EFFECTS OF ANTIBIOTICS ON THE ANAEROBIC DIGESTION PROCESS

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ABSTRACT

The key product from anaerobic digestion (AD) is biogas, which is used to generate heat and/or electricity. Anaerobic digestion involves degradation and stabilisation of the feedstock by microorganisms, leading to the formation of biogas and a digestate residue, which is used as a fertiliser. Contaminants entering the system in the feedstock may limit biogas yield if functioning of the methanogenic archaea is disrupted. Digestate quality may also be compromised. Approximately 40% of the current UK biogas plants are farm-based, using manures and slurry as the main feedstock. Veterinary medicines are excreted in dung of treated animals and if used prophylactically, concentrations in the faeces or urine may be significant. Contaminated animal waste is therefore likely to be used as a feedstock in AD. Since digestates are commonly used as fertilisers, contaminant loading and fate must be understood to prevent transfer into crops, reductions in soil microbial activity, increased antibiotic resistance and detrimental effects to livestock if digestates are applied to fodder crops or pasture. It is unlikely that the use of veterinary pharmaceuticals will be reduced whilst livestock farming is intensifying and the current demand for meat is growing, therefore understanding the operational processes of AD that influence the persistence of commonly used veterinary medicines and subsequent toxicity are crucial to minimising potentially detrimental effects.

Research was undertaken using laboratory-scale digestion vessels to quantify the effect of the commonly used veterinary antibiotics, oxytetracycline and tylosin, when added to naïve (organic) cattle dung or to slurry from a conventional dairy farm. Anaerobic digestion units were spiked with either oxytetracycline or tylosin at low (environmentally realistic) and artificially high concentrations, either at start-up (day 0) or once the system was producing

gas (day 15). Biogas production was measured and gas collected every 5 days to quantify the temporal effect of the antibiotics on methane production.

Oxytetracycline and tylosin significantly reduced both biogas quality and quantity, with the extent of the effect differing with each feedstock. In organic cow dung, the low (4.33 mg L^{-1}) and artificially high (86.63 mg L^{-1}) concentration of oxytetracycline added on day 15 to organic cow dung caused an overall drop in biogas production of 12% and 25% respectively, whilst the same concentrations incorporated at start-up caused a drop of only 4% and 18% respectively. Both the low and artificially high concentrations of tylosin added on day 15 caused a 33% drop in biogas production, whilst the same concentrations incorporated on day 0 caused a drop of 15% and 42% respectively. In conventional dairy slurry, low (4.33 mg L^{-1}) and artificially high (86.63 mg L^{-1}) concentrations of oxytetracycline caused an overall 3% and 10% drop in biogas production respectively, with tylosin amendment causing a decrease in total biogas production of 7% and 22% respectively.

Feedstock origin affected biogas production and quality when the system was challenged by antibiotic inputs. These data highlight the complex interactions that can occur between feedstock and exposure to veterinary pharmaceuticals.

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LIST OF ABBREVIATIONS

AD	-	Anaerobic Digestion
C/N	-	Carbon/Nitrogen
DM	-	Dry Matter
EU	-	European Union
FID	-	Flame Ionization Detector
IBC	-	Intermediate Bulk Container
LSD	-	Least Significant Difference
OTC	-	Oxytetracycline
SR	-	Solids removed
TCD	-	Thermal Conductivity Detector
TN	-	Total Nitrogen
TOC	-	Total Organic Carbon
TS	-	Total Solids
TYL	-	Tylosin
VS	-	Volatile Solids

CHAPTER 1 - INTRODUCTION

1.1 CONCEPTS AND USE OF ANAEROBIC DIGESTION

Anaerobic digestion involves degradation and mineralization of organic materials by microorganisms under anaerobic conditions (without oxygen), leading to the formation of a mixture of primarily methane (CH_4) and carbon dioxide (CO_2), in addition to low concentrations of hydrogen sulphide, hydrogen, nitrogen and oxygen and biomass residue known as digestate (Kelleher *et al*., 2002). The collected biogas, a renewable source of energy, can be used directly by burning within a boiler to heat buildings/water for domestic use. It can also be used in a Combined Heat and Power (CHP) unit, which allows the additional benefit of electricity generation, either to be used directly at source or if connected to the grid, exported. The resulting digestate, is high in nutrients and can be used as a fertiliser. Germany is currently the largest biogas producer in the EU with 7,515 biogas units in 2012, corresponding to a national electricity contribution of 3.85% with a total installed electricity capacity of 3,200 MW (Facherband-Biogas, 2012). In India, anaerobic digestion is by no means a new, or novel way to produce energy, with fossil fuel prices and demand increasing, attempts are being made to meet energy demands from renewable sources (Charters, 2000). It has been estimated that family sized anaerobic digestion units utilizing dung in India in 2010 was approaching 12 million units (Venkateswara *et al*., 2010). The UK is rapidly increasing the number of biogas units with 129 biogas units functional by the end of 2013 (excluding those dedicated to sewage treatment), an increase of over 25% from the previous year. In September 2016 however, this figure had increased to a total figure of 300 biogas units (excluding sewage works), an increase of 132% when compared to 2013 numbers (Biogas-Info, 2013). This number includes 254 on-farm digesters, the rest being associated with processing food waste. The UK is still far behind Germany in terms of the number of anaerobic digestion units, however numbers are increasing at a rapid rate, mainly driven by

