidified slurry (Table 4.6) in both winter and summer experiments. The inhibition of CH$_4$ emissions in ‘self-acidified’ slurries was related to the higher ORP levels. At ORP levels below -100 mV (i.e. in anaerobic conditions), methanogens produce CH$_4$ through the acetoclastic pathway from organic compounds such as VFAs (Mah and Smith, 1981; Gerardi, 2003) but the ‘self-acidification’ following the addition of brewing sugar to slurry increased the ORP levels and oxic slurry level subsequently indicating the electron acceptor availability for oxidation reduction activity (Gerardi, 2003). Moreover, the high lactic acid content subsequently converted to propionic acid which acted as a precursor to acetoclastic methanogenesis inhibition, and thus the acidic environment may inhibit methanogen (except where there are acid tolerant groups). Acid tolerant methanogens belongs to the orders Methanomicrobiales and Methanobacteriaceae, which produce CH$_4$ through the hydrogenotrophic pathway (Horn et al., 2003).

The lower cumulative CH$_4$ emissions in the summer may have been the result of differences in the cattle’s diet between winter and summer, as diet is known to influence methanogen activity (Hindrichsen et al., 2005). In addition, cattle were probably grazing for most for the summer period, hence the slurry collected may not have been as fresh as the slurry collected from the reception pit during the winter period. In addition, Monteny et al., (2006) suggested that the proportion of psychrophilic and mesophilic methanogens present during in the slurry, could associate with the mixed up with an older slurry during winter climates (Petersen et al., 2013). The lower CH$_4$ flux were also observed at 35°C compared to 5 and 25°C although 25°C indicates higher emission than 5°C (Pereira et al., 2012).

Carbon dioxide emission in both storage experiments was very different. The cumulative CO$_2$ emission were between 70.5 and 166.2 g CO$_2$ kg$^{-1}$ VS during winter, meanwhile during the summer they were 0.8 and 1.0 g CO$_2$ kg$^{-1}$ VS. The possible
reason for this is that crust that formed on the slurry surface area during the winter was a suitable medium for microbial respiration, resulting in large CO$_2$ emission. Figure 4.3c and Figure 4.6c indicated CO$_2$ fluxes observed were relatively lower during summer and this suggests that CO$_2$ produced were immediately used up by the methanogen as a C source in hydrogenotrophic pathway methanogenesis thus the cumulative CO$_2$ emission is much lower (Ferry, 2010, 2011). Other studies on slurry GHG emission from slurry have not quantified CO$_2$ fluxes as a part of the suite of GHG emission measurements (CH$_4$ and N$_2$O), it could be due to CO$_2$ being present in the atmosphere and can be removed through natural biological process (Aguerre et al., 2012; Mathot et al., 2012; Fangueiro et al., 2015).

Nitrous oxide is a very important greenhouse gas with a long atmospheric lifetime of about 121 years (Myhre et al., 2013). The N$_2$O emission from our experimental slurry stores was very small/negligible in both the winter and summer storage periods (Figure 4.3e and Figure 4.6e), as there was little opportunity for nitrification of slurry NH$_4^+$ to NO$_3^-$ to occur under the anaerobic conditions (Dinuccio et al., 2008; Molodovskaya et al., 2008; Rodhe et al., 2009b). However, N$_2$O emissions can become significant when slurry particles, organic matter in slurries float or are forced to the surface by bubbles of biogas and create crusts. The crust represents a suitable medium for oxygen diffusion, and hence conditions can become favourable for bacterial nitrification denitrification (Sommer and Hutchings, 2001; Clemens et al., 2006; Rodhe et al., 2009b; Sommer et al., 2009).

4.4.3 Net greenhouse gas and inhibition capability

The GHG emission from both climates was not compared as the slurry was obtained at different times, which may have been influenced by various factor and variables. In addition, both slurries indicate differences in their physiochemical characteristics. Slurry storage with addition of brewing sugar significantly inhibited net GHG CO$_2$ eq. emission by ‘self-acidification’ of slurry to pH <4.5. Furthermore, we observed that slurry pH could decrease to 3.5 within 21 days during the summer climate. It is observed that the percentages GHG CO$_2$ eq. inhibition from the self-acidified slurry was almost similar in both climates within 73 to 85% range. Although summer climate is known to promote microbial activity for greater biological degradation, higher CH$_4$ flux emission and resulting greater GHG CO$_2$ eq., (Clemens et al., 2006; Monteny et al., 2006; Sommer et al., 2009; Pereira et al., 2012), it is not
shown in this study as other factors also simultaneously influence the degradation and the methanogenesis pathway processes. In fact that, the ambient temperature during summer did not reach >30°C for optimal mesophiles.

The AFEM addition had an inconsistent effect on CH₄ fluxes in both storage seasons. The addition of AFEM to slurry stores was not as effective as the addition of glucose in terms of GHG mitigation, although lower (non-significant) emissions were measured from the AFEM treated slurries in the winter experiment. Net GHG emission (CO₂ eq.) were inhibited by 19% during winter, but adversely increased the net GHG emission by 66% during summer (Table 4.6). This level of reduction was far higher than results by Amon et al., (2005) where a 1.8% increased in CH₄ emission was observed after the use of EM during a 120 days slurry storage trial. This result is unexpected, but could be explained by the differences in slurry composition, and the microbial community structure following higher rates of EM addition, which could have displaced harmful microbes by beneficial microorganisms, thus stabilizing the slurry ecosystem (Higa and Parr, 1994). The maggot ‘invasion’ during the summer storage period prevented crust formation, resulting in higher NH₃ and CH₄ losses. Smith et al., (2007) reported that up to 22% of slurry lagoons within England and Wales do not develop crusts. This is important as slurry crusts act as a primary physical barrier reducing both NH₃ and CH₄ emissions from slurry stores. The crust can also act as a CH₄ sink as the crust environment is favourable for methanotrophs, but it may also be a suitable medium for nitrification and denitrification to take place, which release N₂O to the atmosphere.

The ‘self-acidification’ subsequently caused lower on the calculated GHG (CO₂ eq.) and NH₃ emissions in both climates suggest that this is a potential alternative approach to mitigate these emissions from slurry stores. However, the effectiveness and practicality will need to be evaluated at the lagoon / larger slurry tank scale. The ‘self-acidification’ process overcomes the health hazard issue associated with handling concentrated acid. The uses of brewing sugar as fermentable carbohydrate source in this study may not be suitable for all farmers, as this is likely to incur additional costs. Other substrates or products such as wheat flour, maltose or household waste may be useful to promotes slurry self-acidification (Clemens et al., 2002; Nykanen et al., 2010). Finally, the impacts of spreading acidified slurry onto soil and crops needs to be assessed from both agronomic and environmental perspective.
4.5 Conclusion

In conclusion, results from contrasting storage periods (winter and summer) showed that glucose addition induced ‘self-acidification’ by lactic acid production and inhibited NH$_3$ and total GHG (CO$_2$ eq.) emissions during slurry storage. Between 56-76% of NH$_3$ and 72-85% GHG (CO$_2$ eq.) emissions were reduced by ‘self-acidification’ compared to untreated slurry during these relatively small-scale slurry storage experiments. ‘Self-acidification’ of livestock slurries may replace the current practice of chemical acidification with mineral acids, with the reduced risk of injury to farming operators. Biological additives (AFEM) had an inconsistent effect in both storage periods. Greenhouse gas and NH$_3$ losses were relatively high during warmer conditions. There is now a need to address the practicalities of this potential mitigation practice at greater scales, and to explore cheap and abundant sources of carbohydrate to induce ‘self-acidification’ at these scales.
4.6 Appendix

Appendix 4.1: The slurry crust surface observed at end of the winter storage period.

Note:

i) Crust formed with 4 different amendments (AFEM, Glucose, Glucose + AFEM, Control);

ii) Closer observation of slurry crusts (Top, Ctrl); Bottom, with glucose added);

iii) Crust formation with glucose added is thin, has a flat surface, yellow-brownish colour, soft surface layer (easily pressed);

iv) Crust formed on slurry without amendment has a hard surface layer (not able to pressed), irregular surface, grey-black in colour.
Appendix 4.2: Maggot ‘invasion’ on slurry during the summer storage observation.

The cap size 3.5cm in diameter.
CHAPTER 5

5 Ammonia and greenhouse gas emissions from slurry storage in response to the addition of agriculture food chain by-products

Abstract

Animal excreta collected during the housing period is commonly stored as slurry in lagoons and above ground tanks, which are known to be a key source of concurrent emissions of ammonia (NH₃) and greenhouse gases (GHG), especially methane (CH₄). Slurry acidification is known to reduce production of these gases and their emission from slurry stores. Self-acidification can be promoted through the addition of a carbohydrate source. However, the use of agricultural by-product as the carbohydrate substrate to induce acidification has not been well explored. In this study, the sugar content of six agricultural food chain by-products (AFBP) was determined and screened for CH₄ inhibition activity. A regression analysis was used to determine the acidification rate of slurry following the addition of pineapple waste (PP), brewery spent grains (BSG) and milk (MK). The acidification rate of PP, BSG and MK were found to be at -0.009 pH mg CHO⁻¹; $R^2=0.962$, -0.0054 pH mg CHO⁻¹; $R^2=0.951$ and -0.0051 pH mg CHO⁻¹; $R^2=0.954$, respectively. The result revealed the ability of each substrate to acidify the slurry to the lowest pH level of 5.0, in which BSG’s biomass for acidification requirement is 7.1% FWt, followed by 14.2% and 22.8% FWt for PP and MK, respectively. In another study, a significant reduction ($P<0.01$) in CH₄ fluxes was observed, with BSG addition at 1% sugar content resulted in between 3 and 124 times lower CH₄ emissions than from untreated slurry during a 90 day storage period. The cumulative CH₄ emissions were 5.2 ±1.6 and 74.5 ±9.0 g CH₄ kg⁻¹ VS for the BSG treatment and the untreated control, with significant CH₄ inhibition (>90%) observed upon the addition of BSG. The BSG addition resulted in a low slurry pH due to lactic acid production, which subsequently led to significant lower net GHG CO₂ eq. emission (by 86%) following addition of the BSG, compared to the non-amended slurry.

Key words: Slurry storage, agriculture by-products, acidification, ammonia emission, greenhouse gases.
5.1 Introduction

Organic waste production from the agricultural food chain can be transformed into beneficial goods for the farmer, consumer (either for human or for animal) or the environment (Feedpedia, 2014; Siegmeier et al., 2015; Pleissner, 2016). However, the recycling or reuse of agricultural by-products is not as advanced as it could be, possibly because ‘out of sight is out of mind’. It is important to change this mindset and increase the awareness of the potential to divert and upcycle agricultural biomass into goods for economic and environmental benefit. Transformation of by-products was listed by Feedpedia, (2014), covering over 169 plants, plants by-products and 40 animal by-products to indicate the various utilities of these by-products. Some agriculture wastes are shown to be cost effective in reducing the existence of heavy metal ions in wastewater. Minimising the content of heavy metal is crucial in overcoming heavy metal pollution in water (Khan et al., 2004). With the absorption capability, biomass products such as sugarcane bagasse, sawdust, rice husk, coconut husk and oil palm shell may replace the activated carbon in the future. On the other hand, high fibrous agricultural wastes such as sugar bagasse, coconut oil fibre, coconut fibre and straw are useful as thermal insulators (Manohar, 2012). There are many more organic by-products being reused for various purposes, such as lactic acid (C₃H₆O₃), citric acid, other acid products, anti-dyeing agent (Upadhyay et al., 2011), biochar (Rehra et al., 2014), bio-cover (a biological waste used to cover land field to increase CH₄ oxidation) (Siva Shangari and Agamuthu, 2012) fertilizer or compost, and wood ash (Muchovej and Pacovsky, 1997). Yet, the effectiveness of agriculturally associated by-products as additives in mitigating greenhouse gas (GHG) and ammonia (NH₃) emissions is still unclear.

The use of natural additives with livestock slurries is not a new approach. Two studies were conducted to determine the effect of organic additives on NH₃ volatilisation, in which both studies used fibrous material produced by the agricultural industry. Luo et al. (2004) used fibrous material from bark to mitigate NH₃ emission. Bark waste at 20 and 40% (w/w) successfully reduced NH₃ emissions from cattle manure (in storage) by 80 and 85%, respectively, in comparison to soil, sawdust or wood shaving. Nykanen et al. (2010) also reported the use of milled wheat, potato starch and carbohydrate additives to reduce NH₃ emissions from stored pig slurry. However, a study by Clemens et al. (2002) on the addition of sugar beet waste to...
stored dairy slurry at 33 and 333 g L⁻¹ showed that neither concentration exhibited any potential to reduce GHG emissions. The inability to reduce GHG emissions is probably because the treatments did not result in slurry acidification.

In Malaysia, there are no reports on the use of organic by-products as additives or substrates for addition to livestock manures. The current focus of such by-products in Malaysia is only for animal feeding, due to the high cost of grain for livestock, especially for poultry and ruminant feeds (Zahari and Wong, 2009). Specifically, by-products for this purpose include palm kernel cake (PKC), rice straw, tapioca, sorghum, maize stalks and oil palm fronds (OPF) (Zahari and Wong, 2009). By-products use is largely restricted to the location of where they are produced and the cost of transporting the materials to where they could be used. Production focuses on certain areas around Malaysia, e.g. pineapple waste in Simpang Renggam, Johor (Malaysia), while cocoa waste is produced in Sabah, (east peninsular of Malaysia). Most by-products used for ruminant feeds are location specific, such as cocoa pod husk, coconut cake, bagasse, sugar cane tops, maize stover, cassava leaves, sweet potato leaves, groundnut cake, pineapple waste, coffee seed hulls and various others. The processing costs and use of the by-products for animal feed are most likely to increase if the by-products contain high moisture content because of complications during the storage and transportation phases. The use of by-products with high moisture content is limited, not to mention the additional drying expenses. With the exception of transportation costs, by-product usage could be promoted if they are targeted for alternative uses (other than animal feed). This is another challenge to recycle with the ‘3R’ (reduce, reuse and recycle) system approach. The ‘3R’ approach is the best way to manage the increasing biomass production in the agricultural industry.

There are very few reports of by-product use in slurry management, despite their high carbohydrate content and the potential to induce slurry acidification. Therefore, this chapter focuses on recycling agricultural food-chain-based by-products (AFBP) and reusing them in slurry storage as a potential strategy to reduce emissions of NH₃ and CH₄. It is hypothesised that slurry microbes rapidly utilise the simple carbohydrates available in the by-products. So, the aim of this chapter is to evaluate the AFBP capability as slurry additives towards induced acidification with regard to mitigating NH₃ loss and GHG emission.
5.2 Materials and methods

5.2.1 Agricultural food-chain by-products (AFBP)

Selection of AFBP in this chapter is based on their availability and production in Malaysia and United Kingdom (UK). Six (6) types of AFBP were selected for the study. The AFBP used in this experiment were; (i) brewery by-product known as brewery spent grains (BSG) from beer manufacturing, (ii) apple pulp (AP) a cider making by-product, (iii) pineapple peel (PP) from pineapple canning manufacturing, (iv) copra meal (CM) waste from the coconut milk extraction industry, (v) expired wheat flour (WF), and (vi) pasteurised milk (MK). Due to unavailability of the by-products during the experiment, the AFBP were prepared in the laboratory from their main ingredients purchased from nearby supermarket, except for BSG (known as Trafford Gold) that was obtained from the manufacturer Cargill Sweeteners (Manchester, UK). The preparation of the by-product is elaborated below:

I. Coconut by product (Copra meal - CM)

Copra meal (CM) was obtained by extracting coconut milk from desiccated coconut. A desiccated coconut was moistened with warm water and coconut milk was squeezed by hand through 400 µm metal sieves. Coconut milk was discarded and CM was stored at <4°C until further use.

II. Pineapple waste (PP)

Sweet pineapples at the ripening index of between 2 to 4 (Abu Bakar et al., 2013; Appendix 5.1) were bought from a local supermarket. Pineapple waste (PP) comprised pineapple outer skin (peel) and the core without the crown. About 0.5-1.0 cm thick of the outer pineapple skin was removed while the middle core was about 2-3 cm in diameter. The PP was then chopped into small pieces (0.2-0.5 cm) using a food processor (Cookworks, UK).

III. Apple pulp (AP)

Three kilograms of purchased Gala apples were rinsed with tap water and sliced into four. Apple seed, skin and stalk were not removed and processed in the fruit extractor (Breville Pro-Kitchen, Oldham, UK). The apple juice was separated and discarded from the pulp during the process. The amount of apple pulp gained after being processed was 1.1 kg (30.5%) on a fresh weight basis.
IV. Brewery spent-grains (BSG)

BSG as mentioned earlier was obtained from the manufacturer and stored <4°C until further use.

V. Wheat flour (WF) and milk (MK)

Wheat flour (WF) and MK were obtained from the local supermarket. These products were used with the assumption that their production cost is low in UK and they could be used for such purpose after their sell-by dates.

5.2.2 Carbohydrate (extractable sugar) extraction and analysis

The AFBP extractable sugar content was determined following the method mentioned by Chow and Landhäusser (2004). Briefly, 0.5 g of by-product was soaked in 5 mL of 80% ethanol (Sigma-Aldrich, Dorset, UK) and heated in a water bath at 85°C for 10 minutes before centrifuged (Eppendorf, USA) at 2500 rpm for 5 minutes. The sugar content was measured using the phenol-sulphuric acid method (Dubois, 1956) by microplate as described by (Masuko et al., 2005). Fifty (50) µL samples were mixed with 30 µL of 5% phenol and 150 µL sulphuric acid in 96 wells (microplate) and incubated at 80°C for 10 minutes. The sugar content was measured colorimetrically at 490 nm using Biotek Power Wave XS and further analysed by Gen 5 software Biotech (Instruments, Inc. USA).

5.2.3 Experimental Design

I. Effect of cattle slurry amended with various by-products on GHG emissions

All the AFBP and slurry for this experiment are characterised based on the procedures described earlier in previous chapters. The initial cattle slurry characteristics were 15.1 ±0.8 % DM FWt, 71.2 ±6.6 % VS DM⁻¹, 4.9% C FWt, 0.5% N FWt and pH 7.3. The high DM content in this experiment was due to slurry management practice (scraping), types of animal feed provided and collection point as it was collect at reception pit of the entry point. The experiment was carried out using all by-products listed earlier. The application rate to the slurry was based on 1% (w/w) addition based on the carbohydrate content of each AFBP. Each by-product (PP, AP, CM, BSG, WF and MK) was weighed and added to
100 g of fresh slurry, while the control (Ctrl) did not receive any additional treatment. The mixture was then diluted to 300 g by adding distilled water, which caused the extractable carbohydrate content to be diluted to 333.3 mg (0.3% w/w). The mixture was transferred into a 700 mL containers with easy lock airtight lids fitted with rubber butyl septa for gas sampling. There was four replicates for each treatment. Slurry pH and ORP were recorded on days 0, 1, 4 and 7. Samples of GHG gas were obtained from container headspace through the rubber butyl septa after the lids were attached during a 30 minute closed period. Headspace CH₄, CO₂ and N₂O concentrations were analysed using the Perkin Elmer Clarus 580 Gas Chromatograph.

II. Evaluating the minimal addition of by-product addition required to cause slurry acidification

A similar experiment was designed and conducted to the one above. Approximately 200 g slurry was incubated with addition of 0.05, 0.1, 0.5, 1.0 and 3.0% extractable sugar content of by-product (BSG, PP and MK). Slurry mixtures were incubated under cover at Henfaes Research Centre for 11 days. Initial DM and VS content were carried on 20 g subsamples, and the slurry pH and ORP were recorded daily. Prior to the sampling at the end of the observation period, the foam or floated material on the slurry surface was removed. The target slurry pH was pH 5.0, which was considered as the minimal requirement in acidification process through anaerobic fermentation to result in reduced NH₃ and CH₄ emissions.

III. Greenhouse gas mitigation by BSG addition

Fresh slurry (lower dry matter than previously used; 5.6% DM) was collected from the same farm and screened to remove coarse material such as uneaten hay. Brewery spent grains was added based on 1% (w/w) sugar content to 20 kg slurry in 30 L (High-density polyethylene) HDPE plastic drums (n=6). Ammonia loss and GHG emission were measured periodically during a 2 month storage period (from 27th April 2015) using the methods described in Chapter 4.
5.2.4 Statistical analysis

Slurry pH changes as a result of the AFBP additions were analysed by one-way ANNOVA. Later, the GHG fluxes from AFBP slurry were compared using the general linear model (GLM) by Minitab 17 statistical software, (Minitab Ltd. Coventry, UK). The Tukey’s post-hoc test was used to test for statistical significance ($P<0.05$).

The minimal concentrations required to induce the target acidification were determined by linear regression (Minitab 17) based on pH data obtained on day 11. The data represent the estimated maximal substrate amount needed to achieve slurry pH 5.0. The effect of BSG on NH3 and GHG emissions during the 90 day storage period was analysed using the student t-test, utilising the similar statistical software, Minitab 17.

5.3 Results

5.3.1 By-products sugar content and characterization.

The by-product properties and their extractable sugar contents are listed in Table 5.1. The highest sugar content was recorded in AP at 72.8 mg g$^{-1}$ FWt, followed by BSG at 58.1 mg g$^{-1}$ FWt. However, BSG showed at least 3 times lower sugar content compared to AP, based on a DM basis. Wheat flour (WF), MK and CM had the lowest sugar concentration between 15.5 to 17.5 mg g$^{-1}$ FWt. Of all the by-products, PP was most acidic, with a pH of 3.5 to 3.9 pH compared to AP and BSG which had a pH of 3.8 and 3.9, respectively. Pineapples waste (PP), AP and MK contained the highest moisture content with 86.4, 80.1 and 76.7%, respectively. Low moisture and sugar content indirectly caused slurry thickening prior to incubation and increased slurry DM percentage.
Table 5.1 Characteristics of the agricultural by-products used during the study.

<table>
<thead>
<tr>
<th>Agriculture food by-product types</th>
<th>pH</th>
<th>Electrical Conductivity (µS cm(^{-1}))</th>
<th>Dry matter (% FWt)</th>
<th>Volatile solids (% DM(^{-1}))</th>
<th>Extractable Sugar content (mg g(^{-1}) FWt)</th>
<th>Extractable sugar content (mg g(^{-1}) DM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pineapples by-product (PP)</td>
<td>3.5 ±0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1205.0 ±15.37&lt;sup&gt;e&lt;/sup&gt;</td>
<td>13.4 ±0.17&lt;sup&gt;c&lt;/sup&gt;</td>
<td>90.4 ±1.21&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>17.2 ±5.4</td>
<td>1.3</td>
</tr>
<tr>
<td>Apple by-product (AP)</td>
<td>3.8 ±0.08&lt;sup&gt;de&lt;/sup&gt;</td>
<td>628.3 ±8.01&lt;sup&gt;de&lt;/sup&gt;</td>
<td>19.1 ±0.13&lt;sup&gt;c&lt;/sup&gt;</td>
<td>93.8 ±0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>72.8 ±9.5</td>
<td>3.8</td>
</tr>
<tr>
<td>Copra meal (CM)</td>
<td>7.7 ±0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1490.3 ±20.24&lt;sup&gt;a&lt;/sup&gt;</td>
<td>44.6 ±0.36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>95.7 ±0.32&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>17.5 ±3.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Brewery spent grains (BSG)</td>
<td>3.9 ±0.03&lt;sup&gt;de&lt;/sup&gt;</td>
<td>5230.0 ±26.14&lt;sup&gt;d&lt;/sup&gt;</td>
<td>46.1 ±0.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>98.0 ±0.84&lt;sup&gt;d&lt;/sup&gt;</td>
<td>58.1 ±8.7</td>
<td>1.3</td>
</tr>
<tr>
<td>Wheat flour (WF)</td>
<td>6.2 ±0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>780.5 ±9.95&lt;sup&gt;c&lt;/sup&gt;</td>
<td>87.4 ±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>97.2 ±0.89&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>16.3 ±0.7</td>
<td>0.2</td>
</tr>
<tr>
<td>Milk (MK)</td>
<td>6.9 ±0.01&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5.0 ±0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.3 ±5.26&lt;sup&gt;c&lt;/sup&gt;</td>
<td>96.6 ±3.71&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>15.5 ±3.9</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Means with different letters within the same column are significantly different (\(P<0.01\)) Values represent means (\(n=3\)), standard error of the mean (±SEM).
5.3.2 Effect of cattle slurry amended with various by-products.

1. Observation on the slurry DM, pH and ORP.

Table 5.2 shows different DM, VS, pH and ORP values at the start and end of observation. There was no significant difference in the moisture loss (DM) for each treatment due to the drying effect, but each treatment showed different solid-liquid surface and physical observation (Appendix 5.2). By-products used in this experiment had different acidity levels with the exception of the copra meal (CM), which was neutral in nature (pH 7.66). Pineapple waste had the lowest pH (3.5), followed by AP, BSG, WF and MK (3.8, 3.9, 6.2 and 6.9), respectively. Their different acidities significantly changed ($P<0.01$) the slurry start-up pH upon mixing, with the exception of MK (Table 5.2).

The initial pH levels were recorded to be between pH 6.6 (BSG) and pH 7.7 (control slurry). The pH of cattle slurries receiving AFBP significantly ($P<0.05$) reduced during the incubation period (Figure 5.1). The pH of by-product amended slurries showed negative slopes over 7 day incubation, indicating production of organic acid during the anaerobic fermentation. Slurry had dramatically decreased on its pH in the first 3 days of incubation and showed slower rates of decrease from day 4 onwards. The pH in the AP gradually increased from day 3 to day 7 by 0.5 pH unit (Appendix 5.3). Slurries with WF amendment showed the lowest pH after 7 day storage period, followed by PP, BSG and MK. Copra meal reached the lowest pH on day 7 at pH 5.2 comparable to 3.9 and 4.6 of WF, BSG and MK, respectively.

Meanwhile, the potential of oxidation redox potential (ORP) during the slurry incubation with by-product substrates is shown in Figure 5.1b and Appendix 5.4. The opposite trend to pH was observed for ORP. Despite no significant effect ($P>0.05$) on the initial ORP level of the PP, AP, CM, BSG and WF treatments, the ORP level continued to increase until the end of the incubation period (day 7). However, AP showed an exception with a slight decrease from -233 mV to -284 mV. Highest ORP values were achieved on day 7 in the WF treatment, followed by PP, MK and BSG. They were recorded at +37 mV, -44 mV and -49 mV respectively, compared to the control (Ctrl) that was at -327 mV.
Table 5.2 Slurry characteristics at the start and end of the incubation period.

