ENHANCED METHANE PRODUCTION FROM PIG MANURE IN COVERED LAGOONS AND DIGESTERS

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Final report prepared for the Cooperative Research Centre for High Integrity Australian Pork

By

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Executive Summary

Methane production in covered anaerobic ponds or anaerobic digesters is a natural process whereby microorganisms convert manure into methane. Manure methane is increasingly used at Australian piggeries to produce heat and generate electricity. Currently, about 13.5% of total Australian pork production captures manure methane, and the majority use it in engine generators or hot water systems. System design and operation, the methane potential of the waste (e.g. manure or other) and the health of the microorganisms, dictate the performance of a covered anaerobic pond or anaerobic digester in terms of methane production.

A common strategy to produce more methane is anaerobic co-digestion, whereby two or more wastes are simultaneously digested. Abroad, a number of carbon-rich wastes are regularly co-digested with animal manures. Examples include vegetable by-products, industrial organic wastes, agricultural industrial by-products and residues, food wastes, fodder and brewery wastes, organically rich industrial wastewaters, and biofuel and biorefinery by-products. Methane yields vary widely between different wastes. Some wastes are excellent methane boosters, being highly biodegradable and/or are very concentrated. However, co-digestion should be carefully managed to prevent unsafe organic loading rates and/or to prevent chemical inhibition. Some other wastes require much longer treatment times than pig manure to be adequately converted into methane. The availability of co-digestion wastes is an on-going challenge in Australia. Transport costs and the availability of wastes dictate cost feasibility. A major future incentive could be the revenue from gate fees to divert waste away from landfill and instead sustainably converting them into methane for beneficial use. Unfortunately, in Australia, the imposition of landfill levies that drive such gate fees is a relatively recent and uncertain phenomenon, and varies from state to state. Unique waste handling infrastructure may be required for co-digestion (e.g. high solids) and should be considered during the planning and design stages of a biogas project. Systems designed for piggery loadings may have limited capacity to co-digest other wastes.

Desludging of covered ponds has been an on-going concern with biogas use in the Australian pork sector. Settled sludge eventually displaces active pond volume and requires removal. The present research project measured residual methane potential in sludge samples extracted from covered ponds at Pork CRC demonstration piggeries. At these piggeries, sludge is readily extracted by pumping via extraction ports, do not require removal of the pond cover, and the pond can remain in full operation whilst being desludged. The results showed that the sludge samples were reasonably stable, with over 50% of the organic matter already converted into methane. This was also the case for ponds with very short desludging periods of 1 year. It could be beneficial to decrease frequency of desludging to every 2 years. However, some piggeries may elect to desludge more frequently to manage water balances during wet/dry seasons and to sustainably apply sludge nutrients to cropland. Sludge that is extracted less frequently than every 2 years could become excessively consolidated inside the covered pond, and could then become more difficult to pump/extract.

Chemical inhibitors, such as sodium or ammonia nitrogen, can affect anaerobic digestion of pig manure. In this research, inhibition resilience and adaptation of microbial

communities were assessed using inoculum samples from full-scale and pilot scale digesters, and separately by subjecting microbes in continuous digesters to chronic high levels of inhibitor. The aim was to identify intervention options to promote inhibition resilience. The results showed that inhibition resilience varied moderately between different inoculum sources, with some microbial communities being more resilient than others. Also, subjecting microbial communities to chronic inhibitor stress showed clear acclimation. These results were encouraging, because accordingly, microbial communities could adapt to inhibitors. Unfortunately, no statistical links were found between intervention strategies and inhibition resilience. Instead, acclimation occurred naturally, and could not be greatly promoted by intervention. The only way was to chronically expose microorganisms to high levels of inhibitor, which then promotes adaptation, but the negative impacts on digestion performance would probably negate this option in practice. In general, when an inhibitor is unavoidable, it should be gradually introduced into the covered pond or digester, to allow time for natural microbial acclimation.

Overall, there is considerable opportunity for co-digestion in Australia, including as a way to deal with chemical inhibition. Future research is needed to better understand the effects of temperature and organic loading rate on co-digestion performance, especially because ambient temperature systems are common in Australia because of a temperate climate. It is also worth exploring in future research, the growing of microbial inoculums in smaller dedicated systems under a stress condition such as inhibitor presence, and then using these adapted microbial inoculums to seed a larger digester or covered pond in preparation for exposure to the same stress condition.

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Nomenclature list

AD	Anaerobic digestion
AcoD	Anaerobic co-digestion
BMP or B_0	Biochemical methane potential (L CH_4/kg VS fed)
CH_4	Methane gas
CO_2	Carbon dioxide gas
CAP	Covered anaerobic pond
COD	Chemical oxygen demand, a measure of chemical energy (also tCOD for total fraction, or sCOD for soluble/filtered fraction)
C/N	Carbon to nitrogen ratio
FOG	Fat Oil and Grease
GC	Gas chromatography
GHG	Greenhouse gas
H_2	Hydrogen gas
HA	Humic acid
ISR	Inoculum to substrate ratio
K_a	The acid-base equilibrium coefficient
k	First-order kinetic rate coefficient (d^{-1})
KI_{min}	Lowest inhibitor concentration that causes notable inhibition
KI_{max}	Inhibitor concentration corresponding to complete inhibition (i.e. SMA being NOT significantly different from zero).
KI_{50}	Inhibitor concentration at which the measured biological rate is half of the measured maximum rate.
I_i	Inhibition term, describing the fraction of the maximum metabolic uptake rate that is measured under inhibited conditions, with a value between 0 for completely inhibited and 1 for no inhibition.
N_2	Nitrogen gas
Na^+	Sodium ion
NH_3	Free ammonia
NH_4^+	Ammonium ion
NSW	New South Wales, A state in Australia
OTU	Operational taxonomic unit
PCA	Principal component analysis
QLD	Queensland, A state in Australia
SMA	Specific methanogenic activity (g COD_{CH4}/g $VS_{inoculum}$/d)
S_i	Concentration of dissolved compound i
NaCl	Sodium chloride
NH_4Cl	Ammonium chloride
t_d	Initial start-up time delay for anaerobic digestion (d)
T	Temperature (˚C)
TS	Total solids
TAN	Total ammonical-nitrogen
UASB	Upflow anaerobic sludge blanket
UQ	The University of Queensland, Brisbane, Australia
VFA	Volatile fatty acid
VS	Volatile solids
VIC	Victoria, A state in Australia
WA	Western Australia, A state in Australia
X_i	Concentration of particulate compound i

1. Introduction

Manure methane is increasingly used at Australian piggeries to produce heat and generate electricity. Currently, about 13.5% of total Australian pork production captures manure methane, and the majority use it in engine generators or hot water systems. By capturing and burning the methane in a generator, flare or hot water system, greenhouse gas (GHG) emissions can also be reduced by up to 64% across an Australian pork supply chain (Wiedemann et al., 2016). Covered anaerobic ponds (CAPs) or in-vessel anaerobic digesters produce manure methane via a natural microbial process called anaerobic digestion (AD). The performance of a CAP or in-vessel anaerobic digester (in terms of methane production) is dictated by:

1. the design and operation of the CAP or digester; i.e. dimensions, loading rate, hydraulic retention time;
2. the methane potential of the waste (e.g. manure or other) being treated in the CAP or anaerobic digester; and
3. the health of the micro-organisms responsible for the AD.

The present project investigates the latter two aspects, with the aim to enhance methane production from CAPs and digesters.

Methane potential refers to the amount of methane recoverable from a particular waste (e.g. pig manure) treated by a CAP or digester. Methane potential is a conserved quantity, meaning that it can be mass-balanced across a CAP or digester. This is shown in Figure 1.1, wherein waste (e.g. pig manure) that enters a CAP or anaerobic digester carries with it a certain methane potential (A). Some of this methane potential is converted into actual biogas methane, which can be captured and used (B) (*a desired outcome*). However, some of the methane potential is lost by:

- flowing out as partially treated organic matter in the effluent (C);
- uncaptured gas leaks, such as via an emergency vent (D)
- premature extraction of settled sludge that still contains digestible organic matter (E); or
- the formation of a floating crust layer inside a CAPs or digester that is inaccessible to AD (F).

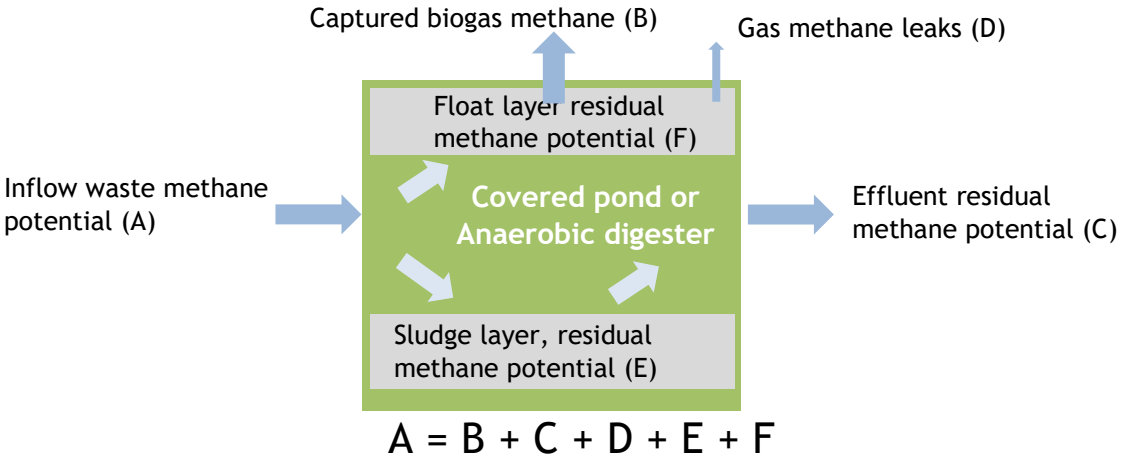


Figure 1.1 – Schematic illustration of the conservation of methane potential across CAPs or anaerobic digesters

In accordance with Figure 1.1, methane production from CAPs or anaerobic digesters could be enhanced by:

1. increasing the inflow of methane potential A (Figure 1.1). This can be done by adding other wastes together with the pig manure, and is commonly called anaerobic co-digestion (AcoD). ***The addition of other wastes must not exceed safe organic loading limits of the CAP or anaerobic digester***.

   Section 2 of the report reviews AcoD options and considerations.

2. managing the extraction of sludge from a CAP to minimize the losses of residual methane potential in E (Figure 1.1).

   Section 3 of the report addresses residual methane potential in pond sludge.

3. increasing the conversion of A (methane potential in the inflow) into B (methane in biogas), by creating favourable AD conditions that minimize the impact of inhibitors or enhance microbial activity, or by pretreating the waste (e.g. particle size reduction) to make it more accessible to AD microbiology.

   Section 4 of the report investigates strategies to mitigate chemical inhibition.

2. Anaerobic co-digestion review

2.1 Background

Anaerobic co–digestion (AcoD) involves the simultaneous AD of two or more wastes (e.g. pig manure and dairy processing waste) (Mata-Alvarez et al., 2014). In relation to the pork industry, AcoD increases methane production by:

- co-digesting pig manure with wastes of a higher methane potential than pig manure; and/or
- by increasing the total amount of waste being co-digested, thereby increasing the overall methane potential.

A number of piggeries in Australia operate as farrow-to-wean (breeder) units with progeny grown to slaughter weight at offsite grower units. Without the pig manure of the grower-finisher herd, these breeder piggeries may not be able to produce enough biogas methane to meet onsite energy demands for electricity and heat. In such cases, other wastes sourced from other off-farm industries, could be co-digested with pig manure to boost methane production (Skerman, 2017a).

Australian producers are already being approached by local industry offering by-products rich in organic matter, such as glycerol or whey, to enhance methane production. Moreover, some producers in Queensland (QLD) and Victoria (VIC) are currently routinely co-digesting pig manure with paunch from a nearby abattoir (http://bettapork.com.au/biogas-plant/), decanned fruit and vegetable waste (RIRDC Biomass Producer), or past use-by-date dairy products, which are greatly boosting methane production (https://aussiepigfarmers.com.au/). Co-digestion increases the inflow of organic matter to a CAP or in-vessel digester and thereby increases methane production (A, Figure 1.1). Co-digestion can also be beneficial in creating favourable digestion conditions to further enhance microbial activity and methane production. However, it is important not to exceed safe organic loading limits with the addition of other wastes, to protect the stability and performance of the CAP or in-vessel digester.

A wide range of wastes may be suitable for AcoD with pig manure, including:

- Agricultural products – other animal manures and slurries, vegetable by-products, crop residues and energy crops.
- Industrial products - organic wastes, by-products and residues from agro-industries, food industries, fodder and brewery industries, organic wastewaters and sludges from industrial processes, and organic by-products from biofuel production and bio-refineries.
- Municipal products - source-separated household waste, sewage sludge, municipal solid waste and food residues.

Usually the selection of waste mixtures to co-digest with pig manure is based on:

1. Economic considerations such as availability, transportation costs, and alternative revenue streams such as from waste diversion away from levied landfills;
2. Anaerobic digestion performance impacts because of the AcoD, such as potential inhibition risks or organic loading risks; and
3. Materials handling considerations, such as a need for pre-treatment (e.g. particle size reduction or sterilization).

To date, the selection of wastes for AcoD has been somewhat arbitrary, and generally based on availability. A literature review in this section addresses AcoD opportunities in the Australian pork sector, and aims to provide an overview of important considerations and guidance in the selection of AcoD wastes.

2.2 Economic considerations and environmental benefits

Anaerobic co-digestion can produce more methane from existing on-farm infrastructure (CAP or digester) or by using new purpose-built infrastructure for AcoD. The increased methane production can be used to generate more electricity and/or produce more heat for use at the piggery.

The cost feasibility of AcoD is largely determined by the costs of transport to bring wastes to the site (Mata-Alvarez et al., 2014). However, the quality of residue remaining after AcoD (termed digestate) is also important (Mata-Alvarez et al., 2014). This is because, ideally, digestate is beneficially reused as a nutrient fertilizer, and if digestate quality is compromised, then it may instead need to be disposed of at additional cost. For example, when sewage sludge is co-digested with manure in other countries, it improves biogas yield and process stability (Al Seadi et al., 2013b), but strict requirements limit the application of the digestate as a fertilizer, because of heavy metals, persistent organic pollutants and pathogens (Al Seadi et al., 2013b).

Anaerobic co-digestion offers environmental benefits by diverting waste from conventional disposal such as landfilling and incineration (Nghiem et al., 2017). The diversion of wastes away from landfills can generate revenue from gate fees, in the case where a landfill levy is in place (Al Seadi et al., 2013b). A gate fee system requires an accurate measurement of the quantity of waste received. Diversion can save landfill space, reduce whole of life impacts of landfilling and reduces post-closure maintenance (Nghiem et al., 2017). In Australia, landfill levies and diversion targets are implemented on a state by state basis; however, state-based legislation is informed by a collaborative committee consisting of members of state governments and the federal government (Edwards et al., 2015). The landfill levy is still a new approach in Australia, but is already helping diversion rates, especially in New South Wales (Edwards et al., 2015). Unfortunately, a general lack of clear, robust and nation-wide waste diversion policy is a major hurdle to widespread adoption of AcoD in Australia (Nghiem et al., 2017).

2.3 Co-digestion waste types and effects on performance

Classified by sector, waste types that can be co-digested include vegetable by-products and residues, industrial organic wastes, by-products and residues from agricultural industrial origin, food wastes, fodder and brewery wastes, organically rich wastewaters from industrial sectors, and organic by-products from biofuel production and biorefineries (Al Seadi et al., 2013b). Co-digestion mixtures of other wastes together with pig manure can reduce inhibition and enhance AD. This is achieved by co-digesting pig manure with preferably carbon-rich wastes, i.e. wastes having a high carbon to nitrogen ratio or C/N ratio (Astals et al., 2011). When such wastes are co-digested with pig manure, the pig manure increases alkalinity, which stabilizes digestion pH, and the pig manure provides macro or micro-nutrients, whilst the other wastes dilute strong inhibitors such as ammonia (Mata-Alvarez et al., 2014) and increase active biomass (Al Seadi et al., 2013b). Overall, these effects can make AcoD more resilient to inhibitors, thereby enhancing digestion stability and performance, and increasing methane production (Al Seadi et al., 2013b).

Plant residues are commonly used in AcoD with animal manures (Al Seadi et al., 2013b), including various vegetable agricultural by-products and harvest residues, plants and plant parts, low-quality or spoiled crops, fruit and vegetables, and spoiled feed silage (Al Seadi et al., 2013b). For example, high consumer expectations on quality of bananas in Australia has led to approximately 30% of harvested bananas being rejected as packing shed waste, then providing a steady supply of banana waste for local AcoD (Clarke et al., 2008). Most plant residues require some form of pre-treatment before feeding to a digester. Currently,

a producer in VIC has a CAP also co-digesting waste by-products not suitable for use in pig feeds. These include past use-by-date canned vegetables and fruit which are decanned or depackaged at the piggery (RIRDC Biomass Producer).

Pretreatment may include simple mechanical reduction of particle size down to 1 cm to facilitate proper handling and mixing, or could involve more intensive thermal or chemical treatment to make recalcitrant wastes more bioavailable for digestion (Al Seadi et al., 2013b). Intensive pre-treatment might not be cost-feasible in the Australian pork industry, because of high capital and operating costs. Interestingly, a study on AcoD of pig manure and algae (Scenedesmus sp.) showed that pretreatment of algae for extraction of valuable lipids and proteins, can increase methane potential of the processed algae as compared to raw algae (Astals et al., 2015b). This aligns with the future concept of a bio-refinery where nutrients and carbon are utilized in a sustainable and cost-effective way. Spent bedding is particularly interesting as a pseudo-plant residue, because some pre-conditioning of the bedding already occurs in a pig shed (e.g. by pigs chewing and fermenting the bedding in the presence of moisture and manure). This can improve AD of spent straw bedding as compared to unused straw (Tait et al., 2009). However, not all spent bedding types are favourable. For example, wheat straw spent bedding has a high methane potential, but rice husk spent bedding has a very low methane potential (Tait et al., 2009). Table 2.1 above provides literature values for biochemical methane potential (BMP) of spent litter and various other wastes.

Table 2.1 – Methane potential and other relevant characteristics of AcoD wastes

Waste type	Dry matter (%)	Volatile solids (VS, organic matter) (% of dry matter)	Biochemicial methane potential or BMP (L CH_4/kg VS fed)	Known inhibition or other risk[#]	Reference
Piggery shed effluent	1.7-6	64-84	150-640		(a), (c), (f)
Apple pulp, apple waste			306, 317		(a), (b)
Asparagus peels			219		(a)
Alcohol*	40	95	400		(c)
Banana peels			289		(a)
Beef feedlot manure (fresh)	20-22	79-88	230-360		(e)
Brewers spent grains	20	90	330		(c)
Barley straw	81	89	229-230		(b), (d)
Confectionary			320		(b)
Corn Stover			360		(b)
Cotton residues			365		(b)
Carrot peels			388		(a)
Citrus			473		(a)
Chocolate			370		(b)
Cattle manure	20	80	200		(c)
Fruit wastes	15-20	75	250-500		(c)
Fruit and vegetable waste			470		(b)

Waste type	Dry matter (%)	Volatile solids (VS, organic matter) (% of dry matter)	Biochemicial methane potential or BMP (L CH_4/kg VS fed)	Known inhibition or other risk[#]	Reference
Fish oil*	90	90	500	Lipid inhibition	(c)
Fish waste			390		(b)
Garden waste	60-70	90	200-500	Materials handling	(c)
Grape			231		(a)
Gelatin			100-150	Ammonia	(b)
Glucose			335		(b)
Grass			128-374		(b)
Kitchen waste			370-450		(b)
Leachate of food waste			478		(b)
Mango peels			370-523		(a), (b)
Mixed food waste			472		(a)
Paper			84-369		(b)
Potato peel			267		(b)
Poultry droppings	5	80	300	Ammonia	(c)
Rice straw			270-367		(b)
Rice husks	90	73	90		(d)
Ryegrass			360		(b)
Stomach/intestinal content, cattle	12	80	400		(c)
Stomach/intestinal content, pig	12	80	460		(c)
Concentrated whey (20-25% protein)	5	90	330		(c)
Source-separated organic fraction of municipal solid waste			275-410	Heavy metals	(a)
Spent wheat straw pig deep litter	35-61	68-74	250-270		(d)
Spent rice husks pig deep litter	47-49	45-48	160-190		(d)
Spent barley straw pig deep litter	17-85	89-93	100-120		(d)
Switch grass			191-309		(b)
Wheat straw			227-333		(b)

(a) (Lesteur et al., 2010); (b) (Raposo et al., 2011); (c) (Al Seadi et al., 2013b); (d) (Tait et al., 2009); (e) (Gopalan et al., 2013a); (f) (Gopalan et al., 2013b); [#]Organic loading limits apply to most substrates; *Methane booster.

Note that methane yields measured in the laboratory are influenced by test conditions and may be higher or lower in practice depending on a number of factors, such as adapted microbiology (Shrestha et al., 2017), organic loading or solids loading, and the presence of inhibitors or recalcitrant ingredients. Waste variability in terms of methane potential and composition (e.g. inhibitors) is usually important. Consequently, it is generally recommended that tests on the wastes be determined on an individual/site specific basis (Bond et al., 2012). However, at the time of writing, typical costs for testing of methane yields by robust methods in 2017 ranged from $1,000 upwards per waste sample tested. The test type used for this, measures methane potential, and is different from the Pork CRC test used for measurements of anaerobic microbial activity and chemical inhibition (Astals et al., 2015a).

Amongst the various wastes that can be co-digested with pig manure, glycerol from biodiesel production has attracted much interest, being highly concentrated and nearly 100% convertible into methane (Mata-Alvarez et al., 2014). A minimal amount of added glycerol can greatly boost methane production. The biggest concerns with glycerol and other similar high-strength wastes is the lack of knowledge about inhibitors present in such wastes and the high risk of exceeding safe organic loading limits (Mata-Alvarez et al., 2014). For example, glycerol can be inhibitory due to the presence of methanol from biodiesel production or high monovalent cation loads (sodium). With other AcoD wastes, examples include desugared molasses and dairy wastes such as whey processing wastes, which can be rich in sodium and potassium posing a salt inhibition risk (Mata-Alvarez et al., 2014; Nghiem et al., 2017). Distillery wastewater can be inhibitory due to phenolic compounds, citric waste can be inhibitory due to limonene, and some high-sulphur wastes such as seaweed can exacerbate sulphide content and hydrogen sulphide in biogas (Mata-Alvarez et al., 2014).

Legislation may restrict the flow of wastes onto a piggery and may limit the use of certain wastes in AcoD mixtures in order to minimise the risk of disease transmission to humans or livestock (Skerman, 2017a). Specific materials of animal origin (e.g. slaughterhouse and rendering wastes) or certain food residues may require sterilization or pasteurization prior to anaerobic digestion, but this depends on the waste type. For example, certain European regulations classify “manure and digestive tract content from slaughterhouse” as lower risk requiring no pre-treatment, whereas “slaughterhouse wastes from animals fit for human consumption” require pasteurization (Al Seadi et al., 2013b).

In general, the use of agricultural residues is probably preferred over energy crops (as used in parts of Europe, e.g. Germany), because when using agricultural residues, the methane (energy) production is not competing with food production (Pietsch, 2014).

2.4 Anaerobic co-digestion microbiology

Anaerobic digestion and AcoD is reliant on a balance of biological reactions carried out by various functional groups of microorganisms. These microorganisms progressively break down complex composite organic matter into simpler intermediate products and ultimately into methane, carbon dioxide and other trace by-products (Figure 2.1). Upsets and failure in the AD or AcoD process is typically caused by an imbalance between (Chen et al., 2008):

(a) upstream acid-forming microbial reactions carried out by bacteria that are responsible for the upstream reactions of hydrolysis, acidogenesis and acetogenesis; and

(b) downstream methane-forming reactions carried out by methanogenic archaea.

An imbalance in these upstream and downstream microbial reactions typically leads to an accumulation of volatile organic acids (Ahring et al., 1995), which can depress pH, cause inhibition of the biological reactions and can ultimately result in system failure (Chen et al., 2008; Demirel & Scherer, 2008; Sträuber et al., 2012). It can be difficult or costly to recover from such failures, because it may require alkali dosing to increase the pH of the liquid content in the failed CAP or digester (Tait et al., 2009). Poor selection of AcoD waste mixtures or increasing the organic loading rate of the CAP or digester performing the AcoD can lead to this imbalance and system failure.

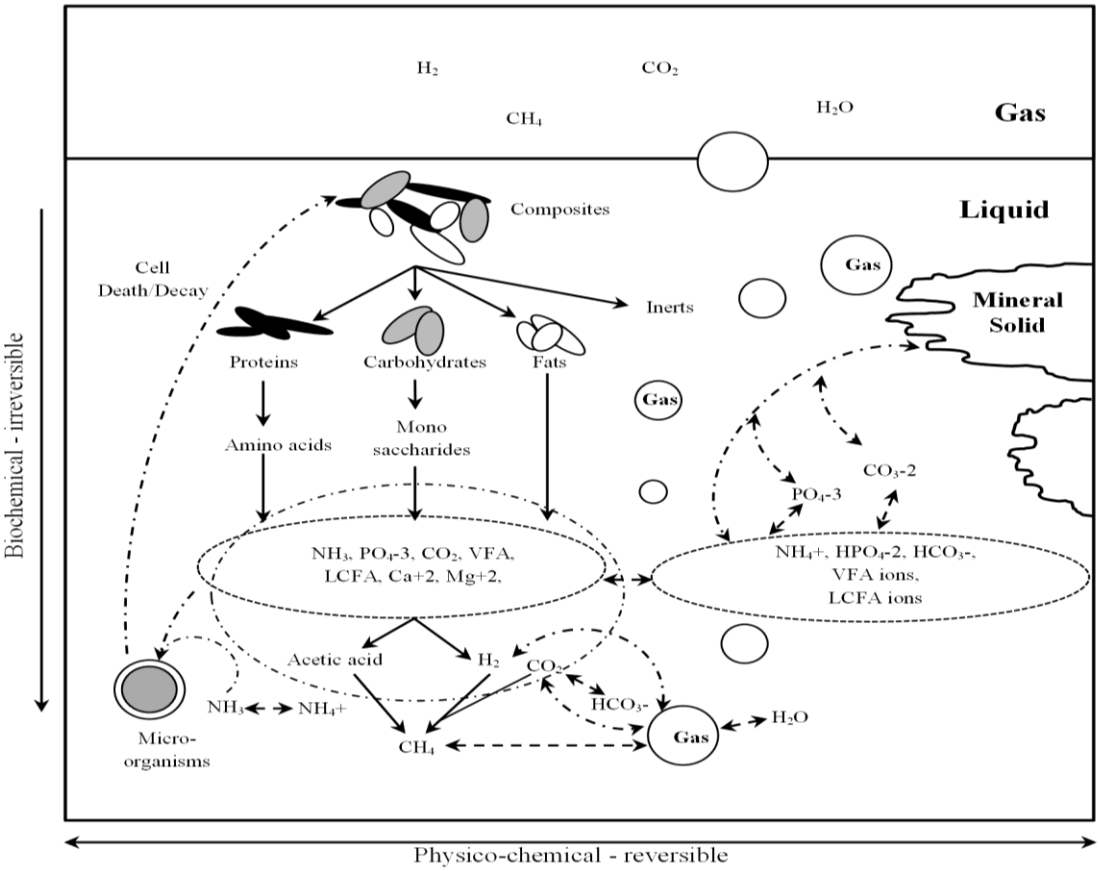


Figure 2.1 – A schematic of key anaerobic digestion reactions, progressively breaking down composite particulate wastes into soluble intermediate products and ultimately into methane. Adapted from (Batstone et al., 2002).