the push for renewable energy within the UK. Anaerobic digestion in the 1970s utilised both municipal and industry organic wastes as a management strategy for the increase in waste and rising environmental awareness (Steffen *et al*., 1998). Suitable biomass for biogas production includes agricultural matter (animal manure/slurry, animal bedding, vegetable by-products and energy crops), industrial waste (e.g. brewery industries, food industries, organic wastes, agro-industries, wastewaters, bio-refineries) and municipal waste (separated household waste, sewage sludge, municipal solid waste and food residue) (Seadi *et al*., 2013). Biogas feedstock substrates are however primarily derived from the agricultural sector (Steffen *et al*., 1998).

1.2 THE ANAEROBIC DIGESTION PROCESS

The key aim of anaerobic digestion is to produce biogas, specifically CH_4. Biogas mainly consists of CH_4 (50-75%), CO_2 (25-45%) and H_2S (10 - 10000 ppm). Methane fermentation can be divided up into four phases, hydrolysis, acidogenesis, acetogenesis and methanogenesis, as shown in Figure 1 (Gujer and Zehnder, 1983). Acetogenesis and methanogenesis occurs *via* 3 sequential stages; acetogenic bacteria convert volatile fatty acids into acetic acid and hydrogen (H_2), acetoclastic methanogenic archaea convert acetic acid to CH_4 and hydrogenotrophic methanogenic archaea convert H_2 to CH_4 (Sanz *et al*., 1996). In anaerobic digestion, acetic acid and CH_4 producing microorganisms need different environmental conditions to perform optimally, factors such as physiology, nutritional needs, growth kinetics and sensitivity to environmental condition such as pH, temperature, substrates and the presence of veterinary pharmaceuticals can influence the digestion process (Pohland and Ghosh, 1971; Zayed and Winter, 2000; Lallai *et al*., 2002).

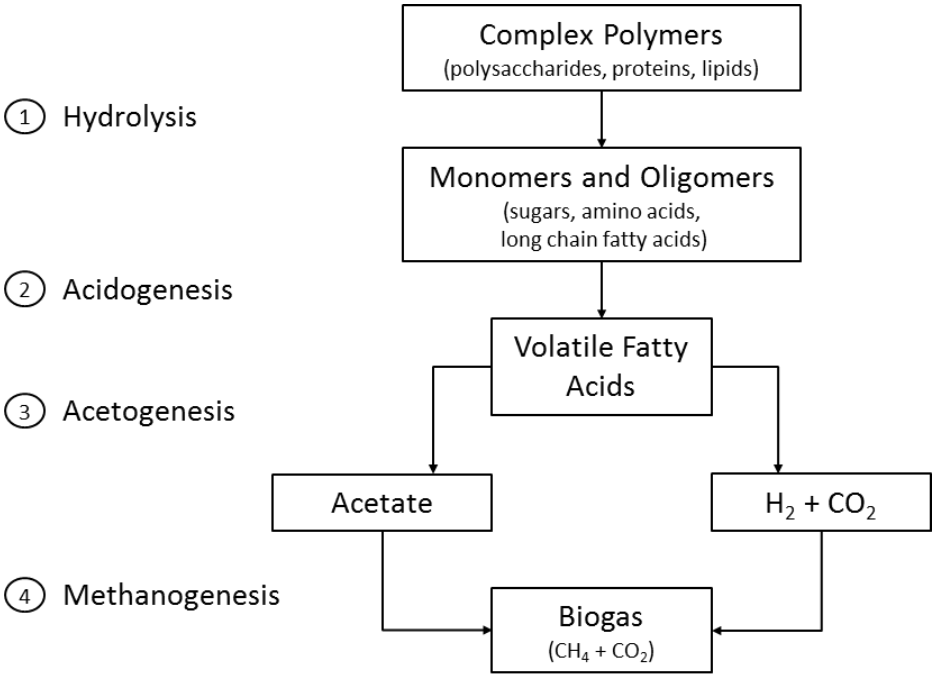

Figure 1-1 The stages of biogas production under anaerobic digestion. (Adapted from Gujer and Zehnder 1983).