<table>
<thead>
<tr>
<th>Agri-food by-product types</th>
<th>Dry matter (% FWt)</th>
<th>Volatile solid (% DM⁻¹)</th>
<th>pH</th>
<th>Oxidation redox potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 0</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pineapples by-product (PP)</td>
<td>9.5 ±1.6&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>70.7 ±0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.7 ±0.04&lt;sup&gt;Ac&lt;/sup&gt;</td>
<td>-344 ±2.3&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Apple by-product (AP)</td>
<td>9.6 ±2.3&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>58.7 ±0.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>7.0±0.02&lt;sup&gt;Ab&lt;/sup&gt;</td>
<td>-359 ±12.7&lt;sup&gt;Abc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Copra Meal (CM)</td>
<td>14.3 ±0.1&lt;sup&gt;Ab&lt;/sup&gt;</td>
<td>85.2 ±0.2&lt;sup&gt;Ab&lt;/sup&gt;</td>
<td>7.1 ±0.05&lt;sup&gt;Ab&lt;/sup&gt;</td>
<td>-351 ±5.7&lt;sup&gt;Abc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Brewery spent grains (BSG)</td>
<td>7.3 ±0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>84.5 ±0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.6 ±0.05&lt;sup&gt;Ac&lt;/sup&gt;</td>
<td>-332 ±3.4&lt;sup&gt;Ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Wheat Flour (WF)</td>
<td>24.1 ±0.2&lt;sup&gt;Aa&lt;/sup&gt;</td>
<td>94.8 ±0.1&lt;sup&gt;Ab&lt;/sup&gt;</td>
<td>7.1±0.04&lt;sup&gt;Ab&lt;/sup&gt;</td>
<td>-306 ±3.4&lt;sup&gt;Aa&lt;/sup&gt;</td>
</tr>
<tr>
<td>Milk (MK)</td>
<td>7.7 ±0.3&lt;sup&gt;Ac&lt;/sup&gt;</td>
<td>78.4 ±0.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.1±0.01&lt;sup&gt;Ab&lt;/sup&gt;</td>
<td>-371 ±3.5&lt;sup&gt;Ac&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control (Ctrl)</td>
<td>5.7 ±0.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>71.2 ±0.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.3 ±0.02&lt;sup&gt;Ab&lt;/sup&gt;</td>
<td>-362 ±1.8&lt;sup&gt;Abc&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Day 7</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pineapples by-product (PP)</td>
<td>9.6 ±0.3&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>85.3 ±0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.2 ±0.02&lt;sup&gt;Be&lt;/sup&gt;</td>
<td>-44 ±30.9&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Apple by-product (AP)</td>
<td>6.8 ±0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>83.8 ±1.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.5 ±0.16&lt;sup&gt;Bb&lt;/sup&gt;</td>
<td>-284 ±10.6&lt;sup&gt;bd&lt;/sup&gt;</td>
</tr>
<tr>
<td>Copra Meal (CM)</td>
<td>17.2 ±0.2&lt;sup&gt;Bab&lt;/sup&gt;</td>
<td>91.1 ±0.5&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>5.2 ±0.04&lt;sup&gt;Bc&lt;/sup&gt;</td>
<td>-234 ±20.3&lt;sup&gt;Bcd&lt;/sup&gt;</td>
</tr>
<tr>
<td>Brewery spent grains (BSG)</td>
<td>9.6 ±0.3&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>82.6 ±0.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.6 ±0.09&lt;sup&gt;Be&lt;/sup&gt;</td>
<td>-143 ±37.4&lt;sup&gt;Bbc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Wheat Flour (WF)</td>
<td>29.6 ±0.2&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>94.3 ±0.0&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>3.9 ±0.04&lt;sup&gt;Bf&lt;/sup&gt;</td>
<td>37 ±37.4&lt;sup&gt;Ba&lt;/sup&gt;</td>
</tr>
<tr>
<td>Milk (MK)</td>
<td>9.1 ±0.1&lt;sup&gt;Bab&lt;/sup&gt;</td>
<td>83.5 ±0.2&lt;sup&gt;B&lt;/sup&gt;</td>
<td>4.6 ±0.02&lt;sup&gt;Bd&lt;/sup&gt;</td>
<td>-49 ±38.6&lt;sup&gt;Bab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control (Ctrl)</td>
<td>5.7 ±0.2&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>78.2 ±0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.0 ±0.04&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>-327 ±6.0&lt;sup&gt;Bd&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Means with different letters within the same column are significantly different (P<0.05) Values represent means (n=4), standard error of the mean (±SEM). Different capital superscript letters within the columns represent significantly different (P<0.05) between Day 0 and Day 7.
II. Greenhouse gas emission during slurry incubation

Figure 5.2 shows the GHG fluxes (CH$_4$, CO$_2$ and N$_2$O) during the 7 day observation period. There were no significant effects on CH$_4$ emission on ‘day-0’ immediately following the addition of the substrates, except for WF ($P<0.05$). However, CH$_4$ emission decreased in the following days except for the control slurry. The CH$_4$ fluxes from the amended slurries were significantly lower than the Ctrl ($P<0.05$), with exception of WF on day 4 and CM and MK on day 7. Highest CH$_4$ fluxes were observed from the Ctrl slurry at day 4, with 0.46 µg kg VS$^{-1}$ hr$^{-1}$. Methane flux was small and near to zero on day 7 for slurries amended with PP, BSG and WF. The CH$_4$ flux was significantly different ($P<0.05$) to those of Ctrl, but it showed no significant difference with the AFBP treatments.

Constant CO$_2$ fluxes from the slurries were observed during the incubation, except for WF and CM. Yet, the treated slurries had similar or higher CO$_2$ fluxes compared to Ctrl slurry. Hence, the observed cumulative emission was highest on WF, CM and PP treatments. Meanwhile, N$_2$O fluxes were very low or negligible during the experiment, despite a higher flux from the PP treatment.

Total GHG emissions were calculated based on the GHG fluxes for the first 4-day period and are summarized in Table 5.3. Data of the fluxes on day 7 were not included as the slurry treatments were affected by drying (loss of moisture). Methane and CO$_2$ emission represented the major contribution to the total GHG
(CO₂ eq.) emission, while the N₂O contributed a negligible amount. The total GHG CO₂ eq. was measured by using the GWP of the different gases, as guided by IPCC, (2013) (Myhre et al., 2013). The net GHG emission indicated that WF had the highest emission (2457.6 µg CO₂ eq. kg⁻¹ VS) followed by Ctrl, (715.0 µg CO₂ eq. kg⁻¹ VS). The percentage GHG CO₂ eq. reduction was calculated by comparing the emission from the treated slurry to the amount of GHG emission of the control (untreated) slurry. Highest GHG reductions were measured from the BSG and AP treatments, at 51.1% and 21.1% respectively. These large reductions were mainly caused by the effect on CH₄ emission, in which they represented 70.2% and 62.1% inhibition level compared to the untreated slurries.
Figure 5.2 Greenhouse gas emissions (CH$_4$, N$_2$O and CO$_2$) fluxes and cumulative emissions during the 7 day incubation from slurry amended with various agri-food chain by-products.
(a) CH$_4$ flux emission; (b) Cumulative CH$_4$ emission; (c) CO$_2$ flux emission; (d) Cumulative CO$_2$ emission; (e) N$_2$O flux emission; (f) Cumulative N$_2$O emission.

Greenhouse emissions after day 4 could be affected by moisture loss. PP, Pineapples waste; AP, Apple pulp; CM, Copra Meal; BSG, Brewery spent grains; WF, Wheat flour; MK, Milk; Ctrl, Control untreated. Values represent means (n=5), vertical bars indicate the standard error of the mean (±SEM).
Table 5.3 Cumulative GHG emissions (CO$_2$ eq.) based on the first 4 days of measurements.

<table>
<thead>
<tr>
<th>Agri-food by-product types</th>
<th>GHG emission (µg kg$^{-1}$ VS)</th>
<th>CH$_4$ Reduction (% compare to control)</th>
<th>Total GHG Reduction (% compare to control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CH$_4$</td>
<td>CO$_2$</td>
<td>N$_2$O</td>
</tr>
<tr>
<td>Pineapples by-product (PP)</td>
<td>10.3$^b$</td>
<td>308.3$^b$</td>
<td>0.164$^a$</td>
</tr>
<tr>
<td>Apple by-product (AP)</td>
<td>15.8$^b$</td>
<td>225.6$^b$</td>
<td>0.003$^c$</td>
</tr>
<tr>
<td>Copra Meal (CM)</td>
<td>17.2$^b$</td>
<td>379.3$^b$</td>
<td>0.004$^c$</td>
</tr>
<tr>
<td>Brewery spent grains (BSG)</td>
<td>8.2$^b$</td>
<td>177.5$^b$</td>
<td>0.003$^c$</td>
</tr>
<tr>
<td>Wheat Flour (WF)</td>
<td>40.9$^a$</td>
<td>1590.8$^a$</td>
<td>0.025$^b$</td>
</tr>
<tr>
<td>Milk (MK)</td>
<td>18.6$^b$</td>
<td>220.2$^b$</td>
<td>0.002$^c$</td>
</tr>
<tr>
<td>Control (Ctrl)</td>
<td>27.3$^b$</td>
<td>141.0$^b$</td>
<td>0.000$^c$</td>
</tr>
</tbody>
</table>

*GHG emissions were converted to CO$_2$ eq. using the GWP of the different gases, as guided by IPCC (2013) (Myhre et al., 2013). Total GHG emission was calculated based on day 0 to day 4 assuming that GHG flux on day 7 was adversely affected by drying.

5.3.3 Evaluating the minimum by-product application rate required to causes acidification

The effect of the different sugar concentrations in PP, BSG and MK treatments on slurry pH is shown in Figure 5.3. Significant ($P<0.05$) pH reduction and increment in ORP level were observed after 24 hours for all treatments. However, slurry with sugar concentration lower than 150 mg 100$^{-1}$ g slurry, showed reverse effect on its pH level after 72 hours of incubation. The data were strengthened by the decrease in ORP level. Fitted linear regression line (Figure 5.4) indicates acidification rate, showing PP had the fastest acidification rate at -0.009 pH mg CHO$^{-1}$ (R$^2$=0.962) compared to BSG (-0.0054 pH mg CHO$^{-1}$, R$^2$=0.951) and MK (-0.0051 pH mg CHO$^{-1}$, R$^2$=0.954). Linear regression was used to estimate the minimal substrate concentration needed to acidify the slurry to the target pH of pH 5.0. It was noticed that 244, 410 and 354 mg extractable sugar from PP, BSG and MK were required to acidify 100 g slurry during the 11 storage days, equivalent to 14.2, 7.1 and 22.8% FWt for PP, BSG, MK, respectively.
Figure 5.3 Effect of different concentrations of agricultural food chain by-products (AFBP) on slurry pH and ORP.

(a) Effect of PP by-product on slurries pH during the 11 day incubation; (b) Effect of PP by-product on slurry ORP during the 11 day incubation; (c) Effect of BSG on slurry pH during the 11 day incubation; (d) Effect of BSG on slurry ORP during the 11 day incubation; (e) Effect of MK on slurry pH during the 11 day observation; (f) Effect of MK on slurry ORP during the 11 day incubation.

PP, Pineapples waste; BSG, Brewery spent grains; MK, Milk. pH and ORP data on PP was stopped at day 3 due to high moisture loss and become unsuitable for reading. Values represent means (n=4), vertical bars indicate the standard error of the mean (±SEM). Note: Arrow (a, b) indicates measurements truncated due to drying effect.
Figure 5.4 Effect of different concentrations of agri-food chain by-products on slurry pH at the end of the 11 day incubation period. Values represent means (n=4), vertical bars indicate the standard error of the mean (±SEM).

5.3.4 Greenhouse gas mitigation by BSG addition

The results presented here (Table 5.4; Figure 5.5) are the comparison between BSG and Ctrl slurry during the initial and at the end of the storage period. The characteristics of BSG slurry at both the start and end of the storage period were significant (P<0.01), with higher DM, VS, ORP and NH₄-N, but lower in pH compared to Ctrl slurry. Meanwhile, the characteristics of the BS slurry at the end of the period were found to be higher in NH₄-N, total C, total N and lower in pH compared to Ctrl slurry.

As seen in Figure 5.5a, slurry temperature followed the ambient temperature, while the slurry pH and ORP changed significantly over time. The BSG pH that started at pH 6.0 immediately decreased to the lowest point at pH 3.9 on day 28, while the ORP, which started at -184.3 mV dramatically increased to -51.7 mV on the same day. However, during the following period there was a gradual increment in pH, while ORP decreased to the level as stated earlier (Table 5.4).
Table 5.4 Comparison of cattle slurry characteristics at the start and end of storage.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>BSG</th>
<th>Ctrl</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Start experiment</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry matter (% FWt)</td>
<td>9.9 ±0.21a</td>
<td>3.8 ±0.09b</td>
</tr>
<tr>
<td>Volatile solids (% DM⁻¹)</td>
<td>87.0 ±0.23a</td>
<td>75.9 ±0.31b</td>
</tr>
<tr>
<td>Oxidation redox potential (mV)</td>
<td>-184.3 ±3.6a</td>
<td>-241 ±5.59b</td>
</tr>
<tr>
<td>Ammonium-N (mg N kg⁻¹ FWt)</td>
<td>419.2 ±6.9a</td>
<td>372.8 ±12.1b</td>
</tr>
<tr>
<td>pH</td>
<td>6.0 ±0.08a</td>
<td>6.9 ±0.03b</td>
</tr>
<tr>
<td>Total C (g C kg⁻¹ FWt)</td>
<td>33.8 ±5.0a</td>
<td>11.0 ±0.5b</td>
</tr>
<tr>
<td>Total N (g N kg⁻¹ FWt)</td>
<td>3.9 ±0.4a</td>
<td>2.4 ±0.4b</td>
</tr>
<tr>
<td><strong>End of storage (90 days)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry matter (% FWt)</td>
<td>11.5 ±0.61a</td>
<td>3.8 ±0.38b</td>
</tr>
<tr>
<td>Volatile solids (% DM⁻¹)</td>
<td>83.8 ±0.90a</td>
<td>68.2 ±1.70b</td>
</tr>
<tr>
<td>Oxidation redox potential (mV)</td>
<td>-195.2 ±11.4a</td>
<td>-249.8 ±8.3b</td>
</tr>
<tr>
<td>Ammonium-N (mg N kg⁻¹ FWt)</td>
<td>1025.2 ±76.8a</td>
<td>331.2 ±9.5b</td>
</tr>
<tr>
<td>pH</td>
<td>6.2 ±0.17</td>
<td>7.2 ±0.08</td>
</tr>
<tr>
<td>Total C (g C kg⁻¹ FWt)</td>
<td>59.5 ±3.9a</td>
<td>11.7 ±0.7b</td>
</tr>
<tr>
<td>Total N (g N kg⁻¹ FWt)</td>
<td>6.4 ±0.2a</td>
<td>3.1 ±0.2b</td>
</tr>
</tbody>
</table>

Means with different letters within the same row are significantly different (P<0.05) Values represent means (n=6), standard error of the mean (±SEM).
Figure 5.5 The slurry temperature, pH and ORP levels during the 90 day storage period.
(a) Slurry and ambient air temperature recorded during the storing period; (b) Effect on slurry pH following BSG amendment; (c) Slurry treatment ORP level. Values represent means ($n=6$), vertical bars indicate the standard error of the mean ($\pm$SEM).
I. Ammonia emission

The relative ammonia fluxes and cumulative emission from both BSG and Ctrl slurry are illustrated in Figure 5.6. Overall, Ctrl slurry showed higher NH$_3$ fluxes than BSG (no significant different; $P>0.05$). The NH$_3$ fluxes trend for both slurries decreased from day 1 to the end of day 90, with a slight increment on day 8, 15 and 30. The relative cumulative emissions during the 90 days storage period from BSG and Ctrl were 18.5 ±1.70 and 18.9 ±4.07 mg NH$_3$ m$^{-2}$, respectively (Figure 5.6b).

![Figure 5.6](image)

Figure 5.6 Slurry ammonia volatilisation from BSG and untreated slurry during the 90 day storage period. (a) Ammonia fluxes; (b) Cumulative ammonia emission. Values represent means ($n=6$), vertical bars indicate the standard error of the mean (±SEM).
II. Lactic acid production

The production of $C_3H_6O_3$ production was detected immediately in BSG (day 0; Table 5.5) but was absent in the Ctrl. The acid levels continued to increase to the highest concentration on day 15, and then decreased on the following day before being depleted.

Table 5.5 Lactic acid concentration in BSG amended slurry during the incubation period.

<table>
<thead>
<tr>
<th>Slurry lactic acid content (g C$_3$H$_6$O$_3$ L$^{-1}$ FWt)</th>
<th>Day</th>
<th>Mean ±SEM</th>
<th>Ctrl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1.77 ±0.08</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.87 ±0.15</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.83 ±0.15</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2.20 ±0.03</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>4.68 ±0.20</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>8.50 ±1.22</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>7.48 ±0.33</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>8.04 ±0.30</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>2.93 ±0.44</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>0.02 ±0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

\(n=6\)

Values represent means \((n=6)\), vertical bars indicate the standard error of the mean \(±SEM\).

III. GHG emission

BSG addition resulted in significant lower CH$_4$ fluxes than in Ctrl slurry \((P<0.01)\) during the entire storage period (Figure 5.7). The difference in the fluxes between the two slurries was comparatively large by about 3 to 124 times during the incubation. The calculated relative cumulative emission was 5.2 ±1.6 and 74.5 ±9.0 g CH$_4$ kg$^{-1}$ VS (BSG and Ctrl), equivalent to 92.9% lower CH$_4$ emission for slurry amended with BSG. Conversely, the slurry treated with BSG indicated higher CO$_2$ fluxes during the same incubation period. Appearance of the CO$_2$ fluxes was only significant during the first 15 days \((P<0.05)\) and decreased on the following days despite the highest CO$_2$ flux was recorded on day 90 from the BSG added slurry at 234.8 ±60.9 mg CO$_2$ kg$^{-1}$ VS hr$^{-1}$. Cumulative CO$_2$ emissions from the BSG and Ctrl's were 205.7 ±32.0 and 104.7 ±7.5 g CO$_2$ kg$^{-1}$ VS, respectively.
In contrast, N$_2$O fluxes were very small and almost negligible (<11.1 µg N$_2$O kg$^{-1}$ VS hr$^{-1}$) in both the BSG and Ctrl slurries. However, there were significant ($P<0.05$) N$_2$O fluxes emitted by the Ctrl slurry on day 90 amount of 62.7 ±17.9 µg N$_2$O kg$^{-1}$ VS hr$^{-1}$, and resulted in a cumulative N$_2$O emission at 30.6 ±7.8 mg N$_2$O kg$^{-1}$ VS. This is in comparison to BSG at 1.1 ±0.3 mg N$_2$O kg$^{-1}$ VS. Looking into the net GHG emission, BSG GHG emission was equivalent to 383.6 g GHG CO$_2$ eq. kg$^{-1}$ VS, 86% lower than those of Ctrl (2645.5 g GHG CO$_2$ eq. kg$^{-1}$ VS).
Figure 5.7 Dynamics of GHG fluxes and cumulative emissions from BSG amended and control slurry during the 90 day storage period.

(a) CH₄ flux; (b) CH₄ cumulative; (c) CO₂ flux; (d) CO₂ cumulative; (e) N₂O flux; (f) N₂O cumulative. Values represent means (n=6), vertical bars indicate the standard error of the mean (±SEM). Note different y-axis scales.
5.4 Discussion

5.4.1 Agricultural food by-products and its sugar contents

Our aim in this chapter was to explore the potential of available, yet unwanted, by-products as additives to reduce GHG and NH₃ emissions during slurry storage. The use of these by-products can enable farmers to enhance their income through bio-reduction and bio-refinery (Chaudhary et al., 2012). Here, we focussed on by-products based on their availability (in the UK and Malaysia), although some substrates are only available in certain region or country. In this case, WF, MK and AP may not be available in large quantities in Malaysia, while CM is not produced in UK. The utilisation of recycled AFBP as value-added substrates during slurry storage could help to reduce GHG and NH₃ emissions.

In Malaysia, certain districts or areas are known to produce pineapple and contributes to pineapple canning industry. It was estimated that the pineapple canning industry produced 17165 t, in 2011 (Lun et al., 2014; Pyar et al., 2014). According to Siti Roha et al. (2013), PP waste represents 35% of the fruits weight. Therefore, there is high chance of obtaining the by-product to be used for this purpose. Meanwhile, brewing waste from brewing industry is only located in Kuala Lumpur, Malaysia and it is not well utilised. Only 15% of BSG were recycled as ruminant feed (Siva Shangari and Agamuthu, 2012). While their use in poultry, pig and fisheries were in a small percentage, the waste was discarded to landfill. Compared to UK, brewers produced over 250 million tonnes of BSG every year and the waste is recycled mainly as cattle feed supplement (Ben-Hamed et al., 2011; Mussatto, 2014). Copra meal waste was produced from coconut pressed in coconut milk industry. It is produced in certain regions in Malaysia, but is also produced in some other tropical countries, such as Indonesia. To date, no published data were recorded on the recycling of CM in Malaysia. However, it is known that CM is recycled as part of ruminant feeds.

The total extractable sugar content in this experiment was used to determine the presence of glucose, fructose and galactose in total sugar (glucose equivalent). The employed method was described by Chow and Landhäusser, (2004) as a simple calorimetric method based on phenol-sulphuric acid reaction. This approach is much faster and cheaper than HPLC, GC and enzymatic methods. Although HPLC or GC methods can specifically determine the actual sugar type (glucose, fructose, etc.), this study only concerned on the total sugar availability in the by-products, especially in plant based substrates that are often interfered by chloroform extraction methods.
(Chow and Landhäusser, 2004). Our finding indicated the highest sugar content was from AP followed by BSG. However, the sugar content in fruit waste is also affected by the maturity index and/or ripening index (Ackermann et al., 1992; Nadzirah et al., 2013). Moreover, low extractable sugar contents and high DM in AFBP reflected the slurries DM content (Table 5.1). These factors caused slurry thickening and indirectly became economically inefficient in self-acidification during slurry storage.

5.4.2 By-products effect on GHG emission

Measured mean GHG emission from slurries amended with various by-products indicated that the main gases contributing to total GHG (CO$_2$ eq.) were CH$_4$ and CO$_2$. The N$_2$O contribution was very small or negligible. Except for WF, other by-product substrates effectively reduced CH$_4$ emission by between 32-70% compared to the untreated (Ctrl) slurry (Table 5.3). The addition of WF immediately increased the CH$_4$ fluxes and this is similar to the observation by Clemens et al., (2006). Meanwhile, the other AFBP treated slurry showed that longer storage period caused the CH$_4$ fluxes to decrease with any increment in the acidity level (Appendix 5.3).

In this context, the GHG total emission and the calculated inhibition percentage may not represent field-scale conditions as the study was conducted at a small scale (300 mL), covering short observation periods and were affected by moisture loss during the day. However, the use of AFBP in this study was proven effective in enhancing organic acid production, subsequently reducing the slurry pH. Slurry acidification is known to be effective at inhibiting CH$_4$ emission by 25 to 96%, depending on the types of acid used (Berg et al., 2006b; Petersen et al., 2012; Wang et al., 2014). Such a finding is believed to be related to the available sugar and DM content from each substrate. Consideration must be taken when using low sugar substrates with high percentage DM as this is likely to cause thickening of the slurry, thus extra energy may be needed for mixing or pumping when emptying the slurry lagoon. Lowering slurry pH by appropriate acid types had been discussed earlier (Chapter 4) and have been practiced by Danish farmers to retain N (reduce NH$_3$ loss) in the form of NH$_4^+$ (Kai et al., 2008).

5.4.3 Minimal rate of amendment needed to acidify slurry

Earlier finding indicated substantial reduction in the GHG emission resulted from acidification caused by the added substrate. This effect was not sustained for the
addition of substrate at low concentrations (Figure 5.3a, c, e), which showed a gradual increase in slurry pH starting on day 4 onwards. The possible explanation of the increasing pH is the amount of acid produced was utilized by the microbes and/or the depletion of supplied sugar (Patni and Jui, 1985). In addition, organic acids may also be lost through volatilisation, a process that is influenced by gas ebullition, higher wind flow or aeration (Cooper and Cornforth, 1978). The calculated amount between 7.1 and 22.8% DM FWt (PP, BSG and MK) is required to acidify the slurry to pH 5. In addition, the BSG biomass amount used was $\frac{1}{3}$ proportion of MK, while PP amount was about $\frac{1}{2}$ of MK biomass. High sugar concentration in substrate will result in lower pH level caused by the organic acid production. Hence, the pH remained lower for longer period where in this study the increment was not observed until day 9 for BSG amended slurry. As a matter of fact, acid-forced acidification by either HCl, HNO$_3$ or H$_2$SO$_4$ has shown identical results on slurry pH dynamics during a long storage period (Petersen et al., 2012). Thus, re-acidification was required to ensure the pH was maintained below the set point. In this case, further substrate addition is required. To avoid such requirement, further processing steps can be practised such as immediate slurry soil application, solid-liquid separation, etc. (Amon et al., 2006; Masse et al., 2008; Fangueiro et al., 2010a; Dinuccio et al., 2011).

5.4.4 Greenhouse gas mitigation by BSG addition

The current study indicates possible mitigation practices using AFBP addition despite the increasing slurry VS content, which is, contradict to Clemens et al. (2006) and recommendation. Adversely, higher VS with sufficient fermentable carbohydrate content induced acidification through anaerobic fermentation. Surprisingly, the effect was observable immediately after the BSG addition with the production of C$_3$H$_6$O$_3$, pH level and ORP emission (Table 5.5; Figure 5.5b, c). Noticeably, the pH level declined further to 3.9 on day 30 as higher C$_3$H$_6$O$_3$ was produced. The temporal C$_3$H$_6$O$_3$ change justified the lower pH level of the slurry. The C$_3$H$_6$O$_3$ content was between 8.0 and 8.5 g C$_3$H$_6$O$_3$ L$^{-1}$ FWt on day 15 and 30 (pH 3.9 and 4.13). However, the C$_3$H$_6$O$_3$ content might not represent the real (average) content as slurries (on the vessel) were not mixed during sampling as to avoid disruption on the experiment. The decreased in C$_3$H$_6$O$_3$ content between day 60 and 90 explained the increased of pH. Similarly, the potential redox demonstrated negative relation with the C$_3$H$_6$O$_3$ content.
In this study, induced acidification through BSG addition did not significantly inhibit the NH$_3$ loss. However, the acidification did cause lower average NH$_3$ loss, in which the total NH$_3$ emission in BSG added substrate was 0.4 mg NH$_3$ m$^{-2}$ lower than Ctrl at 18.9 ±4.07 mg NH$_3$ m$^{-2}$. The observed large variability and insignificant NH$_3$ inhibition were either due to the two different vessels of slurries being used, or the saturation caused by the acid trap. Ammonia diffusion to the atmosphere increases with the decrease of the solubility of equilibrium NH$_3$ as the temperature increases (Sommer and Feilberg, 2013). Higher NH$_3$ fluxes were observed on day 4 to 21 with the increase of ambient and slurry temperature. However, if the slurry is stored in constant temperature, then fluxes tend to decrease as the crust starts to develop unless there is significant disturbances such as slurry mixing or maggot infestation. Daily NH$_3$ fluxes from the Ctrl slurry later decreased as the crust started to form on day 60 and 90. In comparison, BSG resulted in higher NH$_3$ emission, as no crust formed. In addition, ‘rat tailed maggot’ were found in BSG slurry during the storage period, possibly deteriorated the NH$_3$ air–water equilibrium (Sommer and Feilberg, 2013) and disturbed the surface crust formation. Crust formation is a significant low cost barrier for abatement in NH$_3$ (Smith et al., 2007) and could be facilitated by straw, sawdust, wood chipped, leca or artificial cover (Portejoie et al., 2003; Luo et al., 2004; Mieselbrook et al., 2005a; Balsari et al., 2006). Based on the previous study (Chapter 4), the current technique (induced acidification) could possibly mitigate the NH$_3$ emission between 56 to 76%, or higher (chemical acidification) between the range of 81 and 95% (Petersen et al., 2013a; Wang et al., 2014).