Another unwanted side effect of poor or failed digestion is increased odour potential. This is because the digestate then contains a significant amount of residual biodegradable organic matter (Ang & Sparkes, 2000; Mata-Alvarez et al., 2014), which can sustain unwanted microbial activity when the digestate is subsequently applied to crop land (Crolla et al., 2013). It is important that wastes selected for AcoD would digest quicker than pig manure. Otherwise, the retention time of a CAP or digester designed for pig manure digestion, would need to be increased to accommodate the slower degrading waste (Al Seadi et al., 2013b). For example, some wastes containing fats, oils and grease (FOG), are excellent methane boosters because of a high methane potential, but require very long treatment times to convert into methane (Al Seadi et al., 2013b). This is especially the case at lower liquid temperatures in CAPs during the winter months, because the rate of biological reactions slow down considerably at lower temperatures (Batstone et al., 2002). Digestate quality is further addressed in Section 2.6 below.

2.5 Logistics, onsite waste handling and anaerobic co-digestion infrastructure

Wastes and residues to be co-digested may only become available in large intermittent amounts. Delivery and storage logistics of such wastes then become important. Co-digestion wastes necessarily contain organic matter and most are wet or moist (Bochmann & Montgomery, 2013). Consequently, excessive or unsuitable storage can lead to a loss of methane potential, can cause odour by microbial decay leading to septic conditions, and the formation of toxic by-products (Bochmann & Montgomery, 2013). Some wastes are transported and received as liquids or slurries in tankers (Al Seadi et al., 2013b). Others are received as pre-packaged products (Figure 2.2). (Al Seadi et al., 2013b). In some cases, solid-liquid separation is required using, for example, a screen, sedimentation basin or screw-press separator, to remove recalcitrant solids such as sand and grit that can accumulate in a CAP or digester. With liquid wastes containing organic solids prone to settling, mixing may be required prior to digestion to a prevent loss in methane potential. Temporary storage tanks for liquid wastes typically have to hold several days of material, because the waste supply can be intermittent (Al Seadi et al., 2013b) and a digester or CAP requires regular feeding.

Wastes with FOG may require transport to site at elevated temperature, storage at elevated temperature, and feeding at elevated temperature, to prevent solidification and blockage (Nghiem et al., 2017). Also, FOG can cause problems with foul odour generation, adhesion onto bacterial cell structures, flotation of sludge and a loss of active sludge, or can accumulate on the liquid surface of CAPs, forming a recalcitrant scum or 'crust' layer (McCabe et al., 2014).

Figure 2.2 – Past use-by-date dairy products and juices debottled at a New South Wales (NSW) piggery to be used as pig feed ingredients. Some of these by-products, which are not needed for pig diets, end up in a CAP and so are co-digested with pig manure. Source: Australian Pork Limited https://aussiepigfarmers.com.au/

Solid wastes are commonly received in bunker silos (Figure 2.3). Such wastes are often pre-treated by milling or maceration to reduce particle size and improve homogeneity, sieved or screened of inert materials and slurried up as required for feeding (Figure 2.3). The high water content of pig shed effluent can assist with mixing and by improving the homogeneity of AcoD mixtures, because the effluent acts as a suspending medium or solvent for co-substrates (Al Seadi et al., 2013b). Pre-treatment can make organic solids more accessible to microbes for AcoD (Esposito et al., 2012), but can also have a significant parasitic energy load. Milled and screened solid waste can be screw-fed directly into a digester at around 10-12% solids, but the biodegradability of the waste is then important and the resulting organic loading rate must be appropriate for the digester.

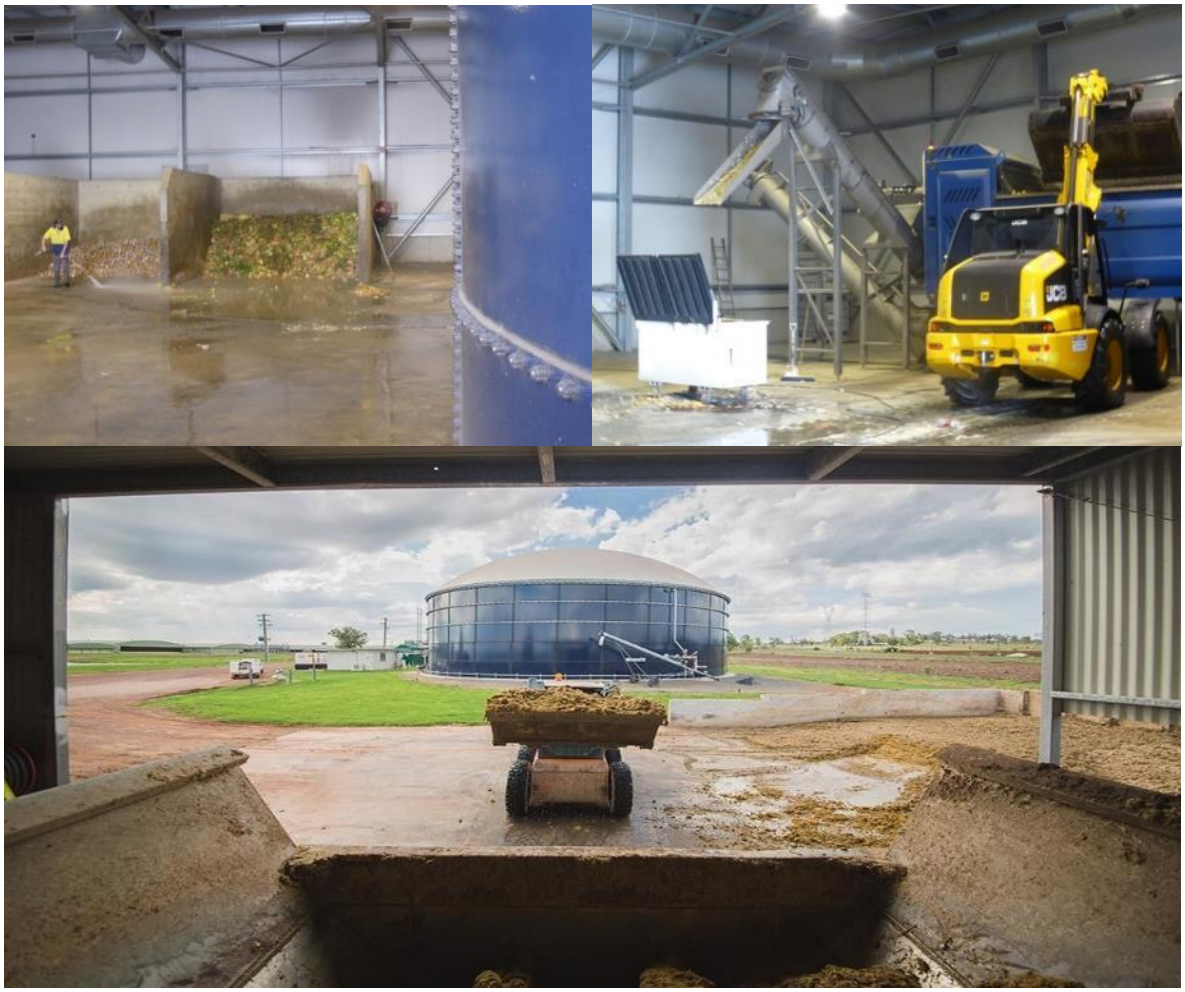


Figure 2.3 – Solid handling facilities associated with anaerobic co-digestion. The top two images are from a Richgro plant in Western Australia that co-digests various industrial wastes (©2017 Richgro, Channel Seven Perth and HOME in Western Australia (WA)). The bottom image is from a piggery in QLD that co-digests solid wastes with pig manure in a mixed heated tank digester (seen in the background). The solid waste is seen loaded into a bin with screw feeders, which force the material through a series of macerator pumps before being subsequently pulped with liquid pig flush manure for feeding into the digesters (http://bettapork.com.au/biogas-plant/).

The waste type that can be co-digested also depends on the digestion system type. For example, CAPs are usually only suitable for wastes with low solids about <3% (Batstone & Jensen, 2011). For higher solids concentrations, a mixed liquid, plug-flow or solid-phase digester is usually required (Batstone & Jensen, 2011). The additional wastes may exacerbate sludge accumulation or residue from AcoD, thereby increasing post-treatment and/or disposal costs. Some particulate wastes may altogether be unsuitable for CAPs, because of the risk of forming float layers (Al Seadi et al., 2013b) under the pond cover that are inaccessible to microbes, may interfere with biogas collection (Bochmann & Montgomery, 2013) or may exacerbate sludge accumulation. Co-digestion also influences important properties of digestate or sludge, changing for example dewaterability (i.e. ability to remove moisture to produce a stackable product), but currently these impacts are poorly understood (Nghiem et al., 2017). Lastly, co-digestion influences the quality of biogas produced and biogas cleaning requirements (Nghiem et al., 2017), with changes in recalcitrant trace ingredients such as hydrogen sulphide or siloxanes, or changing the proportion of methane in the biogas.

2.6 Digestate quality and nutrient loads

The selection of waste mixtures is important for digestate quality (Mata-Alvarez et al., 2014). Digestate quality is important to ensure health and safety and sound environmental practice when reused as a nutrient fertilizer. This includes;

1. being free of physical impurities such as plastics, rubber, metal, sand, stones and glass. For example, straw, garden wastes, crop wastes, energy crops, source-separated organic household wastes and food waste can produce high quality digestate but depending on their origin may contain unwanted physical impurities (Al Seadi et al., 2013a). Upfront physical barriers such as screens, sedimentation, etc. may remove such impurities (Al Seadi et al., 2013a). If particle size is too large, chopping, maceration or similar treatment can be used before AcoD, but these add complexity and cost. The parasitic energy load of such upfront treatment is also important and may require a significant fraction of electricity or heat produced from biogas.
2. being free of chemical impurities. Wastes such as sewage sludge or domestic wastewaters and bulk collected wastes, domestic wastes, and even food wastes, can contain chemical pollutants such as heavy metals or persistent organic pollutants (Al Seadi et al., 2013a). Pesticides and antibiotics can be present in agricultural wastes, with relatively minimal impacts on digestion performance (Al Seadi et al., 2013a), but causing digestate quality issues. Wastes can have high salt loads and salt types that are unwanted from an agronomic perspective (Mata-Alvarez et al., 2014). In some countries, the application of digestate from mixed-collection municipal solid waste to agricultural land is not allowed, because of high contamination risk (Al Seadi et al., 2013b).
3. hygiene, preventing pathogenic effects. Anaerobic digestion is effective at inactivating pathogenic matter such as bacteria, viruses, intestinal parasites, weeds and crop seeds and crop diseases (Al Seadi et al., 2013a). However, it is important to exclude high-risk biological materials (Al Seadi et al., 2013a) and to maintain biosecurity. Digestion temperature and digestion period are important in dictating pathogen destruction (Al Seadi et al., 2013a). At a lower temperature, more time is require for pathogen destruction (Al Seadi et al., 2013a). This is important for CAPs, which typically fluctuate in temperature with seasons.

Nitrogen and phosphorus loads are important when using digestate as a fertilizer. The nitrogen and phosphorus in other wastes mixed with pig manure need to be considered in revised nutrient management plans for a piggery (Tucker, 2015). Anaerobic digestion releases organic nitrogen from wastes into the liquid phase as dissolved ammonia, which is highly mobile. This ammonia can volatilize from uncovered effluent storage dams or when irrigated onto crop land, leading to nitrogen losses and nitrous oxide emissions (a potent greenhouse gas) (Wiedemann et al., 2016). The spreading of digestate can help offset inorganic mineral-based fertilisers (Lijo et al., 2014). However, excessive application of mobile nutrients increases the risk of leaching and damage to surface and/or groundwater (Tucker et al., 2010). Injection style applications of digestate reduces exposure to air, thereby reducing ammonia volatilization and potential for odour (Crolla et al., 2013) (Figure 2.4).

Figure 2.4 – A sedimentation and evaporation pond system (SEPS) (left) and vacuum tanker with slurry applicator (right) used at Australian piggeries for the management of sludge extracted from CAPs.

A large proportion of phosphorus in waste remains sequestered via anaerobic digestion in the form of sludge or digestate (Latif et al., 2017; Tucker et al., 2010) and is available as a fertilizer. For CAPs, when sludge is extracted periodically, the phosphorus accumulated in the sludge over time, becomes available in larger amounts at the times of desludging. Whilst most soils are capable of safely storing some phosphorus, excessive levels of phosphorus applied to soils can cause leaching into groundwater (Tucker et al., 2010), can damage surface water bodies, or can cause land degradation (Mata-Alvarez et al., 2014).

2.7 Summary, opportunities and future research needs

There is substantial opportunity for anaerobic co-digestion of other wastes with pig manure. Abroad, a number of carbon-rich wastes are regularly co-digested with animal manures, and methane production is enhanced by waste mixture properties. The increase in methane production by anaerobic co-digestion with high-energy substrates together with pig manure can leverage existing anaerobic processes and infrastructure (Mata-Alvarez et al., 2011). For example, existing CAPs or anaerobic digesters could be dosed with co-digestion substrates such as solid wastes (crop residues), liquid wastes (Fat Oil Grease (FOG)) or by-products from local industry (glycerol, whey, algae), provided these are suitable for the particular type of digestion system and provided that organic loading limits are not exceeded. In the case of glycerol, for example, organic loading rates and biogas production can increase by 20-50%, even with a moderate increase of 1-2% in the volumetric loading of the digester or CAP. The main factor dictating cost feasibility is transport distance of the wastes to a piggery, and the availability of the waste, essentially free of charge. The availability of wastes in close vicinity to a piggery, is very important because transport costs tend to dictate overall economic feasibility.

Co-digestion offers additional biogas energy for applications where energy demand exceeds supply from pig manure. For example, a number of piggeries in Australia operate as farrow-to-wean, and without the manure of the grower-finisher herd cannot meet onsite demand for heating energy. In such cases, co-digestion can boost methane production to meet the onsite energy demand. Co-digestion can be an environmentally sustainable way to re-direct wastes away from landfill and to beneficial use. Unfortunately, in Australia, landfill levies are still a new concept and vary from state to state. This causes uncertainty about prospects of revenue from diversion of wastes away from landfill. In the selection of wastes, it is also important to maintain digestate quality by avoiding wastes with persistent and problematic contaminants. This then allows beneficial use of digestate as a fertilizer on crop land.

The economics of co-digestion are assessed by comparing an increase in methane production (and the energy value) with the costs associated with increased residual solids to dispose or deal with after digestion. Initial cost-benefit estimates demonstrate that co-digestion of fat oil and grease may provide benefits of approximately 34 GJ/tonne (valued at over $200/ton). Table 2.2 below summarizes important features for co-substrate selection to increase methane production. For example, some substrates such as fat oil and grease and glycerol are excellent methane boosters and are highly biodegradable (high VS destruction), meaning that energy recovery from biogas can be greatly enhanced with minimal increase in residual digestate that requires handling and disposal after digestion. However, such methane boosters require careful management not to exceed safe organic loading limits or not to induce inhibition of the anaerobic digestion microbiology. Other wastes such as paunch or feedlot manure offer comparatively little additional methane and have a relative poor degradability, meaning that digestate amounts could be greatly increased, increasing post-handling and potentially disposal costs. For such co-substrates, the economic feasibility would likely require landfill levy benefits. We note that feedlot manure often has an existing market value as an organic fertiliser/soil amendment. This should be accounted for in any co-digestion economic feasibility assessment. Note that the energy value in Table 2.2 does not include any handling or capital costs, but also does not consider potential revenue from diverting the wastes away from landfill. All these aspects would need to be considered to understand the cost feasibility of specific projects.

Table 2.2 High-level benefit estimate for biogas produced per tonne of co-substrate co-digested

Substrate	Methane Yield (m^3 per tonne dry matter)	Volatile solids Destruction (%)	Energy Value ($ per tonne dry matter)
Fat oil and grease	900	80	$200
Glycerol	460	100	$106
Paunch	250	60	$58
Feedlot Manure	200	40	$44

Basis for calculation:
1. Energy content of methane 34 MJ/m^3
2. Conversion to electricity: 0.35
3. Electricity value: 3.6 MJ = 1 kW.h and 1kW.h = $0.07

Waste handling and digestion infrastructure required for co-digestion is very important and depends on the types of waste to be co-digested (e.g. solid, liquid, pre-packaged). Appropriate storage is also important to provide a steady waste supply whilst minimizing losses in methane potential. Methane potentials vary widely between different waste types, and whilst textbook values are available for many wastes, laboratory testing can resolve important differences and help to identify inhibition risks.

Currently, there has been very little evaluation of co-digestion at temperatures other than 37°C. We believe this increases the risk of co-digestion due to the broad range of anaerobic processes operating at different temperatures. It is known that liquid temperatures in CAPs, without heating, can vary by as much as 20°C between seasons in Australia. Anaerobic degradation rates are expected to change with temperature. There is also a need to better understand the flow-on effects of various co-digestion waste mixtures on digestate characteristics, which affect digestate management (e.g. dewaterability, pumpability, etc.).

Whilst co-digestion generally boosts methane production, it is critical not to exceed safe organic loading limits, otherwise this may cause upset or failure in the microbiology. Interestingly, there is evidence that the maximum sustainable addition rate of a co-substrate is dependent on other components in the substrate mixture. For example, different glycerol overload thresholds of 1% w/w (sewage sludge (Fountoulakis et al., 2010)), 4% w/w (pig manure, (Astals et al., 2011)) and 10% w/w (cattle slurry, (Robra et al., 2010)) have been reported. However, the reasons for this behaviour is a subject of on-going research. Future research should clarify organic loading capacity of co-digestion for specific wastes co-digested with pig manure, including for relevant digester types such as ambient temperature CAPs. With national initiatives such as the Biomass Producer (http://biomassproducer.com.au/) and AREMI (http://nationalmap.gov.au/renewables/), the availability of wastes to co-digest is being mapped and, in the future, such platforms could become increasingly useful to identify and negotiate feedstocks for co-digestion with pig manure on-farm.

Lastly, it is noted that co-digestion should ideally be considered during the planning and design stage of a biogas system/project, because digestion infrastructure designed specifically for piggery manure may later have limited capacity to handle and process other co-digestion wastes. Conversely, if systems are designed for co-digesting pig manure with other wastes, the piggery operator should be confident of a reliable supply of consistent quality wastes into the future, so that the benefits could justify the costs of additional infrastructure required for the co-digestion.

3. Desludging study

This section provides an overview of important issues relating to sludge management in CAPs, and outlines the method and results of a study carried out to quantify potential impacts of desludging on methane yield.

3.1 Background

Desludging is an on-going area of research in pork biogas. The successful operation of CAPs relies on adequate treatment times for conversion of manure organic matter into methane. This often involves natural settling of manure solids inside a CAP (Figure 3.1), so that these settled solids are retained for an adequate treatment time. This is especially important because CAPs generally operate at ambient temperatures, so that AD is usually slower when the manure is at the lower liquid temperatures (especially below 15°C). However, it has been suggested that the prolonged hydraulic and solids retention times in CAPs can somewhat compensate for the lower operating temperatures (Heubeck & Craggs, 2010).

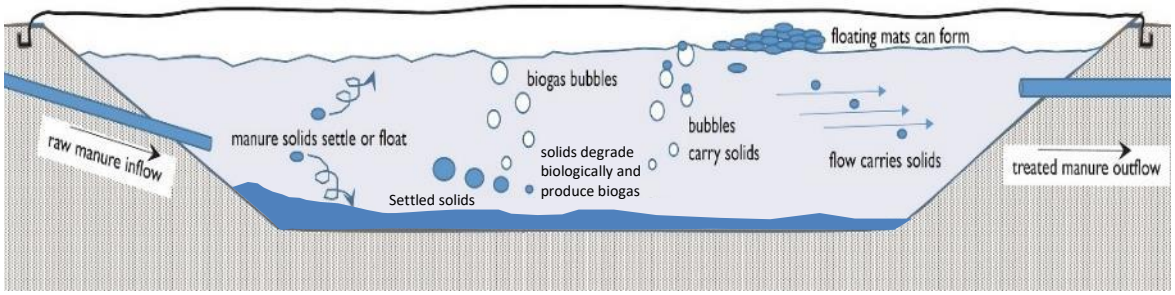


Figure 3.1 – Schematic showing how pig manure solids can accumulate as sludge and scum in a CAP.

Eventually, settled manure solids become biochemically inert as a result of extended AD and are then no longer convertible into methane. These inert settled solids, commonly referred to as sludge, progressively displace active treatment volume in a CAP. The size of CAPs then allows for an additional dead volume to accommodate accumulating inert sludge (ASABE, 2011). This sludge volume allowance can be smaller if sludge is periodically extracted (ASABE, 2011) from a CAP, for example by pumping via sludge extraction pipework (See below).

The contents of a CAP is often described as three main constituents; namely headspace biogas under the cover, liquid supernatant (active volume) and settled sludge (inert volume). The problem with this description is that significant settling of solids occur in as little as 30 minutes after solids enter the liquid under the pond cover (Birchall, 2010), whilst biological activity in the settled solids logically continues for some time afterwards (Birchall, 2010). Consequently, the settled solids consist of active solids with substantial residual methane potential, as well as biochemically inert solids with minimal residual methane potential.

Solids concentrations in sludge generally increase over time (Birchall, 2010) by progressive compaction and consolidation of solids in the settled sludge layer. This is very important, because sludge with a high solids content is much more difficult to pump than sludge with a low solids content (O'Keefe et al., 2013). Sludge therefore needs to be extracted regularly, so that it is pumpable. However, not all sludge should be removed, because it provides a media for growth of anaerobic microbes (Chastain & Linvill, 1999) and if sludge

is extracted too frequently, active solids with significant residual methane potential may also be extracted, thereby leading to significant losses in methane yield from the CAP. There is currently no data available in the literature on residual methane yield in extracted sludge.

In the research described in this section, sludge samples were collected from CAPs of demonstration sites associated with the Pork CRC Bioenergy Support Program. The respective CAPs were being operated with different desludging frequencies. Residual methane potential was measured for the sludge samples by a well-established BMP test protocols, as described below. The residual methane potentials were then used to carry out a methane yield balance and quantify potential impacts of desludging on methane recovery.

3.2 Methods

Sludge was extracted from the base of various CAPs at Australian piggeries A to F. At piggery A (Figure 3.2) in VIC, the CAP had an effective volume of 21.8 ML. It was fed with pig manure from 15,000 standard pig units (SPU) as well as waste by-products. The CAP had a nominal organic loading rate of 0.188 kg VS/m^3/day, a nominal hydraulic retention time of 44 days, top bank dimensions of 44 × 129 m, a depth of 6 m and a compacted clay base. The pond was constructed with a raised bank, and manure was pumped to the pond after coarse screening. Sludge was typically extracted via 300 mm HDPE sludge extraction ports installed angled through the side bank beneath the cover trench and entering the pond near the bottom (~0.5 m above the base). These ports were located approximately 35 m from the pond inlet and approximately evenly spaced 35 m apart after that. A section of suction pipework was connected directly to the flange of the sludge extraction pipe, and created a syphon down to a port to which a vacuum tanker was connected, thereby assisting with the extraction of sludge. The vacuum tanker was positioned down-slope from the point where the sludge extraction pipe exited the pond embankment. In the months leading up to sludge sampling for the present analysis, sludge was being extracted in batches and transported in the vacuum tanker to nearby crop land where it was spread as a fertilizer. The sludge sample from Piggery A was collected from the extraction port closest to the inlet of the pond.

At piggery B (Figure 3.3) located in NSW, the CAP was operating at a relatively low liquid level (approximately 1.5 m below the pond crest). The pond working volume was in question at the time of the site visit, but was thought to be approximately 10 ML (48 m x 60 m x 4 m liquid depth). The pond was receiving direct flushed manure from an estimated 12,780 SPUs of breeder pigs, at a very short hydraulic retention time of 14 days because of high flushing volumes (55 L/SPU/day). The pond had not been desludged since it had been filled with effluent after construction in 2009 and the sludge sample was collected in November 2013 (~4 years without desludging). The sludge sample was collected via a screw-capped vent port on the cover. IMPORTANT, the procedure for performing this sampling was based on a rigorous risk assessment to prevent the hazardous exposure of piggery personnel to toxic biogas. This involved fully vacating the biogas headspace under the cover by operating the biogas flare and dedicated blower at full capacity for a period, so the cover rested on the liquid pond surface preventing the unwanted venting of biogas via the port through which sludge was being collected. The sludge was collected with a stainless steel tube with a ball plug at the end to allow collection of a sample at a desired depth. The sample used in the analysis was from a port on the cover at the centre of the pond, but the sludge level was also checked via ports at other locations on the cover, and was observed to be at the depths indicated in Figure 3.3. In this pond, perhaps due to the high liquid flowrate and thus short hydraulic retention time, sludge tended to accumulated near the pond outlet so that on the days that there was no flushing, the outlet of the anaerobic pond tended to block completely.