1.3 DIGESTER CHARACTERISTICS

Anaerobic digesters can typically be classified into two different categories, continuous flow (single-stage or multi-stage) and batch systems. A continuous single stage process includes one reactor which is continuously stirred, whilst being fed feedstock with relatively low solid content to allow mixing. A continuous multi-stage process typically includes 2 reactors, the first for hydrolysis, acidogenesis and acetogenesis, and the second for methanogenesis, with continuous feeding of higher solid content feedstock. Batch systems are filled once with feedstock (typically >15% dry matter (DM)) and continuously circulated and allowed to go through the entire anaerobic digestion process. Sequential systems of batch processing involve two or more reactors, with leachate re-circulated towards the second reactor to allow methanogenesis to occur. Continuous flow digesters usually incorporate wet feedstock (5-15% DM), whilst batch systems use dry feedstock (<15% DM). Anaerobic digestion units are normally kept within two temperature ranges, thermophilic (50-60°C) or mesophilic (25-

45°C). Thermophilic digestion (8-18 days) operates at a faster rate compared to mesophilic digestion (18-60 days) (Redman, 2010). Mesophilic anaerobic digestion is more widely used than thermophilic digestion due to lower energy requirements and higher process stability (Gavala *et al*., 2003). However, thermophilic anaerobic digestion is characterized by increased reactions, higher growth rate of microorganisms and potentially increased methanogenic potential (Zábranská *et al*., 2000). The vast majority of on-farm digesters are mesophilic, continuous flow wet digesters, as removing and restarting an anaerobic digestion system every few weeks, along with the start-up process would open up to various management issues (Redman, 2010).

1.4 ON-FARM ANAEROBIC DIGESTION

1.4.1 Feedstock and Biogas Production

Anaerobic digestion systems can produce a widely varying amount of biogas depending on feedstock choice, with low-yielding feedstock often having a low cost, or in terms of on-farm availability it could be free. Figure 1.2 demonstrates the expected yield per tonne weight of different feedstocks. Feedstocks vary in potential biogas yield due to their calorific values (Redman, 2010), which is why animal manures are relatively low on the scale, since digestion and absorption has also taken place once already on those feedstocks. Furthermore, slurries and manures in comparison to energy crops have low dry matter content, which results in a low biogas yield. Greenhouse gases emitted by the agricultural sector account for 18% of worldwide production (InfoResources, 2007). A high proportion of these gases are thought to originate from the 13 billion tons of animal waste estimated to be annually produced around the world (Van Horn, 1995; Harkin, 1997). This demonstrates the potential of animal manures and slurries to be used as cheap, readily available feedstock for anaerobic digestion. As Figure 1.2 demonstrates, manure and slurry produce low biogas yields, their ability to

produce biogas is often not economically sustainable, so combined feedstocks are often employed to achieve a higher biogas return (Redman, 2010). However, due to the availability of manures, and the increase in legislation restricting manures to be used directly as fertiliser unless treated and managed by such technology as anaerobic digestion, it can be seen that using manure as feedstock for anaerobic digestion remains an attractive option (Seadi *et al*., 2013).

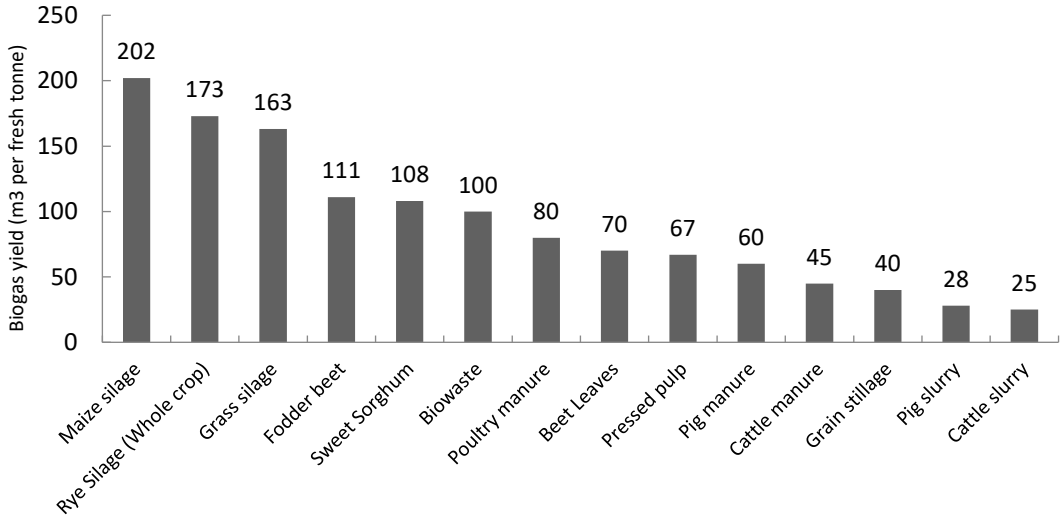

Figure 1-2 Biogas yield for a range of common on-farm feedstocks (Redman, 2010 as cited by Hopwood, 2011).