Upon BSG addition, pH was immediately decreased so as the CH$_4$ fluxes caused by production of C$_3$H$_6$O$_3$ through the anaerobic fermentation. Methane flux indicates methanogenesis process and showed increased over time on Ctrl treatment (Figure 5.7), but decreased on BSG. The BSG CH$_4$ fluxes was recorded lower by 74 to 99% during the storage period and remained between 90.7% and 88.6% lower at day 60 and 90 although the slurry pH increasing gradually at pH 5.0 and 6.2. This lower flux was related to the previously inhibited methanogen by acidic condition thus requires additional lag phase before its CH$_4$ emission increased. This is an advantage of BSG as no additional substrate may be needed until then. As a result, total BSG CH$_4$ emission was surprisingly inhibited by 93% (5.2 g CH$_4$ kg$^{-1}$ VS) compared to untreated (Ctrl) amount of 74.5 g CH$_4$ kg$^{-1}$ VS during the entire storage period.
In the meantime, lower CH$_4$ fluxes in BSG are replaced by higher CO$_2$ fluxes and the total emission was almost twice higher (205.7 ±32.0 g CO$_2$ kg$^{-1}$ VS). The CO$_2$ emissions from BSG were highest (Figure 5.7c, d) between day 60 and 90 as a result of elevated pH level within the period. Meanwhile, the potent gas, N$_2$O, was the least emitted gas from the slurry. Significant fluxes were recorded from Ctrl as the crust was developed and dried, creating mini pores which were excellent microenvironments for nitrification and denitrification (Rodhe et al., 2009b). Slurry N$_2$O emission is considered as negligible with the absence of the crust (Sommer et al., 2009). The crust formed in Ctrl demonstrated the cumulative N$_2$O emission at 30.6 ±7.8 mg N$_2$O kg$^{-1}$ VS. This cumulative emission was as of equal importance to the GHG CO$_2$ eq. contribution.

The net GHG emission from BSG was clearly reduced by 86% or nearly 6.9 times reduction compared to Ctrl during the same period. Looking into the total Ctrl CH$_4$ emission, it represented 95.6% of total GHG CO$_2$ eq., indicating the major contribution in the GHG emission during the storage. Methanogenesis was inhibited immediately as pH dropped upon BSG addition thus CH$_4$ was greatly inhibited. The mitigation was achieved due to the use of fresh slurry as methanogen yet to adapt or establish compared in aged slurry (Sommer et al., 2007; Petersen et al., 2012) where methanogenesis was retarded. It is important to suppress the methanogens immediately and before they enter the log phase of multiplication. In addition, the absence of a crust in BSG was likely to help lower the net GHG CO$_2$ eq. emission. The data suggested that the method showed relatively similar acidification result as those by H$_2$SO$_4$ where it achieved 87% of CH$_4$ reduction (Petersen et al., 2012). However, direct comparison on both studies is inappropriate due to the different experimental designs and perimeters. Reduction in GHG emission was observed in BSG and obviously indicated the valuable approach in mitigation GHG, especially if compared to the typical non-disturbed slurry in storing lagoon. This approach is equally similar to the glucose-induced acidification studied in Chapter 4.
5.5 Conclusion

Biodegradation of cattle slurry with AFBP during storage period led to decreased CH$_4$ emission, mainly due to the induced acidification by the available sugar during anaerobic fermentation. Amongst the AFBP substrates being used, BSG indicated the highest CH$_4$ inhibition by 70.2% and GHG CO$_2$ eq. by 57.4% in this controlled laboratory incubation. Furthermore, the BSG amount needed to induce the acidification process and achieve the targeted pH 5.0 during slurry storage is proportional to 30% and 50% than PP and MK. In the final analysis, BSG addition had a positive inhibition on CH$_4$ and net GHG CO$_2$ eq. emission by 14% and 86% reduction over control during the 90 day storage period. GHG inhibitions were influenced by lower slurry pH caused by the production of lactic acid. The use of AFBP such as BSG requires deeper research on its full life cycle analysis at the farm scale and in terms of the cost effectiveness of the strategy. In addition, after storage management such as slurry soil application also holds the potential for further exploration.
5.6 Appendix

Appendix 5.1: Pineapples ripening index.

Appendix 5.2: Slurry observation (top view) after 7 days of incubation (Slurry amended with various concentration by-product).

Slurry observation after 7 days incubated in an open environment without disturbance.

i) Slurry amended with pineapples by-product 333 mg glucose content

ii) Slurry amended with pineapples by-product 500 mg glucose content (slurry become dry and no liquid observed)

iii) Slurry amended with brewery by-product 333 mg glucose content

iv) Slurry amended with brewery by-product 500 mg glucose content

v) Flocculation observed in slurry with milk with 500 mg glucose content
Appendix 5.3: Observation on slurry pH amended with various agri-food by products during the 7 day incubation period.

<table>
<thead>
<tr>
<th>Agri-food by-product types</th>
<th>Slurry acidity level (pH)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
</tr>
<tr>
<td>Pineapples by-product (PP)</td>
<td>6.7 ±0.04c</td>
</tr>
<tr>
<td>Apple by-product (AP)</td>
<td>7.0 ±0.02b</td>
</tr>
<tr>
<td>Copra Meal (CM)</td>
<td>7.1 ±0.05b</td>
</tr>
<tr>
<td>Brewery spent grains (BSG)</td>
<td>6.6 ±0.05c</td>
</tr>
<tr>
<td>Wheat Flour (WF)</td>
<td>7.1 ±0.04b</td>
</tr>
<tr>
<td>Milk (MK)</td>
<td>7.1 ±0.01ab</td>
</tr>
<tr>
<td>Control (Ctrl)</td>
<td>7.3 ±0.02a</td>
</tr>
</tbody>
</table>

Means within the same column with different letters are significantly different (P<0.05). Data represent mean value ±SEM, n=4.
Appendix 5.4: The effect of agri-food by-products on slurry ORP values during the 7 day incubation period.

<table>
<thead>
<tr>
<th>Agri-food by-product types</th>
<th>Slurry oxidation redox potential level (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
</tr>
<tr>
<td>Pineapples by-product (PP)</td>
<td>-344 ±2.3&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Apple by-product (AP)</td>
<td>-359 ±12.7&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Copra Meal (CM)</td>
<td>-351 ±5.7&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Brewery spent grains (BSG)</td>
<td>-332 ±3.4&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Wheat Flour (WF)</td>
<td>-306 ±3.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Milk (MK)</td>
<td>-371 ±3.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control (Ctrl)</td>
<td>-362 ±1.8&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Means within the same column with different letters are significantly different (P<0.05). Data represent mean value ±SEM, n=4.
CHAPTER 6

6 Slurry crust: Methane sink or nitrous oxide source?

Abstract

Slurry crusts form naturally on slurry surfaces and act as a primary barrier and sink of methane (CH$_4$). The methane oxidising bacteria (MOB) present in the crust metabolizes CH$_4$ as their source of carbon and energy. There is no study evaluating the effect of crust form (intact vs. homogenized) on the CH$_4$ oxidation rate. The oxidation rate is affected by environmental conditions, e.g. temperature. Our interest is look into the different oxidation rate of intact and homogenized crust at different temperatures. Also the effect of bio-augmentation on the CH$_4$ oxidation by applying activated effective microorganism on the crust surface area. There was no significant difference in CH$_4$ oxidation rate of crust form (intact vs homogenize) ($P$>0.05). The CH$_4$ emission from crust was higher than the CH$_4$ consumption by methane oxidising bacteria (MOB). Nitrification and denitrification produced N$_2$O fluxes greater than the CO$_2$ equivalent of the CH$_4$ sink resulting in net greenhouse gas (GHG) emissions of between 8-480 µg g$^{-1}$ DM hr$^{-1}$. The N$_2$O emission rate of effective microorganism treated and non-treated crust was not significantly different ($P$>0.05). It is important to take account of the net GHG emission from slurry crust when reporting the effects of potential strategies to reduce CH$_4$ oxidation rates from slurry stores.

Key words: methane oxidation, nitrous oxide, effective microorganism, crust, nitrification, denitrification.
6.1 Introduction

Methane oxidation is an important aspect in reducing the methane (CH$_4$) concentration in the atmosphere (Petersen and Ambus, 2006; Duan, 2012). Although most (90%) CH$_4$ oxidation occurs in the troposphere (Kirschke et al., 2013), CH$_4$ oxidation by methanotrophic bacteria or MOB by soil (Hanson and Hanson, 1996) and slurry crusts (Petersen et al., 2005; Duan, 2012; Duan et al., 2013) could also be considered important, as we have the ability to manage these CH$_4$ sinks. Methane reduction from livestock farming is possible by the CH$_4$ oxidation from slurry storage by the slurry crust that develops after long storage of undisturbed slurry. This would help to achieve IPCC's, (1995) aim to reduce global GHG emission by 25-40% by the year 2020 compared to year 1990.

The slurry crust is defined as a fibrous layer on slurry the surface, which develops during slurry storage (Pain and Menzi, 2011). Crusts form on cattle slurry by natural processes and can be promoted by adding chopped straw or fibrous material (Sommer and Husted, 1995; Pain and Menzi, 2011; Aguerre et al., 2012). Slurry crusts form due to solids in the slurry being carried to the slurry surface by gas bubbles produced by microbes during microbial degradation of the organic matter (Misselbrook et al., 2005a). Crust formation can be seen as early as 7-10 days after slurry has entered the storage lagoon (Misselbrook et al., 2005a; Wood et al., 2012; Duan et al., 2013) or as late as 30 days depending on the dry matter (DM) content (Misselbrook et al., 2005a). Slurry DM is an important factor in crust formation with higher DM slurries forming faster and with thicker crusts (Misselbrook et al., 2005a). Crusts do not form when slurry DM is <2%, e.g. pig slurries. The DM and particle content (usually <2%) in pig slurry usually settles quickly (Smith et al., 2007). Thus, animal diet and bedding greatly influence crust formation, while environmental factors (temperature, wind speed, rainfall) influence the surface appearance (Misselbrook et al., 2005a). Crusts have been described as having a pH between neutral to mild alkali (Petersen et al., 2005; Duan, 2012) with a dark colour, hard surface and may have an irregular surface. However, crust thickness is affected by higher volume: surface area of the slurry store (Smith et al., 2007).

Methane oxidation by the crust occurs when MOB, specialized methylotrophic prokaryotes establish in the crust and utilize CH$_4$ as a sole carbon and energy source (Hanson and Hanson, 1996). These microbes are either type I or II MOB characterized by defining their ability to produce methane monooxygenases (MMO), an enzyme that
catalyses CH$_4$ oxidation to methanol (CH$_3$OH). The CH$_3$OH produced is further oxidized to formaldehyde (HCHO) by methanol dehydrogenase through either the Rump or the Serine pathway (Hanson and Hanson, 1996).

Methane oxidation by slurry crusts offers a potentially important sink for the CH$_4$ generated by the bulk of the liquid slurry beneath it, but may increase the N$_2$O emission due to nitrification of NH$_4^+$ and subsequent denitrification of NO$_3^-$ in the crust microenvironment (Hansen et al., 2009). Meanwhile CH$_4$ oxidation studies, using bio-cover in landfills, have indicated CH$_4$ oxidation was optimal at 40°C (Siva Shangari and Agamuthu, 2012). Yet no studies have reported the optimum temperature for CH$_4$ oxidation by slurry crusts.

The study in this chapter addresses (1) CH$_4$ oxidation rates by slurry crusts at different forms. Two different forms of crust were used to compare CH$_4$ oxidation rates (intact crust and homogenised/crushed crust). During recent studies, (Petersen and Miller, 2006, Petersen et al., 2005, Duan et al., 2013; Duan, and Yun-feng, 2012), CH$_4$ oxidation rates by slurry crusts were explored using crushed and homogenized crust material, which may not be representative of the actual oxidation rate. It is hypothesized that the actual intact slurry crust will result in lower CH$_4$ oxidation rates than the crushed-homogenized crust, because the crust surface has a capillary action for O$_2$ to diffuse deeper, creating anoxic condition at the base of the crust. Similarly, the CH$_4$ concentration will be higher below the crust compared to the surface thus oxidation rates may be higher at this level. (2) The potential of activated Actiferm effective microorganism (AFEM) to enhance CH$_4$ oxidation in slurry crusts. The effect of effective microorganism (EM) application on soil and wastewater are widely known but their effectiveness on slurry crust processes is unknown. As we know, EM contains a heterogeneous mixtures of microbial species, which by this application may result in shifts of the microbial community within the crust matrix. Although in this chapter, we focussed on the sink of CH$_4$ through oxidation, we also explore net GHG fluxes by quantifying N$_2$O fluxes from the crust during the observation periods, and expressing fluxes as carbon dioxide equivalents (CO$_2$ eq.).
6.2 Methodology

6.2.1 Study 1: Methane oxidation of intact and homogenized crusts at different incubation temperature

I. Slurry crust

The impact of different incubation temperatures and crust form on CH₄ oxidation and net GHG emission was investigated at the laboratory scale. The crust was obtained from the crust formed in the 120 days untreated (Ctrl) stored slurry in Chapter 4. Crust samples from each container represent an individual replicate, with four replicates (n=4) per treatment. Crusts from each container were removed, turned upside down and any loose fibrous material, maggots and wet slurry was scraped away from the crust leaving the crust thickness about 1.5-2.3 cm. Soil bulk density rings (5 cm diameter) were used to cut and obtain 8-10 pieces of intact crusts for the CH₄ oxidation study. The remaining crust material from each container were crushed and cut into small size between 0.5-1.0 cm using a knife and then sieved through a 5.6 mm metal mesh sieve. Crusts were kept in a cold room (4°C) until further use.

Determination of crust pH and electrical conductivity (EC) was carried by suspending 3 grams of crushed crusts into distilled H₂O in 1:5 mL ratio in 50 mL centrifuge tubes. The pH, EC, dry matter (DM), volatile solid (VS) extractable nitrate-N (NO₃-N), ammonium-N (NH₄-N), total carbon (C) and nitrogen (N) were measured using methods described in Chapter 3. The bulk density of crust was measured by the density of weight of crust by the volume of the bulk density mould used.

II. Methane oxidation

Methane oxidation incubations took place over a 48 hr period under controlled conditions in the laboratory. The intact crust (5 cm diameter disc) was weighed and placed upside down in a 700 mL container with an airtight lid. In order to evaluate comparison on CH₄ oxidation from crushed crust (homogenised), similar observation was carried out using homogenised crust at equal weight to the intact crust disc. Pre-incubation with 4% CH₄ for 24-48 hrs at selected incubation temperature [T4, T10, T20 and T30 (°C)] was carried out by injecting 5.1 mL of 4% CH₄ (final CH₄ concentration about 280 µL L⁻¹) through rubber butyl septa on
the lid to mimic the CH$_4$ concentration underneath slurry crust. This experiment was carried out with four replicates ($n=4$) of each crust form (intact and homogenised) with additional empty containers ($n=4$) as negative control at each incubation temperature. Following pre-incubation, the lids were opened to circulate air for 15 minutes before the actual experiments took place. After this time, lids were replaced before injected with another 5.1 mL of 4% CH$_4$. At each sampling point, a 20 mL gas sample was withdrawn from the headspace. Gas sampling was carried out at time 0, 6, 24, 48 ($t_0$, $t_6$, $t_{24}$, $t_{48}$) hours and stored in pre-evacuated 20 mL glass vials until analysed. Gas samples were analysed by Rothamsted Research, North Wyke using a Perkin Elmer Clarus 580 Gas Chromatograph linked to a Perkin Elmer TurboMatrix 110 auto sampler. The GC was equipped with identical megabore capillary Q PLOT columns runs at 50°C and fitted with flame ionization detector (FID) with methanizer detector at 350°C for detection of both CH$_4$ and CO$_2$. While N$_2$O was detected via an electron capture detector (ECD) 375°C with nitrogen (N) gas (O$_2$ free) as carrier gas.

6.2.2 Study 2: Methane oxidation activity on crust amended with EM

This laboratory experiment explored the potential for EM to enhance CH$_4$ oxidation and reduced net GHG emissions from slurry crusts.

I. Slurry crust

Slurry crust was taken from a stored beef slurry that had been stored for 7-8 months in 90 litres barrels (48 cm diameter). The barrels ($n=3$) were setup during winter (December 2013) and placed under a shed at the Henfaes Research Centre, Bangor University. Activated EM (Actiferm EM® later termed as AFEM provided by Effective Micro-organism® Limited, Exeter UK) were sprayed onto the crust surface area at ± 28 mL m$^{-2}$ starting in April 2014, twice weekly for subsequent 8 weeks, while control untreated crust only receiving similar amount of distilled water (H$_2$O). After the 7-8 month storage period, the slurry crust was carefully removed from the barrels and processed as described above in 6.2.1 to produce crushed, homogenized crust only. Intact discs of crust were not taken because of the irregular crust formation that had occurred in the barrels, resulting in variable crust thickness and variable crust wetness.
II. Methane oxidation

Methane oxidation from both treatments was quantified using the methods described above in 6.2.1. The observation focussed on three different temperatures (4, 22 and 30°C). Briefly, 5 g homogenized crushed slurry crust from each treatments were pre-incubated with ca. 280 ppm CH₄, at each incubation temperature before the actual CH₄ oxidation sampling was done. Gas samples were analysed by GC as described earlier.

6.2.3 Data interpretation and statistical analysis

Methane oxidation rates during these experiments were normalized based on knowledge of CH₄ loss rates from empty containers (with 280 ppm CH₄), incubated together during the experiment. Nitrous oxide emission rates were determined by using two sampling points (t₀ and t₁₅) during the incubation. The oxidation rate of CH₄ in different incubation temperatures, and N₂O emission were analysed by General Linear Model, ANOVA using Minitab 16 (USA) software. The significant difference was set at confidence interval at 95%.

6.3 Result

6.3.1 General crusts observation

The crust surface was dry and possessed an irregular surface area (Figure 6.1 and Figure 6.2). The surface crusts colour was grey to black, while underneath grey-brown. No grass or fibrous materials were seen on the surface. The underside of the crust was moist to wet depending on the crust depth and contact with liquid slurry at the bottom. Some crust from “Study 2” seemed to be wetter, because some of the crust had fallen into the slurry. Some hay, fibrous or grains could be seen on the underside of the crust, while maggots were also seen in certain crusts. Crusts background characteristic used during the experimental studies are shown in Figure 6.1, Figure 6.2, Table 6.1 and Table 6.2.
Figure 6.1 Crust formed from container with slurries kept for 120 days.

Photos of intact crust and homogenies crust during oxidation study. A, Original crust obtained from winter storage in PVC container. Left side; crust bottom surface, Right side; top crust surface; B, Homogenized crust that was sieved through 56 mm sieves. C: Intact crust in a disc form with 5 cm diameter prepared by using 5 cm diameter soil metal bulk density ring.

Figure 6.2 Crust formed from 90 litres barrel over 6 months’ periods and treated with AFEM additives.

Note: A, partially breakage crust; B, total submersed crust after felt into crust; C, clear thick regular crust.
Table 6.1 Slurry crust physiochemical properties used in Experiment 6.2.1, obtained from 120 days slurry during winter experimental observation.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>8.2 ±0.03</td>
</tr>
<tr>
<td>Electric conductivity (mS cm(^{-1}))</td>
<td>4.2 ±0.18</td>
</tr>
<tr>
<td>Dry matter (% FWt)</td>
<td>43.7 ±2.3</td>
</tr>
<tr>
<td>Volatile solid (% DM(^{-1}))</td>
<td>79.3 ±1.7</td>
</tr>
<tr>
<td>Total C (g C kg(^{-1}) FWt)</td>
<td>190.3 ±0.3</td>
</tr>
<tr>
<td>Total N (g N kg(^{-1}) FWt)</td>
<td>19.4 ±0.4</td>
</tr>
<tr>
<td>Ammonium-N (g N kg(^{-1}) FWt)</td>
<td>1.08 ±0.13</td>
</tr>
<tr>
<td>Nitrate-N (g N kg(^{-1}) FWt)</td>
<td>0.31 ±0.08</td>
</tr>
<tr>
<td>Bulk density (kg m(^{-3}))</td>
<td>230.7 ±5.4</td>
</tr>
</tbody>
</table>

DM, Dry matter; EC, electrical conductivity; VS, volatile solid; FWt, Fresh matter. Values represent mean ±SEM (n=5).

Table 6.2 Slurry crust physiochemical properties with EM and H\(_2\)O sprayed.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AFEM Sprayed</td>
</tr>
<tr>
<td>pH</td>
<td>9.1 ±0.3</td>
</tr>
<tr>
<td>Electric conductivity (mS cm(^{-1}))</td>
<td>6.7 ±0.5</td>
</tr>
<tr>
<td>Dry matter (% FWt)</td>
<td>57.1 ±1.8</td>
</tr>
<tr>
<td>Volatile solid (% DM(^{-1}))</td>
<td>45.6 ±6.4</td>
</tr>
<tr>
<td>Total C (g C kg(^{-1}) FWt)</td>
<td>149.3 ±9.4</td>
</tr>
<tr>
<td>Total N (g N kg(^{-1}) FWt)</td>
<td>16.1 ±1.3</td>
</tr>
<tr>
<td>Ammonium-N (g N kg(^{-1}) FWt)</td>
<td>0.7 ±0.215</td>
</tr>
<tr>
<td>Nitrate-N (g N kg(^{-1}) FWt)</td>
<td>18.2 ±0.8</td>
</tr>
<tr>
<td>Bulk density (kg m(^{-3}))(^*)</td>
<td></td>
</tr>
</tbody>
</table>

\(^*\)Bulk density was not measured due to crust shape formation and uncontrolled breakage. DM, Dry matter; EC, electrical conductivity; VS, volatile solid; FWt, Fresh matter. (No significant different observed between treatment, \(P>0.05\)); Values represent mean ±SEM (n=3).
6.3.2 Methane oxidation of intact and homogenized crusts and the effect of incubation temperature

I. Methane oxidation and GHG emission

As can be seen in Figure 6.3, CH4 oxidation was not observed during the 48 hr incubation periods. In addition, average CH4 concentration in headspace increased from ±250 ppm CH4 to between 334 and 419 ppm CH4 after 48 hours in both types (intact and crush) of crusts. The CH4 emissions were varied with the crust forms and incubation temperature, although they were not significantly different ($P=0.733; P=0.252$). Emission rate was highest from intact crust at cold temperature with 1.95 ±1.469 ng CH4 g⁻¹ DM hr⁻¹. Although these data obtained did not show a significant difference at a 95% confidence level, these CH4 released indicated opposite of oxidation by higher CH4 emission rather than consumption.

Table 6.3 summarizes the amount of N2O flux observed at each incubation temperature for both crust forms. Lowest emission was from homogenised crust at 4°C incubation. The emission flux increased with higher incubation temperatures. Smaller N2O fluxes were observed at incubation 10 and 22°C. Homogenized crust at 30°C resulted in an emission rate of 532 ng N2O g⁻¹ DM hr⁻¹, the highest among all observations. An important point to note is the N2O flux was far higher than CH4 loss and emission with minimal mean near 26 ng N2O g⁻¹ DM hr⁻¹. Calculated net GHG emission was obtained by the summing the CO2 eq. of CH4 and N2O emissions following IPCC (2013) guidelines (Myhre et al., 2013). Carbon dioxide fluxes were not added to the total CO2 eq. emission calculation in Table 6.3. Homogenized crust resulted in higher total GHG (CO2 eq.) emission at all incubation temperatures. Similarly, highest net GHG emissions were seen from the homogenized crust at 30°C incubation.
Figure 6.3 Methane concentration in container headspace during 48 hours incubation in different incubation temperature.
(a) 4°C incubation; (b) 10°C incubation; (c) 22°C incubation; (d) 30°C incubation. Values represent mean ±SEM (n=4).
Table 6.3 Nitrous oxide flux and net GHG emitted recorded during the same incubation periods.

<table>
<thead>
<tr>
<th>Incubation temperature (°C)</th>
<th>Crust type</th>
<th>CH₄ oxidation rate (ng g⁻¹ DM hr⁻¹)</th>
<th>N₂O emission rate (ng g⁻¹ DM hr⁻¹)</th>
<th>Net GHG emission (CO₂ eq.) (µg g⁻¹ DM hr⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Intact</td>
<td>-1.95 ±1.469</td>
<td>25.7 ±1.47</td>
<td>8.0</td>
</tr>
<tr>
<td></td>
<td>Homogenized</td>
<td>-1.28 ±0.707</td>
<td>24.1 ±0.71</td>
<td>7.5</td>
</tr>
<tr>
<td>10</td>
<td>Intact</td>
<td>-0.70 ±0.463</td>
<td>182.7 ±0.46</td>
<td>56.6</td>
</tr>
<tr>
<td></td>
<td>Homogenized</td>
<td>-0.47 ±0.671</td>
<td>227.7 ±0.67</td>
<td>70.6</td>
</tr>
<tr>
<td>22</td>
<td>Intact</td>
<td>-0.82 ±0.571</td>
<td>367.0 ±0.57</td>
<td>113.8</td>
</tr>
<tr>
<td></td>
<td>Homogenized</td>
<td>0.18 ±0.124</td>
<td>468.6 ±0.12</td>
<td>145.2</td>
</tr>
<tr>
<td>30</td>
<td>Intact</td>
<td>-1.04 ±0.740</td>
<td>348.3 ±0.74</td>
<td>108.0</td>
</tr>
<tr>
<td></td>
<td>Homogenized</td>
<td>-1.20 ±0.939</td>
<td>531.9 ±0.94</td>
<td>164.9</td>
</tr>
</tbody>
</table>

P value | 0.733 | 0.252

Net GHG emission are calculated based on mean CH₄ and N₂O released and were transformed into CO₂ equivalent, using values for equivalent CO₂ global warming potentials for N₂O and CH₄ of 298 and 34 respectively (Myhre et al., 2013). Negative (-) CH₄ oxidation rate indicating emission of CH₄ rather than removed from the headspace. Values represent mean ±SEM (n=4).

II. Methane oxidation potential

Although mean flux obtained indicated CH₄ oxidation was not present in this crust, CH₄ oxidation was recorded in one crust sample from all treatment groups and temperatures, except at 30°C incubation. The oxidation can be clearly seen in Figure 6.4 during 48 hours incubation from 4 and 10°C incubation temperature. Oxidation was very low at higher temperature and did not occur at 30°C. The calculated oxidation recorded from intact crust 4°C (I4), homogenized crust at 4°C (H4), intact crust at 10°C (I10) and homogenized crust at 10°C (H10) were equal to 1.76, 0.35, 0.52 and 1.04 ng CH₄ g⁻¹ DM hr⁻¹, respectively.
6.3.3 Methane oxidation activity in homogenised slurry crust amended with effective microorganism

Methane oxidation rate was hypothesised to differ between crust sprayed with AFEM and crust sprayed with H₂O. Although there was no significant difference between the headspace CH₄ concentrations during this experiment, Figure 6.5 shows CH₄ oxidation activity was present, indicating potential biological (oxidation) activity. Oxidation rates fluctuated during the incubation period and are summarized in Table 6.4. The presented data are the average oxidation rate per hour during each measurement within the 48 hours incubation. The measurements were done on 0-6, 6-24 and 24-48 hour intervals. In most cases, the oxidation rate was highest during first 6 hrs, and decreased after this point, and some cases indicated an emission of CH₄. This is clearly seen at the 22°C incubation temperature. There is no significant difference in oxidation rate during 6, 24 and 48 hours oxidation incubation at confidence level 95% (P=0.051, P=0.097 and P=0.457). Maximum oxidation rate recorded was at 24.89 ng CH₄ g⁻¹ DM hr⁻¹ from the control crust incubated at 30°C (H₂O sprayed).