Figure 3.2 – Desludging and sludge spreading infrastructure at Piggery A in VIC, Australia

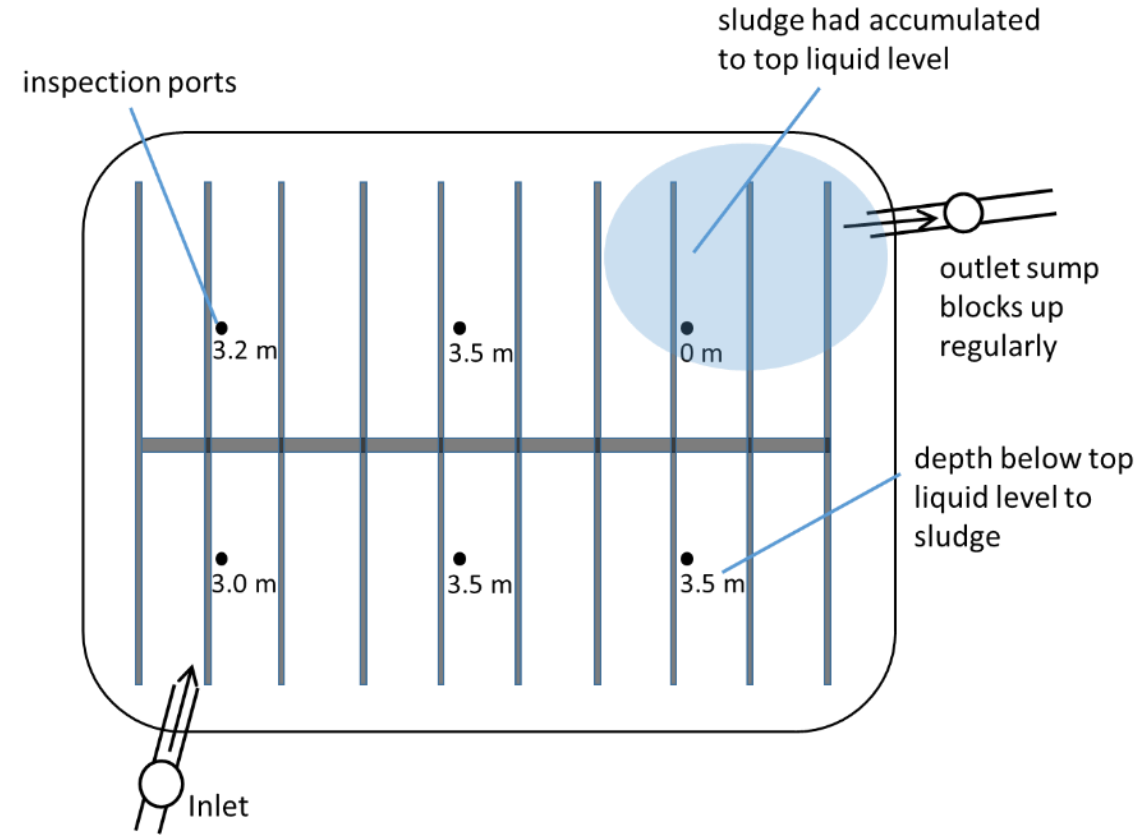


Figure 3.3 – Pond cover infrastructure at Piggery B in New South Wales, Australia, showing 150mm screw cap vent ports through which sludge was collected, with approximate locations on the cover and observed sludge levels

Piggery C (Figure 3.4) located in New South Wales has a CAP of 5 ML fed with direct flush manure from an estimated 4,295 SPU breeder unit (organic loading rate of approximately 0.2 kgVS/m^3/day). Sludge is extracted regularly from this CAP over the 6 months of summer from a PVC sludge extraction pipe protruding through the pond bank at an approximate 45 degrees angle into the base of the pond similar to Piggery A. Sludge was only extracted via one sludge extraction pipe located on the outlet bank of the pond. The sludge was pumped via a smaller HDPE pipe fed into the outer PVC sludge extraction pipe and into a sump from where it flows via gravity to an onsite sedimentation and evaporation basin to be dried and spread on cropland. The desludging at this piggery occurred frequently (essentially yearly). The sludge sample at this piggery was collected via a vent port on the pond cover (Figure 3.5), similar to Piggery B. The vent port used for sludge sampling was that nearest to the pond outlet. A sampling pole was used with a plug at the end that allowed sampling from a defined depth. The sludge samples were collected at depths of 2 m, 4 m and 5 m below the pond cover, with the cover laying on the pond liquid surface. Again, IMPORTANT, the procedure for performing this sampling must be based on a rigorous risk assessment to prevent the exposure of piggery personnel to hazardous biogas.

Piggery D (Figure 3.6) located in WA was a 7,089 SPU breeder unit with an 10ML uncovered pond having a hydraulic retention time of >100 days and 0.2 kgVS/m^3/d organic loading rate planned for future installation of a pond cover. The pond was first filled with effluent in October 2012 and the sludge sample was collected approximately a year afterwards in October 2013. The pond had not been desludged prior to this sampling event. The sludge sampling marked the beginning of a desludging trial carried out at the piggery, whereby sludge was pumped via 1.5" (3.8 mm) diameter flexible suction hose fed through a larger HDPE sludge extraction pipe. The larger extraction pipe in this case was buried at an angle within the pond side bank, had an elbow bend below the pond floor and then protruded through the pond floor near the centre of the pond (Figure 3.7). The sludge was pumped using a 1.5" air-driven diaphragm pump, into an adjacent sludge drying bay (Figure 3.6).

Piggery E was a breeder farm in south-east QLD with a 700 sow (1400 SPU) capacity, previously described by Skerman and Collman (2012). At this piggery, biogas was produced by a 1.7 ML partially covered (~50% of the liquid surface) anaerobic pond (pCAP) treating the manure effluent from a pull-plug system at a nominal organic loading rate of 0.205 kg VS/m^3/d and a hydraulic retention time of 140 days. The sample was collected in August 2014, and the pond was desludged in 2012 (2-year desludge to sample interval). Sludge samples were collected at two locations from the uncovered portion of the lagoon, one location near the outlet, and one location near the inlet. The sampling procedure involved positioning an aluminium boat at the sampling site, before collecting the samples using a sludge sampling pole with an internal cavity that could be exposed by the user to sample sludge from a defined depth. The pond is approximately 2 m deep and the sludge was collected from two nominal depths termed top (1m below liquid level) and bottom (1.5-2 m below liquid level).

Figure 3.4 – Sludge extraction infrastructure at Piggery C in NSW, showing the PVC sludge extraction pipe, with smaller pipe through which sludge was pumped, and the sludge pump from different perspective angles

Figure 3.5 – Vent port on the pond cover of Piggery C in NSW, through which sludge samples were collected.

Figure 3.6 – Desludging infrastructure at Piggery D in Western Australia, with which sludge samples were also collected.

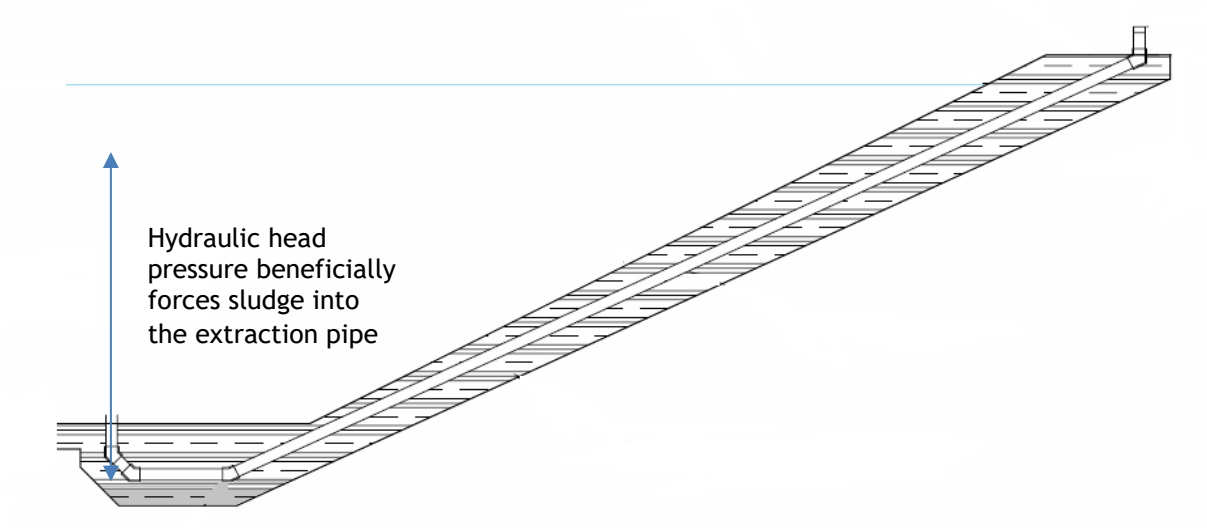


Figure 3.7 – Schematic of desludging pipework at Piggery D in Western Australia. Adapted from McGahan et al. (2012).

In all cases, the sludge samples were sent on ice (but not frozen) by express post to a laboratory at The University of Queensland (UQ) for analysis. Once received, the samples were immediately placed in a refrigerator at 7°C prior to analysis within 1 week. The sludge samples were analysed for TS and VS using Standard Method 2540G (APHA, 2005). Residual methane potential was then measured by placing a known amount of the sludge in a 160 mL glass serum bottle, flushing the headspace of each bottle for 1 min (4 L/min) with 99.99% nitrogen gas (N_2), sealing the serum bottle with a rubber stopper and aluminium crimp and placing the bottles in a temperature controlled incubator at 37°C. Periodically, the bottles were mixed by swirling before samples of headspace gas were collected using a gas-tight syringe and a fine-gauge needle. The headspace gas pressure in each test bottle was then measured as an overpressure on the collected gas sample using a bench-top manometer. The composition of the biogas sample was subsequently analysed by gas chromatography using a Shimadzu GC-2014 equipped with a HAYESEP Q 80/100 packed column, a thermal conductivity detector (100 °C) and Argon as carrier gas (28 mL/min). The measured cumulative residual methane potential was then normalised to the amount of VS present in the sludge sample added to each test bottle. In the case of Piggery C sludge, an inoculum was added to facilitate the digestion of residual organic matter in the sludge. This inoculum was from a mesophilic anaerobic digester at a conventional municipal wastewater treatment plant in QLD fed with mixed sewage sludge (50% primary and 50% secondary sludge on a volume basis) at a hydraulic retention time of 23-24 days and a temperature of 35°C. In this case, a separate control test was run without added sludge to determine background methane production because of residual degradable material in the inoculum. This background methane was then subtracted from methane produced by the test bottle to give the net residual methane potential of the piggery sludge sample. In cases, the sludge samples were diluted with deionized water (never more than 1:3) before being added to the test bottles, to facilitate the subsequent digestion of residual organic matter in the sludge.

The test data were analysed with the package Aquasim 2.1d, fitting data of normalized cumulative methane ($B_{t,res}$) produced over time (t) with a non-linear least-squares fit of a first-order kinetic model (Equation 3.1):

$$B_{t,res} = B_{0,res}\left(1 - e^{-k \cdot t}\right) \quad (3.1)$$

where k is the first-order degradation rate coefficient and $B_{0,res}$ the residual methane potential. $B_{0,res}$ and k have units of m^3 CH_4/kg VS_{fed}, and per day, respectively. A 95 % confidence interval in $B_{0,res}$ was estimated based on a two-tailed *t*-test (5 % significance threshold) with standard error determined using a Secant Fisher information matrix as per Jensen *et al*. (2011).

3.3 Results

Figure 3.8 below presents measured data for cumulative residual methane potential of the various pond sludge samples.

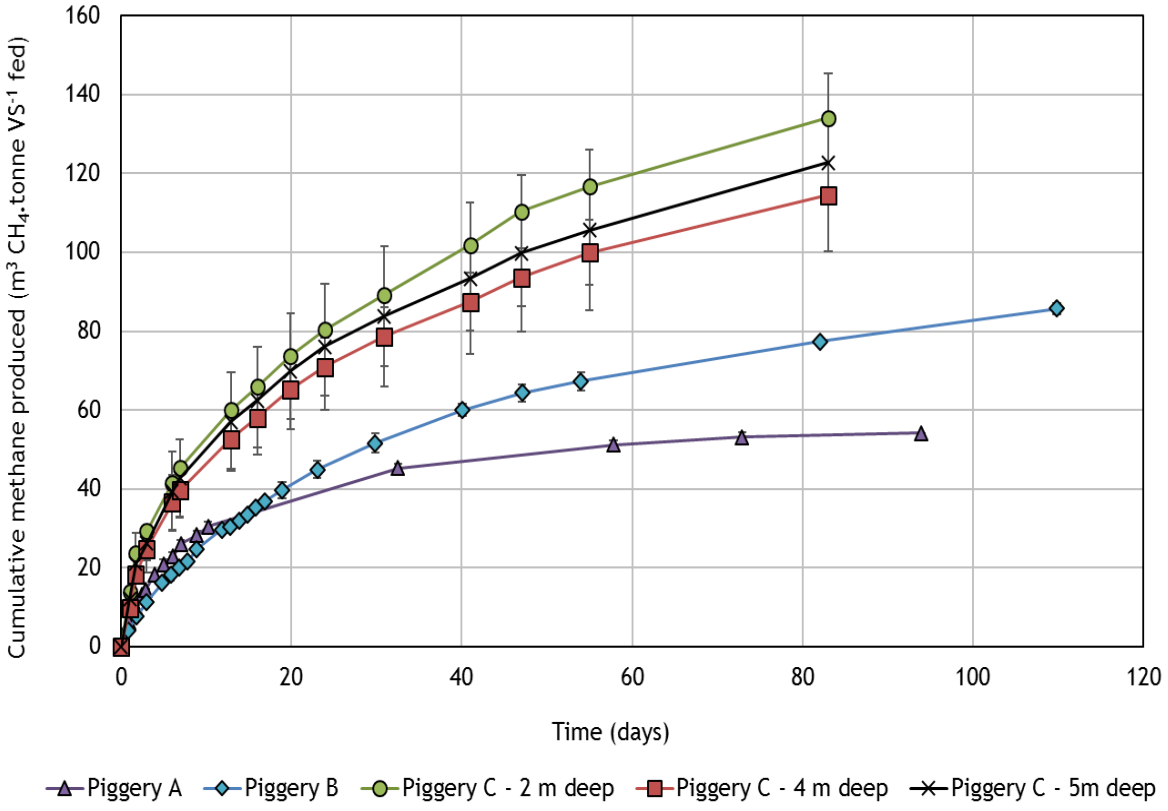


Figure 3.8 - Cumulative normalized methane produced over time by AD of residual organic matter in the sludge samples.

Table 3.1 summarises the results. VS to TS ratio is often used as a proxy for residual methane potential, because VS is a measure of organic matter content. As the manure breaks down via AD in a CAP, the VS is converted into biogas and so the VS/TS ratio progressively decreases. For reference, the VS/TS ratio of pig manure discharged from conventional sheds is typically 0.8 (Gopalan et al., 2013b). Therefore, a settled sludge with a VS/TS ratio of 0.66 would indicate a 51% conversion of VS into methane. A VS/TS ratio of 0.6 would indicate a 63% conversion of VS into methane, a VS/TS ratio in sludge of 0.55 would indicate a 69% conversion of VS into methane, and a VS/TS ratio in sludge of 0.44 would indicate an 80% conversion of VS into methane. An 80% conversion is approaching the maximum practically achievable conversion. This analysis assumes conservation of ash (fixed solids), meaning that the selective migration of ash and/or VS along the length of the CAP is assumed to be negligible, and so the manure is being considered as a single digesting batch of material. The analysis results are also depicted in Figure 3.9.

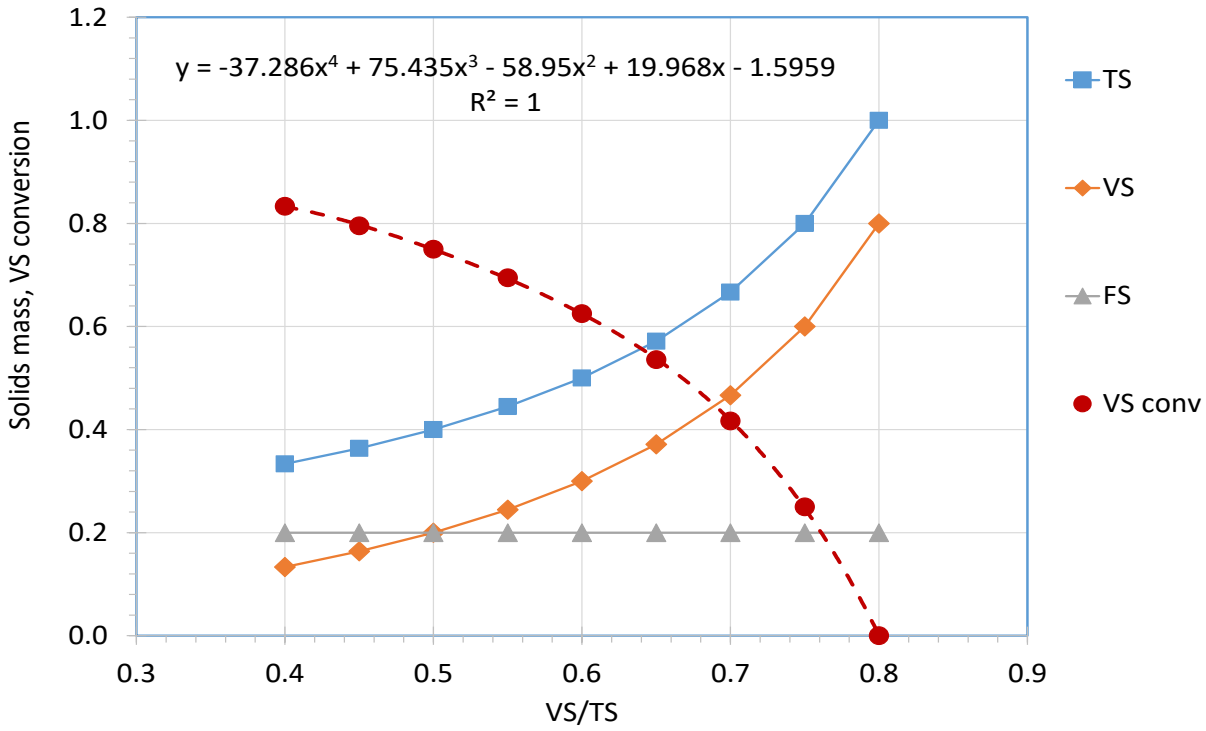


Figure 3.9 – Hypothetical VS to TS ratios, when various proportions of VS in pig manure have been converted into biogas (Skerman, 2017b).

The VS/TS ratios in Table 3.1 indicate that the extent of conversion of VS into methane had been reasonable-to-good, and for Piggery E specifically, conversion had excellent at near 80%. A comparison of VS/TS ratios for samples from piggery C and 4 m and 5 m depths, suggested a sharp transition from less stable sludge at 4 m to the more stable sludge at 5 m depth, with the more stable sludge having the lower VS/TS ratio. However, residual methane yield was not significantly different with sample depth at this piggery. The low residual methane yield in sludge compared to raw manure, indicated that the progressive breakdown of VS in the sludge had left non-biodegradable VS, so that the residual methane yield for the sludge samples (80-130 Nm^3/kg VS fed) was considerably lower than that of raw pig manure (450 Nm^3/kg VS fed). Whilst the data set collected in this study is small, the results in Table 3.1 did indicate some benefit of a 2-year+ desludging frequency compared to yearly desludging, e.g. Piggery B and Piggery E had the lowest VS/TS ratios, corresponding to the longest desludging frequencies. Albeit that Piggery A exhibited a relatively high VS/TS ratio despite a 2 year desludging frequency.

Table 3.1 – Characteristics of sludge extracted from various CAPs at Australian piggeries

Parameter	Piggery A, VIC	Piggery B, NSW	Piggery C, NSW	Piggery C, NSW	Piggery C, NSW	Piggery D, WA	Piggery E, QLD		
Type of piggery	Weaner to finisher	Farrow to wean	Farrow to wean	Farrow to wean	Farrow to wean	Farrow to wean		Farrow to wean	
Approximate sampling depth	5m below cover	3.5m below cover	2m below cover	4m below cover	5m below cover		1.5-2m deep Influent end	1.5-2m deep Effluent end	1m deep
TS (g/kg)	49 ± 3	89±1	60 ± 2	102 ± 1	90 ± 14	75 ± 1	306 ± 4	238 ± 7	129-189
VS (g/kg)	31 ± 3	49±5	40 ± 1	67 ± 1	54 ± 7	47 ± 1	124 ± 8	103 ± 1	66-86
VS/TS	0.63±0.07	0.56±0.01	0.66±0.01	0.66±0.02	0.6 ± 0.1	0.62 ± 0.01	0.41 ± 0.02	0.43 ± 0.01	0.45-0.51
Desludge to sample interval	2 years	4 years	1 year	1 year	1 year	1 year		2 years	
Residual methane potential (Nm^3/kg VS fed)	51±1	82±2	125±14	106±10	112±10	N/M	N/M	N/M	N/M

Estimates of error are given at the 95% confidence level (±), estimated using a two-tailed t-test with appropriate degrees of freedom
N/M - Not measured

3.4 Discussion

Both a lower VS/TS ratio and a lower residual methane potential in the settled sludge help to reduce methane potential losses when a CAP is desludged.

An overall mass balance can then be performed, using a nominal value of 0.8 for the VS/TS ratio of raw pig manure and 360 Nm^3/kg VS fed (Skerman et al., 2016) as the nominal methane potential for raw pig manure (Gopalan et al., 2013a). Also, for this analysis, a high nominal value of 12% was used as the VS concentration of settled sludge and 120 Nm^3/kg VS fed was assumed as a conservatively high methane potential in the settled sludge. The sludge accumulation rate was assumed to be 0.00137 m^3/kg TS fed (ASABE, 2011). The results from this mass balance are depicted in Figure 3.10, which shows that extracted sludge represents 7.3% of the overall methane potential that entered the CAP.

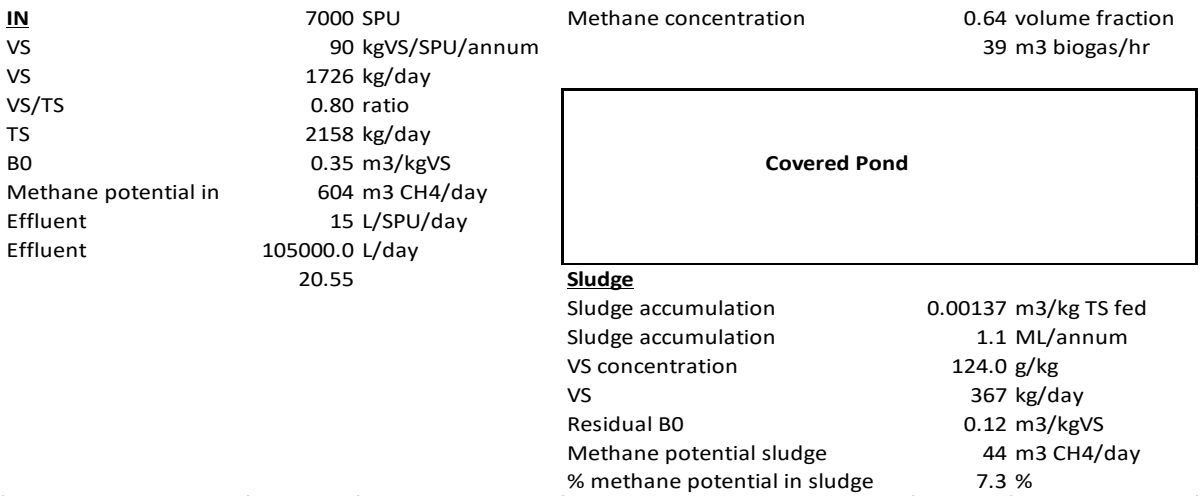


Figure 3.10 – Hypothetical mass balance of methane potential of settled sludge in a CAP

This analysis was encouraging, because it indicated that losses in methane potential by desludging prematurely, could account for a maximum of 10% of the overall methane potential entering the CAP with raw pig effluent. Desludging losses of methane potential are therefore perhaps not a major concern, and sludge should instead be extracted frequently enough to ensure that it is pumpable (Birchall, 2013).

Sludge in the CAPs with 2-year desludging frequencies was thick but not too thick to pump, and so had not consolidated to an extent where pumping would be an issue. For this reason, a desludging frequency of 2 years is in general recommended, to provide longer retention times for conversion of manure solids organic matter into methane, and yet allow reasonably trouble-free desludging. It is important to determine a reasonable estimate of sludge accumulation based on the 0.00137 m^3/kg TS fed guideline noted above, and to base sludge accumulation predictions on a reasonable estimate of manure dry matter (TS) fed to the CAP.

We note that specific piggeries may instead elect to desludge at different frequencies than every 2 years, for example, to manage water balances of sludge drying bays during wet/dry seasons, or to sustainably manage nutrient loads applied to cropland. However, a frequency in excess of 2 years may cause excessive sludge consolidation and make sludge increasingly difficult to pump/extract.

4. Ammonia and sodium inhibition studies

4.1 Introduction

Methane production by AcoD or AD is sensitive to inhibitors that are often naturally present in pig manure and other relevant wastes (Chen et al., 2008). The impact of inhibitors causes sub-optimal digestion performance with low methane yield and digestion process instability. Figure 4.1 illustrates the potential negative impacts, including:

1. a delayed start-up during commissioning of a digester; and/or
2. slower conversion of the waste into methane;
3. reduced conversion of the waste into methane; and/or
4. risk of acidification due to the impact of the inhibitor on methanogens.

Combatting inhibition is therefore an important strategy to enhance methane production from CAPs and anaerobic digesters.