1.4.2 Use of Digestate as a Fertiliser

The literature generally states the benefits of applying anaerobically digested residues to soil; particularly in terms of increased soil fertility, plant growth promotion and improved soil structure (Forge *et al*., 2005). There is increasing experimental evidence that use of anaerobic digestates could suppress plant diseases caused by parasitic nematodes and a variety of phytopathogens (McBride *et al*., 2000; Jothi *et al*., 2003; Cao *et al*., 2013). These controlling effects of digestate have been attributed to components such as ammonia and butyric acid, as well as to the stimulation of naturally occurring nematode antagonists and/or the restructuring of the nematode community (Jothi *et al*., 2003; Oka *et al*., 2007). However,

a recent study found that anaerobic digestion had no effect on the number of antagonistic *Bacillus* sp. present in the digestate (Cao *et al*., 2013).

A recent development and potential problem facing many anaerobic digestion units is the build-up of struvite. Struvite is a phosphate mineral which forms in pipes in sewage farms and anaerobic digestion units and causes pipe blockages. Struvite precipitation occurs when the combined concentrations of Mg^{2+},NH_4^+ and PO_4^{3-} exceed the struvite solubility product, which is a function of the pH (Marti *et al*., 2008). As pH increases, struvite solubility decreases (Marti *et al*., 2008). Struvite, however, is a valuable source of phosphate. Since rock phosphate is not renewable and a valuable nutrient, work has begun to try extract this phosphate, through adding a crystallization process after feedstock digestion (Pastor *et al*., 2010). Struvite can however contain high amounts of micro-organic pollutants which may cause health risks, such as pharmaceuticals and hormones. In a study, pharmaceuticals such as erythromycin and norfloxacin only transferred 4-5% of the original pharmaceutical to the struvite, but 88-98% of oxytetracycline transferred to the struvite (Kemacheevakul *et al*., 2012). The increasing interest in struvite crystallisation for phosphate retrieval from anaerobic digestate highlights the need for monitoring veterinary pharmaceuticals in anaerobic digestion residues in general, but also in novel by-products in which drugs may be concentrated.

One of the worries associated with applying manure and digestate to farm land as fertiliser is the development of antibiotic resistant bacteria in the environment (Beneragama *et al*., 2013) if residues and/or metabolites are present in the dung. The use of antibiotics for treatment of mastitis in cows, as well as the general health of farm animals can lead to antibiotic residues being present in milk, faeces and urine. Not only can manure contain residues of pharmaceutical drugs, but it can contain antimicrobial-resistant bacteria and resistance genes that may survive in the soil or transfer horizontally to indigenous soil

bacteria (Chee-Sanford *et al*., 2001). Multidrug resistance occurs when an organism is resistant to more than one antimicrobial agent *in vitro* (Magiorakos *et al*., 2012). More than 150 antimicrobial drugs are available and with more bacteria gaining resistance to antibiotics, multidrug resistance in many species is increasing (Beneragama *et al*., 2013). Anaerobic digestion is credited with destroying antibiotic resistant bacteria (Beneragama *et al*., 2013) and pathogens (Kearney *et al*., 1993; McBride *et al*., 2000; Jothi *et al*., 2003); however, since it has been shown that some antibiotics survive the AD process, this aspect needs further study.

1.5 LIVESTOCK HUSBANDRY AND WELFARE

1.5.1 Use of Antibiotics and Other Drugs

Veterinary pharmaceuticals are extensively used worldwide for both therapeutic and non-therapeutic purposes. The EU however phased out use of non-therapeutic veterinary pharmaceuticals for treatment of animals in Europe, stopping the sale of the last antibiotic growth promoter on the 1st January 2006 (Castanon, 2007). Antibiotic release into the environment is of great concern due to the persistent nature of many antibiotic residues which can lead to antibiotic resistant bacteria (Chee-Sanford *et al*., 2001), although the EU still allow the continued use of anticoccidials in poultry (Dibner and Richards, 2005). Antibiotics administered to animals are excreted *via* urine or faeces within a range of 17-76% of the administered dose in either an unaltered or metabolised form of the parent compound (Jjemba, 2002). Antibiotics have been used in agriculture and medicine for over 60 years, substantially benefiting agricultural activity and public health (Knapp *et al*., 2010). Veterinary pharmaceutical residues and metabolites in manure can lead to negative effects in anaerobic digesters (Poels *et al*., 1984). In the UK, during 2003 published sales data indicated that 456 tonnes active ingredient (AI) of therapeutic antimicrobials, 241 tonnes AI of coccidiostats and

36 tonnes AI of antimicrobial growth promoters were sold in the UK, whilst tetracyclines accounted for 46% (212 tonnes) of therapeutic antimicrobials sold while macrolides accounted for 4.6% (21 tonnes) of therapeutic antimicrobials sold (VMD, 2004).

1.5.2 Tyslosin and Oxytetracycline: Two Widely Used Antibiotics in Cattle

Oxytetracycline, $C_{22}H_{24}N_2O_9$ (Figure 1.3) is a broad spectrum antibiotic, inhibiting protein synthesis by preventing aminoacle tRNA binding with the bacterial ribosome (Tritton, 1977; Chopra *et al.*, 1992).