Nitrous oxide emissions were also observed during this experiment. Lower rates from the AFEM treated than untreated crust although they were not significantly
different \( P>0.05 \) (Table 6.5). Nitrous oxide emission rates increased with higher temperatures, from 460 to 1551 ng N\textsubscript{2}O g\textsuperscript{-1} DM hr\textsuperscript{-1} at 4°C to 30°C. These high N\textsubscript{2}O flux rates were responsible for the net positive GHG emission, which ranged from 143 to 480 µg N\textsubscript{2}O g\textsuperscript{-1} DM hr\textsuperscript{-1}.
Figure 6.5 Methane concentration in headspace during oxidation observation. (a) 4°C incubation; (b) 10°C incubation; (c) 30°C incubation. Values represent mean ± SEM (n=3).
Table 6.4 Methane oxidation flux recorded from 2 types of slurry crust.

<table>
<thead>
<tr>
<th>Incubation temperature (°C)</th>
<th>Treatment</th>
<th>Average CH₄ oxidation rate during 48 hrs observation (ng g⁻¹ DM hr⁻¹)</th>
<th>Max oxidation rate recorded (ng g⁻¹ DM hr⁻¹)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>24</td>
</tr>
<tr>
<td>4</td>
<td>AFEM</td>
<td>3.06 ±3.26</td>
<td>2.85 ±1.39</td>
</tr>
<tr>
<td></td>
<td>Ctrl</td>
<td>6.71 ±0.77</td>
<td>2.53 ±0.65</td>
</tr>
<tr>
<td>22</td>
<td>AFEM</td>
<td>6.45 ±6.25</td>
<td>-2.10 ±0.58</td>
</tr>
<tr>
<td></td>
<td>Ctrl</td>
<td>-11.01 ±7.21</td>
<td>-5.41 ±4.39</td>
</tr>
<tr>
<td>30</td>
<td>AFEM</td>
<td>-6.85 ±2.02</td>
<td>1.48 ±1.02</td>
</tr>
<tr>
<td></td>
<td>Ctrl</td>
<td>11.86 ±6.66</td>
<td>1.88 ±1.90</td>
</tr>
</tbody>
</table>

*P value (n=3) 0.051 0.097 0.475

Negative (-) CH₄ oxidation rate indicating more CH₄ was released than removed from the headspace.

Maximum oxidation rate based on maximum CH₄ consumed recorded at any point during the study.

Table 6.5 The N₂O flux and net GHG calculated from CH₄ oxidation and N₂O emission.

<table>
<thead>
<tr>
<th>Incubation temperature (°C)</th>
<th>Treatment</th>
<th>N₂O emission rate (ng g⁻¹ DM hr⁻¹)</th>
<th>Net GHG emitted (CO₂ eq.) (µg g⁻¹ DM hr⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>AFEM</td>
<td>460 ±234.4</td>
<td>142.3</td>
</tr>
<tr>
<td></td>
<td>Ctrl</td>
<td>688 ±42.4</td>
<td>213.0</td>
</tr>
<tr>
<td>22</td>
<td>AFEM</td>
<td>1144 ±427.8</td>
<td>354.2</td>
</tr>
<tr>
<td></td>
<td>Ctrl</td>
<td>1200 ±294.8</td>
<td>371.8</td>
</tr>
<tr>
<td>30</td>
<td>AFEM</td>
<td>1444 ±304.3</td>
<td>447.6</td>
</tr>
<tr>
<td></td>
<td>Ctrl</td>
<td>1551 ±412.5</td>
<td>480.2</td>
</tr>
</tbody>
</table>

*P value (n=3) 0.176

Net GHG emission are calculated based on maximum CH₄ oxidation rate recorded (Table 6.4), and N₂O released and were transformed into CO₂ eq. following IPCC 2013 guidelines which indicating GWP for N₂O and CH₄ of 298 and 34, respectively (Myhre et al., 2013). Values represent mean ±SEM (n=3).
6.4 Discussion

6.4.1 Crust variation

Crusts on slurry surfaces during storage act as a natural barrier for emissions of \( \text{CH}_4 \) and provide an opportunity for \( \text{CH}_4 \) oxidation (Petersen et al., 2005; Duan, 2012; Duan et al., 2013; Nielsen et al., 2013). They also represent a source for \( \text{N}_2\text{O} \) emission (Berg et al., 2006a; Petersen and Miller, 2006). The crust formation in the 16 litre containers that was used in Study 1 (Methodology 6.2.1) appeared relatively uniform. Whilst the crust that formed in the larger barrel that was used in Study 2 (Methodology 6.2.2) was more variable in nature. This variation was due to the larger surface area and high moisture loss resulting in immediate slurry level drops. The crust formed in these barrels (Study 2) were concave in shape (seen from the top) and were not completely intact due to moisture loss resulting in crust breakage once it falls into the liquid slurry. Crusts from different vessel were used as replicates to simulate individual sampling points from the field. During the experiments, crust samples were not mixed and homogenized contributing to greater (large) variability (±SEM) on all parameters observed. The slurry crusts used had higher than neutral pH values (weakly alkaline) although liquid slurries are often recorded to be an acidic medium (Petersen et al., 2005; Petersen and Ambus, 2006; Hansen et al., 2009). A similar result was presented by Duan, (2012); Petersen et al., (2005); and Petersen and Miller, (2006). Higher pH crusts were obtained from the longer storage of slurry (methodology 6.2.2) as a result of longer exposure of base salt, \( \text{CO}_2 \) and carbonate salts during slurry storage (Petersen and Ambus, 2006). Crusts are usually rich in inorganic N and the increase of \( \text{NH}_4^+ \) concentrations in crust by capillary action may lead to nitrification, nitrification denitrification potential (Hansen et al., 2009; Nielsen et al., 2010; Duan et al., 2013). Crust formation were not found in slurries with less than 2% DM (Hanson and Hanson, 1996) and influenced by gas bubbling which carries light material to the surface (Wood et al., 2012). There is high tendency of crust formation on slurry stored in the slurry lagoon (Smith et al., 2007). It was recorded that 78% of farms in England and Wales found crust formation on their farms.

6.4.2 Methane oxidation

Methane oxidation from slurry crust is an important microbial process as it is a sink for \( \text{CH}_4 \) in close proximity to the source (liquid slurry). The oxidation of \( \text{CH}_4 \) tends to be higher proportional to deeper depth in slurry crust (Nielsen et al., 2013).
According to Qiang et al., (2011), CH₄ oxidation from slurry crusts has the potential to remove up to 80% of CH₄ emission during slurry storage. Meanwhile Petersen et al., (2005) stated that O₂ level did not change much during a 5 day incubation, therefore we assume that atmospheric O₂ level were not depleted and probably did not affect the CH₄ oxidation rate during our studies. The importance of microclimate condition leads to the observation on the different oxidation flux rate from crust forms (this study). Similarly, AFEM spraying may lead to microclimate change on the crust thus affecting its biological activity.

In the present study, CH₄ oxidation was not clearly observed in the first experiment (Study 1) and there was no significant difference in CH₄ oxidation flux from crust with and without AFEM in Study 2. However, lower oxidation rates were observed in some of the treatments possible indicating reduced oxidation potential from crust treated with AFEM, or possibly higher CH₄ emitted from the crusts. This could be because of the MOB growth and activity is affected by pH, temperature, moisture and substrate availability in the microenvironments (Petersen et al., 2005). Moreover, Duan, (2012) suggested the presence of co-existence of methanogen on anoxic microsites on slurry crust subsequently causing the high variability in oxidation activity. This may explain the CH₄ emission from the crust. The important finding from both observations is that N₂O fluxes outweighed CH₄ oxidation and CH₄ emission.

In our study, the maximum oxidation rate recorded was 24.9 ng CH₄ g⁻¹ DM hr⁻¹, which was lower than those reported by Petersen et al., (2005) and Duan, (2012 and 2013). This lower rate represents an oxidation rate at starting headspace concentration at between 250-300 ppm concentration levels. Petersen (2005) showed the oxidation rate was 0.08-0.4 µg CH₄ g⁻¹ DM hr⁻¹ at normal moisture, and 0.16-1.11 µg CH₄ g⁻¹ DM hr⁻¹ when the crust was partially process (assuming their OM at 79%; data not shown). Similarly if we assuming 1 g equal to 1 cm² surface area, our oxidation rate represents 0.059 µg CH₄ m⁻² day⁻¹, compare to 4.5 µg CH₄ m⁻² day⁻¹ (Petersen and Ambus, 2006).

Oxidation rate fluctuated throughout the season as reported by Petersen (2006). Oxidation rate could be 20x greater in a warm climate compared to winter temperatures. This is relatively similar to data obtained in this temperature incubation study. Types of MOB present and N salts, also influences oxidation rate (Hanson and Hanson, 1996; Duan et al., 2013). In addition, crusts pH indicated at alkaline (pH 9.1) which might impair the MOB activity as optimum pH range was reported at 3.5-8.0 (Hanson and Hanson, 1996).
6.4.3 Nitrous oxide crust emission

The biological activity in slurry crusts has the potential to affect GHG emissions. This can be important for CH₄ reduction via oxidation processes, but N loss through N₂O emissions from crust have been reported (Sommer et al., 2000). The N₂O produced is important not only because of its 298 times GWP potential, but also due to N loss from slurry the crust which play important role as N fertilizer for crops (Defra, 2010a). During slurry storage, N₂O from slurry was emitted at very low or near zero with emission factor 0.0007% which can be considered negligible (IPCC, 2006; Park et al., 2006; Rodhe et al., 2009b; Chadwick et al., 2011; Aguerre et al., 2012). A N₂O emission during slurry store was reported by Amon et al., (2005, 2006); Dinuccio et al., (2008); Aguerre et al., (2012) when a crust developed, however the emission from crust is not well explored. Our finding showed that N₂O emission can be large compared to CH₄ loss in both experiments, on a CO₂ eq. basis.

The crusts in both studies showed the presence of NO₃-N, and was weakly alkaline (pH 8.2 and 9.1) which is within the optimum range (pH 7-9) for nitrifying bacteria (Hayatsu et al., 2008). Nitrous oxide emitted from the crust in this study ranged between 25 - 1551 ng N₂O g⁻¹ DM hr⁻¹ produced during nitrification and denitrification processes in slurry crust (as it is in solid) by nitrifiers including ammonia-oxidizing bacteria (AOB) and the nitrite-oxidizing bacteria (NOB), eukaryote and archaea (Wrage et al., 2001; Hayatsu et al., 2008). Duan (2012) investigated variations in crust O₂ concentration and N₂O distribution on crust using an O₂ micro-sensor, and concluded that N₂O emissions originated in the top part of crust layer, likely to be at range 3-10 mm underneath the surface, depending on the crust thickness. This is possibly because of O₂ levels, which diffuse easily into the crust surface (Duan, 2012). With regard to this, it is creating sub-oxic or anoxic zones in the middle crust and promotes nitrification, and nitrification denitrification activities leading to the accumulation of N₂O at a particular depth. There is the possibility that N₂O emissions appeared in the aerobic part of the crust similar to our finding.

The N₂O flux from a crust is affected by moisture content. Environmental temperatures also reflect the emission rate (Clemens et al., 2006), and this is relatively similar to our finding (Table 6.3 and Table 6.5). Nitrous oxide emission from slurry crust was recorded by Clemens, (2006) between 0.35 to 0.44 µg cm⁻³ day⁻¹ during winter and summer respectively. Considering 1 cm³ (of crust) is equal to 0.45 g, this indicates
their emission rate is lower compared to our finding. A reason for this difference could be due to the surface area, different form and thickness of crust used in the studies thus resulting different rate of oxic-anoxic condition of the crust.

According to a recent study by Petersen (2013), the cumulative amount of N₂O emission from straw crust pig slurry was 40 g m⁻², with the highest rate at 80 mg m⁻² hr⁻¹ during the 58 days summer storing experiment compared to no emission during the winter, possibly due to higher temperature and precipitation during the summer period. In this study N₂O emission was recorded lower on crust with AFEM sprayed, although this was not significantly different due to low replication (n) and high variability (±SEM). Amon (2004) reported similar observation during slurry storage treated with EM at 0.1% (v/v) concentration.

6.4.4 Crust in GHG mitigation perspective

Greenhouse gas emission from slurry is widely known (Rodhe et al., 2009b; Chadwick et al., 2011; Petersen et al., 2012, 2013a; Wang et al., 2014) and in our data it indicated the possibility of CH₄ released from crust when anoxic conditions were prevalent and when there was sufficient available substrate. The CH₄ oxidation in crust was considered a cheap and economic approach for reducing CH₄ emissions from slurry stores (Petersen et al., 2005). The importance of crust as the natural barrier for CH₄ oxidation as explored by Petersen (2005, 2006); and Duan (2012, 2013) is well understood. However, there is possibility that the CH₄ molecule may by-pass crust and released to the atmosphere. Our finding showed that there is a possibility of CH₄ oxidation with concomitant N₂O emission. By including N₂O emissions in the overall GHG budget, the total GHG (CO₂ eq.) calculated indicates minimal emission during cold climate at 8 µg CO₂ eq. g⁻¹ DM hr⁻¹ (CO₂ emission not counted). In order to gain negative total GHG emissions, CH₄ oxidation rates need to counter balance N₂O emissions. Methane oxidation can be promoted by physical cover replacement such as wood chipped, leca, peat or straw (Sommer et al., 1993; Berg et al., 2006a; Hansen et al., 2009). Appropriate inoculum or cultured methanotrophs can be applied with optimum moisture (60-70%) and incubation temperature for maximum oxidation activity (Barasarathi and Agamuthu, 2011). In addition, crust on slurry surface act as barrier for N loss, especially to reduce NH₃ volatilisation (Misselbrook et al., 2005a). This approach is being practiced in livestock farming in Denmark (>80%) to mitigate
NH₃ volatilisation (Petersen et al., 2005) due to regulation imposed by the Danish government.

6.5 Conclusion

In conclusion, CH₄ oxidation in slurry crust is not significant and the crust did not influence the oxidation process during slurry storage, even though positive emission is plausible. Furthermore, the crust is a good medium for nitrification denitrification processes and subsequently emits N₂O to the atmosphere. The effect of AFEM application to slurry crusts does not improve CH₄ oxidation but triggers N₂O emission from slurry crust.
CHAPTER 7

7 The effects of slurry modification on greenhouse gas and ammonia emissions following spreading: a pot study.

Abstract

Any strategy to modify the slurry environment during storage (to reduce e.g. ammonia (NH₃) and methane (CH₄) emissions), also needs to consider the implications of this during subsequent land spreading. Recently, studies have shown that slurry acidification and bio-augmentation, e.g. with effective microorganisms (EM), are the possible approaches to mitigate greenhouse gas (GHG) release during slurry storage. In this study, we measured the agronomic and environmental effects of applying amended slurry treatments, ‘self-acidified’ with brewing sugar and bio-augmented by effective microorganisms (EM), to ryegrass in a 60 day greenhouse pot experiment. Aged non-acidified slurry and an ammonium nitrate fertiliser were included as reference treatments, in addition to a soil which received no slurry treatment. Ammonia volatilisation from the self-acidified slurry treatments were inhibited by 75-89% compared to the volatilisation from the untreated slurry treatment. Meanwhile, the self-acidified slurry treatment also resulted in the reduction of CH₄ emission by about 58% compared to untreated slurry, and 65% compared to the aged slurry treatment. Nitrous oxide (N₂O) emissions from all slurry treatments remained similar throughout the experiment. The cumulative GHG carbon dioxide equivalent (CO₂ eq.) loss (CH₄ and N₂O) was much lower from the slurry treatments than from the N fertilizer treatment. The ryegrass dry matter (DM) yield was lower from the self-acidified slurry treatments, particularly after the first cut. Therefore, although slurry acidification reduces NH₃ and CH₄ emissions from both slurry storage and subsequent land spreading, these benefits did not translate into improved agronomy in this pot trial.

Key words: acidified slurry application, ryegrass, nitrous oxide, methane, ammonia, N offtake, grass yield.
7.1 Introduction

Cattle slurry contains valuable plant nutrients, particularly nitrogen (N), phosphorus (P), and potassium (K), which are useful as substitutes for fertilizer and could save up to £95 ha\(^{-1}\) (£380 ha\(^{-1}\) yr\(^{-1}\)) during application (Defra, 2010a). Additional applications of organic matter via livestock manures could greatly increase soil structure, fertility and water holding capacity (Bot and Benites, 2005; Diacono and Montemurro, 2010). Repeated slurry application over many years has been found to be capable of replacing the amount of N lost by accumulating organic N and subsequent N mineralization (Chadwick et al., 2000; Reijs et al., 2005). Therefore, applying slurries to retain soil fertility and structure is important in maintaining soil quality and long-term agricultural production. However, improper slurry application strategies will result in air and water pollution such as eutrophication (Volterra et al., 2002).

Livestock slurry is a large source of greenhouse gas (GHG) emission, arising from each stage of the manure management chain (Chadwick et al., 2011). Mitigating GHG emissions from all sectors, including agriculture, is an important goal, and is stated in the Kyoto Protocol (1997), with many countries setting national targets for mitigation. Methane and N\(_2\)O are key greenhouse gases from agriculture. Agriculture is also the major source for NH\(_3\) emissions (Misselbrook et al., 2002, 2004, 2005a, 2005b, 2005c; Misselbrook and Powell, 2005). All three gases are lost during storage and following application of livestock slurries to land (Misselbrook et al., 2005c; Chadwick et al., 2011; Feilberg and Sommer, 2013; Samer, 2013; Sommer et al., 2013).

Methane emissions from slurry spreading are generally short-lived, where emissions are high during the first 24 hours (Chadwick and Pain, 1997) before decreasing due to exposure of methanogens to aerobic conditions. However, Burford (1976), reported the presence of CH\(_4\) emissions from slurry 2 weeks after slurry application. The reason is unclear, but the presence is possibly due to the slow slurry infiltration into the soil. In addition, CH\(_4\) emissions are affected by soil type, where emissions are generally highest from clay textured soil (Chadwick and Pain, 1997). Nevertheless, Amon et al., (2006) reported that slurry application only contributed between 1-2% of CH\(_4\) emission compared to the emission level during slurry storage. Similarly, soil type also affects N\(_2\)O emissions after slurry application. Emissions generally higher from clay soils, where N\(_2\)O emissions can reach 5.7 t ha\(^{-1}\) CO\(_2\) eq.
(Leahy et al., 2004). Exposure of slurry-ammonium (NH$_4^+$) to aerobic conditions promotes nitrification of NH$_4^+$ to nitrate (NO$_3^-$) and results in N$_2$O emission during the nitrification process (Bremner and Blackmer, 1978; Firestone et al., 1980; Rahn et al., 1997; Maeda et al., 2010b; Chadwick et al., 2011). In addition, under anaerobic conditions, N$_2$O production and emission also occurs from denitrification processes (Dinçer and Kargi, 2000; Wrage et al., 2001; Hayatsu et al., 2008). Rewetting of slurry promotes further nitrification denitrification and contributes another peak of N$_2$O emission (Meisinger and Jokela, 2000).

Ammonia emission followed by N deposition represents an indirect source of N$_2$O emission, as well as contributing to eutrophication and soil acidification processes (Dise et al., 2011). Ammonia volatilisation from N fertilizer (mainly urea) and slurry application (Beauchamp et al., 1982; Pain et al., 1990; Stevens et al., 1992; Amon et al., 2006) is important as N loss decreases fertilizer efficiency (Thompson et al., 1987; Matsunaka et al., 2006). Nitrogen loss through volatilisation is high during the first 4-12 hours (up to 50% of NH$_3$) after application to soil (Pain et al., 1990; Feilberg and Sommer, 2013). It is important to avoid N losses and retain N in the soil, to improve grass nutrient uptake (Chadwick et al., 2000) as it indirectly contribute to grass quality and nutrient uptake by the cattle. Indeed, high forage quality will also contribute less enteric CH$_4$ emission with higher production efficiency in term of milk yield, carcass gain and lamb birth weight (Hristov et al., 2013).

This study determined the NH$_3$ and GHG emissions following application of amended stored cattle slurry to grass in a 60 d greenhouse pot experiment. In Malaysia and some other tropical countries, slurry application to crops is not always feasible due to slurry transportation costs. Even if such application to croplands do occur, these are often applied to crops and grasses, which are related to the animal feed.

We hypothesise that lower NH$_3$ and GHG emissions from the application of ‘self-acidified’ stored slurry to ryegrass, compared to non-treated slurry. This paper reports the subsequent agronomic and environmental effects of applying the modified slurry treatments from Chapter 4 (summer storage) to ryegrass. Slurry was surface broadcast, as the trailing shoe or injection disc methods are not always appropriate (Jensen and Sommer, 2013) and are not widely practiced by small-sized farms.
7.2 Materials and methods

7.2.1 Soil and Italian ryegrass germination

An Italian ryegrass (ryegrass) (*Lolium multiflorum*) seed mix was sown at 2 g pot\(^{-1}\) in (ca. 1.7 kg ha\(^{-1}\)) in 13 x 13 x 13 cm (0.011664 m\(^{2}\)) pots. Each poly-vinyl chloride (PVC) pot was filled with 1.3 kg (FWt) of 5.6 mm sieved sandy clay loam topsoil (Eutric Cambisols) obtained from Henfaes Research Centre, Bangor University, Abergwyngregyn, Gwynedd, UK (53° 23’ 91.29” N, 4° 01’ 91.09” W). The topsoil physic-chemical characteristics were DM: 86.4%, pH: 6.7 ±0.1, total C, total N, NH\(_4\)-N, (g kg\(^{-1}\) FWt): (21.0 ±0.64, 2.9 ±0.04, 0.85 ±0.11), and EC 290.9 ±102.4 µS cm\(^{-1}\), respectively. Pots were watered daily with deionised water until the seeds germinated and grew to about 5 cm. They were then watered three times per week to maintain at average of 75% water holding capacity (WHC) equal to 69.5% of water filled pore space (WFPS) until the end of the experiment. Ryegrass was allowed to grow for 6 weeks before the first harvest. The entire experiment study was conducted in a temperature controlled greenhouse (average temperature 19.7°C, min; 9.5°C, max; 32°C) located at Henfaes Research Centre.

7.2.2 Slurry-ryegrass application

The slurry treatments came from the modified slurry 60-day summer storage experiment described in Chapter 4 and was stored in cold room (4°C) until the experiment is conducted. Slurry treatments were acidified slurries; FGEM, [Fresh slurry + effective microorganism 5% (\(^{v/\omega}\)) + brewing sugar 10% (\(^{w/\omega}\))]; FG, [Fresh slurry + brewing sugar 10% (\(^{w/\omega}\))] non-acidified slurries, including FEM [Fresh slurry + effective microorganism 5% (\(^{v/\omega}\))], and F Ctrl, [Fresh slurry – untreated]. Prior to slurry application, 3 additional treatments were added; i) neutral pH (pH 6.5-7.5) aged slurry about 4 months old from the same farm collected from the storage tank, ii) N fertilizer (NH\(_4\)NO\(_3\)), and iii) a control of untreated ryegrass, which only received distilled water. Slurries and N fertilizer were manually spread onto the ryegrass at an equivalent rate of 200 kg N ha\(^{-1}\), after the grass was cut to a height of 3.5 cm. Ammonium nitrate (NH\(_4\)NO\(_3\)) was dissolved into distilled water prior spreading. The treatments were later known as; 1- RGEM, 2- RG, 3- REM, 4- RCT, 5- RNAS, 6-RNF and 7- RNC, and are summarized in Table 7.1. The experimental pots were assigned in a randomized complete block design (RCBD) for the experimental period. The physiochemical properties of the slurries are summarised in Table 7.2.
Table 7.1 Slurry and nitrogen fertilizer treatments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ryegrass-slurry application treatment (200 kg N ha⁻¹)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>RGEM</td>
<td>Ryegrass + FGEM treated slurry</td>
<td>Slurries amended with brewing sugar and EM (FGEM)</td>
</tr>
<tr>
<td>RG</td>
<td>Ryegrass + FG treated slurry</td>
<td>Slurries amended with brewing sugar (GEM)</td>
</tr>
<tr>
<td>REM</td>
<td>Ryegrass + FEM treated slurry</td>
<td>Slurries amended with EM (FEM)</td>
</tr>
<tr>
<td>RCT</td>
<td>Ryegrass + F Ctrl untreated slurry</td>
<td>Untreated Slurries (F Ctrl)</td>
</tr>
<tr>
<td>RNF</td>
<td>Ryegrass + Nitrogen fertilizer</td>
<td>N fertilizer (NH₄NO₃ dissolved in H₂O)</td>
</tr>
<tr>
<td>RNAS</td>
<td>Ryegrass + aged slurry (neutral pH)</td>
<td>Aged slurries</td>
</tr>
<tr>
<td>RNC</td>
<td>Ryegrass untreated (control)</td>
<td>Only distilled H₂O</td>
</tr>
</tbody>
</table>

Note: FGEM, [Fresh slurry + effective microorganism 5% (w/v) + brewing sugar 10% (w/w)]; FG, [Fresh slurry + brewing sugar 10% (w/w)]; non-acidified slurries, including FEM [Fresh slurry + effective microorganism 5% (v/w)], and F Ctrl, [Fresh slurry – untreated];

Table 7.2 Slurry physiochemical characteristics prior to slurry-ryegrass application.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>RGEM</th>
<th>RG</th>
<th>REM</th>
<th>RCT</th>
<th>RNAS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium-N (g N kg⁻¹ FWt)</td>
<td>1.12 ±0.05</td>
<td>1.13 ±0.02</td>
<td>1.09 ±0.11</td>
<td>0.80 ±0.09</td>
<td>1.30 ±0.01</td>
</tr>
<tr>
<td>Dry Matter (% FWt)</td>
<td>19.5 ±3.61⁺</td>
<td>13.4 ±0.53ab</td>
<td>10.1 ±1.39b</td>
<td>11.7 ±0.99ab</td>
<td>6.9 ±0.04c</td>
</tr>
<tr>
<td>Volatile solid (% DM⁻¹)</td>
<td>89.9 ±1.59b</td>
<td>88.9 ±1.65b</td>
<td>71.6 ±1.87a</td>
<td>73.4 ±0.52a</td>
<td>77.22 ±0.25a</td>
</tr>
<tr>
<td>pH</td>
<td>4.2 ±0.03c</td>
<td>4.2 ±0.03c</td>
<td>8.4 ±0.09a</td>
<td>8.5 ±0.07a</td>
<td>7.5 ±0.19b</td>
</tr>
<tr>
<td>Oxidation redox potential (mV)</td>
<td>-35.2 ±19.6a</td>
<td>-5.8 ±22.31a</td>
<td>-366.8 ±12.20</td>
<td>-338.0 ±20.3</td>
<td>-305.0 ±36.10</td>
</tr>
<tr>
<td>Electric conductivity (mS cm⁻¹)</td>
<td>9.9 ±0.2b</td>
<td>8.5 ±0.7b</td>
<td>10.7 ±2.10b</td>
<td>10.4 ±1.70b</td>
<td>13.8 ±1.50a</td>
</tr>
<tr>
<td>Total C (g C kg⁻¹ FWt)</td>
<td>66.6 ±4.6</td>
<td>61.7 ±3.10</td>
<td>36.3 ±6.00</td>
<td>39.8 ±5.70</td>
<td>55.7 ±8.30</td>
</tr>
<tr>
<td>Total N (g N kg⁻¹ FWt)</td>
<td>4.1 ±0.3</td>
<td>4.8 ±0.30</td>
<td>4.4 ±0.50</td>
<td>4.4 ±0.20</td>
<td>5.1 ±0.30</td>
</tr>
</tbody>
</table>

Data represent mean ±SEM (n=5). Means within the same column with no common superscript differ significantly (P ≤0.05).
7.2.3 Ammonia and greenhouse gas flux measurement

Ammonia emission was measured by passive diffusion into 0.02 M orthophosphoric acid (H$_3$PO$_4$) solution, and trapping the NH$_3$ as ammonium (NH$_4^+$), in ‘traps’ placed close to the lid during measurement in a closed chamber over a one hour period. In this method, the ryegrass pots were placed in 12 L containers with airtight lids (Figure 7.1). Both NH$_3$ and GHG gas sampling were carried before the watering (in the day where ryegrass watering is needed). Ammonia volatilisation was measured after 1, 24 and 48 hours of slurry application, using fresh acid traps. The NH$_4^+$ content of the orthophosphoric acid H$_3$PO$_4$ was measured using the microplate method as described by Mulvaney, (1996).