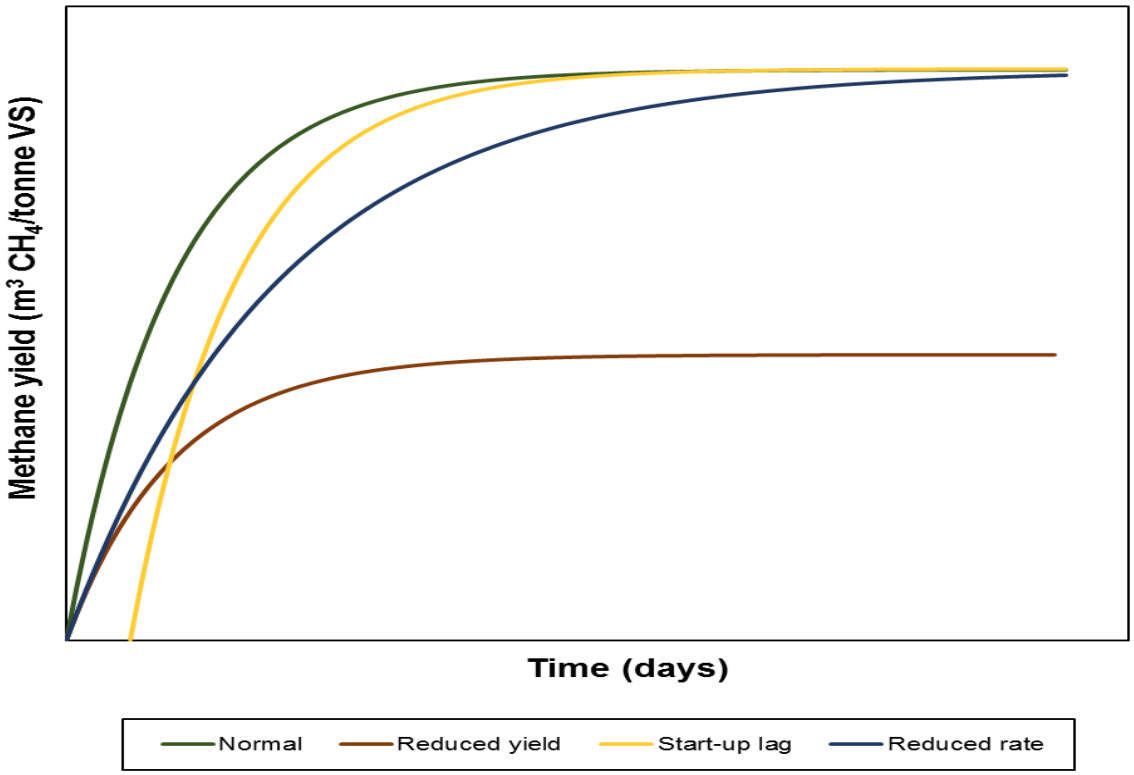


Figure 4.1 - A schematic depicting hypothetical impacts of inhibition on batch anaerobic digestion.

Methane-forming archaea, specifically acetoclastic methanogens, are generally considered most sensitive to inhibitors (Astals et al., 2015a; Chen et al., 2008). This is important, because acetoclastic methanogens typically dominate the archaeal community in many digesters and are responsible for as much as 70% of methane produced by anaerobic digesters (Astals et al., 2015a).

Ammonia nitrogen is a very important inhibitor of AD microbiology, which is naturally produced as nitrogenous organic matter decays in the AD process (e.g. protein, amino acids, urea and nucleic acid) (Chen et al., 2008; Rajagopal et al., 2013; Yenigün & Demirel, 2013b). High ammonia concentrations frequently occur when treating nitrogen-rich substrates such as animal manures or livestock wastes, by increased organic loading rates or by pre-treatment of wastes prior to AD (e.g. thermal-hydrolysis (Batstone et al., 2010)). Inhibition by ammonia threatens many anaerobic digesters and is arguably one of the most widely investigated inhibitors in AD systems.

Ammonia nitrogen is present in anaerobic digesters in two forms, namely free ammonia (NH_3) and ammonium ions (NH_4^+), both of which can be inhibitory (Hadj et al., 2009; Rajagopal et al., 2013). The fractionation into NH_3 and NH_4^+ depends on total ammonia nitrogen concentration (TAN = NH_3 + NH_4^+), pH and temperature (Batstone et al., 2002). The emphasis to date has been on NH_3, because AD is strongly inhibited by NH_3 at much lower concentrations than by NH_4^+ (Kayhanian, 1999; Rajagopal et al., 2013). Most studies have assessed the impact of ammonia in terms of TAN and/or NH_3 concentrations (Rajagopal et al., 2013; Yenigün & Demirel, 2013b). However, publications studying ammonia inhibition at several TAN and pH values have observed that inaccurate descriptions of inhibition result from only considering NH_3 concentration (Hadj et al., 2009; Lay et al., 1998; Nakakubo et al., 2008). This is especially important at high TAN concentrations, when both NH_3 and NH_4^+ can be inhibitory.

Regarding analysis of inhibition, inhibition tests are often performed by measuring the rate of an AD biological process in the presence of various concentrations of a specific inhibitor. Inhibition test data are illustrated in Figure 8, which shows specific methanogenic activity (SMA) test data for sodium as the inhibitor, from the continuous research detailed further below. This test used the Pork CRC inhibition test method reported by Astals et al. (2015a) with acetate as the carbon source. The test recognises that acetoclastic methanogenesis (on acetate) is likely to be a biological step sensitive to inhibition. A linear regression fit of each of the respective methane production data sets in Figure 4.2a, gives the conversion rate of acetate into methane or specific methanogenic activity (SMA, units of gCOD-CH_4/g inoculum VS/d) which is plotted against inhibitor concentration in Figure 4.2b.

Of common interest is the so-called KI_{50} value, which is the inhibitor concentration at which the measured biological rate is half of the measured maximum rate. The KI_{50} value provides a measure of inhibition resilience, with microbial communities having a higher KI_{50} value being more resilient to the particular inhibitor being tested.

However, whilst the KI_{50} value is useful from a comparison perspective, the following values are perhaps of greater interest for the practical operation of a digester or CAP;

a) a lower limit of inhibitor concentration (KI_{min}) whereat no inhibition occurs, so that methane production would not be significantly affected if the digester or CAP was operating at or below this inhibitor concentration, and;

b) an upper limit of inhibitor concentration (KI_{max}) whereat complete inhibition occurs, in order to confine reasonable operating conditions for a CAP or digester.

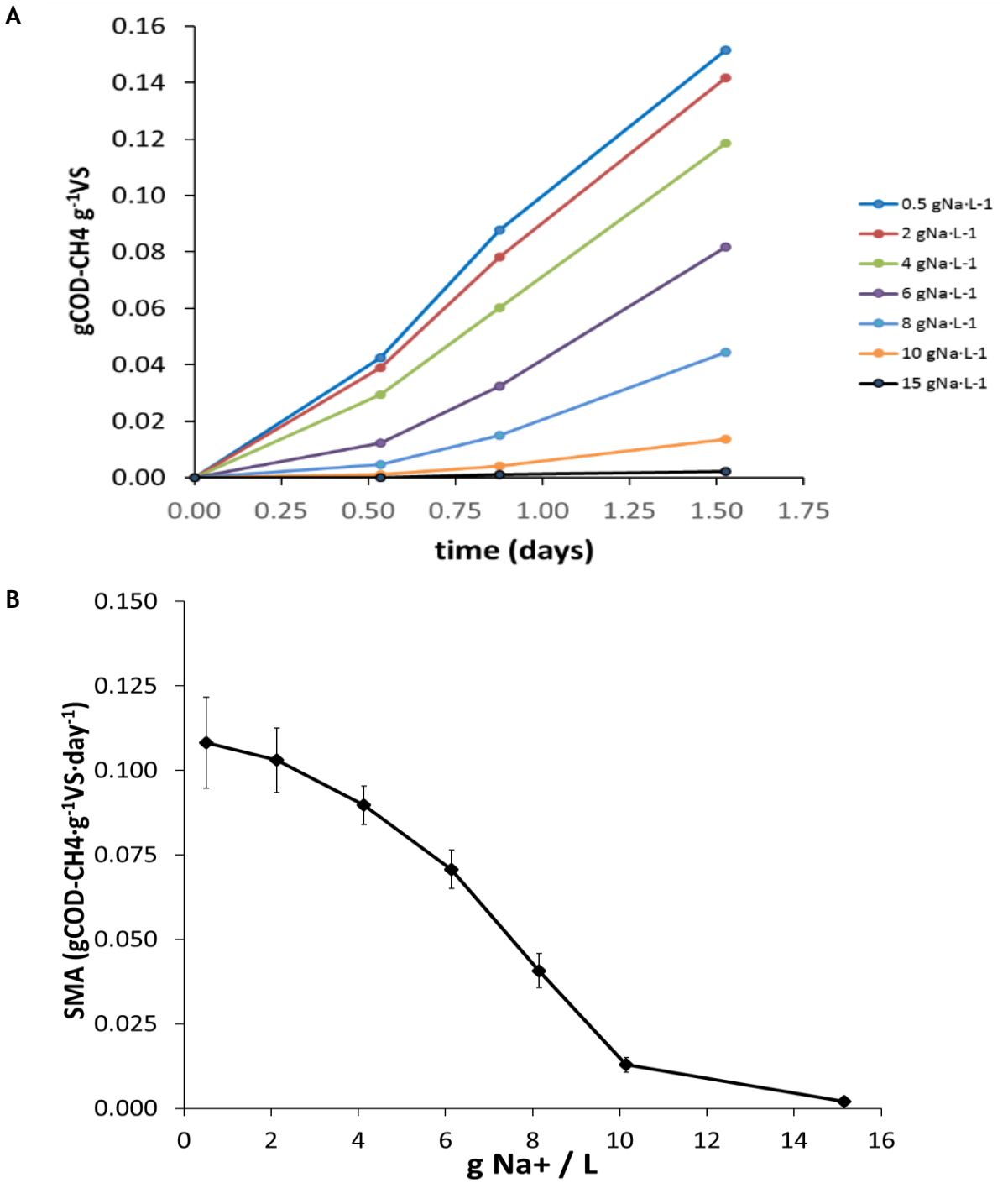


Figure 4.2 - Example inhibition test data, showing (A) Normalised methane production with acetate as carbon source, and at various concentration of an added inhibitor (in this case sodium or Na+), and (B) the calculated specific methanogenic activity (SMA) at the various added Na+ concentrations. This dataset was specifically collected for an effluent microbes from continuous digester R1, operation day 100, as described in a later section below.

4.2 Research aim for this section

Important inhibitors such as ammonia and sodium are present in piggery effluent and are unavoidable. Possible solutions that have been suggested are:

1. to dilute the effluent so that inhibitors remain at tolerable concentrations, but this can require large quantities of clean water that may not be available; or
2. to remove the inhibitor from the effluent before it has a negative impact on AD This can be done by source control, such as separating manure solids from the piggery effluent, but this may not be feasible and can lead to a loss in methane potential. Ammonia can also be removed by physico-chemical methods such as by air stripping, but these can be energy intensive and generally not cost-feasible for on-farm applications.

Because neither of the two above-listed options appeared adequate for piggery biogas in Australia, the present research aimed to examine whether more resilient microbial communities could be fostered/bred/engineered/induced to tolerate higher inhibitor concentrations with less negative impact. With this aim in mind:

1. Thirteen different anaerobic inoculums were collected from very distinct digestion backgrounds, with digesters being fed with a variety of substrate types, and operating at different digester configurations and operating temperatures. Inhibition testing was then performed in lab-scale batch experiments to examine for these inoculums the relationship between (i) digester operating conditions, (ii) microbial community composition, (iii) acetoclastic methanogenic activity, and (iv) inhibition resilience (quantified by measured KI_{50}). The important inhibitor, NH_3, was specifically selected for testing in this study. The study aimed to determine whether practical operating conditions (e.g. pH and temperature) could be used to engineer resilient microbial communities in CAPs or digesters.

   Section 4.3 reports on this inoculums study.

2. Continuous digesters were operated with a pig manure feedstock containing added inhibitors. This allowed a study of changes to microbial communities because of chronic exposure to inhibitors. The response of microbial communities to such stress conditions is a measure of potential resilience and adaptability. Two important and relevant inhibitors were studied in this work, namely sodium and ammonia. KI_{min} and KI_{max} values were used as the measure of inhibition resilience. The study also considered the separate inhibition of the two forms of ammonia nitrogen, namely NH_3 and NH_4^+.

   Section 4.4 describes this continuous digester study.

4.3 Study of microbial community and inhibition characteristics of full-scale and pilot-scale digesters

This research has been documented in a peer reviewed journal manuscript; *Y. Lu, R. Liaquat, S. Astals, P.D. Jensen, D.J. Batstone, S. Tait (2017). Towards understanding the link between ammonia inhibition resilience and microbial communities in anaerobic digesters. In submission.*

4.3.1 Methods

Thirteen different anaerobic inoculums were tested in this study; with 10 inoculums collected from full-scale anaerobic treatment plants and three inoculums collected from pilot-scale reactors (>100 L). The inoculums comprised samples from anaerobic digesters fed with a variety of substrate types, and operating with different digester configurations and different digestion temperatures, as listed in Table 4.1. Five liters of each inoculum was collected in sealed plastic drums and transported to the UQ testing laboratory within 1-3 hours, with the exception of Slurry_1 and Slurry_3 which were cooled before and during transport (using ice bricks) and were received within 36 hours after collection.

Table 4.1 - Operational conditions of the anaerobic digesters from which inoculums were sampled for a study of resilience to ammonia inhibition.

Inoculum	Feedstock substrate	Digester Type	Temperature
Slurry_1	Conventional piggery effluent	CAP	Ambient
Slurry_2	Conventional piggery effluent	CAP	Ambient
Slurry_3	Conventional piggery effluent	Mixed in-ground digester	Mesophilic
Sludge_1	Sewage sludge	CSTR	Mesophilic
Sludge_2	Sewage sludge	CSTR	Mesophilic
Sludge_3	Sewage sludge	CSTR	Mesophilic
Sludge_4	TH WAS*	CSTR	Mesophilic
LBR_1	Crop/manure mix	Batch solid phase leachbed - Pilot (Flood & Drain)	Mesophilic
LBR_2	Crop/manure mix	Batch solid phase leachbed -Pilot (Trickling)	Mesophilic
Slaughter_1	Slaughterhouse wastewater	CAP	Ambient
Slaughter_2	Slaughterhouse wastewater	AnMBR** - Pilot	Thermophilic
Granule_1	Soluble organic wastewater	UASB***	Mesophilic
Granule_2	Soluble organic wastewater	UASB	Mesophilic

*TH WAS: thermally hydrolysed waste activated sludge; **AnMBR: anaerobic membrane bioreactor; ***UASB: upflow anaerobic sludge blanket reactor.

Chemical analysis

In order to measure the characteristics of the inoculums as close to the host environment as possible, chemical analyses were done near immediately on arrival at the laboratory. It is however noted that pH and TAN values may have slightly drifted during transportation. Analyses of total fractions were performed directly on the raw samples as received, and included total chemical oxygen demand (tCOD), TS, VS and pH. For analyses of the soluble fraction, sub-samples were centrifuged at 2,500 × *g* for 5 minutes and the supernatant filtered at 0.45 µm (PES Millipore®) and measurements included soluble chemical oxygen demand (sCOD), TAN and phosphate. TS and VS were measured according to Standard Method 2540G (APHA, 2005). tCOD and sCOD were measured using a Merck COD Spectroquant® test kit and a Move 100 colorimeter (Merck, Germany). pH was measured with a TPS WP-80D multi-parameter meter equipped with a TPS-121210 micro pH sensor. TAN was determined with a Lachat Quik-Chem 8500 flow injection analyser following the manufacturer's protocol. NH_3 concentration was calculated by means of Equation 4.1. For inhibition testing, TAN concentration included the contributions of both added chemical (ammonium chloride) and inoculum background, and the pH used in Equation 4.1 is that measured just before the headspace of each inhibition test bottle was flushed with N_2 (see below).

$$\mathrm{NH_3} = \frac{\mathrm{K_a} \cdot \mathrm{TAN}}{\mathrm{K_a} + 10^{-\mathrm{pH}}} \tag{4.1}$$

The acid-base equilibrium constant for ammonia (K_a) was corrected to the assay temperature (T, in degrees Kelvin) using the Van't Hoff equation (Equation 4.2).

$$\mathrm{K_{a\,(T)}} = \mathrm{K_{a\,(298.15)}} \cdot \mathrm{e}^{\left(\frac{51965}{8.314} \cdot \left(\frac{1}{298.15} - \frac{1}{\mathrm{T}}\right)\right)} \tag{4.2}$$

Biogas composition (CH_4, CO_2, H_2, and N_2) was determined using a Shimadzu GC-2014 equipped with a HAYESEP Q 80/100 packed column, a thermal conductivity detector (100 °C) and Argon as carrier gas (28 mL/min).

Microbial community composition analysis

Sub-samples for microbial analysis were collected immediately on arrival at the laboratory and stored at -20°C before DNA extraction. Genomic DNA was extracted from each inoculum by a FastSpin for Soil Kit (MP-Biomedicals, USA) according to manufacturer's protocol. Pair-end 16S amplicon sequencing was conducted by the Australian Centre for Ecogenomics at The University of Queensland (Australia) with primer sets 926F (5'-AAACTYAAAKGAATTGACGG-3') and 1392wR (5'-ACGGGCGGTGWGTRC-3') (Engelbrektson et al., 2010) by Illumina Miseq Platform. Trimming and quality control were performed on raw paired reads with Trimmomatic (Bolger et al., 2014), Pandaseq (Masella et al., 2012) and FASTX-Toolkit (Pearson et al., 1997). The joined high-quality sequences were analysed by QIIME (v1.9.0) (Caporaso et al., 2010) and singlet reads were removed from the operational taxonomy units (OTUs) table. The raw OTUs table was imported into R (v3.2.3) and rarefied to 10,000 sequences per sample by function "rarefy_even_depth" of package phyloseq (McMurdie & Holmes, 2013). Hellinger adjusted OTU tables were used for Principle Component Analysis (PCA) by function "rda" in the package vegan, to determine overall community characteristics. PCA directly on normalised OTU tables gave similar results, but commonly with weaker correlations. In order to assess inhibition relationships completely, PCA was done separately for the bacterial, archaeal and overall communities. Normalised overall OTU tables were then summarised according to genus and genera with >5% relative abundance used to generate a heatmap by function "heatmap.2" of the package gplots (Libungan & Pálsson, 2015).

Inhibition testing

Inhibition testing was performed as per the method described by Astals et al. (2015a). Inoculums with VS concentration above 10 $gVS \cdot L^{-1}$ were diluted to 10 $gVS \cdot L^{-1}$ with deionised water, whilst inoculums with VS concentration below 10 $gVS \cdot L^{-1}$ were not diluted. The main reason for dilution was to standardise concentrations and to reduce matrix effects from the host environments. This allowed us to isolate the impact of the microbial community composition. However, the inhibition results may not strictly represent performance in the full-sale processes (e.g. inhibitors interaction). The inhibition tests were performed in 160 mL serum bottles containing inoculum (99 mL), with an amount of ammonium chloride added to reach the design TAN concentration, and an amount of acetate solution added to reach an inoculum-to-acetate ratio of 5. Each inoculum was tested at six different TAN concentrations (at 1, 2, 3, 5, 10 and 15 g N/L). A control test was run for each inoculum with only sodium acetate (no added ammonium chloride). pH of each bottle was measured once all reagents were added and then used to calculate the NH_3 concentration, i.e. pH was never adjusted. The headspace of each test bottle was flushed for 1 min (4 L/min) with 99.99% nitrogen gas before sealing the serum bottle with a rubber stopper and aluminium crimp. The gas line was not submerged during flushing. Subsequently, the serum bottles were placed in a temperature-controlled incubator at 37 ± 1 ºC for ambient and mesophilic inoculums and at 55 ± 1 ºC for the thermophilic inoculum.
The SMA of the inoculum at each concentration was determined through three sampling events (0.5, 1.0 and 1.5 days), where biogas headspace pressure (bench-top manometer) and biogas composition (gas chromatography) were quantified. Accumulated volumetric methane is expressed in grams of chemical oxygen demand (g COD_{CH4}) under standard conditions (0 ºC, 1 bar). All conditions were tested in triplicate. SMA was determined as the slope of a linear regression (Analysis Toolpak in Microsoft Excel 2013) applied to the cumulative methane production normalized to VS of added inoculum (y-axis) and time (x-axis), specifically for subsets of data over which the rate of methane production was approximately constant.
Inhibition profile plots of SMA vs. NH_3 concentration were normalised against the measured SMA of the control test (SMA without added ammonium chloride). The slope of the inhibition curve (speed of onset of inhibition) was determined using the Microsoft Excel built-in function "LINEST", applied to subsets of inhibitor concentrations over which the rate of inhibition increase was approximately constant. Standard errors in slopes were analytically calculated by the Microsoft Excel tool from the regression standard error. The 95% confidence interval in slope was determined using a two-tailed t-test with n-2 degrees of freedom where n is the number of data points available for regression. KI_{50} was quantified by determining NH_3 concentration at which measured SMA was reduced by 50% of the maximum measured SMA for each inoculum.

Statistical and correlation analysis

Analysis of variance in mixed categorical/continuous mode (ANCOVA) was performed to test significance of factors on both microbial community composition (assessed by PCA) and NH_3 resilience (assessed as KI_{50}) using the function "anovan" of Matlab (MATLAB version 8.4.0.150421, R2014b). Specifically, PC values of bacterial, archaeal and overall communities were separately used as output with numerical variables pH, TAN and NH_3, treated as continuous factors, temperature as a coded continuous factor (values of '1', '2', or '3' assigned to ambient, mesophilic, and thermophilic respectively), and substrate type as a categorical factor. The full model was tested followed by elimination of non-significant factors to the minimum-parameter, most parsimonious model. Likewise, SMA, slope of the inhibition curve and KI_{50} were tested as outputs against factors as above, but also against PC scores and relative abundance of archaea (both as continuous factors). A significance threshold of 0.05 was applied for rejection of the null hypothesis.

4.3.2 Results

Chemical characterisation

Table 4.2 contains selected characteristics of the 13 inoculums under study. pH values ranged from 7.2 (approximately neutral) to 8.4. Most inoculums, except Sludge_4 and Slurry_3, had TAN concentrations below 1000 mg N/L. Sludge_4 (with thermal hydrolysis pre-treatment) had the highest TAN (2833 mg N/L) and NH_3 (241 mg N/L) concentrations. Leachbed reactor inoculums (LBR 1 and LBR2, Yap et al. (2016)) and Slaughter_2 (the only thermophilic system) had NH_3 concentrations higher than 100 mg N/L. Slaughter_1, Slurry_1, Slurry_2 (all running at ambient temperature) and the granular inoculums had NH_3 concentrations lower than 40 mg N/L. Slurry_3 that operated at pH 7.8 and mesophilic temperature, had a comparatively moderate NH_3 concentration (87.5 mg N/L).

Table 4.2 - Characteristics of the inoculums for which resilience to ammonia inhibition was studied using small laboratory scale batch experiments.

Inoculum	pH	VS	TAN	Background NH_3	KI_{50}	SMA*
		(g/kg)	(mg N/L)	(mg N/L)	(mg NH_3-N/L)	(gCOD-CH_4/gVS/day)
Slurry_1	7.7	9.3	776	21.7	89 ± 11	0.075 ± 0.002
Slurry_2	8.1	98	588	39.6	75 ± 7	0.035 ± 0.003
Slurry_3	7.8	9.3	1384	87.5	169 ± 11	0.093 ± 0.003
Sludge_1	7.7	17	268	13.9	93 ± 3	0.061 ± 0.003
Sludge_2	7.2	26	953	17.4	32 ± 4	0.050 ± 0.005
Sludge_3	7.3	16	949	18.9	75 ± 11	0.132 ± 0.008
Sludge_4	7.9	29	2833	241.3	138 ± 4	0.075 ± 0.002
Slaughter_1	7.3	8.2	255	2.6	64 ± 5	0.136 ± 0.009
Slaughter_2	8.0	11	365	110	118 ± 10	0.138 ± 0.032
LBR_1	8.1	97	803	103	110 ± 4	0.101 ± 0.007
LBR_2	8.4	89.9	985	205	110 ± 8	0.068 ± 0.004
Granule_1	6.7	n.d.**	20	0.1	175 ± 34	0.206 ± 0.012
Granule_2	7.1	n.d.	18	0.3	118 ± 6	0.128 ± 0.003

*Specific methanogenic activity (SMA) from the control test, i.e. without added ammonium chloride; KI_{50} is the inhibitor concentration (in this case ammonia) at which measured SMA is reduced to 50% of that in the control test; ** not determined.

Microbial characterisation

The relative abundance of methanogens (as compared to bacterial) ranged between 10% and 64% for the different inoculums. The abundance of methanogens was highest in the granules (over 60%). Methanogen abundance was also generally high in the Slaughter samples (45-48%), and was generally lower in the LBR samples (22-27%). The relative abundance of methanogens in the Sludge samples was quite variable at 10-47%. *Methanosaeta*, a highly specialized obligate acetoclast, was the major methanogen, except for LBRs inoculums that were dominated by *Methanosarcina*. A number of *Methanosaeta* OTUs were identified, with 7 inoculums dominated by a single common OTU (named *Methanosaeta_1*), whilst Sludge_1, Sludge_4, Slaughter_2 and Granule_1 were dominated by other *Methanosaeta* OTUs. Granule_1 had a diverse *Methanosaeta* population. Slaughter and Granule were the only inoculums where hydrogenotrophic archaea (e.g. *Methanothermobacter* and *Methanobacterium*) represented an overall relative abundance higher than 10%.
Bacterial communities consisted mainly of OTUs from the classes *Anaerolineae*, *Bacteroidia, Betaproteobacteria*, *Clostridia* and *Deltaproteobacteria* (Figure 4.3). The class Bacteroidia and Clostridia, respectively dominated LBR and Slurry inoculums. However, different bacterial classes dominated Sludge, Slaughter and Granule inoculums.