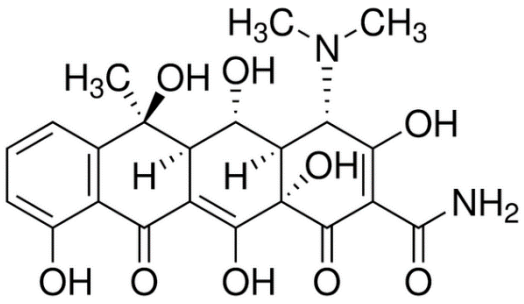

Figure 1-3 Chemical structure of oxytetracycline (molecular weight 460.44 g mol^{-1}).

Oxytetracycline is excreted in dung with detection of approximately 23% of the original dose when administered orally to calves (Arikan *et al*., 2007). Additionally, studies have also reported that wethers (castrated sheep) excreted 21% of an oral dose of tetracycline (cited by Montforts *et al*., 1999), whilst Winckler and Grafe (2001) reported excretion rates depending on dose for swine ranging from 42% - 72%. Oxytetracycline is widely administered to animals to treat both respiratory and intestinal infections (Arikan *et al*., 2007), and oxytetracycline is predominantly administered to whole herds/populations of animals (cattle and pigs), meaning emissions can have a much higher concentration compared to singularly treated animals (Grung *et al*., 2007). The metabolite 4-epi-oxytetracycline has been reported to be the main metabolite of oxytetracycline, whilst the metabolite is also quoted to induce and accelerate further degradation of the parent compound, oxytetracycline (Wang *et al*.,

2015). 4-epi-oxytetracycline had been reported to have a lower antibacterial activity compared to that of the parent compound (Halling-Sørensen *et al*., 2002).

Tylosin, a macrolide, is widely administered to farm animals to control intestinal and respiratory infections (De Liguoro *et al*., 2003). Tylosin predominantly consists of tylosin A (the most active compound), but may also include tylosin B (desmycosin), tylosin C (macrosin) and tylosin D (relomycin) depending on the manufacturer (CVMP, 1996). The mechanism of action of tylosin, $C_{46}H_{77}NO_{17}$ (Figure 1.4) is the inhibition of bacterial protein synthesis by interaction with the 50S ribosomal subunit (Mazzei *et al*., 1993).

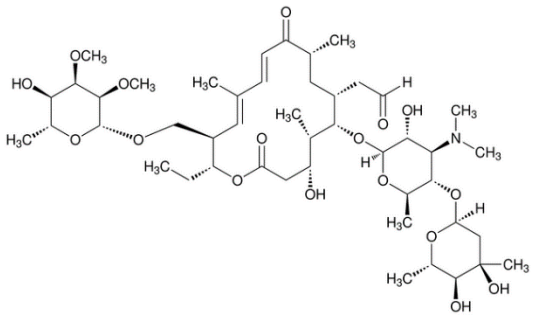

Figure 1-4 Chemical structure of tylosin (molecular weight 916.1 g mol^{-1}).

Tylosin is almost exclusively used as a treatment for whole herds/populations (mainly pigs and poultry) but can also be occasionally used for treatment of individual animals (pigs and cattle) (Grung *et al*., 2007). Grung *et al*. (2007) states that macrolides are metabolised by the treated animals to a very minor extent, with a very high proportion of the administered dose expected to be excreted unchanged. However Boxall *et al*. (2011) states tylosin is reported to be extensively metabolised, but no metabolites have been shown to occur in greater concentrations than that of the parent compound. It is known that the metabolites of tylosin are intrinsically less biologically potent than that of the parent compound, with the metabolite dihydroxydesmycosin having around 15% of the activity of tylosin A (CVMP, 1996).

Capleton *et al*. (2006) conducted a veterinary medicine (AI) prioritisation exercise based on an earlier prioritisation list which was focused on their potential environmental impact (Boxall *et al*., 2003) . Within this prioritisation list oxytetracycline and tylosin were rated as 'high' for both the potential to reach the environment and usage, but rated 'medium' for the toxicity profile classification and the priority for detailed risk assessment (Capleton *et al*., 2006). The prioritisation lists mainly focused upon the parent material, with metabolites of the material only taken into account in a limited manner, this does mean however that for compounds such as tylosin that degrades rapidly, the prioritisation based on the parent compound may not be entirely predictive (Capleton *et al*., 2006). Furthermore, Boxall *et al*. (2011) ranked tylosin and oxytetracycline on potential risk to human health 13th and 25th respectively when based on measured sorption data, and 5th and 27th respectively when based on predicted sorption. The prioritisation exercises along with evidence of potentially high excretion levels in manures offer compelling evidence to suggest that both oxytetracycline and tylosin may have the potential to affect the environment and therefore the anaerobic digestion process.