During the NH$_3$ sampling, air samples were withdrawn by 25G X 1” needle through butyl rubber septa in the container lid following method described in Chapter 4.2.2. The gas samples were placed into 20 mL pre-evacuated gas vials on day 0, 1, 3, 7, 10, 13, 21, 30 and 60, prior to N$_2$O and CH$_4$ analysis by gas chromatograph (GC). Gas samples were analysed using a Perkin Elmer Clarus 580 GC linked to the Perkin Elmer TurboMatrix 110 auto sampler. The GC was equipped with identical megabore capillary Q PLOT columns and fitted with flame ionization detector (FID) with methanizer for detection of both CH$_4$ and electron capture detector (ECD) for N$_2$O detection. Emissions were calculated based on the increase in gas concentration between the T$_0$ and T$_{60}$ samples over the 1 hour period.
Figure 7.1 Ryegrass experimental design for measurements of relative ammonia emissions (note acid traps fixed close to the top of the 12 L vessels) and GHG emissions.

### 7.2.4 Ryegrass yield and total nitrogen content

Ryegrass yield was expressed on a dry matter (DM) basis. The pots were harvested by cutting the grass 3-4 cm above the soil surface before oven drying at 80°C until constant weight. The total N content of the ryegrass was analysed by TruSpec® CN analyser (Leco Corp, St Joseph, MI) on a representative sample of dried and ground material.

### 7.2.5 Statistical analysis

Ammonia emissions, GHG fluxes, grass DM yield and total N content were compared using general linear model (GLM) by Minitab 16 statistical software, (Minitab Ltd. Coventry, UK). Tukey's was also used as a post-hoc test, with $P<0.05$ used as the cut-off for statistical significance. Where necessary, data transformation was carried out to evaluate significant differences between treatments with unequal variances.

The net difference of GHG fluxes during the 60 minutes sampling period was analysed according to the same procedure mentioned above. The cumulative GHG emissions for the 60 days period were calculated by interpolating the area under the curve based using the trapezoidal rule. Total GHG emissions as CO$_2$ eq. were calculated using the global warming potential (GWPs) of 34 and 298 for CH$_4$ and N$_2$O, respectively (Myhre et al., 2013).
7.3 Result

7.3.1 Ammonia volatilisation

For the first 24 hours after application, the NH₃ fluxes varied depending on the different treatments (Figure 7.2a). Self-acidified slurries (RGEM and RG) resulted in significantly lower \( (P<0.05) \) NH₃ emissions, in contrast to the greater NH₃ emissions from the REM, RCT and RNAS treatments. Ammonia fluxes from the REM, RCT and RNAS treatments were 2.1, 1.1 and 2.6 mg N m\(^{-2}\) hr\(^{-1}\) in the first hours, being >10 times higher than the fluxes from the self-acidified slurries (RGEM, RG). The mean emissions from REM and RNAS were significantly greater \( (P<0.001) \) at 1 and 24 hours. However, their fluxes did not differ from other treatments after 48 hours. Fluxes had decreased and were low at 48 hours, between 45 to 103 µg NH₃ m\(^{-2}\) hr\(^{-1}\). Notably, the total (cumulative) NH₃ loss during this 48 hr period was the highest in the RNAS treatment (21.8 mg NH₃ m\(^{-2}\)) followed by REM, RCT and RNF (19.0, 10.7 and 3.7 mg NH₃ m\(^{-2}\)), respectively (Figure 7.2b).
7.3.2 Greenhouse gas fluxes

As expected, acidified slurries resulted in negligible CH₄ fluxes after application. An immediate flux of CH₄ was recorded from the RNAS, REM and RCT treatments (Figure 7.3a). Fluxes were 3.8 ±0.97, 4.1 ±0.56 and 3.4 ±0.35 g CH₄ m⁻² hr⁻¹ (RNAS, REM and RCT), significantly higher ($P<0.05$) than fluxes from other treatments. Later, the fluxes decreased to <0.15 µg CH₄ m⁻² hr⁻¹ on the subsequent days, being similar to the other treatments. Meanwhile, as shown in Figure 7.3c there was no significant difference ($P=0.404$) in the N₂O fluxes between treatments during the entire experiment. Nevertheless, the mean emission level on day 60 was greater than on other sampling days, being significantly different ($P<0.05$) than fluxes observed on day 0.

Cumulative GHG (CH₄ and N₂O) emissions (Figure 7.3b and Figure 7.3d) emitted over the whole experimental period are summarised in Table 7.3. The
cumulative CH₄ emission was greatest from the RNAS treatment at 142.8 ±12.51 mg CH₄ m⁻², which was significantly greater than the cumulative emissions from the acidified slurry treatments which were at 50.4 ±5.36 and 54.1 ±21.35 mg CH₄ m⁻² (RGEM and RG). It is interesting to note that the cumulative emissions of CH₄ from the RGEM and RG treatment showed the lowest CH₄ emissions from all treatments, including the untreated control (RNC). Indeed, the cumulative CH₄ emissions from the RGEM and RG treatments were 7.3% and 13.6% lower than emitted from the control treatment. Other treatment groups showed higher cumulative CH₄ emissions (between 33.8% and 144.9%) compared to the control (RNC). There was no significant difference \((P>0.05)\) in the total N₂O emissions between the different treatments. The cumulative emissions ranged between 63.8 and 102.9 mg N₂O m⁻².

The total GHG emissions, expressed as CO₂ eq. were greatest from the RNF treatment, at 33.3 g CO₂ eq. m⁻². In fact, the total GHG emissions in CO₂ eq. compared to the untreated ryegrass (RNC) indicate that all slurry treatments and the N fertiliser application resulted in positive emissions.
Figure 7.3 Greenhouse gas emissions following slurry treatment applications to ryegrass. 
(a) CH₄ flux; (b) cumulative CH₄ emissions; (c) N₂O Flux; (d) cumulative N₂O emissions. Vertical bars indicate the standard error of the mean (SEM). Values represent means ±SEM (n=5). Note: -1; (‘-ve 1’ on ‘x’ axis) =1 hour after slurry-ryegrass application. Incubation period did not on a scale to show clarity.
Table 7.3 The total GHG emission during 60 days observations after slurry application on ryegrass.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CH₄ (mg m⁻²)</th>
<th>N₂O (mg m⁻²)</th>
<th>Total GHG emission* (CH₄ &amp; N₂O) CO₂ eq. (g m⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RGEM</td>
<td>50.4 ±5.36ᵇ</td>
<td>88.6 ±14.42</td>
<td>28.1</td>
</tr>
<tr>
<td>RG</td>
<td>54.1 ±21.35ᵇ</td>
<td>98.4 ±11.29</td>
<td>31.2</td>
</tr>
<tr>
<td>REM</td>
<td>115.4 ±33.32ᵇ</td>
<td>66.1 ±12.55</td>
<td>23.6</td>
</tr>
<tr>
<td>RCT</td>
<td>119.8 ±10.15ᵇ</td>
<td>76.8 ±19.94</td>
<td>27.0</td>
</tr>
<tr>
<td>RNAS</td>
<td>142.8 ±12.51ᵃ</td>
<td>80.2 ±14.15</td>
<td>28.8</td>
</tr>
<tr>
<td>RNF</td>
<td>78.0 ±15.24ᵃᵇ</td>
<td>102.9 ±15.15</td>
<td>33.3</td>
</tr>
<tr>
<td>RNC</td>
<td>58.3 ±19.68ᵃᵇ</td>
<td>63.8 ±12.79</td>
<td>21.0</td>
</tr>
</tbody>
</table>

Percentage GHG reduction compared to RNC (%)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CH₄</th>
<th>N₂O</th>
<th>CH₄ + N₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>RGEM</td>
<td>13.6</td>
<td>-38.9</td>
<td>-33.9</td>
</tr>
<tr>
<td>RG</td>
<td>7.3</td>
<td>-54.1</td>
<td>-48.3</td>
</tr>
<tr>
<td>REM</td>
<td>-97.9</td>
<td>-3.6</td>
<td>-12.5</td>
</tr>
<tr>
<td>RCT</td>
<td>-105.5</td>
<td>-20.4</td>
<td>-28.4</td>
</tr>
<tr>
<td>RNAS</td>
<td>-144.9</td>
<td>-25.7</td>
<td>-36.9</td>
</tr>
<tr>
<td>RNF</td>
<td>-33.8</td>
<td>-61.3</td>
<td>-58.7</td>
</tr>
<tr>
<td>RNC</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Total GHG emission from ryegrass applied with various slurry treatments and nitrogen fertilizer during 60 days observation. *GHG emission were expressed as CO₂ eq. by calculating to GWP as guided by IPCC (2013) (Myhre et al., 2013). Negative (-ve) percentage indicates emission rather than reduction. Means in columns with superscripts of different letters are significantly different (P<0.05).

7.3.3 Ryegrass yield and ryegrass total nitrogen content

Initial ryegrass yields (pre-harvest) prior to the slurry application were similar among treatments (Table 7.4). Following slurry and N fertilizer application, as expected, the RNF treatment resulted in the highest DM yield on both the first and second harvest, at 584 and 424 g DM m⁻². Ryegrass that received acidified slurry treatments had the lowest yields at first harvest, 29.5 and 42.0 g DM m⁻² (RGEM, RG), respectively. However, their yield increased significantly (P<0.05) at the second harvest. Total DM yield amount from two harvests show that RNF produced the highest yield with 1008.0 g DM m⁻², followed by RNAS, REM, RCT, RGEM, RG and RNC at 502.2, 378.1, 346.0, 272.3, 272.1 and 111.6 g DM m⁻², respectively.

Ryegrass total N concentration and content from the net DM yield are summarised in Table 7.5. At the first harvest, ryegrass with RNF had the highest N concentration, followed by the acidified slurry applications (RNF, RGEM, RG; 25.9,
18.5, 14.2 g N kg\textsuperscript{-1} DM) (Table 7.5). However, the concentration decreased significantly ($P<0.05$) to between 10.1 and 6.9 g N kg\textsuperscript{-1} DM in the following harvest. Other treatment groups showed a somewhat similar total N content with the untreated ryegrass (RNC). The ryegrass N offtake was significantly ($P<0.05$) greater for RNF followed by RNAS during the first harvest, while RGEM and RG showed the lowest N offtake from acidified slurries which is related to the DM yield. Later, it was found that the total N content in ryegrass from RGEM and RG groups increased to a similar range of other slurry treatments, but the total N content remained significantly greater ($P<0.05$) for the fertiliser treatment. The N uptake of the ryegrass receiving the slurry treatments at the second harvest ranged from 1.4 to 1.7 g N m\textsuperscript{-2} and altogether the total N uptake was between 2.1 and 3.7 g N m\textsuperscript{-2}. Yet the total uptake was significantly ($P<0.05$) highest in the fertiliser treatments, at 19.1 ±0.5 g N m\textsuperscript{-2}. The net N uptake efficiency reached its highest by the RNF, followed by RNAS and other slurry treatments groups (Table 7.5).

Table 7.4 Ryegrass dry matter yield during 1\textsuperscript{st} and 2\textsuperscript{nd} harvest after slurry application.

<table>
<thead>
<tr>
<th>Ryegrass treatment</th>
<th>Dry matter yield (g m\textsuperscript{-2})</th>
<th>Pre harvest</th>
<th>1\textsuperscript{st} harvest (a)</th>
<th>2\textsuperscript{nd} Harvest (b)</th>
<th>Total yield (a + b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RGEM</td>
<td></td>
<td>92.4 ±7.00</td>
<td>29.5 ±5.43\textsuperscript{d}</td>
<td>242.8 ±35.88\textsuperscript{bc}</td>
<td>272.3 ±40.07\textsuperscript{cd}</td>
</tr>
<tr>
<td>RG</td>
<td></td>
<td>110.6 ±8.33</td>
<td>42.0 ±7.10\textsuperscript{d}</td>
<td>230.1 ±30.58\textsuperscript{b}</td>
<td>272.1 ±28.67\textsuperscript{cd}</td>
</tr>
<tr>
<td>REM</td>
<td></td>
<td>99.6 ±8.80</td>
<td>177.0 ±6.50\textsuperscript{c}</td>
<td>201.1 ±40.55\textsuperscript{bc}</td>
<td>378.1 ±46.66\textsuperscript{bc}</td>
</tr>
<tr>
<td>RCT</td>
<td></td>
<td>109.1 ±7.01</td>
<td>150.7 ±8.13\textsuperscript{c}</td>
<td>195.3 ±24.07\textsuperscript{bd}</td>
<td>346.0 ±31.67\textsuperscript{bc}</td>
</tr>
<tr>
<td>RNAS</td>
<td></td>
<td>104.3 ±7.81</td>
<td>251.2 ±14.36\textsuperscript{b}</td>
<td>251.0 ±40.10\textsuperscript{b}</td>
<td>502.2 ±48.38\textsuperscript{b}</td>
</tr>
<tr>
<td>RNC</td>
<td></td>
<td>109.1 ±4.35</td>
<td>41.5 ±2.80\textsuperscript{d}</td>
<td>70.1 ±30.42\textsuperscript{c}</td>
<td>111.6 ±32.26\textsuperscript{d}</td>
</tr>
<tr>
<td>RNF</td>
<td></td>
<td>100.0 ±7.51</td>
<td>583.7 ±34.89\textsuperscript{a}</td>
<td>424.4 ±28.27\textsuperscript{a}</td>
<td>1008.1 ±38.44\textsuperscript{a}</td>
</tr>
</tbody>
</table>

Mean\textsuperscript{s} in columns with no common superscript differ significantly ($P\leq0.05$). Values represent means ±SEM, $n=5$. 
Table 7.5 Total nitrogen content and nitrogen offtake of the harvested ryegrass.

<table>
<thead>
<tr>
<th>Ryegrass treatment</th>
<th>Total N (g N kg⁻¹ DM)</th>
<th>Nitrogen offtake (g N m⁻²)</th>
<th>Nitrogen uptake efficiency** (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-harvest</td>
<td>1st harvest</td>
<td>2nd harvest</td>
</tr>
<tr>
<td>RGEM</td>
<td>8.2 ±0.25</td>
<td>18.5 ±1.88b</td>
<td>7.0 ±0.32b</td>
</tr>
<tr>
<td>RG</td>
<td>8.3 ±0.26</td>
<td>14.2 ±1.37b</td>
<td>6.9 ±0.28b</td>
</tr>
<tr>
<td>REM</td>
<td>8.0 ±0.13</td>
<td>7.4 ±0.11c</td>
<td>7.1 ±0.45b</td>
</tr>
<tr>
<td>RCT</td>
<td>8.3 ±0.13</td>
<td>7.7 ±0.32c</td>
<td>7.5 ±0.44b</td>
</tr>
<tr>
<td>RNAS</td>
<td>8.3 ±0.33</td>
<td>7.8 ±0.21c</td>
<td>7.0 ±0.30b</td>
</tr>
<tr>
<td>RNF</td>
<td>8.8 ±0.15</td>
<td>25.9 ±2.13a</td>
<td>10.1 ±0.79a</td>
</tr>
<tr>
<td>RNC</td>
<td>8.6 ±0.24</td>
<td>8.3 ±0.97c</td>
<td>9.2 ±1.01ab</td>
</tr>
</tbody>
</table>

Means in columns with no common superscript differ significantly (P≤0.05). Values represent means ±SEM, n=5.

Note: **Nitrogen uptake efficiency is calculated based on available NH₄-N in slurry m⁻²

7.4 Discussion

7.4.1 Limitation of measurement

There are several factors to be considered in evaluating the measurements made in this experiment. The measurements of NH₃ and GHG (CH₄ and N₂O) during the slurry-ryegrass application in this Chapter were influenced by large headspace volume and small experimental surface area. The amount of NH₃ loss during volatilisation was measured by the amount of NH₄⁺ that was dissolved into H₃PO₄ solution. This process indirectly represents the lost NH₃. This study measured NH₃ losses in a static airflow as compared to other studies (Fangueiro et al., 2008a; Lovanh et al., 2010; Carozzi et al., 2013; Bourdin et al., 2014), which may not represent the actual loss in the static chamber and may underestimate the NH₃ loss by up to 70% (Jantalia et al., 2012; Miola et al., 2015). In addition, the large headspace and side-space on the vessel (refer to Figure 7.1a, b) during gas sampling may result in a large standard error of the mean on gas sampling due to the low chamber volume to surface area ratio (10.5:11.6; mL: cm²) (Baker et al., 2003; Sapkota et al., 2014).

As recommended by Baker et al., (2003) and De Klein and Harvey, (2012), the static chamber should not be deployed for more than 60 minutes to reduce the effect...
of O\textsubscript{2} depletion, thus our one-hour sampling period may not be enough to trap the accumulated gases from the small measurement area. Therefore, the results are better used for comparative purposes within the experiment.

7.4.2 Ammonia loss during slurry-ryegrass application

Ammonia loss during slurry application is unavoidable, but it can be reduced by managing a few factors such as slurry pH, slurry DM, timing and method of application. Estimated NH\textsubscript{3} loss is relatively high immediately after the application, but will decrease within 48 hours. In this study, the pH level for the acidified slurries (RGEM and RG) was at 4.2. The pH level was much lower than the pH range (between pH 5.5 and pH 6.3) of mineral acid acidified slurries used in other studies (Stevens et al., 1992; Clemens et al., 2002; Ottosen et al., 2009; Sørensen and Eriksen, 2009). This resulted in an average 89\% relative inhibition compared to the non-modified aged slurry (RNAS). The percentage was moderately greater than described by earlier authors at 18 and 75\%, but equally similar to the untreated RCT slurry (76\%) obtained from the same storage of induced acidified slurries. Meanwhile, the N loss in the form of NH\textsubscript{3} from the N fertiliser treatment was relatively equal to the amount lost in acidified slurry as N fertilizer was dissolved in distilled water and immediately infiltrated the soil. In addition, NH\textsubscript{3} losses from N fertiliser were low, between 3.0-7.7\% (Misselbrook et al., 2010). High N losses were seen from the non-modified slurry (RNAS) compared to the inorganic N fertilizer (RNF), indicating the importance to reduce the N loss during slurry application.

In earlier observations during the slurry storage experiment in Chapter 4B, large number of maggots were found in the non-acidified (FEM and F Ctrl) slurries during summer storage, and a crust was not well formed. The slurry pH was slightly alkaline (pH 8.5) and DM relatively high (between 11.7 to 19.5\%). However, the NH\textsubscript{4}+N content was much lower than in the RNAS treatment, which subsequently caused significantly greater NH\textsubscript{3} volatilisation compared to the acidified slurry following slurry application. De Baere et al., (1984) reported the presence of free NH\textsubscript{3} in the total NH\textsubscript{3}-N at pH 7.0 of 1.1\%, in comparison to 10.2\% of free NH\textsubscript{3} when the pH level was increased to 8.0. Yet, this study shows slightly higher pH levels at 8.4 and 8.5 for REM and RCT, respectively. Significant NH\textsubscript{3} loss (P<0.05) was also observed in the RNAS treatment, which was similar to RCT treatment as both treated samples had a pH of 7.5.
Following slurry application, the reported NH₃ loss is influenced by a few factors such as ambient air temperature or environment weather and wind disturbances (Kai et al., 2008). In fact, losses can reach up to 100% of the slurry NH₄-N content under certain circumstances (Misselbrook et al., 1996). Slurry DM content and soil moisture conditions influence slurry infiltration rate into the soil; faster slurry infiltration will result in lower NH₃ volatilisation. Moreover, slurry infiltration is much slower in soils with high moisture content, especially after rainfall (Sommer and Jacobsen, 1999). Diluted slurry or lower DM content slurries infiltrate the soil quicker, as demonstrated by Rubæk et al., (1996) who reported 35% of NH₃ emission from the digested slurry compared to 45% of NH₃ emission from non-digested slurry. Alternatively, the infiltration process could be further boosted by actively placing slurries on a few mediums such as on the soil surface (trailing shoe or hose), in the soil (injection), or mixing into the soil (harrow) (Misselbrook et al., 1996; Smith et al., 2000; Wulf et al., 2001b; Rodhe et al., 2006; Carozzi et al., 2013). These approaches are found to be effective to reduce NH₃ volatilisation and increase the available N of slurry for crop uptake. However, are these approaches viable in small-medium scale farms?

7.4.3 Greenhouse gas emission

I. Methane and nitrous oxide emission

Methane emission is short-lived following slurry application, and this was clearly seen with immediate CH₄ fluxes observed from the RNAS, REM and RCT treatments, demonstrating a typical emission after slurry application. These fluxes were probably related to the CH₄ trapped in the slurry during the storage period. Chadwick and Pain, (1997) suggested that the CH₄ released from the slurries follows an organic decomposition of the volatile fatty acid fraction, before being further oxidized to CH₄ by methanogens existing in the anaerobic environment. Further, several studies also found similar findings (Chadwick and Pain, 1997; Wulf et al., 2001a, 2001b; Cardenas et al., 2007; Fangueiro et al., 2008a). Rodhe et al., (2006) and Fangueiro et al., (2008b) reported longer periods of CH₄ emission following slurry application, possibly the result of continued anaerobic fermentation after 48 hours of slurry application (Wulf et al., 2001b).

Very low, or no CH₄ is emitted after 48 hours as reported by Fangueiro et al., (2008a) which is similar to our finding. This is due to the diminishing slurry
anaerobic microenvironment due to soil infiltration and exposure of methanogens to O$_2$ (Wulf et al., 2001b). Meanwhile, very low emission levels in RGEM and RG were related to the high H$^+$ concentration (and low slurry pH) inhibiting methanogenesis. Acidification causes major reduction in the amount of CH$_4$ emitted (Berg et al., 2006a; Petersen et al., 2012; Wang et al., 2014).

In a previous study (Chapter 4), the addition of fermentable carbohydrate (brewing sugar) helped promotes acidification of the slurry during the storage period and resulted in a significantly acidic slurry (pH 4.2). As a result, the fluxes were 98 times lower compared to RNAS and RCT. Further, the acidified slurries (RGEM and RG) emitted cumulative emission less than half of the CH$_4$ than non-acidified slurry (REM, RCT, RNAS) indistinguishable to the untreated ryegrass treatment (RNC). Meanwhile, the higher total CH$_4$ emission by RNF compared to RNC was associated with the increase of atmospheric CO$_2$ (Saarnio et al., 2000) and inhibition of CH$_4$ oxidation (Kara and Özdilek, 2010). On the other hand, negative fluxes were recorded in day 30 and 60 from REM and RCT treatments (Appendix 7.1a). The possible reason for the negative fluxes was that simultaneously oxidation in the soil as emission occurred (Kammann et al., 2001; Bourdin et al., 2014).

There was a slight increase of N$_2$O fluxes from the slurries and N fertilizer applications on day 1, compared to the untreated ryegrass treatment (Appendix 7.1b). Nitrous oxide fluxes were higher from the slurry treatments than the N fertilizer, although the differences were not significant ($P=0.052$). Later, the N$_2$O fluxes remained similar for all slurry groups for the entire experiment, with the exception for the fluxes on the 60$^{th}$ day. These fluxes may have been influenced by drying over the warm weekend which preceded the gas sampling. As a result, a reduction in the percentage soil water filled pore space (WFPS) occurred before the next watering schedule. A lower % WFPS could have resulted in greater O$_2$ diffusion into the soil, favouring nitrification and production of N$_2$O (Klemmedtsson et al., 1988; Bateman and Baggs, 2005), and/or generation of NO$_3^-$ that was subsequently denitrified in anaerobic microsites.

Cumulative N$_2$O emissions were numerically greater from the treated pots than the controls (RNC). This could possibly be explained by the presence of the available C to stimulate the denitrification activity and O$_2$ consumption (Velthof et al., 2003). Data in Figure 7.3c and Figure 7.3d showed no significant difference
in this small-scaled pot study, even though a larger sampling area may produce result that best represent the N\textsubscript{2}O emissions of a grassland area (Velthof et al., 2003). In addition, slurry broadcast onto the soil surface results in high NH\textsubscript{3} loss reducing the pool of NH\textsubscript{4}\textsuperscript{+} for subsequent N\textsubscript{2}O emission. Hence, N\textsubscript{2}O fluxes are considered to be lower from surface broadcast slurry compared to the close-injection of slurry into the soil (Rodhe et al., 2006). With attention to environmental issues, avoiding N\textsubscript{2}O emission is important since N\textsubscript{2}O constitute potent gas with a radiative force 298 greater than CO\textsubscript{2} on per unit mass in a 100-year time horizon (Myhre et al., 2013).

II. Total GHG emission

This study showed that the total GHG CO\textsubscript{2} eq. (CH\textsubscript{4} and N\textsubscript{2}O) emissions were greatest in the treatment applied with N fertilizer (RNF) at 58.7% higher than RNC (Table 7.3). In comparison, ryegrass that received slurry treatments emitted lower total GHG CO\textsubscript{2} eq. than RNF, between 12 to 48%. These emissions from the slurry treatments were about 7 to 29% lower if compared to the RNF treatment. The application of N fertilizer in agriculture has been related to a decrease in CH\textsubscript{4} oxidation in soil, by about 30% (Ojima et al., 1993; Kammann et al., 2001). Although this study could only demonstrate a few sampling points that showed oxidation activity, it does not indicate that there was no oxidation process at all. The low oxidation activity could be attributed to the high CH\textsubscript{4} production rate as a result from the increasing number of easily degradable organic C in the soil.

Total GHG emission following slurry application was influenced by CH\textsubscript{4} emission, with lower emission rates from the acidified slurry treatments. Moreover, although the recorded N\textsubscript{2}O emission was very small, it contributed to at least 83% of the GHG CO\textsubscript{2} eq. emission. This percentage is higher than the figure reported by Fangueiro et al., (2008b) where N\textsubscript{2}O was only responsible for up to 75% of the total GHG CO\textsubscript{2} eq.

Although a full-scale integrated evaluation of GHG emissions from slurry storage and application is needed to produce robust and credible results, data obtained from this pot experiment suggest that self-acidified slurry (by anaerobic fermentation of brewing sugar) can strongly reduce the net GHG emission relative to untreated or non-acidified slurry. Earlier, the net GHG emitted by ‘self-acidified’ slurry during storage (summer storage; Chapter 4.3.2) showed GHG emission
was lower by ca. 73% to 82% (specifically CH₄ emission) and later lower emission between 54.9% and 64.7% during ryegrass-slurry application. This could be further explored at the farm scale. Furthermore, Martyniuk et al., (2002) and Chadwick and Laws, (2002) outlined the benefits of slurry application practice to boost soil OM, total bacteria number, total N and soil respiration, and to recycle nutrients, reducing the chemical fertilizer input and reducing farm spend on chemical fertilizers.