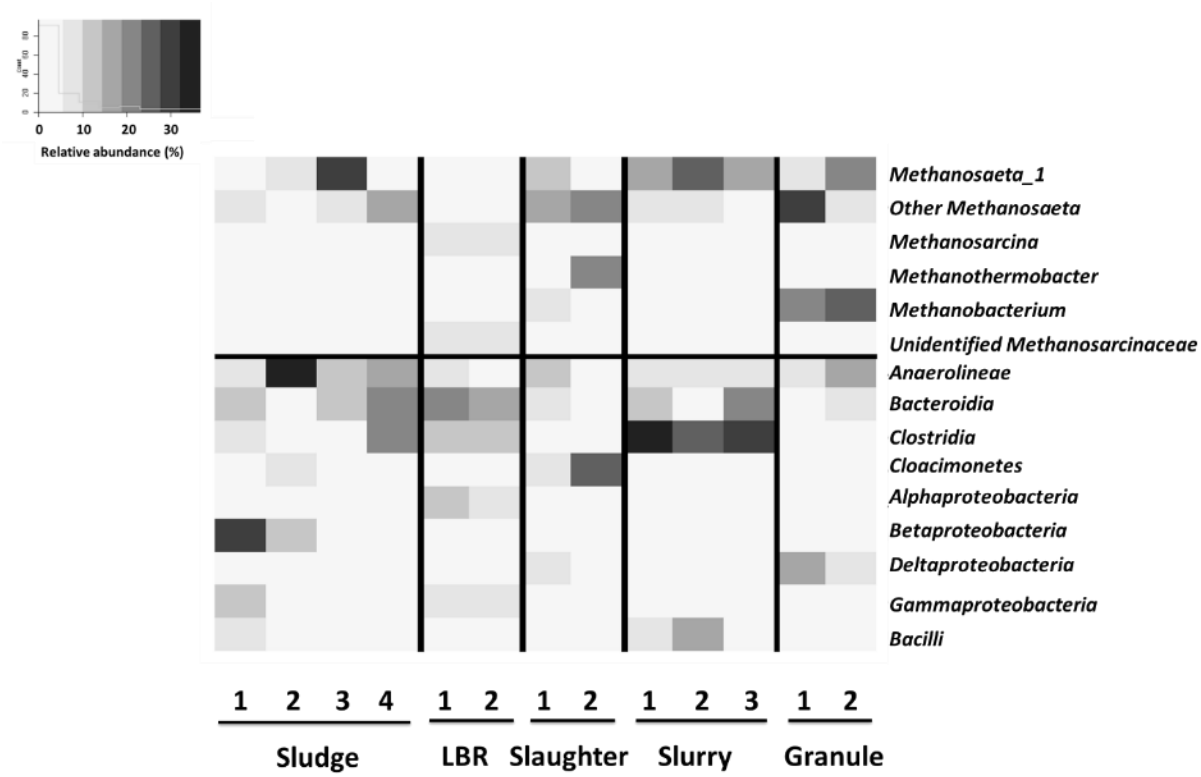


Figure 4.3 – Heatmap of the distribution of major microorganisms (>5% relative abundance) for the inoculums under study (as columns), including archaea (labelled with genus identification, rows 1 to 6) and bacteria (labelled with class identification, rows 7 to 15).

Inhibition resilience

Specific methanogenic activity values for the control tests (without added ammonium chloride) are also shown in Table 4.2 and ranged from a low value of 0.04 (Slurry_2) to a moderate-to-high value of 0.14 gCOD-CH_4/g VS/d (Slaughter_1 and Slaughter_2). KI_{50} values ranged from 56 to 175 mgNH_3-N/L (Sludge_1 and Granule_1, respectively) (Table 4.2). This variability was only moderate in terms of NH_3 resilience as compared to variability reported elsewhere in the literature (Rajagopal et al., 2013; Yenigün & Demirel, 2013a), and was somewhat surprising considering the very diverse backgrounds of the various inoculums.

Figure 4.4 presents inhibition test data as normalised SMA at various NH_3 concentration. The SMA values being presented in each case are normalized to allow better comparison of inhibition resilience between the various inoculums, despite largely differing background SMA values for the various inoculums (Table 2.1). This comparison is valid, because inhibition resilience is a measure of response to an inhibitor relative to a respective background microbial activity.

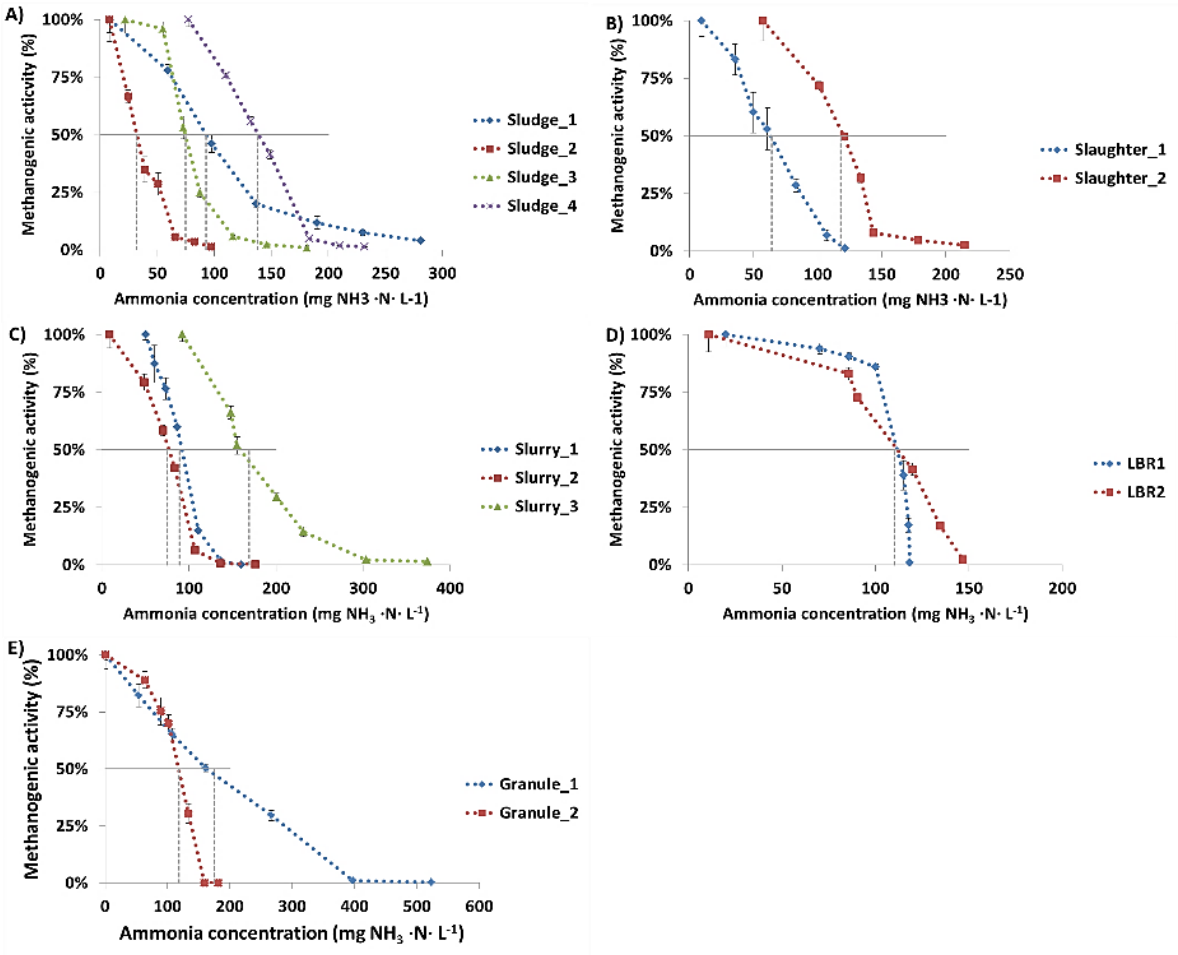


Figure 4.4 - Inhibition test data presented as normalised SMA at various NH_3 concentration in Sludge (A), Slaughter (B), Slurry (C), LBRs (D) and Granule (E) inoculums. The KI_{50} value is indicated in each plot as an intercept of a vertical and horizontal line.

KI_{50} values varied significantly across substrates and within a substrate type (Figure 4.4). For instance, KI_{50} values of Sludge and Slurry inoculums varied from 56 to 153 mgNH_3-N/L and from 89 to 169 mgNH_3-N/L, respectively. Regarding the shape of the inhibition threshold curves, most inoculums were inhibited as soon as the NH_3 concentration increased from its background value, i.e. the inhibition curve had a concave shape. However, three inoculums (Sludge_3, LBR_1 and LBR_2) showed a sigmoidal shape with a greater range of NH_3 concentrations over which inhibition did not drastically change.

Factors affecting microbial community composition and inhibition resilience

The statistical analysis showed that substrate type (the digester feedstock material) was the strongest factor correlated with microbial community, significantly with overall community PC1 (p=9×10^{-4}) and PC2 (p=6×10^{-4}) and bacterial community PC1 (p=2×10^{-7}) and PC2 (p=3×10^{-5}). Substrate type was also a predictor for archaeal community PC1 (p=0.035, considering also temperature as a predictor) and archaeal community PC2 (p=0.025, with no other predictors). Temperature was the most significant predictor for archaeal community PC1 (p=0.018) and also a predictor for overall community PC1 (p=0.015) when considered in conjunction with the dominant predictor of substrate type. Archaeal community PC1 was significantly correlated with pH and NH_3, only when considered in isolation (p=0.001 and 0.007 respectively), but not when the primary factors of substrate type and temperature were also included.

A plot of major archaeal community ranked from the most to least resilient to NH_3 (i.e. highest to lowest KI_{50} value) is shown in Figure 4.5. Archaeal communities were more variable in inoculums with KI_{50} higher than 100 mg NH_3-N/L. Slurry_3 was dominated by *Methanosaeta_1*. Granule_1 and Sludge_4 had OTUs affiliated with different *Methanosaeta*. Slaughter_2 and Granule_2 archaeal community had *Methanosaeta* and hydrogenotrophic methanogens (*Methanothermobacter* and *Methanobacterium*), while LBRs inoculums were dominated by *Methanosarcina* (LBRs). It is worth mentioning that the highest KI_{50} was not obtained from the inoculums with the highest background NH_3 (Sludge_4 and LBR_2), but rather with Granule_1 and Slurry_3 collected from digesters with NH_3 concentrations of 0.1 and 87.5 mg NH_3-N/L, respectively.

Somewhat disappointingly, overall, no microbial factors or other operational factors were correlated with NH_3 resilience, as quantified by KI_{50} values. The total relative abundance of archaea also did not appear to affect inhibition resilience (p=0.5369). Archaeal relative abundance and archaeal community PC2 were predictors for SMA (p=0.0107 and p=0.0242 respectively), and archaeal community PC2 was primarily correlated with substrate type. However, substrate type was not correlated with SMA. In addition, substrate type (p=0.01), temperature (p=0.02) and NH_3 (p=0.005) were significantly correlated to the slope of the inhibition curve, i.e. the extent at which SMA decreased with increasing NH_3 concentration (Figure 4.4). Bacterial community PC1 was correlated to the slope of the inhibition curve only when considered in isolation (p=0.0119). However, this significance was dependent on LBR_1 for which a certain threshold NH_3 concentration caused a sharp decrease in activity (increase in inhibition), and once LBR_1 was omitted, the PC1 significance was lost.

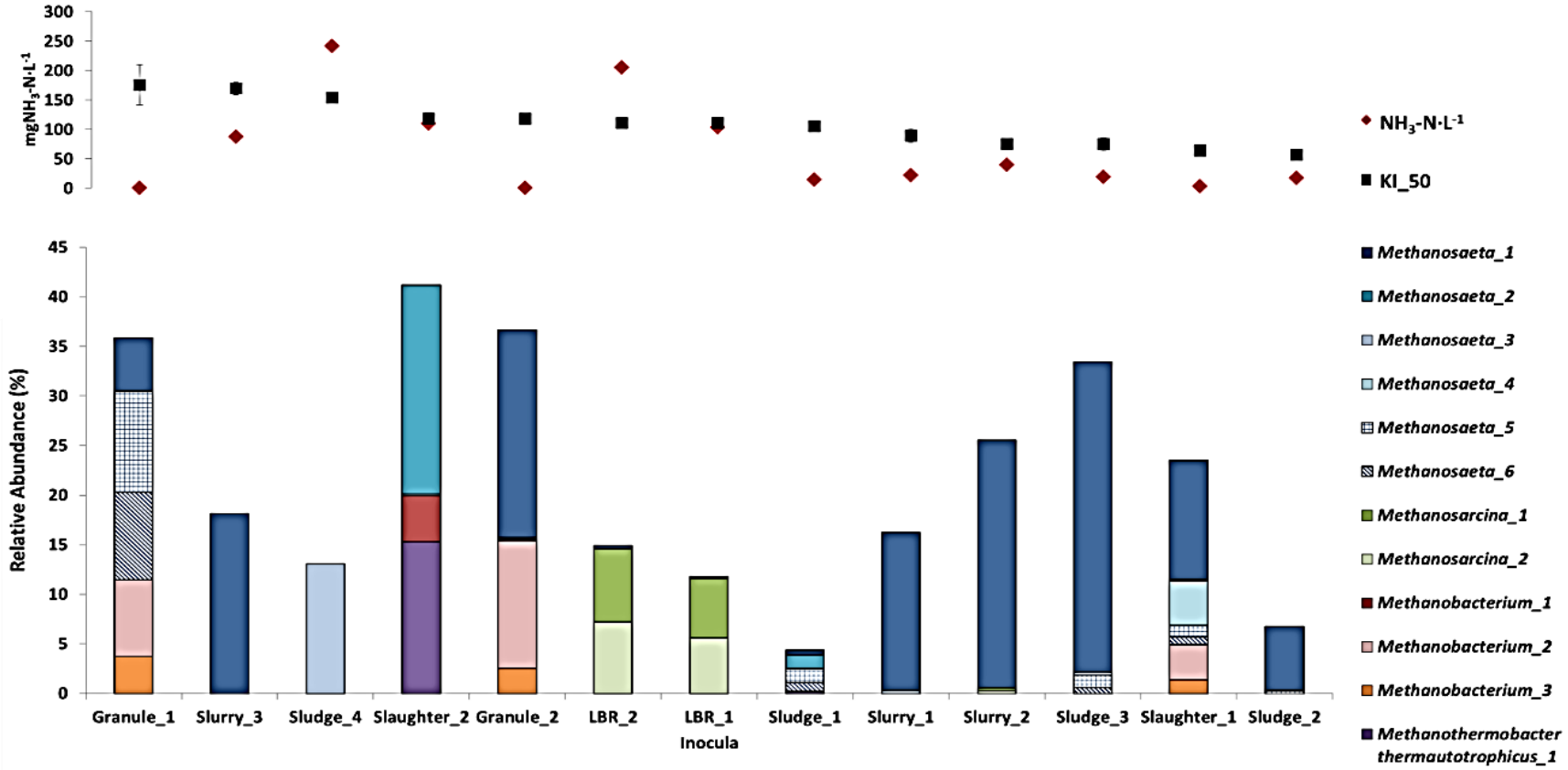


Figure 4.5 - The combination of major archaeal abundance (>1%, as bar chart), inoculum native NH_3 concentration (as red diamond) and KI_{50} (as black box) for the inoculums under study. Samples from left to right are in order of descending KI_{50} values.

4.3.3 Discussion

Substrate type and temperature were the primary factors influencing microbial community composition. pH and NH_3 concentration were secondary rather than primary factors for archaeal community, because significance was lost when variance was proportioned to substrate type or temperature. These findings were as expected, because microbial communities typically need to adjust functionally to their feedstock, to cope with the specific complex composition of proteins, amino acids, lipids and carbohydrates. In addition, temperature affects microbial growth rates.

The obligate aceticlast *Methanosaeta*, dominated as methanogen in most cases, indicating that the Pork CRC inhibition test method was appropriate to assess inhibition resilience. In the literature, there is evidence that microbial acclimation can lead to enhanced NH_3 and TAN resilience (Rajagopal et al., 2013). Acclimation of microbial communities to high NH_3 has been primarily attributed to a change in methanogenic pathways (from acetate cleaving to syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis). However, it is not clear if individual organisms are shifting pathways (Zhang et al., 2014a) or if the shift results from a transition to more resistant microorganisms with different metabolic capability (Fotidis et al., 2014; Niu et al., 2015). In the present case, the lack of link between microbial community composition and inhibition resilience could be due to:

(i) NH_3 concentrations in the digesters sampled being generally lower than in previous microbial acclimation studies (Angelidaki & Ahring, 1993; Fotidis et al., 2013; Regueiro et al., 2016). Perhaps the microbial communities in the many full-scale and pilot scale digesters that were sampled, were not under selective stress and thus NH_3 was not a key driving factor for microbial profile; or

(ii) NH_3 resilience may not be directly reflected by microbial community profile, if general shifts in microbial community are somehow decoupled from overall microbial function. This is suggested by Zhang et al. (2014a) who reported a strong transcriptional response to NH_3 stress without a change in the microbial community structure.

Overall, the observation that no operational or microbial factors led to higher NH_3 resilience, was somewhat disappointing, because it suggested that there was minimal opportunity for practical intervention to engineer more resilient microbial communities (e.g. by heating a digester). Instead, the moderate variability in inhibition resilience across the various inoculums that were tested, indicated natural variability in inhibition resilience.

4.4 Continuous digester microbial acclimation study

This research is being documented in a peer reviewed journal manuscript; *S. Astals, Y. Lu, D.J. Batstone, P.D. Jensen, S. Tait. Exposure to inhibitors is the key to induce microbial adaptation and improve inhibition resilience in anaerobic systems. In preparation.*

4.4.1 Methods

Fresh pig manure was collected from the concrete floor of a specialised breeder piggery located in South East QLD, Australia, consisting of mainly faeces but also with some urine, water, straw and spilt feed. After collection, the pig manure was frozen at -20 °C until use. Multiple batches of pig manure were prepared at various times throughout the study, and the BMP of the different batch samples was measured as described in Section 4.2, and was observed to be reasonably consistent over time, at between 310 and 345 L CH_4/kg VS fed. An inoculum was used to seed the initial start-up of a set of lab-scale continuous digesters. This inoculum was a sample of settled sludge collected from the bottom (~2 m) of a partially-covered anaerobic pond located treating unscreened flushed pig manure at the abovementioned piggery (Skerman et al., 2011) and at ambient temperature. The manure feedstock for the lab-scale continuous digesters were prepared every fortnight by grinding thawed pig manure lots in a food processor, diluting it with water down to a volatile solids concentration of 10 g VS/L to prevent blockages during the experiment and to improve the ease of pumping and mixing. This feedstock was then stored at 4 °C until use, typically within the subsequent fortnight.

Four 6-L continuous stirred tank digesters were constructed for the project (Figure 4.6 and Figure 4.7). Temperature of the liquid contents in each digester was maintained with a heating jacket around each digester vessel and circulating hot water between the jacket and a heated water bath. Each digester vessel was continuously stirred at 60 rpm using an overhead stirrer unit (F) and an anchor impeller with only a small gap between the vessel inner wall and the outer edge of the impeller. Each impeller also had a small scum/foam breaker plate on the impeller shaft at the interface between the liquid surface and the gas headspace. Approximately three times per day, the digesters were simultaneously fed from a reservoir bottle (C) stirred with a magnetic stirrer unit (D) and drained into a separate reservoir bottle (E), using a dedicated peristaltic pump with two pump heads (B) for each digester (one for feeding and one for discharge). The digesters were operated at mesophilic temperature (37 ± 1°C) with a liquid working volume of 4-L each, a hydraulic retention time (HRT) of 20-22 days and an organic loading rate (OLR) of 0.50-0.55 g VS/L/d.

The relatively large HRT, the low ORL and the low feedstock solid concentrations were chosen to avoid secondary inhibitory mechanisms (e.g. overloading, concentration hotspots due to poor mixing or biomass washout) as well as to minimise the inhibitor concentrations (Na+ and nitrogen) contributed by the manure background. The total operational period was 305 days, over which biogas volume produced was routinely measured using a tipping bucket gas meter (A), and biogas composition was analysed three times per week on gas samples collected from the digester headspace and using a gas chromatograph.

The four digesters were operated in three distinct stages, each with different operating conditions: (i) Stage Ia – Initial convergence period - uninhibited, (ii) Stage Ib – Operating at uninhibited steady state, and (iii) Stage II – Inhibited operation. Table 4.3 summarises the respective inhibitor concentrations in each digester. pH of the R3 and R4 feedstocks was adjusted with 4 M NaOH back to the pH of the control digester, because of a pH decrease caused by added NH_4Cl. The final target inhibitor concentrations in each experimental continuous digester (R2-4) were selected based on the SMA inhibition testing from Stage Ib. The elected concentrations were those that resulted in a 90% acute inhibition of SMA during

the inhibition testing of Stage IIB. Specifically, R2 was subjected to the 90% inhibition concentration of Na^+ (added as sodium chloride salt or NaCl), R3 was subjected to the 90% inhibition concentration of ammonia (NH_4^+ and NH_3) (added as ammonium chloride salt or NH_4Cl), and R4 received both Na^+ and NH_4^+ and NH_3 inhibition (added as both NaCl and NH_4Cl). In R4, the 90% inhibition concentration for the two inhibitors was estimated as a combination of the 70% inhibition concentration for Na+ and the 70% inhibition concentration for ammonia (NH_4^+ + NH_3) (30% residual activity × 30% residual activity = 10% residual activity or 90% inhibition). It is noted that these values are extreme compared to typical values observed in CAPs at Australian piggeries (<1000 mg N/L and <1200 mg Na/L), but these very high levels were specifically selected to force microbial acclimation.

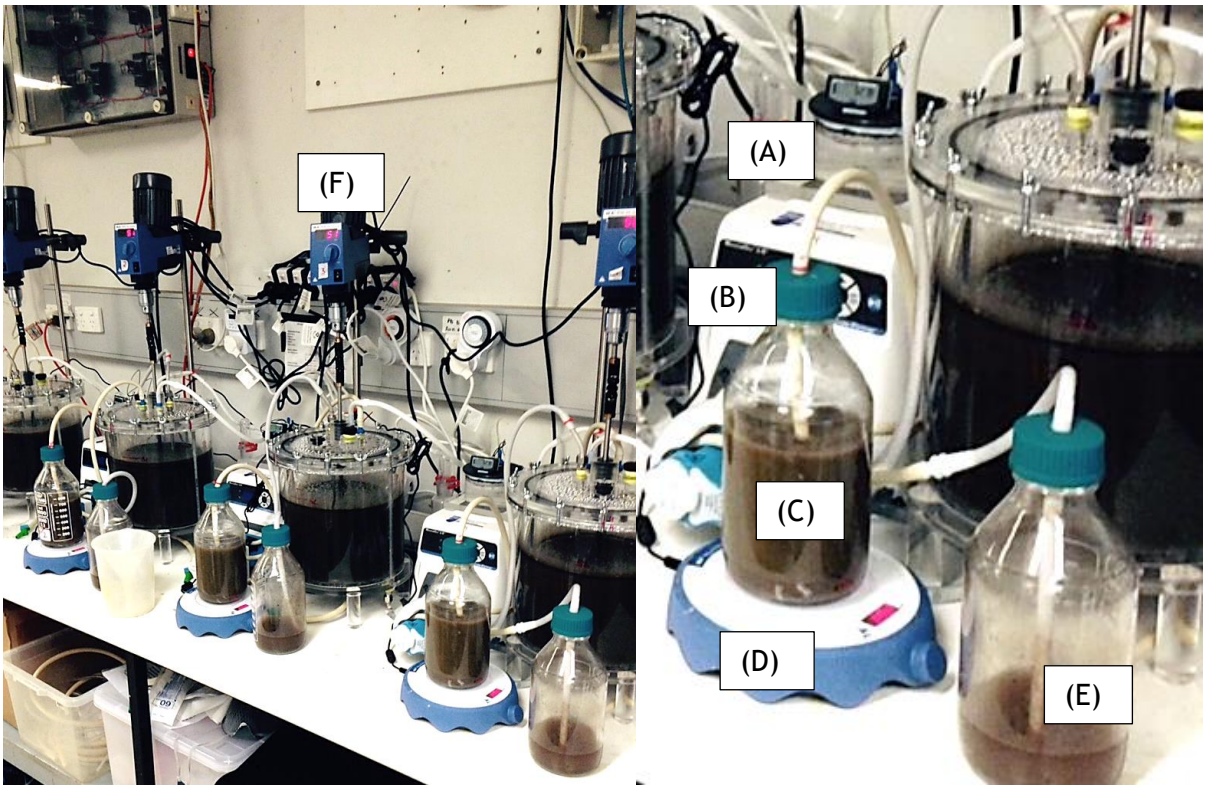


Figure 4.6 – Continuous digester apparatus. Photos of the four continuous 6-L digesters while operational on the laboratory bench, showing Schott glass bottles (C) at the front on magnetic stirrer plate (D) from which pig manure was fed, and on the bench (E) into which the effluent discharged. The digesters were stirred with overhead stirrers (F). Biogas production was measured using tipping bucket flowmeters (A).