1.6 EFFECTS OF VETERINARY PHARMACEUTICALS ON THE ANAEROBIC DIGESTION PROCESS

Anaerobic digestion utilises a range of animal wastes in addition to other agricultural residues. The primary feedstock and farming methods used will dictate the amount and variety of drug residues entering the anaerobic digestion unit and the duration of addition. Tylosin is a pharmaceutical that has been widely studied (Poels *et al*., 1984; Sanz *et al*., 1996; Massé *et al*., 2000; Loftin and Henny, 2005; Angenent *et al*., 2008; Shimada *et al*., 2008, 2011; Mitchell *et al*., 2013). Tylosin reportedly does not affect CH_4 production from swine waste at

concentrations up to 16.7 mg L^{-1} (Poels *et al*., 1984; Massé *et al*., 2000; Angenent *et al*., 2008; Stone *et al*., 2009), although Loftin and Henny (2005) demonstrated inhibition of CH_4 production at tylosin concentrations of 1, 5 and 25 mg L^{-1}. These results are summarised in Table 1.1. Tylosin however has been reported to decrease biogas production by 10 and 20% due to tylosin addition of 130 mg L^{-1} and 260 mg L^{-1} respectively to cow manure. Most research papers focus on the effects of tylosin on swine manure, with very few looking at cow manure derived feedstock.

Authors that reported a tylosin-related decrease in biogas production spiked feedstock prior to AD, whilst those that did not observe a decrease used dung from animals that ingested it *via* their feed, although others also spiked the AD feedstock. Published data relating to tylosin are very variable, highlighting the number of environmental and operational factors that contribute to biogas production.

Table 1-1 Summary of biogas reduction in swine and cattle manure in the presence of tylosin

Compound	Addition	Manure	Conc. (mg L^{-1})	Biogas production decrease (%)	Reference
Tylosin	Diet	Swine	1.1	No Inhibition	Stone *et al*. (2009)
Tylosin	Diet	Swine	-	No Inhibition	Massé *et al*. (2000)
Tylosin	Diet	Swine	1.6	No Inhibition	Angenent *et al*. (2008)
Tylosin	Spiked	Swine	1.7	No Inhibition	Poels *et al*. (1984)
			8.3	No Inhibition	
			16.7	No Inhibition	
Tylosin	Spiked	Swine	1	32	Loftin and Henny (2005)
			5	30	
			25	29	
Tylosin	Spiked	Cattle	130	10	
			260	20	Mitchell *et al*. (2013)

Oxytetracycline concentrations fall within a range of 0.21 and 136 mg L^{-1} in pig manure, although Winckler *et al*. (2003) found a maximum concentration of just 29 mg L^{-1} on 4 farm

sites in Germany (Winckler *et al*., 2003; Martínez-Carballo *et al*., 2007). Massé *et al*. (2010) demonstrated that tetracycline reduced CH_4 production in pig slurry and in a similar study, tetracycline adversely affected initiation and total volume of CH_4 produced during anaerobic digestion of spiked pig manure (Shi *et al*., 2011). Similarly, CH_4 production decreased by 56%, 60% and 62% with the addition of oxytetracycline and chlorotetracycline at concentrations of 10, 50, and 100 mg L^{-1} respectively (Álvarez *et al*., 2010). However, Lallai *et al*. (2002) reported that oxytetracycline at a concentration of 125 and 250 mg L^{-1} did not affect biogas production and concluded that acid-forming (acetogenic bacteria) and CH_4-forming (acetoclastic and hydrogenotrophic archaea) bacteria were not affected by the presence of oxytetracycline.

The inconsistencies in data from studies using oxytetracycline may be due to different feedstock history, operational conditions maintained, antibiotic concentration, reactor size and reactor type (continuous versus batch) (Álvarez *et al*., 2010). Álvarez *et al*. (2010) suggest that the absence of biogas inhibition reported by Lallai *et al*. (2002) was because they used swine manure originating from antibiotic treated pigs. However, Lallai *et al*. (2002) clearly stated that the slurry came from pigs that had not been dosed with antibiotics for a considerable time. Lallai *et al*. (2002) also found that both thiamphenicol and amoxicillin reduced CH_4 production. Table 1.2 summarises the tetracyclines discussed together with other results. Lallai *et al*. (2002) highlighted that demonstrating antibiotic-related inhibition of biogas production, whilst interesting, does not identify which part of the microbial community (CH_4 or acid producing bacteria) was affected.

Oxytetracycline at high concentrations of 50, 100 and 200 mg L^{-1} produced a biogas decrease of 41%, 57% and 61% respectively over a 60-day period in cow manure (Coban *et al*., 2016). A decrease in the generation of CH_4 and CO_2 by 27.8% and 28.4% respectively due to the

presence of chlorotetracycline at a concentration of 28 mg L^{-1} in swine manure was reported (Stone *et al*., 2009). Chlortetracycline at a concentration of 100 mg L^{-1} and above inhibited anaerobic digestion in a volatile fatty acid mixture, causing complete inhibition of the acetoclastic methanogenic archaea (Sanz *et al*., 1996).