7.4.4 Ryegrass yield and nitrogen uptake

Application of self-acidified slurry to the ryegrass results in acute acidity and subsequently led to acute stress and death of some of the grass sward, due to the very low pH soil (<5.5) (Hart et al., 2011). The acute acidity however is temporary and can possibly reduce by reducing the rate from 200 kg⁻¹ N to 100 kg⁻¹ N or lower concentration with double or higher application number of spreading to the ryegrasses. Later, gradual alkalisation resulting from decarboxylation re-stabilised the pH level to a normal range (Rukshana et al., 2011). In response to the high acidity, low DM yields were measured from the RGEM and RG treated ryegrass during the first harvest. Nevertheless, the yield levels increased at the following harvest (2nd), which is similar to the findings by Misselbrook et al., (1996). This recovery of yield at the second harvest appeared to be the result of longer and wider leaf areas of the grass in the acidified slurry pots. Higher yield reflects conservation of slurry-NH₄⁺ in the soil and N immobilisation, which is later made available for uptake by mineralisation process after the first harvest. The highest total DM yield was observed from the N fertiliser treatment at 1008 g DM m⁻², as a result of N uptake from the supplied N. Similarly, higher DM yields compared to RNC was also observed in other slurry treatments while the lower DM yield in the RNC indicates suppressed or poor N availability in the soil.

During the study, the ryegrass total N content from REM, RCT, RNAS treated was similar to RNC as the N was lost earlier through volatilisation during storage and/or during spreading. The volatilisation rate was about 94% higher than the acidified slurries applied during the first hour after application. Volatilisation was also indirectly influenced by higher pH level above pH 6.5 (Dai and Blanes-Vidal, 2013). The net N offtake from the second harvest indicated N fertilizer (RNF) applied ryegrass had the greatest total N value at 4.2 g N m⁻², whilst the ryegrass applied with slurries had an N value within the range of 1.4 to 1.7 g N m⁻². The N offtake in RGEM and RG could have
been higher if the ryegrass had not been affected by the low slurry pH. Overall, the total N uptake was equally similar to the other slurry treatments (REM, RCT), with slightly lower compared to RNAS ryegrass treated. The low percentage of net N uptake efficiency from acidified slurries were related to the ryegrass DM yield which was caused by the acute death.

7.5 Conclusion

Findings from this study show that stored acidified slurry produced by the fermentation process of brewed sugar significantly retained N and resulted in minimal NH$_3$ loss after application to ryegrass, with NH$_3$ emission reductions of 84% and 32% compared to the aged slurry (neutral pH; RNAS). Similarly, the acidified slurry also reduced CH$_4$ emissions by between 30% and 100% within the same period. The highest total ryegrass yield from the two harvests were significantly greater from the N fertilizer treatment, ca. 8 times higher than the untreated ryegrass treatment, followed by the ryegrass applied with non-acidified slurry, with the acidified slurries resulting in the lowest yield. Nonetheless, it is possibly to manage slurry acidity level by either controlling the induced acidification process or by increase the slurry pH prior to spreading (naturally or additives such as limestone), or applying slurry via injection of band spreading, to avoid reduction of the ryegrass yield. This mitigation study is worth pursuing in other crops or grass types which can tolerate reduced slurry pH.
### 7.6 Appendix

Appendix 7.1: GHG fluxes from various amended slurries and N fertilizer application

a) Methane dynamics following slurry treatment applications to ryegrass during the 60 days experiment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>hour</th>
<th>Day</th>
<th>1</th>
<th>0</th>
<th>1</th>
<th>3</th>
<th>6</th>
<th>10</th>
<th>13</th>
<th>30</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>RGEM</td>
<td>75.3 ±23.64</td>
<td>34.2 ±29.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-14.9 ±11.80</td>
<td>35.6 ±16.67</td>
<td>84.0 ±18.90</td>
<td>96.0 ±17.32</td>
<td>94.6 ±24.16</td>
<td>7.1 ±12.88</td>
<td>11.9 ±9.87</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RG</td>
<td>75.0 ±22.34</td>
<td>-22.4 ±17.75&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32.7 ±17.09</td>
<td>32.0 ±11.79</td>
<td>58.4 ±8.24</td>
<td>87.4 ±41.20</td>
<td>64.6 ±19.88</td>
<td>39.9 ±34.54</td>
<td>-1.6 ±3.84</td>
<td></td>
<td></td>
</tr>
<tr>
<td>REM</td>
<td>71.4 ±30.76</td>
<td>3823.2 ±976.67&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>52.1 ±24.52</td>
<td>29.6 ±39.33</td>
<td>41.9 ±12.99</td>
<td>93.2 ±66.92</td>
<td>91.5 ±49.50</td>
<td>-29.7 ±19.59</td>
<td>4.7 ±25.32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RCT</td>
<td>49.6 ±11.38</td>
<td>4054.5 ±562.12&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>129.4 ±50.32</td>
<td>59.3 ±35.18</td>
<td>62.2 ±20.37</td>
<td>36.0 ±16.02</td>
<td>87.1 ±23.15</td>
<td>-24.2 ±13.22</td>
<td>-6.7 ±31.01</td>
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<td></td>
</tr>
<tr>
<td>RNAS</td>
<td>8.9 ±19.82</td>
<td>3442.1 ±349.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>95.2 ±20.87</td>
<td>43.9 ±18.53</td>
<td>57.5 ±19.77</td>
<td>67.5 ±12.25</td>
<td>91.1 ±36.26</td>
<td>34.0 ±17.11</td>
<td>6.5 ±7.79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNC</td>
<td>61.3 ±44.42</td>
<td>30.7 ±8.83&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>54.7 ±40.15</td>
<td>38.0 ±15.07</td>
<td>35.2 ±12.75</td>
<td>28.4 ±18.84</td>
<td>107.0 ±35.84</td>
<td>39.9 ±21.13</td>
<td>-4.6 ±19.62</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNF</td>
<td>73.9 ±40.52</td>
<td>3.5 ±21.16&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>23.8 ±19.31</td>
<td>31.1 ±12.13</td>
<td>42.4 ±14.18</td>
<td>150.2 ±25.52</td>
<td>88.2 ±29.98</td>
<td>49.5 ±26.49</td>
<td>22.7 ±14.02</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P value (n=5)*

|       | 0.889 | <0.001 | 0.121 | 0.737 | 0.767 | 0.127 | 0.799 | 0.333 | 0.140 |

Means in columns with no common superscript differ significantly (P≤0.05). Values represent means ±SEM, n=5
b) Nitrous oxide dynamics following slurry treatment applications to ryegrass during the 60 days experiment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>hour</th>
<th>Day</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>6</td>
<td>10</td>
<td>13</td>
<td>30</td>
<td>60</td>
</tr>
<tr>
<td>RGEM</td>
<td>17.2 ±6.34</td>
<td>3.2 ±18.07</td>
<td>72.0 ±28.15</td>
<td>11.9 ±11.91</td>
<td>16.2 ±15.36</td>
<td>50.2 ±20.89</td>
<td>71.9 ±15.66</td>
<td>26.8 ±21.71</td>
</tr>
<tr>
<td>RG</td>
<td>-27.9 ±23.91</td>
<td>-3.2 ±14.51</td>
<td>57.3 ±8.18</td>
<td>18.6 ±18.59</td>
<td>59.3 ±10.77</td>
<td>19.5 ±15.75</td>
<td>35.7 ±13.01</td>
<td>68.6 ±20.05</td>
</tr>
<tr>
<td>REM</td>
<td>39.6 ±10.47</td>
<td>-13.0 ±6.07</td>
<td>98.2 ±23.14</td>
<td>28.2 ±28.17</td>
<td>34.0 ±9.70</td>
<td>-2.7 ±24.46</td>
<td>59.0 ±32.34</td>
<td>5.7 ±8.83</td>
</tr>
<tr>
<td>RCT</td>
<td>-1.5 ±9.62</td>
<td>8.1 ±19.20</td>
<td>36.8 ±17.29</td>
<td>27.9 ±27.93</td>
<td>47.3 ±23.99</td>
<td>46.5 ±30.69</td>
<td>36.4 ±10.87</td>
<td>30.1 ±22.15</td>
</tr>
<tr>
<td>RNC</td>
<td>7.9 ±10.79</td>
<td>-19.5 ±25.86</td>
<td>4.1 ±31.83</td>
<td>25.3 ±25.26</td>
<td>25.4 ±12.82</td>
<td>14.0 ±18.75</td>
<td>34.4 ±20.85</td>
<td>13.8 ±9.80</td>
</tr>
<tr>
<td>RNF</td>
<td>-18.3 ±25.79</td>
<td>-12.2 ±6.95</td>
<td>8.2 ±9.45</td>
<td>18.3 ±18.31</td>
<td>93.3 ±20.88</td>
<td>36.7 ±21.31</td>
<td>55.3 ±27.34</td>
<td>45.8 ±29.68</td>
</tr>
</tbody>
</table>

*P value (n=5)*  
0.877 0.052 0.112 0.069 0.069 0.640 0.640 0.423 0.756

Means in columns with no common superscript differ significantly (P≤0.05). Values represent means ±SEM, n=5.
CHAPTER 8

8 Do microbial diversity dynamics help explain the possible mechanism of self-induced acidification of slurry?

Abstract

Storing slurry in a lagoon or storage tank is known to contribute to GHG emission through the release of CH$_4$ as the end product of anaerobic fermentation. Previous findings illustrate the mitigation of CH$_4$ emission by self-induced acidification was related to the high lactic acid production following addition of a carbohydrate source. In this study, microbial diversity and dynamics (bacteria and archaea) in stored slurry at $<15^\circ$C amended with brewing sugar and stored were evaluated using a metagenomics approach through next generation sequencing (NGS) using an Illumina MiSeq platform. The 16S ribosomal ribonucleic acid (rRNA) sequences data from the two sampling points (0d and 30d) revealed the presence of the Order of Lactobacillales that was responsible for the lactic acid production. The operational taxonomic units (OTUs) abundance indicates the dynamics of the slurry bacteria over the storage period. The methanogenic community was dominated by hydrogenotrophic methanogens from the member Order of Methanobacterales, Methanomicrobiales, and Methanosarcinales. The decrease of intolerant methanogen OTUs abundances in acidified slurry was probably the main reason for the lower CH$_4$ emission during the storage period. The findings of this study confirm the mechanism of inhibiting CH$_4$ production at the microbial level during slurry storage under a temperate climate. Further research is required to determine the dynamics of this CH$_4$ inhibition mechanism under warmer, mesophilic, conditions.

Key words: slurry storage, microbial diversity, sequencing, lactic acid bacteria, archaea, hydrogenotrophic methanogen
8.1 Introduction

The world reached its historic 7 billion human population in 2011 and recorded over 7.34 billion in 2015 (Haub and Gribble, 2011; Population Reference Bureau, 2016). However, this planet is actually dominated (numerically) by microorganisms whose populations are umpteenth times greater than the human population. Microorganisms are important in regulating carbon and nutrient cycles within terrestrial ecosystems and are also considered as important drivers in regulating the earth’s biogeochemical cycles (Van Der Heijden et al., 2008). Moreover, the extinction of microorganisms may possibly end human civilization.

It is known that microbes carry out diverse switchable metabolic modes in their normal life cycle, and within a staggering range of physiochemical conditions. Microorganisms respond to surrounding factors such as nutrient concentrations, acidity, temperature and interaction with other microorganisms and higher organisms. They produce metabolites, enzymes, peptides, antibiotics, and lipids. However, any intolerable physiochemical changes may lead to microbial diversity changes. Furthermore, abundance of certain species may become highly competitive, thus decreasing abundance and/or activities of other species (Hooper et al., 2005). Microbial diversity and richness are characteristic of complex structured communities in terms of their resource usage, nutrient retention and productivity rates (Hooper et al., 2005). Studying microbial diversity from any source or location results in: i) an understanding of the microbial richness or total number of species, and ii) knowledge of the distribution of individual species (Fakruddin and Mannan, 2013). This information can be used to explain the functional traits, types and diversity of microorganisms within different ecosystems (Hooper et al., 2005).

Microbial diversity measurements may not completely discover the entire community’s diversity and richness. In addition, any method or technology used is not without their own limitations (Kirk et al., 2004). The global ecosystem microbial diversity is broad, thus metagenomics studies focus on specific areas such as soil, marine, agriculture and livestock (Fierer et al., 2007; Ekkers et al., 2012; Kodzius and Gojobori, 2015). In livestock; (more specifically in rumen); gastro-intestinal microbial diversity is explored to help understand the microbe’s functional traits during fermentation and nutrient uptake. Meanwhile, microbial diversity in manure has been extensively explored to provide insights into factors controlling degradation or biodecomposition of organic matter (Ouwerkerk and Klieve, 2001; Tiquia, 2005; Peu et
al., 2006; van Vliet et al., 2006; Wang et al., 2009; Song et al., 2010; Liu et al., 2011; Scherr et al., 2012; St-Pierre and Wright, 2013; Zhao et al., 2013). However, information on microbial communities during cattle slurry storage is less well known. Previously, van Vliet et al., (2006) conducted a semi-quantitative study using the denaturing gradient gel electrophoresis (DGGE) method to determine the bacterial community in cattle slurry. Through this approach, microbial diversities were classified and grouped but not identified. On the other hand, some researchers have categorised the manure microbial diversity by DNA sequencing, but these studies only focussed on pig and poultry faeces and manure (Whitehead and Cotta, 2001; Snell-Castro et al., 2005). Other studies have focussed on understanding the microbial diversity of compost (Ouwerkerk and Kliese, 2001; Tiquia, 2005; Liu et al., 2011; Zhao et al., 2013).

As slurries are stored for certain periods, anaerobic fermentation occurs and the number of pathogenic bacteria decreases. This indicates the ever-changing microbial communities (Wentzel and Joergensen, 2015), despite the unknown required time shift. However, PCR–single-strand conformation polymorphism of pig slurry has showed changes in the dominant bacterial community during the first two weeks of slurry storage (Peu et al., 2006), but there is no similar published information for cattle slurry. Although there are a few studies on optimisation of biogas production that have explored microbial dynamics associated with bacterial degradation and methanogenesis (Dhadse et al., 2012; Abubaker et al., 2013; Zhao et al., 2013), mechanisms remain unclear. The microbial diversity and dynamics of cattle slurry are important and could be extremely useful in mitigating greenhouse gas (GHG) emissions from beef and dairy farms in the future.

We hypothesise that the slurry’s microbial diversity and relative function changes after the addition of a fermentable carbon source, which results in the abundance of lactic acid producing microbes. Subsequently, the pH shift (decrease) will affect the diversity of the methanogenic archaea communities and methanogenesis pathway. The study was carried out on slurry stored at temperatures typical of UK conditions (psychrophilic), as in Chapters 4 and 5. The results will possibly reveal the functional groups and diversity, as well as the microbial shift of the bacteria and archaea, specifically the methanogens after addition of a fermentable carbon source.
8.2 Materials and methods

Fresh slurry was obtained from a commercial farm near Henfaes Research Centre, near Bangor in North West Wales, and kept in a 250 L plastic container prior to removal of any large particulates of uneaten straw and hay. Slurry was then transferred into 30 L high-density polyethylene (HDPE) plastic drums, such that each drum contained a final weight of 20 kg with and without 10% \(^{w/w}\) of brewing sugar (Ritchie Products Ltd, Staffordshire, UK) addition. There were 4 replicates of two treatments: slurry + brewing sugar (10% \(^{w/w}\)) (BS), and untreated slurry (Ctrl). The experiment was carried out during the autumn season, at the end of October 2015. Initial slurry characteristics were 9.1 ±0.2% DM, 68.1 ±0.3% VS, 17.3 ±2.1 g C kg\(^{-1}\) FWt, 0.33 ±0.2 mg NH\(_4\) -N kg\(^{-1}\) FWt, 4.2 ±0.4 g N kg\(^{-1}\) FWt, and pH 7.0.

8.2.1 Greenhouse gas, pH, ORP and lactic acid measurement

Ammonia (NH\(_3\)) and methane (CH\(_4\)) emissions were measured on days 1, 4, 7, 14 and 30. These gaseous emissions were measured simultaneously during a 1-hour period when the drums containing the slurry treatments were fitted with lids and the same measurement methods used as described in previous chapters.

Meanwhile, slurry pH, ORP and temperature were measured in situ at 10-15 cm in depth using an electrode probe (Hanna HI 991003, Hanna Instrument, USA). Slurry subsamples (3 mL) were taken from the same spot without shaking or disturbing the formed slurry crust. The subsamples were used for lactic acid determination. Additional samples (2 mL) were obtained on 0 d and 30 d for metagenomics analyses.

8.2.2 Metagenomics of the slurry treatments

I. DNA extraction,

DNA was extracted from the sub-samples of the slurry treatments on 0 d and 30 d samples using a PowerFecal DNA Isolation kit (MO BIO, California USA). The quality of the extracted DNA was quantified using Nano Drop 1000 (Thermo Scientific, UK) and stored at -20°C. The best DNA quality of three samples from each treatment from 0 d and 30 d were used for further DNA sequencing. However, due to low DNA quality on 0d samples, only two Ctrl and four BS samples were used (Ctrl, \(n=2\); BS \(n=4\)).
II. Amplicon library design, barcoding and sequencing

The library design, polymerase chain reaction (PCR), and barcoding were prepared and conducted by the Centre of Genomic Research, Liverpool, UK. In brief, double PCR steps were carried out to amplify the targeted V4 region of 16S ribosomal ribonucleic acid (rRNA) genome, followed by incorporation of the sequence barcode for tagging purpose. The Universal PCR primers targeting V4 region used in the study known as F515/806R, following Amore et al. (2016) and Caporaso et al., (2011), while the 8 base pair (bp) barcode sequence tagging (N501F and N701R) is illustrated in Figure 8.1. The PCR reactions for V4 region amplification were held at initial denaturation temperature of 98°C for 2 min, amplification at 95°C for 20 seconds (s), 65°C for 15s, 70°C for 30 s, and final extension of 5 min at 72°C. Following the PCR amplification, the genome sequence was analysed by an Illumina MiSeq paired-end (2x250PE) sequencing platform on a MiSeq flow cell after pooling the amplicon samples. This identification sequence was on ‘read’ bases (A, T, C and G bases) of the oligos RNA on the targeted RNA region.
A: Universal PCR primers sequence
515F:
5’ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNGTGCCAGCMCGCCGGTAA3’

806R:
5’GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGACTACHVGGGTWTCTAAT3’

B: Barcode tagging sequence
N501 F:
5’ AATGATACGGCGACCACCGAGATCTACAC TAGATCGCAACTCTTTCCCTACACGACGCTC 3’

N701 R
5’ CAAGCAGAAGACGGCATACGAGATCGCCTTA GTGACTGGAGTTCAGACGTGTGCTC 3’

Figure 8.1 The forward and reverse (A) universal PCR primers and (B) tagging barcode sequence used during the next generation sequencing by Illumina MiSeq platform.
Note: The primers targeted region sequences are in BOLD (A); The ITALIC underline sequences are the 8 bp barcode used for sample tagging and identification (B).
8.2.3 Data analysis

I. Physiochemical changes, ammonia and methane emission

Any observed changes on the physiochemical characteristic (pH, ORP, dry matter: DM, volatile solid: VS, lactic acid content) were analysed using a t-test using Minitab 17 (Minitab Ltd. Coventry, UK) statistical software. The confidence interval of these analyses was set at 95%. Similarly, the NH₃ and CH₄ emission fluxes and cumulative emission were analysed using the same statistical procedure.

II. Microbiological observation and analysis

Following PCR genome amplification and identification of RNA oligos by the Illumina MiSeq 250PE sequencer analyser, millions of reads, possibly gigabase (Gb) sequenced data were trimmed to remove the sequence adapter. This provided cleaned and quality data for sequence mapping and pipeline analysis. The bioinformatics and ANOVA multivariate analyses between ‘treatments’ and ‘period’ (days) categories were conducted by the Centre of Genomic Research, Liverpool, UK.

Briefly, the obtained sequence data were trimmed using the Cutadapt version 1.2.1 (Martin, 2011) and Sickle version 1.200 after removing any reads shorter than 10bp, thus providing between 6.4 x10⁵ and 2.6 x10⁶ number of reads. Polished or filtered sequenced data were later analysed for metagenomics analysis using QIIME 1.9.0 by clustering similar sequences into groups to define the operational taxonomic units (OTUs) at 99% similarity. The relative abundance of each OTU is clustered using a minimum similarity threshold of 97% for the entire sequences length. The obtained result is assigned to ribosomal RNA (rRNA) gene reference using greengenes 13.8 database for OTUs. Taxonomic cluster and group were also assigned. The sequencing bioinformatics and explorative analyses of the OTUs were simplified in the diagram below (Figure 8.2).
Figure 8.2 The bioinformatics analysis process and explorative analysis of the OTUs.
8.3 Results

8.3.1 Physiochemical observation and greenhouse gas emission

As expected, the addition of the carbohydrate source (brewing sugar) resulted in rapid lactic acid production and the subsequent lactic acid accumulation. Hence there was a significant decrease ($P<0.05$) in the slurry pH and an increase of slurry ORP ($P>0.05$) (Table 8.1; Figure 8.3a, b). BS slurry remained acidic even until 70 d (pH 3.3; Table 8.1), i.e. a considerable time after the 30 d sampling for metagenomics analysis. During the storage period, a large amount of lactic acid was generated within the treated slurry (2.3 mg kg$^{-1}$ FWt; $P<0.05$), compared to the untreated Ctrl treatment (Table 8.2).

The estimated NH$_3$ loss through volatilisation was lower from BS, even though the losses were not significantly different between the two treatments at most measuring points, except 7 d (Figure 8.3c) during the first week storage compared to Ctrl slurry. Yet, the cumulative NH$_3$ emission was significantly lower ($P<0.05$) from BS than Ctrl, a reduction of ca. 60% during the 30 d storage period. The BS and Ctrl cumulative NH$_3$ emissions were 174 ±58 µg NH$_3$ m$^{-2}$ and 435±78 µg NH$_3$ m$^{-2}$, respectively (Figure 8.3d). Similarly, the CH$_4$ fluxes from BS were dramatically reduced ($P<0.05$) by an average of 97% compared to Ctrl after the slurry pH decreased to below pH 5.0 (4 d: pH 4.7; Figure 8.3a). The differences in cumulative CH$_4$ emission between both BS and Ctrl were found to be 95% times lower in BS (0.01 g kg$^{-1}$ VS; $P<0.05$) than Ctrl, which had a yield of 12.9 g kg$^{-1}$ VS (Figure 8.3e, f).
Table 8.1 Slurry physiochemical properties before and after storage period.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (Ctrl)</th>
<th>Brewing sugar (BS)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Start experiment -day 0</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.0 ±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.7 ±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Oxidation redox potential (mV)</td>
<td>-279 ±5.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-243 ±6.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dry matter (% FWt)</td>
<td>9.0 ±0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14.7 ±0.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Volatile solids (% DM&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>680.1 ±3.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>81.6 ±0.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>NH&lt;sub&gt;4&lt;/sub&gt;-N (µg N kg&lt;sup&gt;-1&lt;/sup&gt; FWt)</td>
<td>326.1 ±17.58&lt;sup&gt;a&lt;/sup&gt;</td>
<td>360.0 ±3.98&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total C (g kg&lt;sup&gt;-1&lt;/sup&gt; FWt)</td>
<td>17.3 ±2.31&lt;sup&gt;b&lt;/sup&gt;</td>
<td>49.0 ±2.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total N (g kg&lt;sup&gt;-1&lt;/sup&gt; FWt)</td>
<td>4.2 ±0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.6 ±0.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Subsample -day 30</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>6.5 ±0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.4 ±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Oxidation redox potential (mV)</td>
<td>-232 ±11.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-62 ±15.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>NH&lt;sub&gt;4&lt;/sub&gt;-N (µg N kg&lt;sup&gt;-1&lt;/sup&gt; FWt)</td>
<td>350.9 ±23.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>394.0 ±7.17&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>End of experiment -day 70</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.2 ±0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.3 ±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Oxidation redox potential (mV)</td>
<td>-232 ±16.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-35 ±11.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dry matter (% FWt)</td>
<td>5.3 ±0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.7 ±5.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Volatile solids (% DM&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>66.4 ±0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>81.9 ±0.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>NH&lt;sub&gt;4&lt;/sub&gt;-N (µg N kg&lt;sup&gt;-1&lt;/sup&gt; FWt)</td>
<td>394.0 ±36.6</td>
<td>265 ±51.7</td>
</tr>
<tr>
<td>Total C (g kg&lt;sup&gt;-1&lt;/sup&gt; FWt)</td>
<td>15.9 ±0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>37.2 ±1.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total N (g kg&lt;sup&gt;-1&lt;/sup&gt; FWt)</td>
<td>2.9 ±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.5 ±0.52&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table 8.2 Temporal lactic acid dynamics in slurry amended with brewing sugar during the storage period.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Lactic acid content (Day / µg kg&lt;sup&gt;-1&lt;/sup&gt; FWt)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>BS</td>
<td>4.3 ±0.18</td>
</tr>
<tr>
<td>Ctrl</td>
<td>1.4 ±0.17</td>
</tr>
</tbody>
</table>
Figure 8.3 Brewing sugar effect on slurry pH, ORP, NH₃ and CH₄ fluxes.
8.3.2 Bioinformatics analysis and OTUs observation.

The twelve samples of sequenced data are produced from 1 lane MiSeq PE sequencing (Illumina) and they varied from 645,154 to more than 2,000,000 raw reads, followed by further quality step and filtering to obtain the sequences with average quality of 99% (Table 8.3). These sequences consist of single pair which overlap almost entirely to its other pair by read pair alignment. They produce single sequence from each pair and denoted as R1/R2 on the Table 8.3. Any error or undetectable sequence, which does not have similarity to the database, is known as singleton and found to be low, with the highest rate at a mere 0.1% compared to the obtained read pairs. The number sequences of read that matched the greengene 13.8 database showed a high clustered percentage with an average 99% aligned sequences (Table 8.3). The pooled pair sequences assembled specific OTU found that 72,161 taxa were assigned, which represent nearly 70,000 bacterial taxa and over 1,100 archaeal taxa, as shown in Table 8.4. The OTU richness index of individual slurry sample or known as the ‘alpha diversity’ is explained by the metrics in Figure 8.4. All samples were found to be highly and equally diverse. However, a sample labelled as ‘Cattle slurry 5c’ is not shown, as it failed to reach the best trade off species richness during the rarefaction process. Hence, it was dropped from the taxonomic coverage assessment (Appendix 8.1). The OTU abundancy statistical analysis is unique and is shown in Table 8.5, Table 8.6 and Table 8.7. Comparison of the abundance of OTU between Control and BS slurry at 0 d, shortly after the brewing sugar had been added to the BS slurry, showed significant composition \( (P<0.05) \) of *Bacteroides coprosuis* in Ctrl. However, the Control slurry did not show any significant differences between 0 d and 30 d. Meanwhile, a family of *Methanomassilicocccaceae* of the OTU48982 was found to be significantly different in the BS slurry between 0 d and 30 d samples. A comparison between the storage period of 0 d and 30 d found 10 significantly different OTUs. The OTUs matched to the one Order of *Clostridiales*, four Order of *Bacteroidales* and each phylum of *Euryarchaeota* and *Spirochaetes* (Table 8.7).
Table 8.3 Summary of sequence data for each sample, before and after adapter and quality trimming.