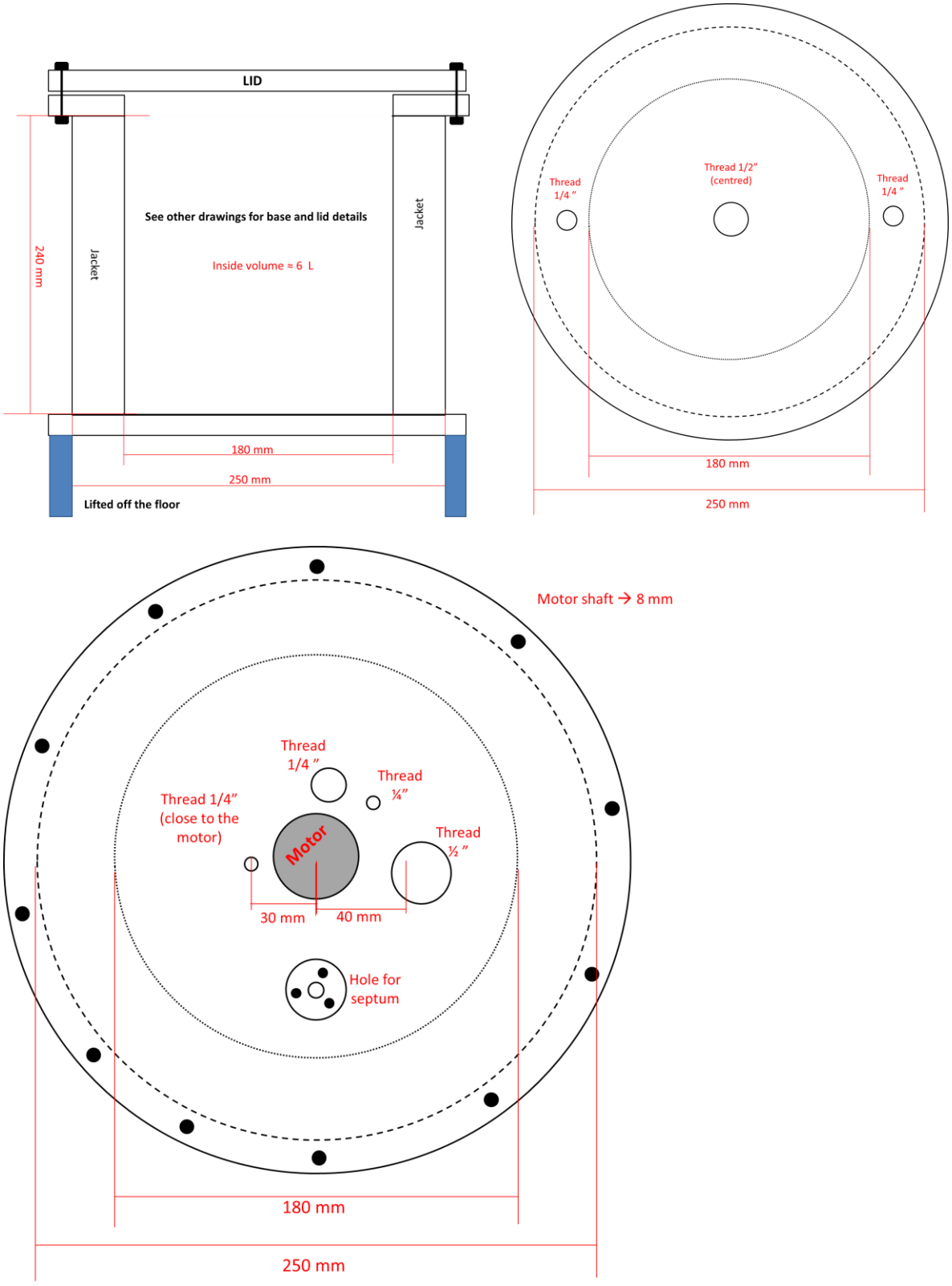


Figure 4.7 - Continuous digester apparatus. Schematic design drawings of the digester, showing heating jacket, and feed and discharge ports that were located specifically to ensure that manure being fed was rapidly mixed into the digester contents and that digestate being withdrawn was representative of the mixed digester contents.

Table 4.3 – Digesters media inhibitors concentration of each reactor at each stage

	Stage Ia	Stage Ib	Stage II			
	R1 – 4 (no inhibition)	R1 – 4 (no inhibition)	R1 (control)	R2 (Na^+)	R3 (NH_4^+, NH_3)	R4 (Na^+, NH_4^+, NH_3)
Operation time (days)	0 - 99	150 - 225	226 – 305			
Na^+ (mg Na/L)	117±1	96±1	84±1	11,607±142	112±1	10,011±160
NH_4^+ (mg N/L)	155±2	217±1	206±2	208±7	3,386±31	2,698±21
NH_3 (mg N/L)	4.2±0.1	7.7±0.2	4.3±0.2	4.3±0.3	58 ±2	34±2

During Stage Ia and Ib, all four digesters (named R1, R2, R3 and R4) were only fed with diluted pig manure (no added inhibitor). Stage Ia ensured that all four digesters converged to a similar performance and steady state. During Stage Ib inhibition testing was performed to check for similarity between the digesters in terms of acute inhibition response and over an extended time period (6 × HRTs). The interval between Ia and Ib (designated by a break in the horizontal axes in Figure 9) was a period over which the digesters were operated to continue stabilizing but were not monitored. In Stage II, R1 was maintained as a control digester (no added inhibitor), whilst R2, R3 and R4 started receiving inhibitor added as NaCl (Na^+ inhibition) and/or as NH_4Cl (ammonia inhibition) to the manure feedstock. Inhibitor was not acutely and directly spiked at high concentration into the digester, but instead was gradually fed with the feedstock. For this reason, the inhibitor concentration in the experimental digesters gradually increased over time, as the digester contents were being replaced. This mode of operation represents chronic exposure to an inhibitor, which is more representative of full-scale digesters.

At various times during the operational stages, samples of digestate effluent were collected from each digester for chemical and microbial composition analysis and for offline inhibition testing in small batch tests. Chemical constituents and microbial community composition were analysed as described in Section 4.3.1 above, and VFAs were measured as described in Section 5.1 below. Prior to inhibition testing, the digestate was diluted as needed to 10 gVS/L as per the method described by Astals et al. (2015a). The inhibition testing was performed in 120 mL serum bottles, typically containing 80 mL of inoculum and an amount of sodium acetate solution (200 $g_{acetate}$/L) to give an inoculum-to-acetate VS ratio of 5. A predetermined amount of ammonia nitrogen was added as crystalline ammonium salt (i.e. ammonium chloride or ammonium bicarbonate). Control tests without added ammonium salt (inoculum and sodium acetate only) were run for each inoculum and each experimental condition. pH of each serum bottle was measured once all reagents had been added, before the headspace of the serum bottle was flushed for 1 min (4 L/min) with 99.99% N_2. During flushing with N_2, the gas line was not submerged into the test medium. The bottle was then promptly sealed with a rubber stopper and aluminium crimp seal. The serum bottles were placed in a temperature-controlled incubator at 37 ± 1 ºC. Headspace gas samples were collected at 0.5, 1.0 and 1.5 days with a gastight syringe and fine gauge needle. The pressure of the biogas headspace was measured with a bench-top manometer and methane concentration by gas chromatography as per the method in Section 4.3.1 above. After the final sampling event, the serum bottles were opened and pH again measured without delay.

All inhibition testing was performed in triplicate and error bars presented are ±95% confidence intervals in mean values, estimated using a two-tailed student t-distribution with two degrees of freedom ($t_{2,\ 0.025}$ = 4.303). Values of SMA were determined as described in Section 4.3.1. To quantify inhibition resilience, the SMA/inhibition test data was fitted with a model accounting for the separate inhibition of NH_3 and NH_4^+ using the respective inhibition terms I_{NH3} and I_{NH4+} with values ranging between 0 (completely inhibited) and 1 (no inhibition).

$$\mathrm{SMA} = \mathrm{SMA_{max}} \cdot \mathrm{I_{NH3}} \cdot \mathrm{I_{NH4+}} \tag{4.3}$$

and the inhibition terms were modelled using a threshold inhibition function (eq. 4.4):

$$\mathrm{I_{NHx}} = \left(1_{[\mathrm{I}] \leq \mathrm{KI_{min}}}, \left(e^{-2.77259\left(\frac{([\mathrm{I}]-\mathrm{KI_{min}})}{(\mathrm{KI_{max}}-\mathrm{KI_{min}})}\right)^2} \right)_{[\mathrm{I}]>\mathrm{KI_{min}}} \right) \tag{4.4}$$

where KI_{min} is the lowest inhibitor concentration that causes notable inhibition, and KI_{max} is the inhibitor concentration corresponding to complete inhibition (i.e. SMA being NOT significantly different from zero).

4.4.2 Results

Figure 4.8A shows that in Stage Ia and Ib the specific methane production of all four digesters were statistical identical, as desired. The addition of the inhibitors during Stage II caused a notable decrease in methane production in R2 (Na+), R3 (TAN) and R4 (TAN and Na+), as expected, whereas the performance of the control digester remained similar to during Stage I. Once the digesters had reached a new partially inhibited steady state (constant inhibitor concentration, ~day 275) the specific methane production of the control reactor was 113±6 mL CH_4/L/day. The specific methane production of the inhibited reactors was significantly lower at 79 ± 7 mL CH_4/L/day for R2 (Na+ inhibited), 61 ± 3 mL CH_4/L/day for R3 (ammonia inhibited) and 52 ± 3 mL CH_4/L/day for R4 (both Na+ & ammonia inhibited). Interestingly, the results showed that despite the exposure to 90% acute inhibition concentrations, the inhibited digesters maintained ~30% (R2), ~55% (R3) and ~45% (R4) of the specific methane production in the control digester (R1). This could indicate microbial adaptation as a result of gradual increases in inhibitor concentration and/or could result from AD limited by hydrolysis instead of acetoclastic methanogenesis (as measured in the acute inhibition testing). However, Figure 4.8B shows that volatile fatty acids (VFAs) did eventually accumulate in R3 and R4 during Stage II, indicating that methanogenesis was inhibited to an extent where the acute inhibition test results were relevant. In contrast, R2 did not show VFA accumulation during Stage II. VFA that accumulated was mostly propionate, being a clear indicator of acute inhibition. The relatively low VFA accumulation in R2 indicated that various microbial reactions may have been inhibited along the AD pathway from hydrolysis through to methanogenesis (Figure 2.1). As expected, VFA levels during Stages Ia and Ib (no added inhibitor) were negligible at below 10 mg/L, indicating robust operation of all four digesters at the outset of the test.

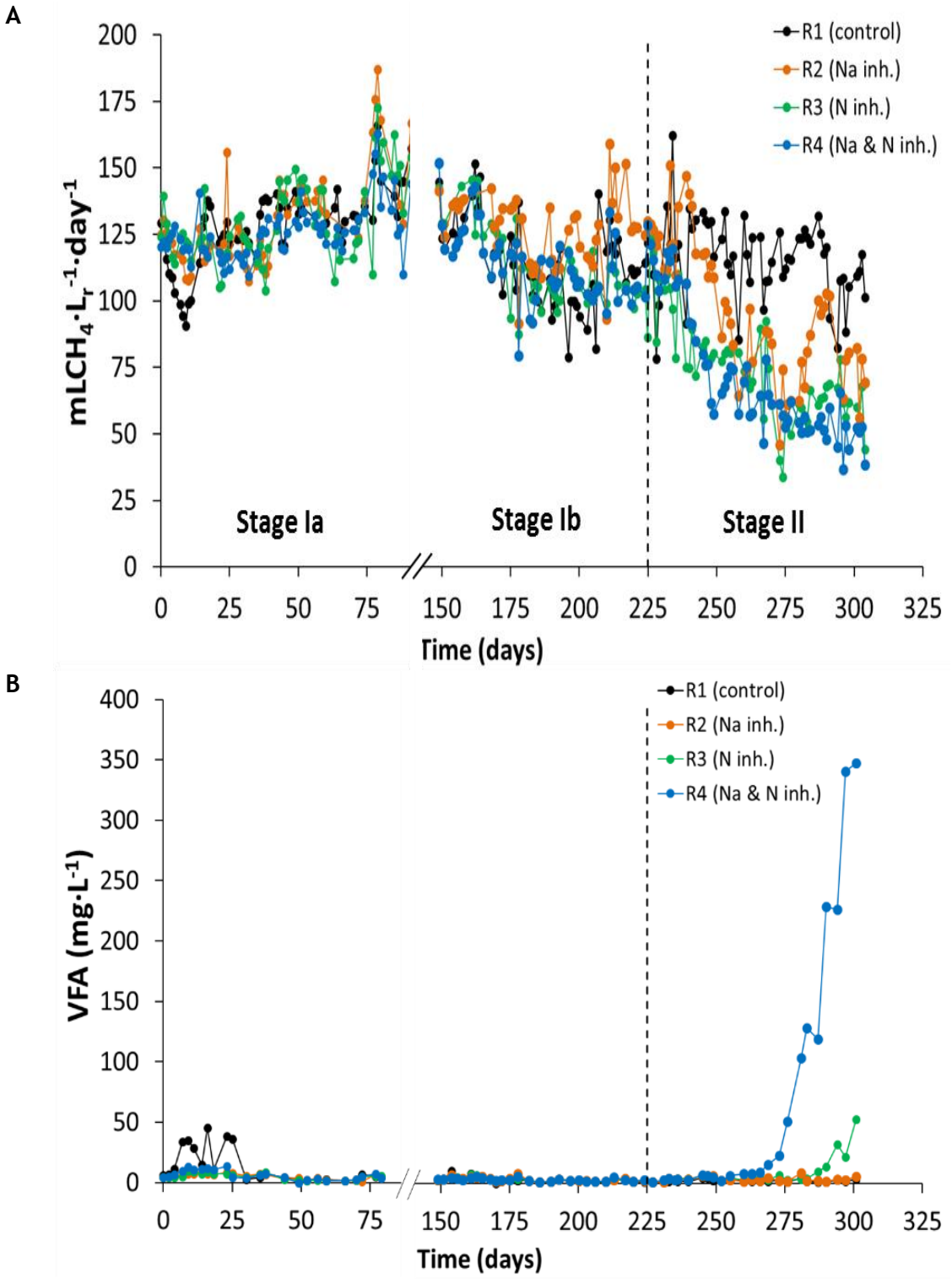


Figure 4.8 – (A) Specific volumetric methane production and (B) volatile fatty acid concentration of the four reactors over time. Vertical dashed line indicates when inhibitor addition started.

Figure 4.9 shows the inhibition threshold values (KI_{min} and KI_{max}) determined from SMA and acute inhibition testing performed on effluent collected from each digester at Stages I and II and for sodium and ammonia nitrogen. Higher KI_{min} and KI_{max} values indicate that a microbial community is more resistant to inhibition and therefore has greater inhibition resilience. Uncertainty at the 95% confidence level (error bars in Figure 4.9) was greater for ammonia nitrogen than for sodium. This was likely because model analysis on ammonia had to separately consider the inhibition by NH_3 and NH_4^+ (Section 4.1). This meant that the model analysis for ammonia used four fitted parameters instead of two in the case of Na+, so that the ammonia analysis had less residual degrees of freedom.

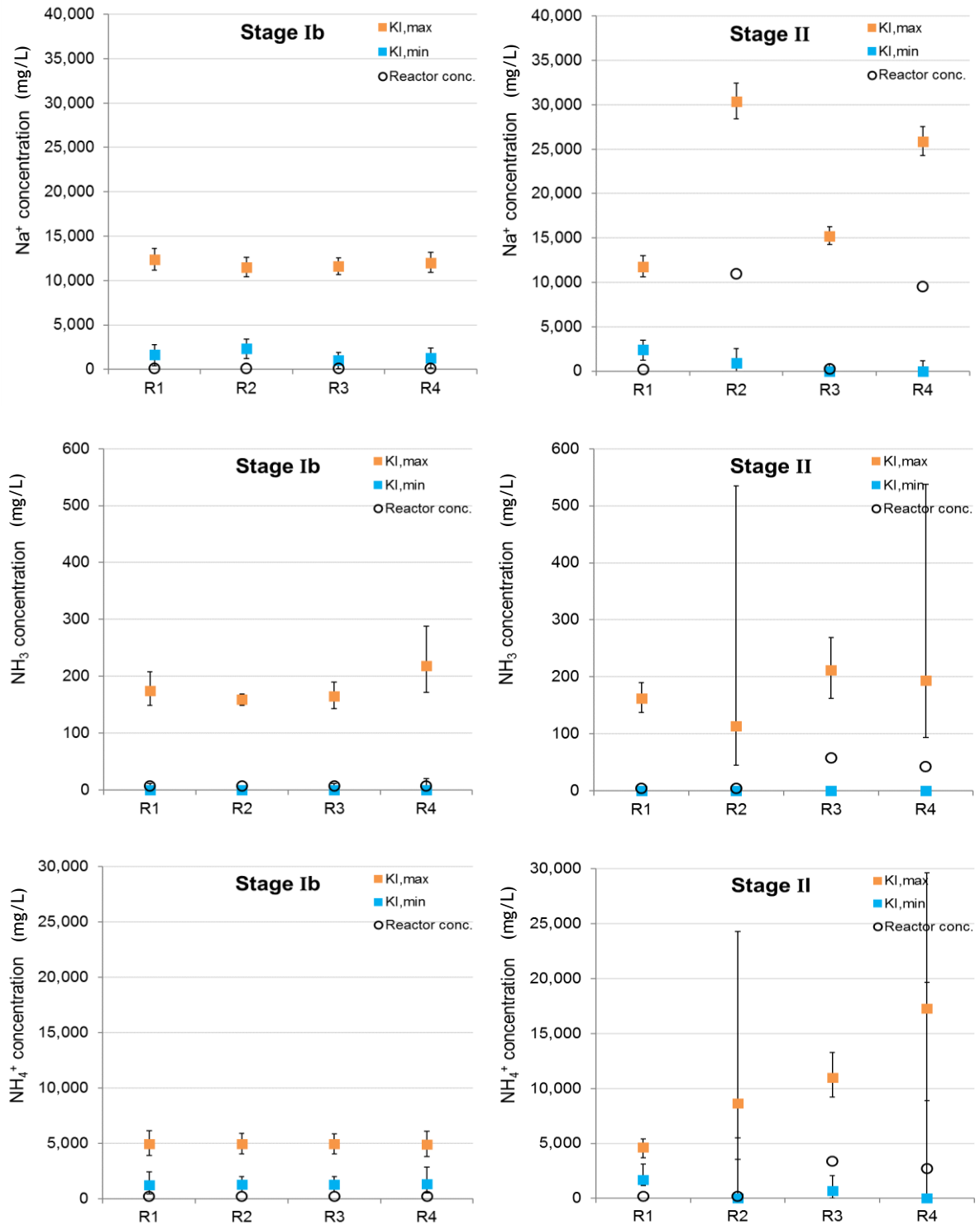
The results in Figure 4.9 show that during Stage Ib (no added inhibitors), KI_{min} and KI_{max} values were statistically identical between the four digesters. This indicated that inhibition resilience of the microbial communities were identical in the four digesters, which is expected because of the identical inoculum and identical feedstock. In addition, a comparison of KI_{min} and KI_{max} values of the control digester between Stage Ib and Stage II shows that inhibition resilience was consistent over time, and that the inhibition resilience of microbial communities remain stable in the absence of inhibition pressure. This suggested that the approaches used in this study were robust.

KI_{max} values measured during Stage II (with added inhibitors) for Na+ were statistically different between the control (R1) and the experimental digesters (R2 and R4). This observation supported that the decrease in specific methane production in digesters R2 and R4 (Figure 4.8) was due to added inhibitor and not random factors. The introduction of Na+ in R2 and R4 appeared to boost the inhibition resilience towards Na+, as shown by higher KI_{max} values for R2 and R4 in Stage II as compared to R1. From Stage Ib to Stage II, the KI_{max} value of R3 for Na+ did not increase as much as with R2 and R4, possibly because Na+ was not added to R3 and therefore did not impose strong selective pressure for Na+ in R3.

For ammonia, there was weak evidence that the experimental digesters (R3 and R4) had greater inhibition resilience to NH_4^+ than did the control digester (R1), with a slightly but statistically higher KI_{max} value for R3 and R4 as compared to R1. However, this inhibition resilience could not be resolved by comparing R2 (no added ammonia nitrogen) with R3 and R4. There is very limited evidence in the literature that the presence of Na+ decreases the severity of inhibition by ammonia (an antagonistic interaction) (Chen et al., 2008). It is possible that the presence of Na+ in R2 had this effect, thereby decreasing the sensitivity of R2 to ammonia.

Interestingly, KI_{min} values were statistically identical in most cases between the control (R1) and experimental digesters (R2-R4), indicating that low concentrations of inhibitor will still cause some inhibition of acetoclastic methanogens, albeit at a minimal extent.

The inhibitor concentration in each of the experimental digesters (R2-R4, Figure 4.9) was above the KI_{min} value and below the KI_{max} value. Thus, some extent of inhibition was expected and was indeed observed from a decrease in specific methane production (Figure 4.8). However, because of adaptation of microbial communities in R2 and R4 (evidenced by higher KI_{max} values for Na+ as compared to R1), the inhibition extent was less than expected from the acute inhibition testing during Stage Ib.



R1 (control); R2 (Na+); R3 (ammonia nitrogen); R4 (ammonia nitrogen and Na+)

Figure 4.9 – Inhibition threshold parameters (KI_{min} and KI_{max}) determined for the four continuous digesters as measured by offline acute inhibition SMA tests, for sodium and ammonia nitrogen separately as added inhibitor, and for inoculum collected as effluent (digestate) from the digesters at operation day 225 (Stage Ib) and day 305 (Stage II) (left to right). Circles (○) indicate the inhibitor concentrations as added in each of the digesters. Error bars show 95% confidence intervals on the parameter values.

In terms of microbial community composition, Figure 4.10 presents a PCA plot that considered the entire microbial community (bacteria and archaeal) in each of the continuous digesters during Stages Ia, Ib and II. The results showed similar microbial community composition in all four digesters during Stage I (before added inhibitor), with a general clustering in the top left corner of the PCA plot. The introduction of inhibitor caused a shift in microbial community of R2, R3 and R4, being distinct in the presence of Na+ (R2 and R4, green and purple symbols, Figure 4.10) as compared to in the presence of ammonia only (R3, blue symbols, Figure 4.10). In comparison, the microbial community in R1 (orange symbols, Figure 4.10) remained largely unchanged. These results indicated that the presence of inhibitor was a major driver for a shift in microbial community, and that Na+ had a distinct effect as compared to ammonia-only.

The addition of inhibitor led to a less diverse archaeal community, which shifted from *Methanobrevibater*, *Methanolinea* and *Methanosaeta* to *Methanocelleus* and *Methanocorpusculum*. A disappearance of *Methanosaeta* and the flourishing of *Methanocelleus* is in agreement with previous research. Bacterial abundance was also affected by the inhibitors, but, this was an overall change in relative bacterial abundance rather than a change in particular bacterial strains.

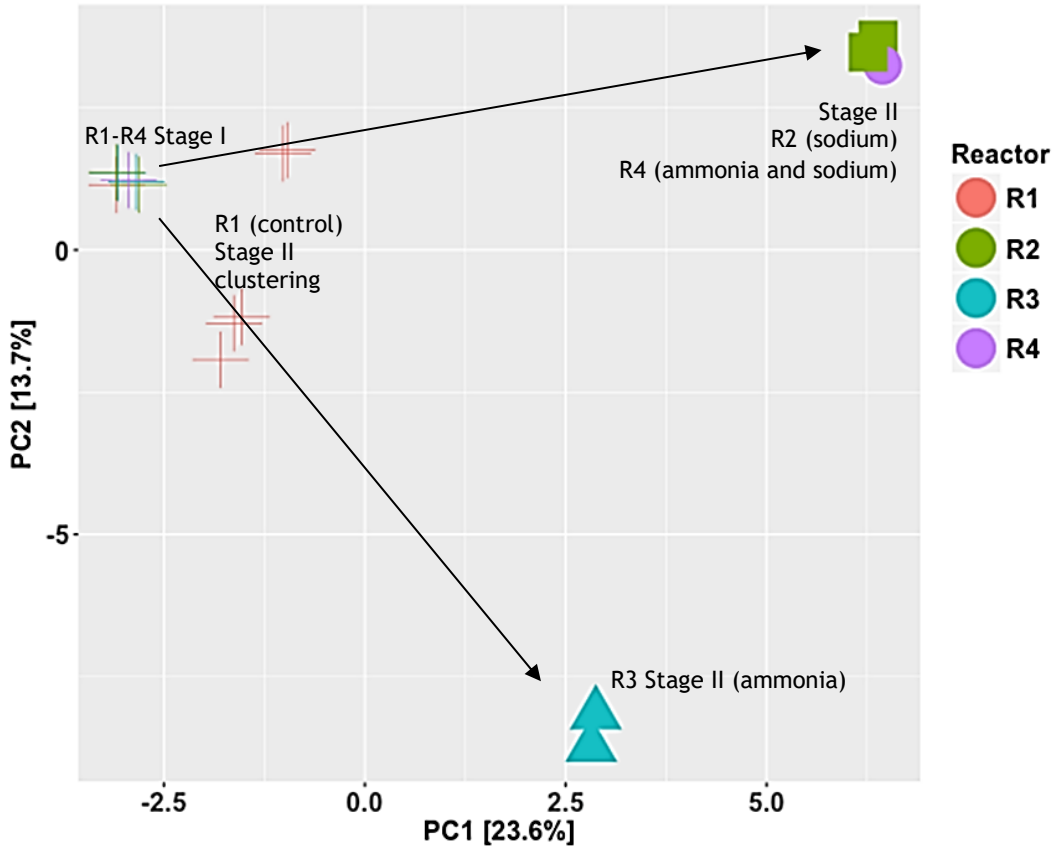


Figure 4.10 – Principal component analysis of the reactors. Multiple data points for the same digester and Stage I are for samples collected at various times during the respective operational stages.

4.4.3 Discussion

Unlike the inoculums analysed in Section 4.3, the continuous digesters in the present work showed clear adaptation to chronic exposure to inhibitors. This was most notable for Na+. The inhibitor response also caused clear changes in microbial community composition.

Changes in microbial community composition and the corresponding increases in inhibition resilience were occurring simultaneously, but it seems unjustified to imply a direct relationship between microbial community composition and inhibition resilience, because of the findings in Section 4.3.

In terms of practical applications, gradual exposure to an inhibitor would allow a CAP or digester to better adaptation of the microbial community than with acute exposure. For example, piggeries that use a high proportion of recycled flushing water at sites with low rainfall and high evaporation would gradually build up salinity concentration, instead of experiencing shock loads as with the feeding of large proportions of highly saline AcoD wastes. Anecdotal evidence suggests that highly salinity in uncovered anaerobic ponds continue to function satisfactorily without excessive odour emission, sludge deposition or floating crust formation, all of which would may indicate failure of the anaerobic digestion process. Based on the present results for the continuous digesters, similar adaptation and sustained performance would be expected for CAPs and anaerobic digesters.

4.4.4 Conclusion

Based on the findings of Sections 4.3 and 4.4, it is concluded that microbial community is not a predictor for inhibition resilience, but that microbial communities do respond naturally to inhibitors. Whilst chronic exposure to inhibitors did increase inhibition resilience, it could not be identified whether this is due to microbial factors or other causes. Regardless, the improved resilience induced by exposure to inhibitors would not likely justify the negative impacts on methane production that results from the exposure to inhibitors.