Table 1-2 Summary of biogas reduction in swine and cattle manure in the presence of tetracyclines.

Compound	Addition	Manure	Conc. (mg L^{-1})	Biogas production decrease (%)	Reference
Tetracycline	Diet	Swine	-	25%	Massé *et al*. (2010),
Tetracycline	Spiked	Swine	25	*	Shi *et al*. (2011),
			50	*	
Oxytetracycline	Spiked	Swine	125	No Inhibition	Lallai *et al*. (2002),
			250	No Inhibition	
Oxytetracycline + chlorotetracycline	Spiked	Swine	10	45.2	Alvarez *et al*. (2010),
			50	56.5	
			100	64.1	
Chlortetracycline	Diet	Swine	28	27.80%	Stone *et al*. (2009)
Chlortetracycline	Diet	Swine	-	20%	Fedler and Day (1985)
Chlortetracycline	Spiked	Swine	-	No Inhibition	Fedler and Day (1985)
Oxytetracycline	Feed	Cattle	3.1	27	Arikan *et al*. (2006)
Oxytetracycline	Injected	Cattle	1 – 3.3	50-60	Ince *et al*. (2013)
Oxytetracycline	Injected	Cattle	3.1	35	Akyol *et al*. (2016a)
			3.07	47	
Oxytetracycline	Injected	Cattle	2	10-30	Akyol *et al*. (2016b)
Oxytetracycline	Spiked	Cattle	50	41	Coban *et al*. (2016)
			100	57	
			200	61	

Manure containing 2 mg L^{-1} of chlortetracycline from medicated pigs reduced CH_4 production by 20%, however, in the same trial when chlortetracycline was added directly into digesters, there was no reduction in CH_4 production (Fedler and Day, 1985). This was speculated to be caused by metabolites of chlortetracycline produced as a product of the swine

gastrointestinal tract rather than the parent compound chlortetracycline (Fedler and Day, 1985). There have however been numerous studies reporting a decrease in biogas production from the direct addition of veterinary pharmaceuticals, meaning that metabolites may be less detrimental than the parent drug, although this is something that deserves further study (Álvarez *et al*., 2010; Shi *et al*., 2011).

Oxytetracycline was measured in dung of animals for 20 days after they were intramuscularly injected; concentrations ranged from 1 - 3.3 mg L^{-1} and were associated with a 50-60% reduction in biogas production (Table 1.2) when the dung was used in an AD unit (Ince *et al*., 2013). In contrast, a 27% decrease in biogas production was recorded when dung from beef calves treated orally was used as an AD feedstock, despite the measured concentration of oxytetracycline (3.1 mg L^{-1}) being similar (Arikan *et al*., 2006) to that of the injection formulation (Table 1.2).

The study by Ince *et al*. (2013) went further than previous oxytetracycline studies (Massé *et al*., 2000; Arikan *et al*., 2006; Álvarez *et al*., 2010) investigating the effect of oxytetracycline on the microbiology of manure digesters after the authors noted a knowledge gap in this specific area (oxytetracycline). Active microbial groups in serum bottles were characterized using fluorescent tRNA-targeted oligonucleotide probes specific for phylogenetically defined groups of methanogens and total bacteria (Ince et al., 2013). Methanogens detected belonged to the groups of Methanobacteriales, Methanomicrobiales and Methanosarcinales, a similar community structure to that reported in previous anaerobic digestion studies (Karakashev *et al*., 2005; Angenent *et al*., 2008; Ike *et al*., 2010). In a similar experiment, using T-RFLP analysis, methanogen populations were found to belong to the order Methanobacteriales and Methanosacinals (Stone *et al*., 2009). Methanomicrobiales were not present at detectable levels in the study by Stone *et al*. (2009), despite being

expected and reported by other authors (Padmasiri *et al*., 2007; Ince *et al*., 2013). Redundancy analysis showed that biogas production was positively correlated with total bacteria and the Methanomicrobiales, while Methanobacteriales and Methanosarcinales explained less of the variance in the data (Ince *et al*., 2013). Ince *et al*. (2013) showed that a predominant amount of the CH_4 production was accomplished through the hydrogenotrophic pathway of Methanomicrobiales. A simultaneous inhibitory effect of oxytetracycline on both methanogens and other bacteria could be assumed, due to the stability of the percentage CH_4 in the biogas produced (Ince *et al*., 2013).