<table>
<thead>
<tr>
<th>No</th>
<th>Library</th>
<th>Treatment</th>
<th>Barcoding Index</th>
<th>Raw reads</th>
<th>Trimmed reads (%)</th>
<th>R1/R2</th>
<th>Singletons (%)</th>
<th>Number of assembled sequences to taxa (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sample_1</td>
<td>Cattle_slurry_2b</td>
<td>BS 0d</td>
<td>CGAGGCTG-TAGATCGC</td>
<td>1,663,286</td>
<td>1,661,146 (99.87)</td>
<td>829,820</td>
<td>1,506 (0.09)</td>
</tr>
<tr>
<td>2</td>
<td>Sample_2</td>
<td>Cattle_slurry_3b</td>
<td>BS 0d</td>
<td>CGAGGCTG-CTCTCTAT</td>
<td>1,413,048</td>
<td>1,411,437 (99.89)</td>
<td>705,071</td>
<td>1,295 (0.09)</td>
</tr>
<tr>
<td>3</td>
<td>Sample_3</td>
<td>Cattle_slurry_4a</td>
<td>Ctrl 0d</td>
<td>CGAGGCTG-TATCCTCT</td>
<td>1,455,572</td>
<td>1,454,239 (99.91)</td>
<td>726,454</td>
<td>1,331 (0.09)</td>
</tr>
<tr>
<td>4</td>
<td>Sample_4</td>
<td>Cattle_slurry_6a</td>
<td>Ctrl 0d</td>
<td>CGAGGCTG-AGAGTAGA</td>
<td>1,510,522</td>
<td>1,509,076 (99.90)</td>
<td>753,822</td>
<td>1,432 (0.09)</td>
</tr>
<tr>
<td>5</td>
<td>Sample_5</td>
<td>Cattle_slurry_7b</td>
<td>BS 0d</td>
<td>CGAGGCTG-GTAAGGA</td>
<td>1,712,748</td>
<td>1,710,719 (99.88)</td>
<td>854,539</td>
<td>1,641 (0.10)</td>
</tr>
<tr>
<td>6</td>
<td>Sample_6</td>
<td>Cattle_slurry_8b</td>
<td>BS 0d</td>
<td>CGAGGCTG-CTCTCTAT</td>
<td>2,002,366</td>
<td>1,999,663 (99.87)</td>
<td>998,911</td>
<td>1,841 (0.09)</td>
</tr>
<tr>
<td>7</td>
<td>Sample_7</td>
<td>Cattle_slurry_1c</td>
<td>Ctrl 30d</td>
<td>CGAGGCTG-AAGAGTA</td>
<td>1,881,444</td>
<td>1,879,388 (99.88)</td>
<td>938,831</td>
<td>1,726 (0.09)</td>
</tr>
<tr>
<td>8</td>
<td>Sample_8</td>
<td>Cattle_slurry_2d</td>
<td>BS 30d</td>
<td>CGAGGCTG-CTCTCTCT</td>
<td>1,910,738</td>
<td>1,908,390 (99.89)</td>
<td>953,304</td>
<td>1,782 (0.09)</td>
</tr>
<tr>
<td>9</td>
<td>Sample_9</td>
<td>Cattle_slurry_3d</td>
<td>BS 30d</td>
<td>AAGAGGCA-TAGATCGC</td>
<td>2,612,486</td>
<td>2,609,354 (99.88)</td>
<td>1,303,547</td>
<td>2,260 (0.09)</td>
</tr>
<tr>
<td>10</td>
<td>Sample_10</td>
<td>Cattle_slurry_4c</td>
<td>Ctrl 30d</td>
<td>AAGAGGCA-CTCTCTAT</td>
<td>1,725,442</td>
<td>1,723,023 (99.86)</td>
<td>860,686</td>
<td>1,651 (0.10)</td>
</tr>
<tr>
<td>11</td>
<td>Sample_11</td>
<td>Cattle_slurry_5c</td>
<td>Ctrl 30d</td>
<td>AAGAGGCA-TATCCTCT</td>
<td>645,154</td>
<td>642,264 (99.88)</td>
<td>321,850</td>
<td>564 (0.09)</td>
</tr>
<tr>
<td>12</td>
<td>Sample_12</td>
<td>Cattle_slurry_8d</td>
<td>BS 30d</td>
<td>AAGAGGCA-AGAGTAGA</td>
<td>2,074,284</td>
<td>2,072,113 (99.90)</td>
<td>1,035,103</td>
<td>1907 (0.09)</td>
</tr>
</tbody>
</table>

1 After adapter and quality trimming.
2 Percentage of the trimmed reads that are singletons.
### Table 8.4 Number of sequences match to OTU selection.

<table>
<thead>
<tr>
<th>Number of sequences</th>
<th>Number of clusters with taxon assignment</th>
<th>Number of Bacterial taxa found</th>
<th>Number of Archaeal taxa found</th>
<th>Number of Eukaryotic taxa found</th>
</tr>
</thead>
<tbody>
<tr>
<td>10,203,597</td>
<td>72,161</td>
<td>70,838</td>
<td>1,165</td>
<td>0</td>
</tr>
</tbody>
</table>

### Table 8.5 The abundance of OTU difference between Control and BS slurry at 0d (a vs b).

<table>
<thead>
<tr>
<th>OTU</th>
<th>Test-Statistic</th>
<th>P</th>
<th>Bonferroni_P</th>
<th>a_mean</th>
<th>b_mean</th>
<th>Taxonomy</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTU1890</td>
<td>14112</td>
<td>3.01 x10^{-8}</td>
<td>0.002</td>
<td>63.5</td>
<td>0.5</td>
<td>k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Bacteroidaceae; g__Bacteroides; s__coprosuis</td>
</tr>
</tbody>
</table>

Note: k, Kingdom; p, Phylum; c, Class; o, Order; f, Family; g, Genus; s, Species.

### Table 8.6 The abundance of OTU difference between BG slurry at 0d and 30 d storage period (b vs d).

<table>
<thead>
<tr>
<th>OTU</th>
<th>Test-Statistic</th>
<th>P</th>
<th>Bonferroni_P</th>
<th>b_mean</th>
<th>d_mean</th>
<th>Taxonomy</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTU48982</td>
<td>3298</td>
<td>3.03 x10^{-8}</td>
<td>0.002</td>
<td>3.3</td>
<td>46</td>
<td>k__Archaea; p__Euryarchaeota; c__Thermoplasmata; o__E2; f__[Methanomassiliicoccaceae]; g__vadinCA11; s__</td>
</tr>
</tbody>
</table>

Note: k, Kingdom; p, Phylum; c, Class; o, Order; f, Family; g, Genus; s, Species.
Table 8.7 The abundance of OTU difference between 0 d and 30 d.

<table>
<thead>
<tr>
<th>OTU</th>
<th>Test-Statistic P</th>
<th>Bonferroni_P</th>
<th>30d_mean</th>
<th>0d_mean</th>
<th>Taxonomy</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTU60066</td>
<td>288 3.84 x10⁻⁸</td>
<td>0.003</td>
<td>60</td>
<td>122</td>
<td>k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Porphyromonadaceae; g__; s__</td>
</tr>
<tr>
<td>OTU40499</td>
<td>232 9.88 x10⁻⁸</td>
<td>0.007</td>
<td>53</td>
<td>17</td>
<td>k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__; s__</td>
</tr>
<tr>
<td>OTU35460</td>
<td>220 1.24 x10⁻⁷</td>
<td>0.008</td>
<td>256</td>
<td>16</td>
<td>k__Archaea; p__Euryarchaeota; c__Thermoplasma; o__E2; f__[Methanomassiliicoccaceae]; g__vadinCA11; s__</td>
</tr>
<tr>
<td>OTU72</td>
<td>188 2.44 x10⁻⁷</td>
<td>0.016</td>
<td>201</td>
<td>56</td>
<td>k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__; s__</td>
</tr>
<tr>
<td>OTU39063</td>
<td>185 2.65 x10⁻⁷</td>
<td>0.018</td>
<td>308</td>
<td>627</td>
<td>k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Porphyromonadaceae; g__; s__</td>
</tr>
<tr>
<td>OTU43363</td>
<td>161 4.80 x10⁻⁷</td>
<td>0.032</td>
<td>178</td>
<td>352</td>
<td>k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Porphyromonadaceae; g__; s__</td>
</tr>
<tr>
<td>OTU67426</td>
<td>152 6.06 x10⁻⁷</td>
<td>0.041</td>
<td>112</td>
<td>233</td>
<td>k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Porphyromonadaceae; g__; s__</td>
</tr>
<tr>
<td>OTU43134</td>
<td>151 6.27 x10⁻⁷</td>
<td>0.042</td>
<td>247</td>
<td>41</td>
<td>k__Bacteria; p__Spirochaetes; c__MVP-15; o__PL-11B10; f__; g__; s__</td>
</tr>
<tr>
<td>OTU4497</td>
<td>149 6.61 x10⁻⁷</td>
<td>0.045</td>
<td>1139</td>
<td>3857</td>
<td>k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Moraxellaceae; g__Psychrobacter</td>
</tr>
<tr>
<td>OTU51183</td>
<td>148 6.84 x10⁻⁷</td>
<td>0.046</td>
<td>54</td>
<td>156</td>
<td>k__Bacteria; p__TM7; c__TM7-3; o__; f__; g__; s__</td>
</tr>
</tbody>
</table>

Note: k, Kingdom; p, Phylum; c, Class; o, Order; f, Family; g, Genus; s, Species.
Figure 8.4 The alpha diversity measured for each slurry samples after rarefaction step.
8.3.3 Bacteria phylogenetic diversity richness and taxonomic abundances

The microbial abundant of taxa in each sample at Order level indicates some similarity and diversity among the treatments and sampling periods (0d; 30d). Each band presented in the barplot (Figure 8.5) represents a specific group of taxonomic rank. In general, there are three major bands across the samples, which represent the main Orders within the microbes community (Figure 8.5)

The major bands were found to be as Clostridiales in the greatest abundant, followed by Bacteroidales and Actinomycetales. However, members of the Flavobacteriales, Pseudomonadales and Xanthomonadales Order were also observed across the samples in lower relative of OTU abundances. Similarly, the OTU abundance at Family level showed higher total number of bands in each sample, representing specific OTUs taxa (Figure 8.6) with the most abundant OTUs found to be similar across the samples. At the Family level, greater differences among samples were observed even within the same treatment. The abundances of Genus and Species level resembles the abundances in the Family level, more complex and are not further discussed (Appendix 8.2 and Appendix 8.3).

Meanwhile, the relative comparison of the OTU abundance between treatments at 0 d indicates small difference at Order level on the Lactobacillales and Fibrobacteria Order but greater differences were seen at Family level where Streptococceae, Enterococceae, Fibrobacteriaceae and Carnobacteriaceae are present in the BS treated slurry. This indicates a shift of bacterial communities, even within the short period after the brewing sugar was added, that resulted in the increase in the Order Clostridiales and decrease of Psedomonadales in Ctrl slurry. Meanwhile, Lactobacillales in BS increases over the 30 d storage with exception of the Cattle slurry 4c sample. Comparison on the abundancy of the OTUs communities is explained by the non-metrics multidimensional scaling (NMDS) analysis, which separated the 0 d samples from 30 d samples and Ctrl and BS samples (Figure 8.7). However, 30 d samples seem to be segregated from the rest. The NMDS and the OTUs abundances chart suggest that 4c and 3d Cattle slurry were outliers within their own groups.
Figure 8.5 Relative OTU abundance shift over time and treatment at Order level across slurry samples revealed by 16S rRNA data.

Note treatments label: a, 0d Control; b, 0d BS; c, 30d Control; d, 30d BS
Figure 8.6 Relative OTU abundance shift over time and treatment at Family level across slurry samples revealed by 16S data.

Note treatments label; a, 0d Control; b, 0d BS; c, 30d Control; d, 30d BS
Figure 8.7 Non-metrics multidimensional scaling analysis of OTUs abundances of slurry samples.
8.3.4 Archaea diversity and abundances

There are relative increases in Archaea community over the storage time (0 d and 30 d) and with the addition of brewery sugar, as can be seen in Figure 8.8. The increase indicates the diversity in the Archaea community across the samples. Yet, in this study, the focus microbes are of the Order of methane producing archaea. We observed that *Methanosarcinales* was the dominant methanogen at the initial starting point, and that the abundances of Order of *Methanococcales*, *Methanobacteriales* and *Methanosarcinales* in Ctrl slurry increased over time. *Methanococcales* was also found to increase in BS, but the other 2 Order decreased on 30 d samples with exception of the 3d Cattle slurry sample.
Figure 8.8 Relative OTU abundance shift over time and treatment of Archaea at Order level across slurry samples revealed by 16S data.

Note treatments label: a, 0d Control; b, · · · 0d BS; c, 30d Control; d, 30d BS
8.4 Discussion

8.4.1 Mitigation by induced acidification

As has been noted, the result of adding a fermentable carbon source, such as brewing sugar to slurry promotes self-induced acidification through the large production of lactic acid and subsequent decrease of the slurry pH. This greatly reduced CH$_4$ emission by 62 times, concurrent with the reduction of estimated NH$_3$ loss by 2.5 times from the slurry surface over the 30 d period. The recorded cumulative CH$_4$ emission remained 93% lower over the 70 d observation, suggesting that the emission remained low as long the slurry pH did not exceed pH >6.2 which is required for optimal methanogen growth (Mah and Smith, 1981; Jones et al., 1987; Garcia et al., 2000). This inhibition in CH$_4$ and (estimated) NH$_3$ emission were similar to previous finding (Chapter 4 and 5) and Petersen et al. (2012, 2013) and Wang et al. (2014). Another key point to note is the growth in the temperature condition during this experiment which was at an average of 11°C; suitable for psychrophiles or psychotolerant methanogen and CH$_4$ production by the hydrogenotrophic path such as *Methanomicrobiales* (Garcia et al., 2000, 2006; Nozhevnikova et al., 2003; McKeown et al., 2009; O'Reilly et al., 2010).

8.4.2 The 16S rRNA metagenomics observation approach

Microbial genome identification through 16S rRNA is a next generation method as it requires small sample, involves no isolation and cultivation, and also provides a true pictures of organism presence in the sample (Pace, 1997; Shokralla et al., 2012). In fact, some microbes are difficult or impossible to be cultured in the laboratory e.g. some methanogens (Mah and Smith, 1981; Jones et al., 1987; Garcia et al., 2000). By this approach, the microbial diversity is observed based on genomic profile by characterising their conserved rRNA genes. This rRNA hypervariable region is specifically similar among the species but differ between genus or microbes. Hence, this is a useful method to accurately identify and study the microbial phylogeny and diversity (Van de Peer et al., 1996; Chakravorty et al., 2007; Amore et al., 2016). The microbial diversity in the next generation sequencing (NGS) usually targets the area between V3-V4 region of the 16S rRNA sequences, known as the best target for microbes identification in a microbial community (Chakravorty et al., 2007) although other regions may be more appropriate for specific species or certain archaea (Yu et
al., 2008). In addition, there is no single genetic marker that is capable to completely diagnose an organism or microbial diversity (Hartmann et al., 2010).

In this molecular observation, the selection and assignment of the OTUs to the greengene databases showed that microbial diversity in the Order level of microbes was similar to the predominant microbes in livestock manure, especially in cattle manure (Ouwerkerk and Kliewe, 2001; Maeda et al., 2010a; Liu et al., 2011; Shanks et al., 2011). The genus and species levels are not explored in detail in this Chapter, but some bacteria found in pig manure which might be similar to manures contained the following microbes; Clostridium sp., Streptococcus sp. Pseudomonas sp., Lactobacillus sp., Megasphaera sp., Roseburia sp., (Whitehead and Cotta, 2001). Meanwhile, parallel microbes found in cattle manure compost are a member of the Actinobacteria, Clostridia, Cyanobacteria, Euryarchaeote, Fibrobacteres Lentisphaerae, Proteobacterium, Sphingobacteriaceae, Spirochaetes and Verrucomicrobia (Ouwerkerk and Kliewe, 2001; Maeda et al., 2010a; Liu et al., 2011; Shanks et al., 2011). This 16S rRNA genomic study is not limited to unexplored microbial diversity and ecological characteristic of the community, but also to analyse multiple microbial genomes while simultaneously allow phylogenetic discovery. Such findings allow the quantification of a microbial shift dynamic or 3D maps of microbial communities across many sites (Caporaso et al., 2011; Poretsky et al., 2014).

8.4.3 Bacteria richness, diversity and shift

Changes in the microbial community pattern or microbial dynamics occur when the ecology or environment is changed, disturbed or modified by any factors (Herrmann and Shann, 1997). In this study, an immediate bacteria shift in BS was detected through the lactic acid production and changes in the slurry pH. These changes were proven by the presence Order of Lactobacillales and the high OTUs level on 30 d samples (Figure 8.5). The presence of lactic acid producing organism (Order of Lactobacillales) at 0 d can be explained by the immediate uptake and metabolism of the additional C source and the prolong sample processing before the DNA extraction. Five lactic acid producing bacteria Families were found in this study and exhibited the possibility of both homolactic or heterolactic fermentation path (Rogers et al., 2013; Sikora et al., 2013). Any of these families could be the major lactic acid producer in this observation. After all, nearly 400 lactic acid producing bacteria were identified but are not showcased on the barplot chart as it may already
represented in the Order and Family level (Sikora et al., 2013; Zhang and Cai, 2014). As lactic acid producing bacteria, they are tolerable to lower pH environments, which explains the presence of high lactic acid concentration in the acidic slurry (Shah and Jelen, 1990; Assohoun-Djeni et al., 2016). This is another factor influencing the absence of the other member of Order such as Rhizobiales and Pirellulales in the acidic condition. In addition, some lactic acid producing bacteria may produce substances that act as bactericidal, bacteriocins or inhibitors. These substances are important proteinaceous toxins and organic metabolites, which are capable to kill or inhibit the growth of certain microbes (Assohoun-Djeni et al., 2016). In contrast to the Order of Lactobacillales, there was a relative decrease in the Actinomycetes and Pseudomonales Order after 30d under both treatments. These microbial shifts also occurred in other environment such as sandy soil (Abubaker et al., 2013).

By comparing the distinct bacterial community found in Ctrl slurry at later storage point (30 d), although the diversity was found as likely similar, it obviously showed an increase in the abundances of Order Synergistales, Clostridales and Bacteroidales. These bacterial Orders were fairly easy to be found in cattle manure as a sign of microbial community changes over the storage time (Ouwerkerk and Klieve, 2001; Maeda et al., 2010a; Liu et al., 2011; Shanks et al., 2011).

8.4.4 The methanogen diversity and shift

The diversity and dynamic of methanogen community can be observed in the barplot at Genus level (data not shown), but the following discussion opts to exclude any further elaboration regarding the community. In fact, the Euryarchaeota phylum barplot indicates the presence of a member of the Order Methanomicrobiales, Methanobacteriales and Methanosarcinales and absence of Order Methanococcales and Methanopyrales (Garcia et al., 2000; Demirel and Scherer, 2008; Liu and Whitman, 2008; Nazaries et al., 2013). All members in the Order of Methanomicrobiales, Methanobacteriales and Methanosarcinales are known to be involved in the methane production by the hydrogenotrophic pathway. The later Order is also possibly using other methanogenesis path; the acetoclastic pathway. In the hydrogenotrophic methanogenesis, methanogen utilised H₂ and reduced CO₂ during the metabolism process (Garcia et al., 2000; Liu and Whitman, 2008; Ferry, 2011). In contrast, an acetate as substrate for methanogenesis was cleaved in the acetoclastic pathway and reduced the methyl group to CH₄ (Garcia et al., 2000; Liu and Whitman,
Abundance of the Methanomicrobiales over time in the control (Ctrl) and the acidic slurry suggested a shift from both methanogenesis pathway to hydrogenotrophic pathway after long storage duration. The slurry storage experiment was conducted below the 11°C threshold which is suitable for psychrophilic methanogen such as Methanomicrobiales which may become dominant methanogenic community at low temperature (McKeown et al., 2009; O’Reilly et al., 2010).

The increased Methanomicrobiales in BS and the presence of other remaining methanogens are possibly related to the acid tolerant methanogen. The Order of Methanosarcinales methanogens found in the acidic slurry could possibly gain its energy by utilising the acetate or methyl group (the acetoclastic pathway) rather than hydrogenotrophic path; despite the absence of any scientific evidence to support this claim in this study. Nevertheless, it is clear that major CH₄ production in this storage condition was from hydrogenotrophic pathway as the total OTUs of Methanomicrobiales and Methanobacteriales exceeded the Methanosarcinales. In addition, the optimum pH range for acetotrophic methanogenesis was known to be at between pH 6.6 and pH 7.3 and inhibited below a pH of 6.2 (Chen et al., 2008; Demirel and Scherer, 2008). Presence of dominant hydrogenotrophic group methanogen under low temperature in this study is supported by the other findings such as at artic peat soil (Horn et al., 2003; Høj et al., 2008), low temperature granular sludge bed (McKeown et al., 2009) and low temperature granular wastewater (O’Reilly et al., 2010).

8.5 Conclusion

The experiment concludes that the slurry’s microbial community shifts over time and the low pH slurry in BS was caused by the increased population of Order Lactobacillales which was also presumably responsible for the lactic acid production. Microbial decomposition of organic materials in stored slurry often produce CH₄ gas as the result of the growth and activity of psychrophilic methanogens and is influenced by the diversity of methanogen community. The methanogen OTUs abundance level suggests that the methanogenesis from stored slurry at low temperature (<15°C) was mainly through the hydrogenotrophic pathway. The low CH₄ emission from self-induced acidified slurry (BS) was caused by the decreasing methanogen abundance in the microbial community. The psychrophilic methanogen and acid-tolerant methanogens identified in this study are the phylogenetic members Order of the
Methanobacteriales, Methanomicrobiales, and Methanosarcinales. This study demonstrates the dynamics of the microbial diversity, and the study should be expanded to slurry stored at higher temperatures (±30°C) to observe the microbial dynamics especially the mesophilic methanogens.
8.6 Appendix

Appendix 8.1: Rarefaction of individual slurry sample measured for alpha diversity at many sequence coverage level.
Appendix 8.2: Relative OTU abundance of Genus level across slurry samples

Note treatments label; a, 0d Control; b, · · · 0d BS; c, 30d Control; d, 30d BS
Appendix 8.3: Relative OTU abundance of Species level across slurry samples.

Note treatments label; a, 0d Control; b, 0d BS; c, 30d Control; d, 30d BS
CHAPTER 9

9 General discussion

9.1 Introduction

Greenhouse gas emissions from livestock are on the rise due to the increasing number of animals needed to meet the demand for livestock products. Nearly two decades ago, the Kyoto protocol (1997) was established following the UNFCCC which focused on global climate change. The first national greenhouse gas (GHG) inventory was later established (in 1998), and the amount of reduced GHG was monitored. Currently, the importance of reducing GHG has become a global concern. In 2009, the Global Research Alliance (GRA) was established with over 45 member countries worldwide, together with some organisations contributing to the workgroup (GRA, 2015). The Alliance establishment is to deepen the area and increase the research efforts within the agricultural sub-sectors of paddy rice, crops and livestock that will help producing more food. The workgroup actively share their knowledge, expertise, capacity and fellowship opportunity in its quest towards lower GHG emission.

One of the barriers in reducing agricultural GHG emissions is the lack of knowledge of practical and cost-effective manure management practices that would deliver lower GHG emissions, especially for small-medium scale farmers in developing economies. Despite the available integrated manure management practices, farmer’s cooperation in this matter requires awareness in term of social responsibility and safety. Local support is needed to facilitate awareness of manures as a source of GHGs, and to support the adoption of cost-effective mitigation strategies, e.g. in the form of policies and infrastructure. In this regard, the local support may come in the form of adequate expertise and investment funds and subsidies by government agencies are greatly needed. In addition, farmers (and policy) need evidence-based guidance for a range of GHG mitigation strategies that are both practical and affordable.

The preceding chapters demonstrate two potential alternative strategies in mitigating GHG from cattle farming, specifically during slurry storage. This thesis has demonstrated the potential of ‘self-acidification’ during slurry storage to reduce
ammonia (NH₃), methane (CH₄), and total GHG emissions. Similarly, the co-effect of bio-augmentation by effective microorganism (EM) was evaluated during slurry storage, with and without the carbohydrate addition also the bio-augmentation effect on the crust. The mechanism responsible for this self-acidification was revealed, through exploration into the slurry microbial genome profiles. Furthermore, GHG emissions were assessed following the spreading of ‘treated’ slurry to grassland.

In the discussion below, I start by synthesising the slurry storage data with the slurry spreading data in order to understand the effects of carbohydrate and EM addition towards the total GHG and NH₃ emission from the entire storage-spreading phases. Later, I continue to discuss the potential of the observed mitigation chain, mitigation complexity, practicality and wider mitigation scope for farmers of small-medium scale systems.

9.2 Complete sustainable mitigation of ammonia and GHG emission

The complete view of the mitigation process during slurry management is important to guide farmers and encourage the participation rate in reducing GHG emission. Successful mitigation of NH₃ and GHG emission is only achievable if farmers from all types of systems are involved in this program. Hereby, complete data analysis on the potential of mitigated NH₃ and GHG during slurry storage and slurry spreading (in the preceding chapters) are synthesised and summarised in Table 9.1, Table 9.2 and Table 9.3. The NH₃ and GHG emission during both periods (storage and spreading) were normalised to slurry spreading area (m² basis at slurry application rate 200 kg N ha⁻¹) as the end-emission calculation unit. The total NH₃ emission in both periods (storage and spreading) were found to be at least four times lower from the glucose added treatment (induced-acidified) than the untreated treatment (Ctrl). In the final analysis, the effect of induced acidification by a simple carbohydrate was found to result in marked NH₃ inhibition, by an average of 76%. Meanwhile, complete analysis (storage and spreading) of the GHG emissions, based on kg⁻¹ VS slurry, indicates that CH₄ emissions represented the majority of the GHG CO₂ eq. emission during the storage period, which was responsible for 92 - 99% of the total emission (Table 9.2). In contrast, it was clear that major proportion of GHG CO₂ eq. during the slurry application was from N₂O emissions (83 - 94%). A significant CH₄ emission was observed immediately after applying the slurry to the sward, with the exception of the acidified slurry. As shown in Table 9.3, induced acidification resulted in lower GHG
CO₂ eq. during both phases (slurry storage and spreading), which subsequently reduced the net GHG CO₂ eq. kg⁻¹ VS slurry by between 34% and 61%. The importance of this study lies in the GHG mitigation potential from the slurry management during storage and spreading as summarised in Figure 9.1 and Table 9.4.