5. Humic substances as inhibitor in spent litter anaerobic digestion

This research has been submitted as a peer reviewed journal manuscript to the journal Water Research and is under review; *S.D. Yap, S. Astals, Y. Lu, P.D. Jensen, D.J. Batstone, S. Tait. Humic acid inhibition on hydrolysis and methanogenesis with different anaerobic inocula.*

Whilst aceticlastic methanogenesis is commonly considered the most sensitive biological step to inhibitors, some chemical inhibitors such as humic and fulvic acid can have a greater impact on hydrolysis (See Figure 2.1) than on methanogenesis (Ghasimi et al., 2016; Khadem et al., 2017a). This is important for anaerobic digestion of particulate residues such as spent bedding, where the overall speed of methane production is typically dictated by the speed of hydrolysis (Vavilin et al., 2008). Humic acid (HA) is a product of the decay and/or polymerisation of organic matter (Veeken & Hamelers, 1999). Previous studies have reported inhibition of hydrolysis by humic substances within a relevant concentration range of 0.5 to 5.0 $g \cdot L^{-1}$ (Fernandes et al., 2015; Ghasimi et al., 2016). Humic acid inhibition may be especially relevant when considering solid-phase AD and/or associated pre-treatment of high-solids wastes. In such cases, HA can progressively accumulate up to relevant concentrations 10 $g \cdot L^{-1}$ as the wastes is pretreated or degraded (Dwyer et al., 2008b; Fernandes et al., 2015), including when spent piggery litter is digested in solid phase digesters such as trickling leachbeds (Yap et al., 2017; Yap et al., 2016).

Whilst there is an abundance of literature on ammonia inhibition, research on inhibition by humic substances is relatively scarce. Prior studies on HA inhibition have mainly emphasized physico-chemical methods to mitigate HA inhibition, such as precipitating or complexing the HA with metal salts (Azman et al., 2015; Brons et al., 1985) to prevent it from inhibiting the microbiology. Other studies have sought to remove HA by ion exchange (Boyer & Singer, 2006; Fearing et al., 2004). Whilst such methods are reasonably successful, they are unlikely to be cost-feasible for decentralized farm-scale digestion in the Australian pork industry.

Differences in digester configuration, AD operating conditions and AD substrate type could be selectors for microbial composition and microbial diversity in anaerobic digesters (McHugh et al., 2003; Vanwonterghem et al., 2014; Zhang et al., 2014b), and prior inhibitor stress may translate into increased inhibition resilience (Section 4.4). Other studies have suggested that microbial community composition does affect inhibition resiliance to HA. For example, a review by Smith et al. (2005) suggested that bacteria in the rumen of livestock overcome HA inhibition by producing altered enzymes and/or through membrane modification and repair. This is of particular interest for solid-phase digesters such as a leachbed (illustrated in Figure 5.1), where a high solids waste, such as spent piggery litter is contacted with a water leachate to induce AD (Yap et al., 2016). A leachbed operates as a batch process, meaning that leachate from a previously completed digestion batch could be reused in a subsequent fresh batch. This can provide inoculum with a microbial community previously exposed to HA in the old leachbed batch. In this way, inoculation could enhance inhibition resilience and methane production in a subsequent fresh leachbed batch. However, this concept has not been previously tested.

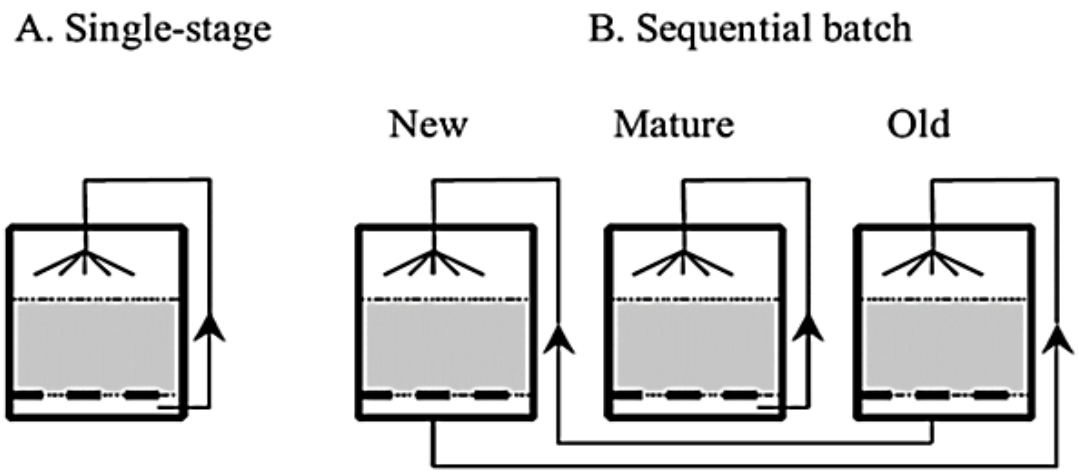


Figure 5.1 – Configuration of leachate recirculation patterns in different leach bed systems Source: Vandevivere et al. (2003). In the single-stage configuration (A), the leachbed is inoculated by the native inoculum present on the waste being digested. In the sequential batch mode (B), a new leachbed batch is first inoculated by exchanging leachate with an old leachbed for which digestion had already finished. Then, once mature, the leachbed is disconnected and operates as a standalone batch, because then it has a balanced and resilient microbial community for the remainder of its digestion time.

The research in the present section used inoculums collected from full-scale anaerobic digesters in smaller scale batch inhibition tests, to identify links between microbial community differences and inhibition resilience specifically for HA. The target application was solid-phase anaerobic digestion of spent piggery litter in a leachbed (See Project 4C-111) because a leachbed can ease material handling for AD of spent piggery litter and can minimize water use for the AD process as compared with complete mixed tank digestion systems. The aim of the present research was to determine whether inoculation could be used as a strategy to counter inhibition by HA.

5.1 Methods

All substrates were analytical reagent grade, purchased from Sigma-Aldrich. Sodium acetate anhydrous, gelatin and α-cellulose were added to represent acetate, protein and carbohydrate substrates, respectively. The inhibitor HA was added as a sodium salt (CAS number 68131-04-4, lot number 16308-048). The four inoculums studied were:

1. DSS: digestate from a 5500 m^3 mesophilic digester (35 °C) at a domestic wastewater treatment plant (WWTP) in South East QLD, treating a mixture of primary and secondary sludge at a hydraulic retention time (HRT) of 23-24 days.
2. THD: digestate from a 2250 m^3 mesophilic (37 °C) anaerobic digester at a centralised municipal biosolids processing facility in South East QLD, fed with thermally hydrolysed sludge from a CAMBI® process (155 °C and 4.5 bar).
3. PLS: sludge extracted from the base of a CAP treating coarse-screened flush manure from grower-finisher pig sheds in VIC, Australia. The sludge was extracted using a vacuum tanker connected to sludge extraction ports through the side banks of the lagoon near the inlet end of the lagoon.
4. PPDS: digestate from a completely mixed tank digester located at a piggery in QLD Australia, treating a mixture of macerated paunch from a nearby abattoir together with pig flush manure, at 25 °C and a HRT of about 15 days. The facility produces approximately 130 kWe and 70 kWe from digestion of the manure fraction and

paunch fraction, respectively. These generation capacities are based on the typical continuous operation of the biogas system, but it is noted that intermittent loads may differ greatly.

The inoculums were characterised for pH, color ($mgPtCO \cdot L^{-1}$), TS and VS, tCOD, sCOD, VFAs and TAN as described in Section 4 and below. Inoculum composition is summarised in Table 5.1. Microbial community composition of each inoculum was characterised as described below.

Table 5.1 - Physico-chemical composition of the inoculums used in the study on HA inhibition

Parameter	DSS*	THD	PPDS	PLS
pH	7.00 ± 0.03	6.98 ± 0.03	7.92 ± 0.01	6.99 ± 0.04
Total solids (TS, g/kg)	30 ± 2	49 ± 3	28 ± 2	52 ± 3
Volatile solids (VS, g/kg)	21 ± 2	31 ± 3	19 ± 2	40 ± 2
Total Chemical Oxygen Demand (tCOD, g/L)	33 ± 3	53 ± 4	41 ± 4	64 ± 4
Filtered Chemical Oxygen Demand (soluble, sCOD, g/L)	0.2 ± 0.1	5.2 ± 0.4	2.8 ± 0.1	0.6 ± 0.1
Total Volatile fatty acids (VFAs, mg/L)	61 ± 10	97 ± 22	445 ± 111	87 ± 18
Total ammonia nitrogen (NH_4-N, mg N/L)	210 ± 12	2,665 ± 70	1,648 ± 12	612 ± 36
Color (mg PtCO/L)	2,667	13,581	2,558	10,490

Estimates of error are given at the 95% confidence level (±), estimated using a two-tailed t-test with appropriate degrees of freedom; *DSS means digested sewage sludge inoculum, THD means thermal hydrolysed digestate inoculum, PPDS means pig manure and paunch digested sludge inoculum, and PLS means piggery lagoon sludge inoculum.

Inhibition testing

Two sets of inhibition tests were conducted. In Set 1, all four inoculums were tested with added HA concentrations of 0 to 2 g HA/L. In Set 2, only DSS was tested for a broader range of added HA concentrations of 0 to 20 g HA/L.

Hydrolytic inhibition tests were performed in 160 mL glass serum bottles (working volume 100 mL) at 37 ± 1°C. For this, the inoculums were pre-diluted to 10 g VS/L with deionised water to minimize interference by background inhibitors. Cellulose or gelatine were then added as digestion substrates at an inoculum-to-substrate ratio (ISR) of 5 on a VS basis. Prior to the test, the inoculums were typically stored at 37 ± 1 °C for 5 days to de-gas. Six replicate bottles were run. At each sampling event, 1 mL liquid samples were withdrawn from three of the six test bottles, while biogas samples were collected from the remaining three bottles. The withdrawn liquid samples were analysed for VFAs and sCOD as described below. To quantify net effects in the test condition batches, background VFAs, sCOD and methane production from substrate-free blanks were subtracted from data of the test condition batches. The test bottles were mixed by inverting once before every sampling event. Biogas volume was measured using a water displacement manometer and biogas composition determined by gas chromatography as described in Section 4.3. The batch

experiments were terminated when the net methane produced was no longer significant (daily methane production below 1% of the cumulative methane production for three consecutive days) (Holliger et al., 2016).

SMA was determined according to the method of Astals et al. (2015a) with acetate as the carbon substrate and at an inoculum-to-acetate ratio of 5, again with each inoculum being pre-diluted to 10 g VS/L with deionised water. Cumulative methane production was determined using a displacement manometer and GC as described above.

Microbial community analysis

To characterise microbial community composition, genomic DNA was extracted from each inoculum prior to the inhibition testing, using a FastSpin for Soil Kit (MP-Biomedicals, Santa Ana, California, USA) in accordance with the manufacturer's protocol. 300 ng DNA of each sample was provided to the Australian Centre for Ecogenomics (ACE, The University of Queensland) to perform 16S Amplicon sequencing using an Illumina Miseq Platform with a 926F (5'-AAACTYAAAKGAATTGACGG-3') and 1392wR (5'-ACGGGCGGTGWGTRC-3') primer set (Engelbrektson et al., 2010). The microbial concentration was estimated by ACE using a qPCR method described by Vanwonterghem et al. (2014) and the primer sets 1406F (5'-GYACWCACCGCCCGT-3') and 1525R (5'-AAGGAGGTGWTCCARCC-3'). Raw paired reads were first trimmed by Trimmomatic (Bolger et al., 2014) to remove short reads (less than 190bp) and low quality reads (lower than Phred-33 of 20). The trimmed paired reads were then assembled using Pandaseq (Masella et al., 2012) with default parameters. The adapter sequences were removed by the FASTQ Clipper of the FASTX-Toolkit (Pearson et al., 1997). The joined high quality sequences were analysed using QIIME v1.8.0 (Caporaso et al., 2010) with an open-reference operational taxonomic unit (OTU) selecting strategy by uclust (Edgar, 2010) at 1% phylogenetic distance and assigned taxonomy by uclust against the greengenes database (13_05 release, McDonald et al. (2012)). OTUs with only one read were filtered from the OTUs table by command filter_otus_from_otu_table.py in QIIME.

Chemical Analysis

Analyses of total fractions (particulate plus soluble) were conducted on samples as collected, without pre-treatment. For soluble fractions, samples were filtered through 0.45 µm syringe filters (PES membrane) and the filtrate stored at 4 °C prior to analysis. VFAs and alcohols were measured with an Agilent 7890A gas chromatograph and an Agilent DB-FFAP column. TAN and phosphate were measured using a Lachat Quik-Chem 8000 Flow Injection Analyser (Lachat Instrument, Milwaukee). TS and VS were measured according to Standard Methods procedures 2540G (Franson et al., 2005) and corrected for potential volatile losses (Peces et al., 2014). tCOD and sCOD as well as color (mg $PtCO \cdot L^{-1}$) were determined using a Merck Spectroquant® cell and a Spectroquant® Move 100 mobile colorimeter (Merck, Germany). Biogas composition (CH_4, CO_2 and H_2) was determined using a Shimadzu GC-2014 equipped with a HAYESEP Q80/100 column using Argon as carrier gas. pH was measured using a Hannah Instrument HI8614LN Transmitter and calibrated HI2910B/H pH probe.

Data Analysis and statistics

The package Aquasim 2.1d was used to analyse data for net cumulative methane produced (B_t) over time (t) for each inoculum and substrate (i.e. cellulose and gelatine). The analysis performed a non-linear least-squares fit using a first-order kinetic model with a lag-phase (t_d, units of d, a fitted parameter) (Equation 5.1):

$$B_{t,i} = \begin{cases} 0, & t < t_d \\ B_{0,i}\left(1 - e^{-k_i \cdot t}\right), & t \geq t_d \end{cases} \qquad (5.1)$$

where $B_{0,i}$ is the fitted maximum methane yield (g COD/gVS_{fed}) and k_i is a fitted first-order hydrolysis rate coefficient (d^{-1}). Subscript i is either ch or pr representing cellulose and gelatine (protein), respectively.

SMA was determined as the slope of a linear regression fit (analysis Toolpak in Microsoft Excel 2010) to the cumulative methane produced over time for tests with acetate as described in Section 4.3.

Where measured data are presented below with error bars or error (±), these are average values ($n \geq 3$) with error estimates representing 95% confidence intervals based on a two-tailed t-test (5% significance threshold) with appropriate degrees of freedom (n-1). Where model parameter errors are presented, these are 95% confidence intervals based on a two-tailed t-test (5% significance threshold) based on a standard error determined from the Secant Fisher information matrix as used by Jensen et al. (2011). Where relevant, errors were analytically propagated as described by Batstone (2013).

For microbial community analysis, OTU tables were normalised and a square root transformation applied to emphasise comparison of niche populations over dominants. A principal component analysis (PCA) was performed according to the method described by Batstone et al. (2015).

5.2 Results

5.2.1 Hydrolysis

Low HA concentration (0 – 2 g/L)

The results showed that cellulose tests with THD and PPDS as inoculums were affected by HA over the range 0 – 2 g/L. The presence of HA induced a time lag-phase significantly longer than in the control tests. The length of the lag-phase increased linearly with increasing HA concentration (Figure 5.2, Table 5.2). For DSS and PLS, a lag-phase was also observed at HA concentrations up to 2 $g \cdot L^{-1}$, but was not significantly different from that of the control tests (Figure 5.2). After the initial lag-phase, kinetics (k_{ch}) and B_0 (~0.79 g $COD_{methane}$/g VS_{fed}) of cellulose digestion in the presence of HA was not significantly different from that in the control tests (Figure 5.3; Table 5.1). These results indicated that bacterial activity, in terms of enzyme release, was not directly affected by HA up to 2 g/L. This contrasts with mechanisms suggested by Smith et al. (2005), where humic substances bind directly to and disrupt cell membrane integrity/transport. Analysis of soluble fractions for tests with HA up to 2 g/L did not show accumulation of VFA or sCOD over time, indicating that downstream microbial processes (i.e. fermentation, acetogenesis and methanogenesis) were not rate limiting.

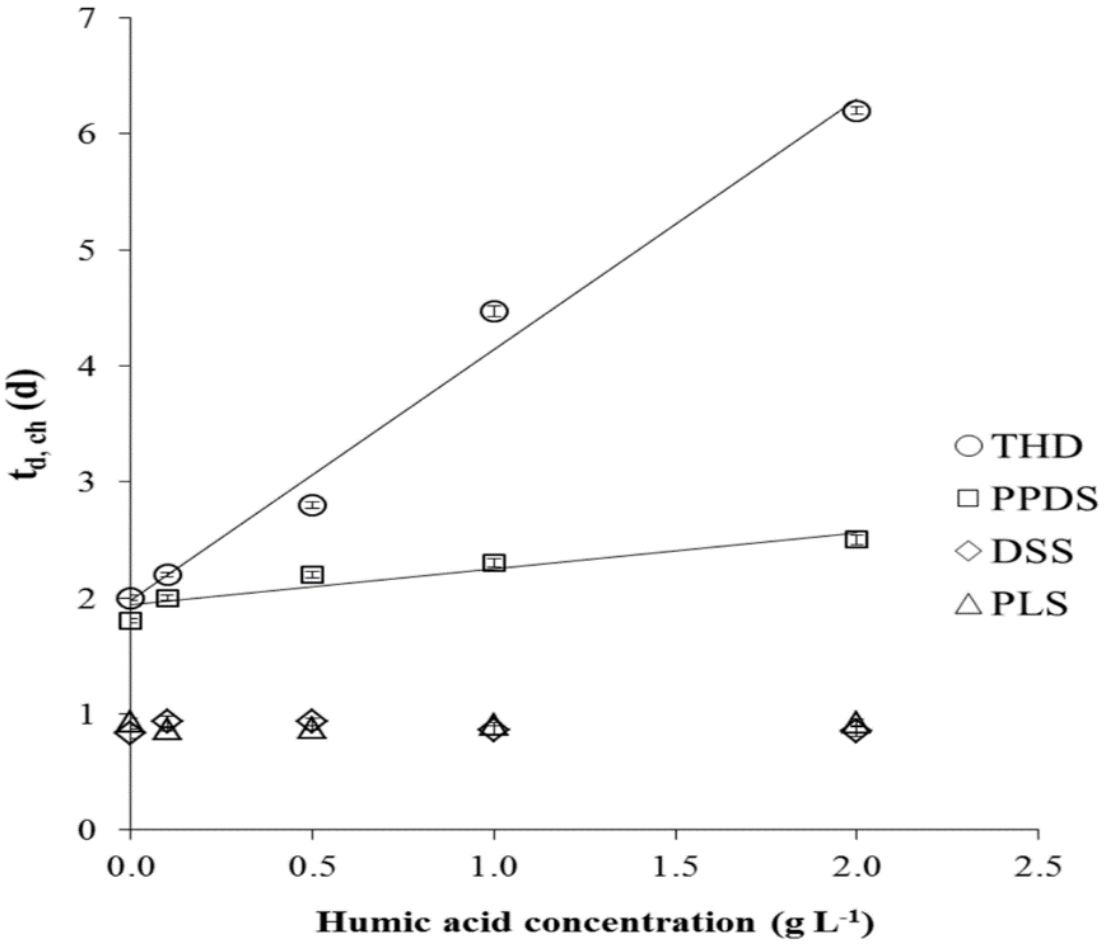


Figure 5.2 – Cellulose degradation, initial lag-phase ($t_{d,ch}$) for different humic acid concentrations (0 - 2 g/L) and for the four inoculums under study.

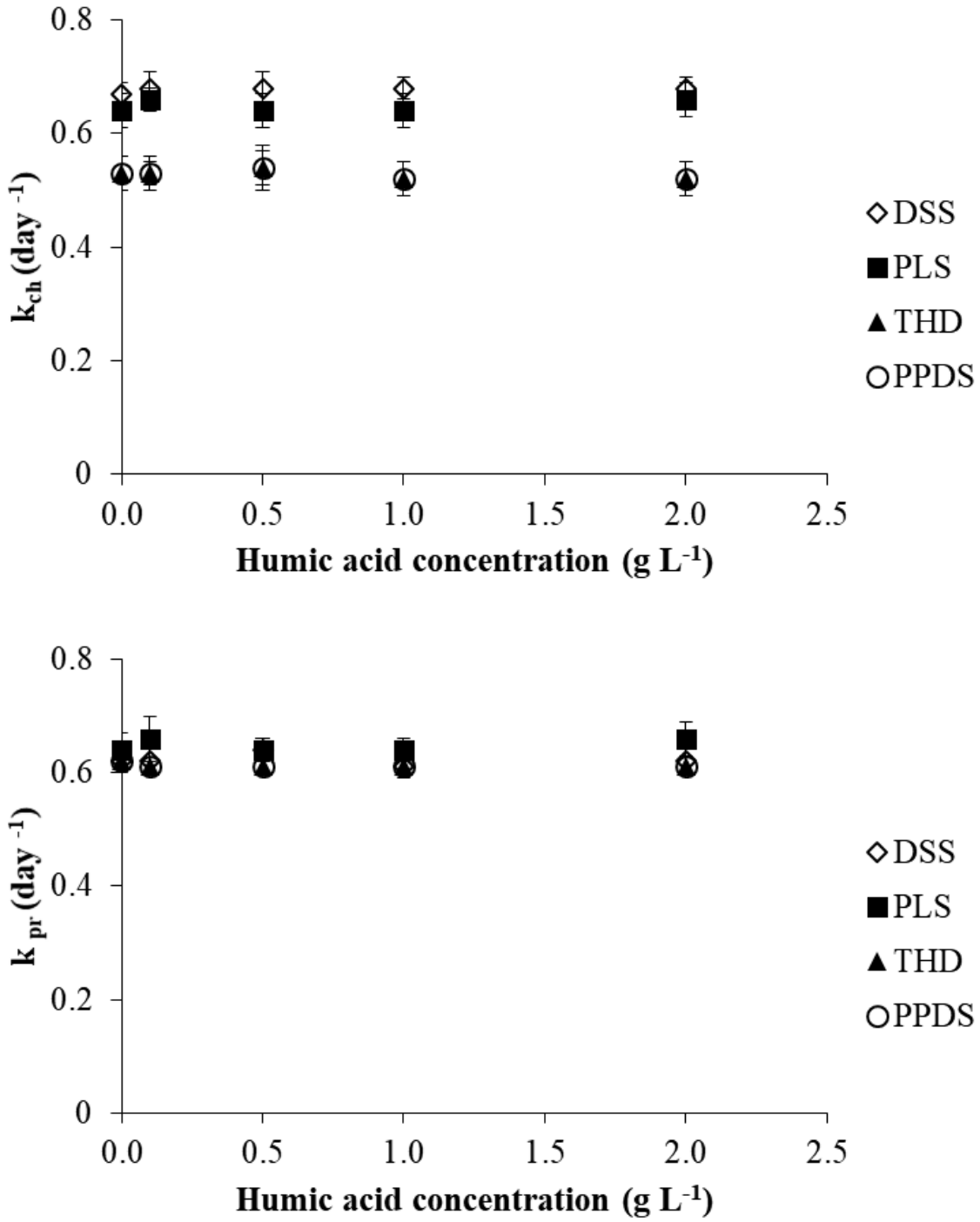


Figure 5.3 - Fitted first-order hydrolysis rate coefficients (k) for hydrolysis of (top) cellulose and (bottom) gelatin at various added humic acid concentrations (0 - 2 g/L).

In contrast to the cellulose tests, protein degradation was not significantly influenced by HA up to 2 g/L, with no significant lag-phase (i.e. not significantly different from zero) and no significant impact on k_{pr} or B_0 (~0.85 g $COD_{methane}/gVS_{fed}$) compared to the control tests without HA (Table 5.2). In contrast, Brons et al. (1985) found that HA inhibited protein hydrolysis at concentrations as low as 0.25 g/L, but humate compositions and microbial community composition could have been different in their study.

<u>High HA concentration (0 – 20 g/L)</u>

For DSS as inoculum and HA concentrations between 5 – 20 g/L, both cellulose and gelatine digestion were significantly influenced, as evidenced by a significantly longer lag-time and decreased degradation rates (k_{ch} and k_{pr}) compared to control tests without HA (Figure 5.4). At a HA concentration between 5 and 10 g/L, no sCOD or VFA accumulation was detected, so fermentation, acetogenesis and methanogenesis were not rate limiting. However, at a HA concentration of 20 g/L, VFAs accumulated and measured methanogenic activity on acetate was essentially nil, indicating strong inhibition of methanogenesis.

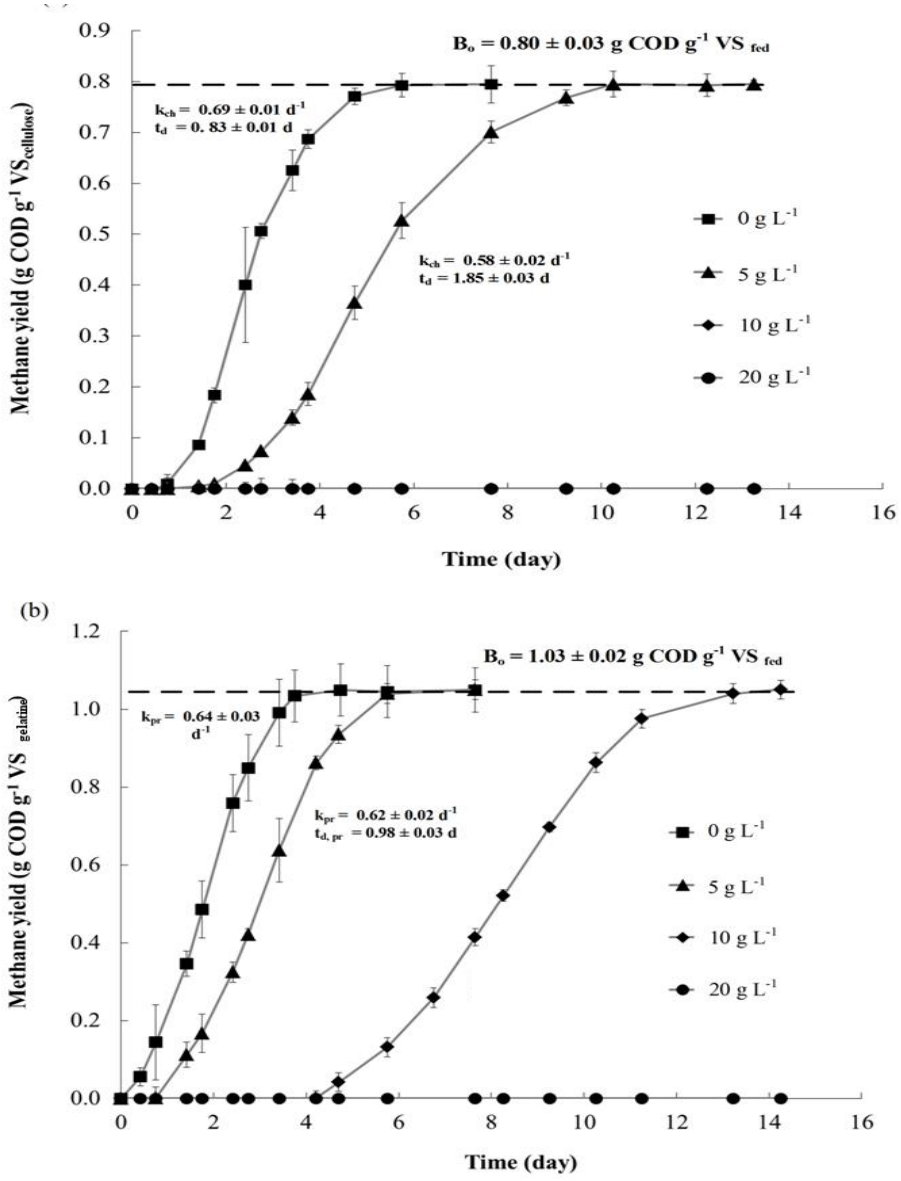


Figure 5.4 – Hydrolytic activity plots and model outputs for hydrolysis of (a) cellulose and (b) gelatin, with DSS as inoculum and at various humic acid concentrations (0 - 20 g/L).