Table 9.1 The total ammonia emission following storage and spreading of cattle slurry treatments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Slurry storage</th>
<th>Slurry spreading</th>
<th>Net NH₃ emission</th>
<th>Percentage inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Duration 60 days [mg m⁻²] (A)</td>
<td>Duration 48 hr [mg m⁻²] (B)</td>
<td>(A+B) [mg m⁻²]</td>
<td></td>
</tr>
<tr>
<td>GEM</td>
<td>19.8</td>
<td>2.4</td>
<td>22.2</td>
<td>77.3</td>
</tr>
<tr>
<td>G</td>
<td>21.4</td>
<td>2.8</td>
<td>24.1</td>
<td>75.3</td>
</tr>
<tr>
<td>EM</td>
<td>96.8</td>
<td>19.0</td>
<td>115.8</td>
<td>-18.6</td>
</tr>
<tr>
<td>Ctrl</td>
<td>86.9</td>
<td>10.7</td>
<td>97.6</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Treatment abbreviation; G, Glucose; EM, effective microorganism; GEM, Glucose + effective microorganism; Ctrl, untreated.
Table 9.2 Proportion of GHG emission from storage and spreading of cattle slurry treatments, based on 1 kg volatile solid of cattle slurry.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GHG CO(_2) eq. (g kg(^{-1}) VS)</th>
<th>Percentage contribution (%)</th>
<th>GHG CO(_2) eq. (g kg(^{-1}) VS)</th>
<th>Percentage contribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CH(_4)</td>
<td>CO(_2)</td>
<td>N(_2)O</td>
<td>Total CO(_2) eq.</td>
</tr>
<tr>
<td>GEM</td>
<td>17.7</td>
<td>0.7</td>
<td>0.0</td>
<td>18.5</td>
</tr>
<tr>
<td>G</td>
<td>11.2</td>
<td>1.0</td>
<td>0.0</td>
<td>12.2</td>
</tr>
<tr>
<td>EM</td>
<td>111.2</td>
<td>0.9</td>
<td>0.0</td>
<td>112.0</td>
</tr>
<tr>
<td>Ctrl</td>
<td>66.6</td>
<td>0.9</td>
<td>0.0</td>
<td>67.4</td>
</tr>
</tbody>
</table>

Treatment abbreviation; G, Glucose; EM, effective microorganism; GEM, Glucose + effective microorganism; Ctrl, untreated.

Note: CO\(_2^*\) emission is not included in GHG CO\(_2\) eq. as the emission is affected by the ryegrass photosynthesis (Leahy et al., 2004).
Table 9.3 The net GHG emission following storage and spreading of cattle slurry treatments, based on 1 kg volatile solid of cattle slurry.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Slurry storage</th>
<th>Slurry ryegrass application</th>
<th>Net GHG emission [g kg(^{-1}) VS] (A+B)(^{*})</th>
<th>Inhibition percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total GHG during storage [g kg(^{-1}) VS] (A)(^{*})</td>
<td>Weight applied during spreading [kg m(^{-2})]</td>
<td>Total GHG during slurry spread [mg m(^{-2})]</td>
<td>Total GHG emission [g kg(^{-1}) VS] (B)(^{*})</td>
</tr>
<tr>
<td>GEM</td>
<td>0.52 0.86 0.00 18.51</td>
<td>5.6 1.9 50.4 88.6 28121 0.10 - 0.17 53.09</td>
<td>0.62 0.86 0.17 71.59</td>
<td>60.7</td>
</tr>
<tr>
<td>G</td>
<td>0.33 1.01 0.00 12.18</td>
<td>8.7 3.5 54.1 98.4 31154 0.19 - 0.34 107.68</td>
<td>0.52 1.01 0.34 119.86</td>
<td>34.3</td>
</tr>
<tr>
<td>EM</td>
<td>3.27 0.90 0.00 112.02</td>
<td>13.7 4.8 115.4 66.1 23623 0.55 - 0.32 113.55</td>
<td>3.82 0.90 0.32 225.57</td>
<td>-23.7</td>
</tr>
<tr>
<td>Ctrl</td>
<td>1.96 0.91 0.00 67.43</td>
<td>11.6 4.3 119.8 76.8 26968 0.51 - 0.33 114.90</td>
<td>2.47 0.90 0.33 182.33</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Treatment abbreviation; G, Glucose; EM, effective microorganism; GEM, Glucose + effective microorganism; Ctrl, untreated.

Note: (B)*: data used for calculating the net GHG emission kg VS\(^{-1}\) from both (storage and spreading) studies; CO\(_2\)\(^{*}\) emission is not included in GHG CO\(_2\) eq. as the emission is affected by the ryegrass photosynthesis (Leahy et al., 2004).
Figure 9.1 The percentage greenhouse gas and ammonia mitigation reduction potential observed in the preceding chapters.

#Note: w, winter; s, summer; B, brewery-spent grains; EM, effective microorganism; n.d, not detected.
Table 9.4 Overview on the potential mitigation methods in the reduction of the GHG emission.

<table>
<thead>
<tr>
<th>Mitigation methods / observation period</th>
<th>Additive</th>
<th>Season</th>
<th>Mitigation methods</th>
<th>Additive</th>
<th>Season</th>
<th>Potential GHG % inhibition</th>
<th>Additive</th>
<th>Season</th>
<th>Potential GHG % inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>NH₃</td>
<td></td>
<td></td>
<td></td>
<td>CH₄</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Storage - additive addition</td>
<td>Glucoseᵃ</td>
<td>Winter</td>
<td>56</td>
<td>97.2</td>
<td>83.6</td>
<td></td>
<td>58</td>
<td>98.1</td>
<td>85.4</td>
</tr>
<tr>
<td></td>
<td>Glucose+EMᵇ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EM</td>
<td></td>
<td>-</td>
<td>12.6</td>
<td>19.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glucoseᵃ</td>
<td>Summer (A)</td>
<td>75.4</td>
<td>83.2</td>
<td>81.9</td>
<td></td>
<td>77.2</td>
<td>73.5</td>
<td>72.6</td>
</tr>
<tr>
<td></td>
<td>Glucose+EMᵇ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EM</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Brewery spent grains 1% sugar content basisᵃ</td>
<td>Spring</td>
<td>-</td>
<td>93.0</td>
<td>93.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crust - methane oxidation</td>
<td>Bio-augmentation (EM)</td>
<td>Spring</td>
<td></td>
<td></td>
<td></td>
<td>CH₄ oxidation- n.d + N₂O emission</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glucoseᵃ</td>
<td>Temperature controlled (glass house) (B)</td>
<td>74.3ᵃ</td>
<td>54.8</td>
<td></td>
<td></td>
<td>77.6ᵃ</td>
<td>57.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glucose+EMᵇ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td></td>
<td>EM</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glucoseᵃ</td>
<td>A + B</td>
<td>75.3</td>
<td>75.7</td>
<td>60.7ᵃ</td>
<td></td>
<td>77.3</td>
<td>79.1</td>
<td>34.3ᵃ</td>
</tr>
<tr>
<td></td>
<td>Glucose+EMᵇ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Note: *, CO₂ emission during slurry spreading is not included; ᵃ, induced acidified; ᵇ, measurements during first 48 hr; EM, Actiferm effective microorganism; n.d, not detected.
9.3 The potential for additives and self-acidification to reduce GHG emissions

9.3.1 Bio-augmentation and other biological additives

The emergence of various bio-augmentation additives in the market requires scientific evidence to prove their claims. The use of effective microorganism, indigenous microorganism or beneficial microorganisms (Andrews et al., 2012; Zuraihah et al., 2012; Lakshman and Sai, 2015) as additives in the bio-augmentation approach are to gain positive effect through their application. However, the effect of each additive might vary and do not represent other additives. The results from this project demonstrate that the usefulness of the activated effective microorganism (Actiferm EM®; AFEM) is still uncertain, with different results obtained from different scales and environments (cold/warm). Even though the functional microorganism (AFEM) rate applied in slurry was higher than the recommended dosage, these microorganisms still need to compete with slurry microbes, mainly the rumen microorganism, that are discharged and influenced by the animal breed, age and diet (Külling et al., 2003). The key point in using EM is the microbial synergism activity and the close relation between the beneficial microbes within the five microbial groups in EM that extensively compete for available nutrient in the slurry. These microorganisms have been claimed by the manufacturers and some consumers, to reduce unpleasant odours with less flies, improve nitrogen retention, lower grass burn when slurry is spread and improve the microbial quality in soil (EM Effective Micro-organisms Ltd, 2013). EM have also been specifically developed to enhance CH₄ production via anaerobic digestion, through ‘fortification’ with other microbes, e.g. C. ultunense, Methanoculleus C. proteolyticusor hemicellulolytic bacteria (EM Effective Microorganisms Ltd, 2013; Louis, 2016). Recently, known specific pure culture microorganisms have been used as microbial agents (Bacillus licheniformis, Bacillus subtilis, Rhodopseudomonas capsulate, Lactobacillus casei) to control odour from pig slurry. They were known to be anaerobic and facultative microbes and have similarly been claimed to improve N retention (reduced NH₃ emissions from slurry stores). Developers of EM are aiming to improve the roles of the microbes, other than their original purposes, which was mainly to reduce pathogen viability and odour emission (Choi et al., 2015).
9.3.2 Acid forced acidification

The use of chemical additives in slurry is nothing new. The technology (acidification) was initially introduced in Denmark by the use of mineral acid. At present, an average volume of 0.5% ($\text{v/w}$) concentrated $\text{H}_2\text{SO}_4$ kg$^{-1}$ slurry is used for acidification purpose. The volume of slurry that will be acidified is projected to increase to 20% in 2014 (Fangueiro et al., 2013). The main objective of the acid utilisation was to reduce NH$_3$ loss and retain more available N for application to soil, and reduce NH$_3$ losses after slurry spreading. Slurry acidification allow the farmers to expand their farms’ capacity and widen the area for slurry application even though slurry soil application through active incorporation is unavailable (Kai et al., 2008; Fangueiro et al., 2015). Regular monitoring of slurry pH in the store would be needed and re-acidification may be necessary, unless an automated monitoring acidification system was established. However, an automated acidification system would require regular mixing, and may not be feasible for small-medium scale farmers. The present study has demonstrated that acidification of slurry by mineral acids can be replaced by ‘induced acidification’, and that slurry pH’s of <5.0 can be achieved in both fresh and aged slurries.

9.3.3 Induced acidification

Induced acidification can be initiated by simple carbohydrate addition such as by adding glucose, or any waste product with a high content of fermentable carbohydrates. If immediate acidification is needed, simple sugar at 10% ($\text{w/w}$) can result in a rapid decrease in the pH level, to as low as pH 4.0. Indeed, carbohydrate breakdown (hydrolysis) is reportedly higher in acidic conditions (Hjorth et al., 2015). The use of 10% glucose might be too high and only useful to initiate the immediate acidic condition. The cost may be reduced if cheaper carbohydrate sources are used, e.g. high fructose corn syrup (HFCS), which is cheaper than the beet/sugar cane. High fructose corn syrup is made up of 55% fructose and 45% glucose, and it is currently found in over 40% of food products (Ruediger, 2010).

9.3.4 Agriculture food chain by-product as potential substances / additive

The use of simple carbohydrates; either in the form of brewery sugar (dextrose) or HFCS; is optional. Otherwise, any organic waste from the manufacturing or
agriculture sector may act as primary cheap source of fermentable carbohydrate (specifically simple sugars) (Weiland, 2010). For example, kitchen waste is often used as a substrate in an anaerobic digester (AD) (Wang et al., 2001; Bo et al., 2007), but it is also found to undergo lactic acid fermentation similar to the ‘induced-acidification’ process (Wang et al., 2001; Bo et al., 2007). Excessive use of kitchen waste or at high loading rate will lead to decreased pH in the digester (Tanimu et al., 2014) with impacts on CH₄ generation. Furthermore, the only probable costs of using food waste as a source of carbohydrates for inducing self-acidification of slurry would be those associated with the collection and transportation processes. So local sources of these substrates would be important. In tropical countries such as Malaysia, there is the potential to recycle local fruit waste during the harvest season, e.g. from mango, pulasan, rambai, rambutan and honey apple (jambu madu). Meanwhile the use of agricultural food waste could benefit the manufacturer in term of providing an outlet of what could otherwise be a waste disposal problem, and sharing of the processing costs through multiple waste streams in a single facility (Alatriste-Mondragon et al., 2006).

9.4 Crust as primary GHG barrier or source

The benefit of retaining a crust or cover on slurry lagoons/ponds, as practiced by Danish farmers, is to retain N from being volatilised in the form of NH₃ (Petersen and Ambus, 2006). This practice was part of the government legislation to optimise the intensive livestock farming. With this, the developed crust acts as the primary barrier for NH₄⁺ from being lost in the gas form. The lagoon depth influences the crust thickness and develops surface area topography (Smith et al., 2007). Smaller surface areas will result in thicker crust, created by the accumulation of fibre carried to the surface by ebullition of gases. The longer the crust is left undisturbed, the thicker the crust will be and subsequently trap and reduce the amount of CH₄ emitted from the slurry through oxidation process by methanotrophs. Although CH₄ oxidation was insignificant in our study, other reports have claimed that oxidation rates can reach up to 80 and 90% (Petersen and Ambus, 2006). The maximal oxidation activity reported earlier (in our study) was 24.9 ng CH₄ g⁻¹ DM hr⁻¹, and is much lower than those reported by Petersen et al., (2005). The oxidation rates recorded by Petersen et al., (2005) were between 0.1 to 1.4 mg kg⁻¹ DM hr⁻¹, and were estimated to oxidise as much as 90% of the CH₄ that could have been emitted (Seger, 1998, reviewed by
However, the net savings in CH$_4$ emission through this oxidation process in GHG CO$_2$ eq. was detrimentally affected by the increased N$_2$O emission from nitrification and denitrification in the crust. In addition, CH$_4$ oxidation in the crust is not effective in all circumstances, e.g. where the crust formation cracks, or collapses and when gaps are created (Nielsen et al., 2010), or when the slurry volume is decreased through evaporation process during the dry season. In such circumstances, the CH$_4$ trapped underneath the crust is directly emitted to the atmosphere and thus reduces the efficiency of CH$_4$ oxidation and triggers higher net GHG emission (Hansen et al., 2009).

On balance however, the amount of N$_2$O produced from slurry crusts are negligible compared to N$_2$O emission from soil. The N$_2$O emission from agricultural soils contribute between 50 to 70% of global N$_2$O emission (Velthof and Mosquera, 2011; Butterbach-Bahl et al., 2013). Moreover, crust acts as passive barrier for NH$_3$ emission where NH$_3$ emission is known to cause soil acidification, eutrophication and further nitrification and denitrification (Eickenscheidt et al., 2014).

In certain cases, infestation by 'rat tailed maggots', or where slurry DM is < 2%, intact crusts do not form. About 20%-30% of slurry lagoons in England reportedly do not form any crust. Crust formation can be actively promoted by adding low cost fibrous materials such as rice straw or Leca into the slurry. This cost may become a burden to small-medium farmers as the minimal amount purchased terms and transportation cost also need to be considered. In term of mitigation, retaining crust results in positive trade of GHG emission from the N$_2$O emission (retained NH$_3$ is not counted), rather than the GHG emission in the reduction process. However, the crust is capable of retaining plant available N in the slurry, which is later useful during fertilizer application (Misselbrook et al., 2005a; Smith et al., 2007).

### 9.5 Benefiting mitigation during slurry grassland application

Spreading the processed or untreated slurry onto pastures or cropland is the last step of slurry management. This is another important practice because it recycles the nutrients and organic matter for plant uptake and growth. The present study showed that ryegrass yield was increased by one to four times following the application of the slurry treatments. If grass scorch (and death) can be avoided, e.g. through shallow injection, incorporation into seed beds of new leys or arable crops, or perhaps by the even use of acid tolerant grasses, then greater yields may be possible from the
low pH slurries. Greater forage yields would benefit farmers who want to reduce the reliance on production from concentrates, especially in a country where pasture, forages and grazing area are limited such as Malaysia. However, the practice is quite unlikely and scarcely practised in Malaysia. The 3rd Malaysia National Agriculture Policy (NAP3) (MOA, 2010) encourages farmers to use animal manure as fertilizer to enhance the soil’s fertility, minimise waste and reduce the dependency on chemical N fertilizer. Only a few intensive farmers in the east peninsular of Malaysia (Sabah and Sarawak) currently apply slurry to land without further treatment or additives during the storage period.

In the UK however, the potential remains to acidify slurry during slurry storage – since many livestock farmers operate slurry-based systems and need to be able to store slurry for considerable periods of time, e.g. as a result of the NVZ Action Plans (Defra, 2015). The UK Farm Practices Survey 2014 reported that 57% of farmers kept their slurry in the farm for four to six months, while 19% stored slurry for seven to twelve months, and 2% stored slurry for over a year (Defra, 2013a). Longer storage period allows more time for biodegradation process and greater potential release of CH₄, and could result in increased N₂O emission from the crust. Even though immediate application to soil or grazing area will reduce both gases during storage, the reduction also reflects the widening managerial cost. Moreover, slurry application rates are limited to crop requirements in order to avoid N loss or pollution. In the UK, the government guidance (Guidance on Nitrate Vulnerable Zones) limits the total amount of slurry or fertilizer to grass, restraining the amount to not more than 250 kg N ha⁻¹ yr⁻¹ (Defra, 2010, 2013b). In addition, excessive fertilizer application increases the percentage of N loss from leachate after harvest (Defra, 2010b) and spurring additional cost.

If we scale the NH₃ emissions from the application of unamended slurry treatment (RCT and RNAS) to the ryegrass in the pot experiment, equivalent emissions fall between the ranges of 1.26 to 2.25 kg NH₃ ha⁻¹. These are low for a typical slurry application (Misselbrook et al., 2005b), and reflect the measurement approach we used, i.e. no air exchange within the headspace of the chambers. Misselbrook et al., (2005b) have shown that NH₃ emission factors (EFs) increase with temperature, hence the UK has different NH₃ EFs for slurry spread in spring compared to the slurry spread in summer (Webb and Misselbrook, 2004). Higher NH₃ emissions are likely to be emitted in temperate/tropical climates (Webb et al., 2002, 2010). The
NH₃ emissions from the self-acidified slurry (in the pot experiment) were reduced by 84%, and similar reductions might be expected under a warmer climate (e.g. Malaysian conditions) from the acidified slurry application.

Based on the UK NH₃ inventory, the estimated emission in 2013 was 271 kt and nearly 82% of it were produced by the agriculture sector (Defra, 2014); with 54% of it coming from livestock farming activities (Misselbrook et al., 2013). Slurry spreading contributed about 23% of the total NH₃-N emitted from agriculture in 2012 (Misselbrook et al., 2013). Greater NH₃ loss from land spreading is also possible. Bourdin et al., (2014) reported that up to 71% of total NH₃-N was emitted from land applied slurry in his study on farms in Ireland. Ideally, storing slurry at an acidic pH effectively reduces NH₃ emission from the store and following spreading. Greater emission reduction can be achieved if rapid soil incorporation is practiced. However, adoption of this technology in Malaysia is doubtful due to the high handling and machinery costs. Also, active slurry incorporated into soil could possibly increase N₂O emissions from soil (Wulf et al., 2001b). Using self-acidified slurry could possibly help the UK government to meet their targets to further reduce its NH₃ emissions, by 8%, (compared to the emission level in 2005) for the following years until 2030, in line with the “The Clean Air Policy Package” proposal by proposed in The National Emission Ceilings Directive 2001/81/EC (NECD) (European Commision, 2016).

Unlike NH₃ volatilisation, GHG emissions from soil following slurry spreading are associated with direct and indirect N₂O emissions, (associated with NH₃ emission, N deposition, and NO₃ leaching) with dissolved and trapped CH₄ within the applied slurry. Acidified slurry is likely to have less dissolved and trapped CH₄ compared to non-acidified slurry. In fact, methanogenesis within the slurry is inhibited after the slurry application due to exposure of the methanogens to O₂.

Malaysia is expected to reduce agricultural CH₄ emissions by 4% in 2015 compared to emissions in year 2000 (Ministry of Natural Resources and Environment Malaysia, 2000). However, this target is being revised as Malaysia’s livestock industry projects a continuous annual growth in livestock (meat) and milk production at 2.7% and 5.8%, until the year 2020, respectively, (MOA, 2011). Meanwhile, N₂O is not directly emitted during the storage, but directly emitted after the slurry infiltrates the soil and provides NH₄⁺ for the nitrification and denitrification processes. Chadwick et al., (2011) reported that N₂O EFs in applied land slurry surface is between 0.1 and 1.0%, whilst Bell et al., (2016) showed that N₂O EFs possibly reach 2.57% when slurry
is applied in autumn season. Much higher N₂O EFs have been reported, e.g. 7 and 14% of N applied in pig slurry (Velthof et al., 2003), but such large N₂O EFs are rare.

Other than application with a carbohydrate source, there is no clear evidence of the benefit of EM as an additive, neither in GHG reduction nor grass DM yield, which is similar to the report by (van Vliet et al., 2006). Another additional point is the tolerance level of the grass; the ryegrass used in the experimental study was intolerant to the acidic slurry (pH <5.5) and consequently resulted in low biomass yields (Department of Health and Ageing Office of the Gene Technology Regulator, 2008). However, typical forages in Malaysia such as the *Panicum maximum*, *Digitaria setivalva*, and *Pennisetum purpureum* are known to adapt in acidic soil of pH 4.5, while the *Bracharia decumbens* and *Brachiaria humidicola* are capable to survive in even lower soil pH (pH 3.5). Both these forages are widely planted as cattle forages (Chin, 2001). Also, acidified slurry could be applied to guinea type grass (*Panicum maximum*), a creeping type grass which may easily recover the acute death grasses area. Alternatively, the volume of applied slurry may be reduced but the application frequency may be increased by up to four times per year with additional managing cost.

The long-term effects of repeated applications of acidified slurry on soil are unknown, and perhaps the soil's inherent buffering capacity would needs to be strengthened by frequent liming. An additional benefit of slurry acidification is that it increases the dissolved P in the slurry and soil (Fangueiro et al., 2015; Hjorth et al., 2015).

9.6 The methanogenesis pathway

Slurry consists of various organic elements including carbohydrates, proteins, lipids, amino acids, sugars and fatty acids, which undergo anaerobic degradation mainly by bacteria, but also by some of the archaea community (from the *Euryarchaeota* Phylum) resulting in biogas production and emission namely CH₄ and H₂ (Demirel and Scherer, 2008). Emission of CH₄ is the result of H₂ utilisation or acetate for energy uptake through hydrogenotrophic or acetoclastic pathway by exclusive Order of *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, *Methanosarcinales*, and *Methanopyrales* (Garcia et al., 2000; Whitman et al., 2006; Demirel and Scherer, 2008; Liu and Whitman, 2008; Nazaries et al., 2013). However, two-thirds of the biological production of CH₄ from methanogenic archaea is through
the acetoclastic pathway at neutral pH (pH 7) (Liu and Whitman, 2008). Only two genera are known to utilise acetate substrate during methanogenesis, Methanosarcina and Methanosaeta, which are both members of the Order of Methanosarcinales. However, both of these genera have distinctive affinity for acetate concentration (Uludag-Demirer et al., 2008; St-Pierre and Wright, 2013).

In normal mesophilic conditions, the major CH₄ producer in stored slurry is thought to be Methanosarcina sp. (Barret et al., 2013). In the precedent study (Chapter 4, 5, 7 and 8), the average slurry and ambient temperature were below than 30°C regardless in summer or winter seasons, indicating that the methanogen responsible was categorised by psychrophilic (<20°C) rather than mesophilic (30-40°C) organisms (Monteny et al., 2006). Therefore, hydrogenotrophic methanogenesis was found to be an important pathway in psychrophilic and low pH condition. This is strengthened by the observed dominant methanogen which belongs to the Order of Methanobacteriales, Methanomicrobiales and Methanosarcinales. Their existence is symbiotic to hydrogen producing-anaerobes or acetogenic bacteria such as Clostridiales and Enterobacteriaceae (Kim et al., 2004; Sikora et al., 2013; Ali Shah et al., 2014).

The methanogen diversity and dynamic is influenced by the environmental changes as well as the microbial community. The changes in slurry environment such as temperature, pH or acetate level can trigger the methanogen dynamics between acetotrophic and hydrogenotrophic groups (Demirel and Scherer, 2008; Lin et al., 2012). Although most methanogen deceases to low pH level in low or medium acidic environment, certain acid tolerant methanogen such as Methanobacterium isolate and a strain of Methanosarcina barkeri are capable to tolerate the pH level of 4.0. However, methanogen dynamics may differ in slurry that was forced-acidified by concentrated acid. The difference is due to their immediate acidification level which affects the microbial activity (Ottosen et al., 2009). The use of different substrate or substrate mixture ratio as co-digestion in slurry also affects the microbial dynamic. In general, unsuitable condition triggers the shift in methanogen community structure (Lin et al., 2012).

9.7 Final conclusion

The data presented in this thesis demonstrate that the addition of a carbohydrate source to slurry causes induced-acidification, and a subsequent benefit
to environment through the reduction of CH₄ production and emission, as well as the lower NH₃ emissions. These findings provide great potential for testing and developing mitigation strategies for GHG and NH₃ emissions from slurry storage at the commercial scale. The slurry acidification was found to be caused by the production and accumulation of lactic acid by the member Order of Lactobacillales. It is plausible that an agriculture food chain by-product with large amount of fermentable carbohydrates e.g. brewery spent grain may act as the co-substrate during slurry storage to initiate self-acidification in slurry. The self-acidification by carbohydrate addition is regarded as a suitable alternative mitigation process of CH₄ and NH₃ emission from the stored slurry compared to mineral acids. It is also deemed as applicable in small and medium scale farms, as well as larger scale farms. The impacts of applying acidified slurries to soil, i.e. on soil health and crop production need further exploration, but it is believed to result in reduced NH₃ losses and increased plant N availability. Yet, the environmental and agronomic benefits of EM addition to slurry remain inconclusive.

9.8 **Suggestion for further exploration and recommendation**

- The current study demonstrates the potential for self-acidification to reduce both CH₄ and NH₃ emissions from laboratory scale slurry stores and reduce NH₃ emissions following slurry spreading at the pot-scale.
- This NH₃ and CH₄ mitigation strategies need to be scaled up and tested at the farm level; either in the small, medium or large farm scales. Furthermore, this study was conducted at small volume and static slurry storage where storage tank or lagoon may continuously receive fresh slurry on daily or weekly basis. In addition, the cost-effectiveness and practicality of adopting self-acidification practice needs to be explored using the full life cycle analysis.
- The effectiveness of this approach is to explore the solid manure during composting period as it also contributes to GHG emission and was widely practiced in Malaysia.
- Further tests need to be conducted to determine how long the slurry remains at an appropriate pH to reduce CH₄ production and NH₃ emission following the addition of a carbohydrate source. Perhaps, multiple carbohydrate additions will be necessary if slurry is stored for prolonged periods.
• The potential for self-acidification to help meeting the national targets to reduce both GHG and NH₃ emissions should be assessed, e.g. through use of models such as Manure-DNDC (Li et al., 2012; Gilhespy et al., 2014). The model are capable of estimating any gaseous emission throughout the whole manure management chain (housing, storage, processing and spreading) from a range of farming types with different infrastructure and management.

• Methane oxidation as a sink of CH₄ is considered as a new focus area that can be further explored, especially in warmer (tropical) climates. Oxidation could be enhanced by the application of specific MOB groups in slurry crusts, while nitrification within the crust could be reduced using an inhibitor such as DCD or DMPP (Pratt et al., 2012; Petersen et al., 2013a). These processes will result in optimal CH₄ sink and lower N₂O loss. However, the effect of rainfall or wet season on CH₄ oxidation should be evaluated as crust moisture contents will affect CH₄ uptake by physiological water stress or diffusion rate of CH₄ and O₂ (Luo et al., 2013).
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