Table 5.2 - Model outputs for cellulose and gelatine hydrolysis and different inoculums at HA concentrations of 0 - 2 g/L.

Humic acid level	DSS				PLS				THD				PDDS			
	Cellulose degradation		Gelatine degradation		Cellulose degradation		Gelatine degradation		Cellulose degradation		Gelatine degradation		Cellulose degradation		Gelatine degradation	
	t_d	k_{ch}	t_d	k_{pr}	t_d	k_{ch}	t_d	k_{pr}	t_d	k_{ch}	t_d	k_{pr}	t_d	k_{ch}	t_d	k_{pr}
g/L	d	d^{-1}	d	d^{-1}	D	d^{-1}	d	d^{-1}	d	d^{-1}	d	d^{-1}	D	d^{-1}	d	d^{-1}
0.0	0.83±0.04	0.67±0.02	0.0	0.62±0.02	0.93±0.03	0.64±0.03	0.0	0.61±0.03	2.01±0.02	0.49±0.03	0.40±0.02	0.53±0.03	1.80±0.02	0.53±0.03	0.0	0.62±0.03
0.1	0.93±0.05	0.68±0.03	0.0	0.62±0.02	0.87±0.02	0.66±0.02	0.0	0.60±0.04	2.20±0.02	0.47±0.02	0.38±0.03	0.55±0.02	2.00±0.02	0.53±0.03	0.0	0.61±0.02
0.5	0.93±0.03	0.68±0.03	0.0	0.64±0.02	0.88±0.02	0.64±0.03	0.0	0.59±0.02	2.80±0.03	0.51±0.04	0.37±0.04	0.55±0.03	2.20±0.03	0.54±0.02	0.0	0.61±0.02
1.0	0.86±0.04	0.68±0.02	0.0	0.61±0.02	0.91±0.01	0.64±0.03	0.0	0.59±0.02	4.70±0.05	0.48±0.03	0.38±0.02	0.55±0.04	2.30±0.04	0.52±0.03	0.0	0.61±0.02
2.0	0.95±0.05	0.68±0.02	0.0	0.62±0.02	0.92±0.03	0.66±0.03	0.0	0.58±0.03	6.20±0.03	0.48±0.03	0.34±0.03	0.56±0.02	2.50±0.04	0.52±0.03	0.0	0.61±0.03

Inhibition mechanisms

A mechanistic hypothesis is stated (Figure 5.5) here to interpret observations for cellulose digestion at HA up to 2 g/L:

(1) Bacteria excrete enzymes at a specific rate to hydrolyse cellulose, however HA complexes with the enzymes, preventing the enzymes from binding with cellulose. This causes the lag-phase.

(2) Once all the HA has been complexed (lag-time), enzymes that are subsequently released can bind with cellulose. Hydrolysis then proceeds unaltered by complexed HA and the rate of hydrolysis is dictated by enzyme excretion and enzyme reaction.

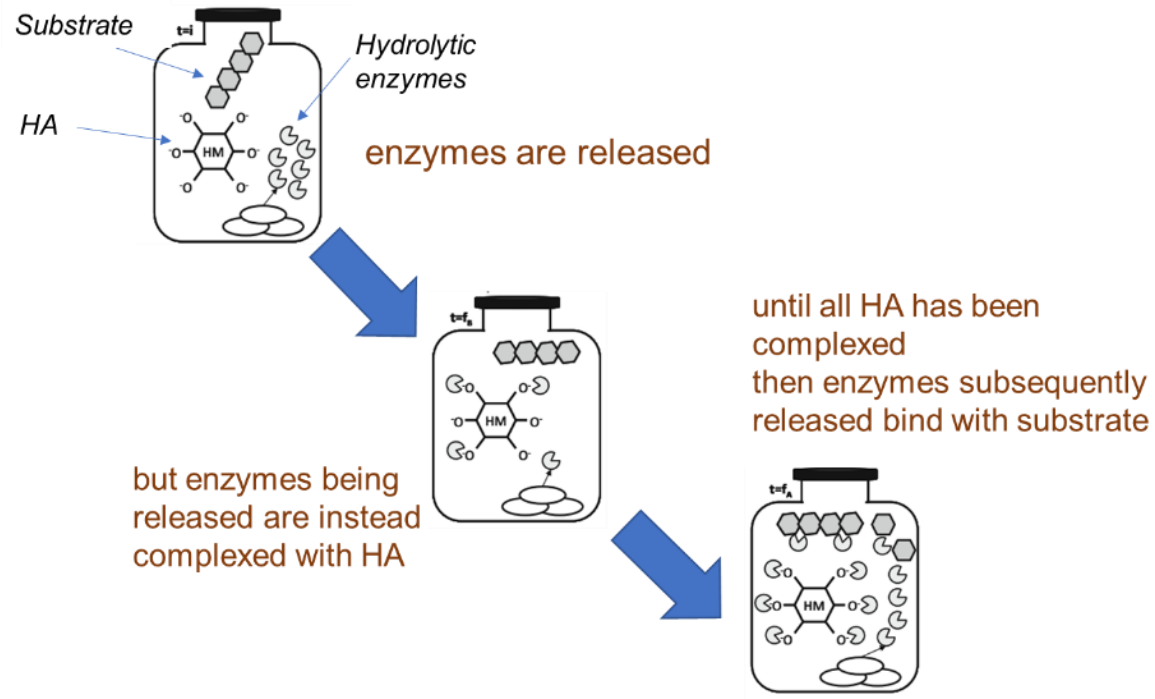


Figure 5.5 – Illustration of potential HA inhibition mechanisms at up to 2 g HA/L. Adapted from Fernandes et al. (2015).

Further testing is required to verify the suggested mechanism; however, this hypothesis is consistent with observations from previous studies. For example, hydrolytic activities have been reduced by increasing HA concentration (Fernandes et al., 2015; Li et al., 2013). Bacterial activity has been positively correlated with enzyme production (Nybroe et al., 1992). Interestingly, DSS and PLS (no lengthened lag-phase at increasing HA concentration) had a higher hydrolysis rate than THD and PPDS (lengthened lag-phase at increasing HA concentration) suggesting a link between microbial activity and inhibition resilience.

The mechanism of HA inhibition at HA up to 2 g/L appeared to be different from that at HA > 5 g/L. HA inhibition at higher concentrations could be caused by higher-order complexing of substrates, enzymes and HA (Li et al., 2013; Tomaszewski et al., 2011), and inhibition of methanogenic activity.

The PCA of the bacterial community indicated occupancy of one of three major spaces (Figure 5.6). The more resilient DSS and PLS inoculums (no increase in lag-time with increasing HA up to 2 g/L) occupied the upper left quadrant, associated with increased relative abundance in the phyla *Bacteriodetes*, *Chloroflexi*, *Planctomycetes* and *Proteobacteria*. The less resilient THD and PPDS inoculums also exhibited lower microbial diversity than DSS and PLS. THD occupied the right hand region with a dominance of phylum *Firmicutes*, while PPDS occupied the lower left region and was dominated by phylum

Spirochaetes with a lesser contribution by *Tenericutes*. A closer examination of bacterial community composition (see heatmap in Figure SII) revealed the presence of bacterial strains of order *Plactomycetales, Sphingobacteriales* and *Syntrophobacterale* in the more resilient inoculums DSS and PLS, which have been previously reported to be humic-resistant (Azman et al., 2015). However, while these differences in microbial community composition aligned with differences in HA resilience, *Plactomycetales*, *Sphingobacteriales* and *Syntrophobacterale* are not known to participate in hydrolysis (Muyzer & Stams, 2008; Scheurwater et al., 2008; van Teeseling et al., 2015) and hydrolytic bacteria with reported humic resistant properties could not be identified in the present study. Increased HA inhibition resilience may also result from higher microbial activity or microbial concentration, independent of differences in microbial community composition.

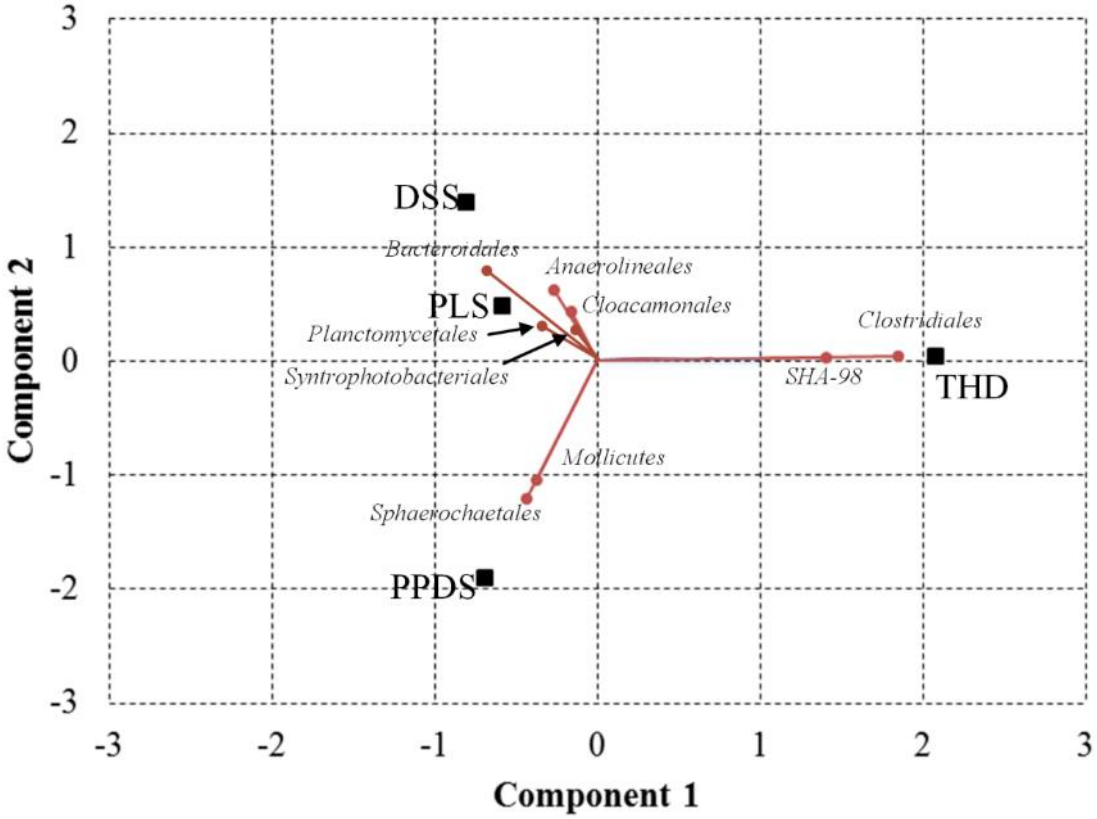


Figure 5.6 – PCA of the bacterial population for the HA inhibition study, indicating major vectors (red) and samples (black) in the PCA space.

5.2.2 Methanogenesis

Measured SMAs of the four inoculums (DSS, THD, PLS and PPDS) were comparable at 0.11, 0.092, 0.095 and 0.075 g $COD_{methane}/gVS_{inoculum}/d$, respectively. The results showed that HA concentrations of 0 to 2 g/L did not have a significant effect on methanogenic activity for any of the inoculums. These findings were consistent with that of Ghasimi et al. (2016) who reported no significant effect of HA on methanogenesis at concentrations up to 2 g/L. However, when HA concentration was further increased to > 5 g/L, the methanogenic activity was reduced by 40% (Figure 5). Methanogenesis was completely inhibited at 20 g/L (Figure 5.6). Whilst the addition of the HA as a salt at 20 g/L equivalent concentration would have corresponded to the simultaneous addition of 2 g/L sodium, notable inhibition of acetoclastic methanogenesis by sodium usually occurs only at much higher concentrations >3 g/L (Astals et al., 2015a; Chen et al., 2008).

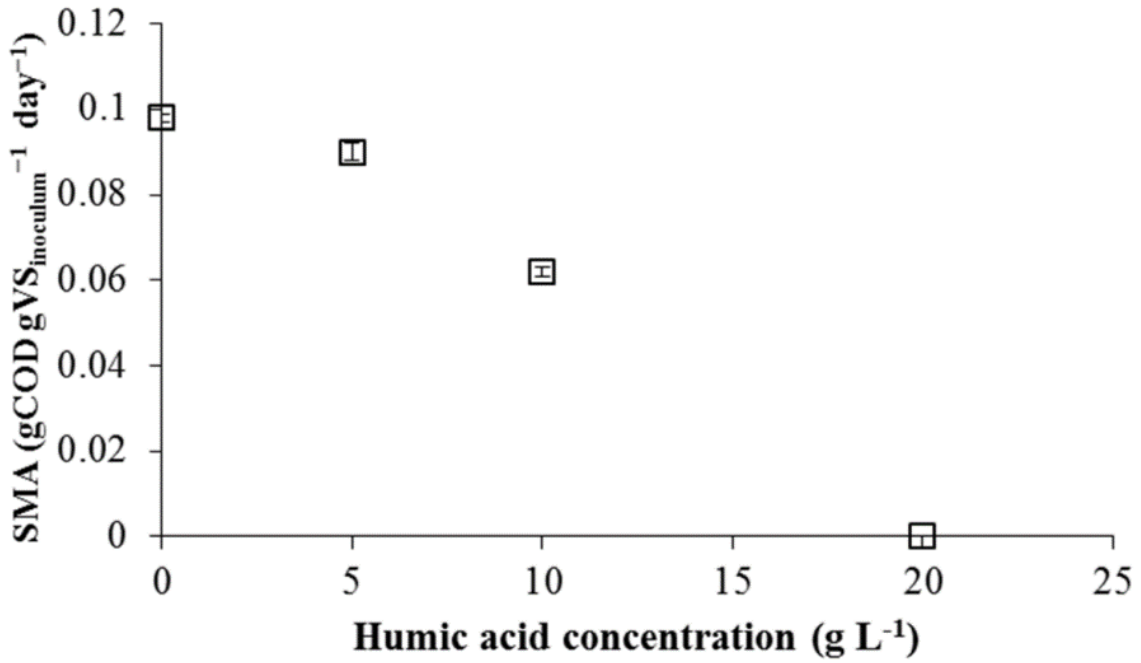


Figure 5.6 – Methanogenic activity (SMA) of DSS at HA concentration range between 0 - 20 g/L**.**

The observed inhibition was therefore more likely caused by HA. HA inhibition of acetoclastic methanogenesis has not been previously reported, but HA concentrations in other studies were much lower (Brons et al., 1985; Fernandes et al., 2015; Ghasimi et al., 2016). Microbial community analysis showed that all the inoculums were dominated by the acetoclastic methanogen *Methanosaeta* (relative abundance between 57 to 66%). Previous works by Khadem et al. (2017b) and Ghasimi et al. (2016) observed that *Methanosaeta* could withstand HA concentrations up to 3 g/L without inhibition. The study by Khadem et al. (2017b) also suggested that *Methanosaeta* has better resistance to HA inhibition than other methanogens, because its cell wall structure.

5.3 Discussion

The results showed that moderate concentrations of HA (<5 g/L) could slow down hydrolysis of carbohydrate-rich substrates and inhibit methanogenesis at concentrations above 10 g HA/L (see Section 3.2). This is relevant for many AD substrates including pig manure and deep litter. Humic acid concentration is rarely monitored in AD processes, partly because no standardised measurement method exists. However, it is expected that HA concentrations in digesters would vary significantly depending on pre-treatment, feedstock and reactor configurations and operating conditions (Dwyer et al., 2008a; Fernandes et al., 2015; Yap et al., 2017). For instance, in agricultural applications, HA inhibition on AD could be exacerbated by HA accumulation up to 5 g/L, because of high proportions of organic matter (Fernandes et al., 2015; Yap et al., 2017).

The results showed clear differences in inhibition resilience between the tested inoculums, possibly due to differences in bacterial activity. However, no definitive link was found between microbial community composition and resilience to HA inhibition. Therefore, it remains unknown if the inhibition resilience is a property of specific hydrolytic bacterial strains, or rather due to microbial activity or microbial concentration differences.

The HA inhibition was reversible, meaning that if a secondary complexing agent such as a metal cation was added to the digestion mixture, the HA may instead bind to the secondary complexing agent and release enzymes to bind to the digestion substrate instead. This was shown by the observation that, when all enzymes had complexed with HA, the rate of hydrolysis subsequently proceeded unaffected by HA. The microorganisms responsible for hydrolysis were not directly affected by HA up to 5 g/L, in contrast to irreversible toxicity which causes cell death. Specifically, subsequent hydrolysis rates and methane yields after the initial lag-phase had passed, were not significantly influenced by HA presence.

Unfortunately, similar to the results in Section 4, no definitive link could be established between microbial community composition and inhibition resilience. Instead, more resilient communities appeared to be those that exhibited higher microbial activity when HA had not been added (control tests).

Future applications, such as with solid-phase digestion in a leachbed, could target inoculation with high activity inoculums to mitigate HA inhibition. Other strategies that have been trialled abroad include the dosing of hydrolytic enzymes to counter the effect of HA on hydrolysis. However, such dosing of commercial enzymes would unlikely be feasible for decentralized AD of spent piggery litter. Dosing of a cation such as calcium can also be detrimental to AD by forming mineral precipitates that cause clogging of AD systems.

6. Application of Research and Conclusion

Methane production from pig manure in covered anaerobic ponds and anaerobic digesters is a natural process carried out by microorganisms converting manure into methane. Manure methane use is increasing at Australian piggeries to produce heat and to generate electricity. Currently, about 13.5% of total Australian pork production captures biogas, and the majority use it in generators or hot water systems. By capturing and burning the manure methane in a generator engine, flare or hot water system, greenhouse gas emissions are also reduced. The performance of a covered anaerobic ponds or anaerobic digester (in terms of methane production) is dictated by the methane potential of the waste (e.g. manure or other) and the health of the microorganisms responsible for anaerobic digestion.

A common strategy to increase methane production is anaerobic co-digestion, whereby two or more wastes are simultaneously digested to produce methane. This increases methane production by increasing organic matter and by creating favourable digestion conditions that increase the conversion of the organic matter into methane. Piggeries can and do already benefit from co-digestion because of part substitution of pig feed ingredients with permissible food by-products (e.g. past use-by-date dairy products). Usually, the unusable portion of the by-products ends up in a digester or covered anaerobic pond, and is then co-digested together with pig manure producing more methane for use at the piggery. Savings in feed ingredient costs probably dictate the economic feasibility of the waste handling, but co-digestion does have the added benefits of increased energy production. As an example, one piggery in QLD boosts its methane energy from 130kW (anticipated from pig manure only) to over 200kW, by co-digesting pig manure together with meat processing wastes. Overall, there is considerable opportunity for co-digestion in Australia.

Whilst desludging has been an on-going concern to pork producers with covered anaerobic ponds, due to potential accessibility issues, there are now clear examples of successful routine desludging. As a result, covered anaerobic ponds could be sized for a nominal sludge accumulation period, rather than excessive sizing to hold sludge for the entire design life of the pond cover. The research in this report suggested that the treatment of manure solids in Australian covered anaerobic ponds is reasonably effective, and consequently, when sludge is extracted from the ponds, the residual methane loss with extracted sludge would likely be minimal (at most 10%). Sludge is therefore ideally extracted frequently to prevent densification and sludge pumpability issues, however the present results may indicate a benefit of extending desludging frequency to 2-yearly intervals to increase methane recovery.

Inhibitors will always be present in pig manure, and co-digestion with other wastes can introduce unique inhibitors that further affect digestion performance. The research herein showed that microbial communities cope with inhibitors to an extent, albeit with reduced performance. However, the results showed that acute short-term exposure to high levels of inhibitor is more detrimental to microbial community health than longer-term gradual increases in inhibitor presence. This is important from an applications perspective, because digesters or covered anaerobic ponds may cope better with increased levels of inhibitor, if the exposure to the inhibitor is gradual, giving the microbial community time to adapt. For a co-digestion waste, this might mean storing the co-digestion substrate with high inhibitor content onsite at the piggery, and gradually feeding it into the CAP and digester whilst carefully monitoring the impact on digestion performance. In summary,

microbial community is not a predictor for inhibition resilience, but there is a (substantial) community response to the presence of inhibitors, including a progressive increase in resilience with chronic exposure. It is not identified whether this progressive increase in inhibition resilience because of exposure, is due to microbial factors (microbial composition). Overall, the negative impacts of inhibition itself are not justified by the improvement in inhibition resilience upon exposure of AD microbial communities to inhibitors.

7. Limitations/Risks

Digestion temperature is very important and influential for methane production. Whilst most digesters are controlled at mesophilic (37°C) or thermophilic (55°C) conditions, ambient temperature covered anaerobic ponds are common in the Australian pork sector because of low cost and complexity. This introduces some degree of technical risk with co-digestion because covered anaerobic ponds in Australia can vary in temperature by up to 20°C. This can affect the biodegradability and methane potential of different co-digestion substrates and can limit the organic loading that can be applied to a covered anaerobic pond or digester, but these aspects are currently poorly understood.

Increased methane production by co-digestion is the result of increased organic loading, but the waste types and organic loading that can be safely applied without causing process upset, is partly dictated by the digester configuration. For example, particulate wastes or high organic loading may increase the risk of forming floating crust layers under the pond cover. Co-digestion also influences properties of the digestate or sludge that remains after digestion, changing important properties such as dewaterability (i.e. ability to remove moisture to produce a stackable product). Currently these aspects are poorly understood and yet will become increasingly important as a greater number of alternative waste options become available for co-digestion at piggeries. Co-digestion also impacts on biogas quality, with potential to change methane concentrations and to increase recalcitrant trace ingredients such as siloxanes (not usually present with straight manure digestion) and hydrogen sulphide (exacerbated by high sulphur wastes).

The covered anaerobic ponds researched in the sludge extraction studies in this report varied considerably in terms of hydraulic design and retention times. However, variability in covered anaerobic pond design and operation would affect sludge accumulation and sludge behaviour. Consequently, sludges in other lagoons may contain considerably more residual methane potential than measured in the present study.

The behaviour observed for sodium and ammonia as inhibitors in the present study may not apply to other forms of inhibition common to pig manure digestion, e.g. dissolved sulphide, volatile fatty acids, pH, and inhibition may be affected by complex interactions between inhibitors leading to increased overall inhibition (as observed for sodium and ammonia in the present study). This may affect the ability of microbial communities in covered anaerobic ponds or digesters to cope with inhibition. Whilst it is suggested in the present work that the gradual introduction of inhibitor into a digester or covered anaerobic pond would allow for adaptation, this may not be possible with all inhibitors relevant to digestion in the pork sector.

The research interpretations in the present study relies on microbial community composition analysis methods, which are known to be sensitive to sample processing methods and operator differences. This is an on-going topic of debate between

microbiology researchers. In addition, the current microbial community analysis techniques only identifies a portion of the total micro-organism population, being known organisms, and the “others” unidentifiable fraction is often substantial. The resulting risk is that interpretations in the present work based on microbial analysis may not be exhaustive.

The continuous digester studies in the present research exposed microbial communities to higher levels of sodium and ammonia than would be typical for covered anaerobic ponds and even mixed heated digester vessels. These high inhibitor levels led to clear adaptation behaviour, which was necessary for the research. However, as observed in the present research for tests on inoculums sourced from real full-scale digesters and covered anaerobic ponds, microbial communities in real systems may not be routinely subjected to inhibitor stress. Consequently, microbial communities in real systems may not be tolerant of future high levels of inhibitors resulting co-digestion scenarios, even if inhibitor concentrations were to be gradually increased via the planned progressive addition of the co-digestion waste containing the inhibitor.

8. Recommendations

As a result of the outcomes in this study the following is recommended:

1. That producers explore opportunities for anaerobic co-digestion to leverage existing biogas infrastructure to boost methane production for energy and to generate additional revenue streams by diversion of wastes away from landfill. It would be worthwhile to advocate for consistent landfill levy policies in all production states of Australia, because such levies can be a key driver for profitability from anaerobic co-digestion.
2. That desludging strategies be designed primarily for pumpability of sludge and sludge inventory, with some potential benefit of extending desludging frequency to every 2 years.
3. That, in cases where inhibitors of anaerobic digestion are of concern or their increased presence is anticipated (such as by the receipt of large quantities of a co-digestion substrate), that the emphasis should be on gradual introduction of the inhibitor into the covered anaerobic pond or digester to allow adequate time for natural microbial acclimation.
4. It would be interesting to explore in future research the dedicated adaptation of a microbial inoculum to be added to the main digester or covered anaerobic pond in preparation for a pending inhibitor load, and in this way try and mitigate microbial inhibition.

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