1.6 ECOTOXICITY

It is well documented that slurry can have measurable levels of veterinary pharmaceuticals present (Hamscher *et al*., 2002; Thiele-Bruhn, 2003; Kümmerer, 2009; Cengiz *et al*., 2010). Oxytetracycline has been reported to have a half-life of 30 days in manure, but can still be detected in aged manure (De Liguoro *et al*., 2003). The low partition coefficient (log K_{ow}) of oxytetracycline reflects its hydrophilic properties and potential excretion not only through faeces but also urine (Boleas *et al*., 2005). Despite the low log k_{ow}, oxytetracycline sorbs strongly onto manure (Loke et al., 2002). Tetracycline has been shown to be excreted by treated animals in a predominantly unaltered parent compound form, representing 50-80% of the applied dose (Halling-Sørensen *et al*., 2002).

Oxytetracycline concentrations of 100 and 1000 mg kg^{-1} caused no significant mortality in earthworms (*Eisenia fetida*) at either concentration although it inhibited respiration rates of soil microorganisms by 16-25% and 25-38% respectively (Boleas *et al*., 2005). This corroborated previous work by Baguer *et al*. (2000) who found no observable effects at environmentally relevant concentrations, although they reported effects at 3000 mg kg^{-1}.

Tetracycline exerted a lethal effect at 8.5 mg kg^{-1} on the microbial community under anaerobic conditions, which led to reduced substrate use and resulted in the total collapse of a reactor and of biogas production (Cetecioglu *et al*., 2013).

Beneragama *et al*. (2013) investigated the survival of 3 groups of multidrug-resistant bacteria (MDRB) to three groups of antibiotics during anaerobic digestion of dairy manure and co-digestion of dairy manure and waste milk for 22 days under mesophilic (37°C) and thermophilic (55°C) conditions, respectively. The antibiotic groups were: (i) Cefazolin, neomycin, vancomycin, kanamycin; (ii) penicillin, oxytetracycline, ampicillin, streptomycin; (iii) cefazolin, neomycin, vancomycin, kanamycin, penicillin, oxytetracycline, ampicillin, streptomycin. At the thermophilic temperature, 100% destruction of the bacteria population was found for all 3 groups of MDRB, while at mesophilic temperature 90% destruction of the MDRB population was achieved, with no significant differences between manure and the co-digested mixture (Beneragama *et al*., 2013). Bacteria can obtain resistance genes through a number of pathways, genes may be inherited, genes may occur due to genetic mutations and finally through horizontal gene transfer from one cell to another (Levy, 1998).

In a recent survey conducted in the UK, 90% of the farmers questioned who fed waste milk to calves used milk from cows undergoing antibiotic treatment to combat mastitis (Brunton *et al*., 2012). The chance of the milk containing antibiotic residues and antibiotic resistant bacteria is therefore a high possibility. Anaerobic digestion of the waste milk in mixture could lead to the destruction of the bacteria but if not, then the possibility of antibiotic resistant bacteria being incorporated into soil when AD residues are used as a fertiliser is real.

1.7 AIMS AND OBJECTIVES

The overall aim of the project is to examine the effect of two commonly used veterinary pharmaceuticals, oxytetracycline and tylosin, on the anaerobic digestion process and to establish if the addition of biochar can help ameliorate the negative effects of pharmaceuticals or help improve biogas production and quality. It is widely acknowledged that pharmaceuticals could play a destructive role in anaerobic digestion, however, currently there are limited reports on the effects of oxytetracycline and tylosin in cow manure feedstocks, with reports often quoting contradictory results. With the current knowledge suggesting contradictory results as to the effects of oxytetracycline and tylosin, the aim of this project will be addressed by the following objectives:

- To investigate the effect of oxytetracycline on the anaerobic digestion process using different sources of cattle derived feedstock (Chapter 3).
- To investigate the effect of tylosin on the anaerobic digestion process using different sources of cattle derived feedstock (Chapter 4).
- To study the effect of anaerobic digestion and antibiotic addition on the quality of digestate (Chapter 3, 4, 5 and 6).
- To establish whether biochar can be used as an antibiotic amelioration additive or improve anaerobic digestion performance (Chapter 6).

The following hypothesis were addressed within this project:

1. Addition of OTC and TYL will reduce biogas production because of negative effects on the microbiology of the system when organic cattle dung is used as the feedstock.

2. Addition of OTC and TYL when dairy slurry is used as the feedstock will reduce biogas production, but not to the same extent as when applied to organic cattle dung. The dairy cattle are treated with antibiotics and therefore microorganisms in the farm slurry tank may have previously encountered OTC and TYL, albeit at low concentrations, making them less susceptible to the antibiotics. Antibiotic resistance resulting from horizontal gene transfer has been shown within the farm slurry tank (Ibrahim *et al*., 2016).

1.8 APPROACH AND THESIS OVERVIEW

Laboratory scale anaerobic digestion units were employed to fulfil the objective throughout the project. **Chapter 1** is an introductory chapter, providing a summary of the literature related to anaerobic digestion and the use of veterinary pharmaceuticals, and outlines the aims and objectives of this project. **Chapter 2** outlines the general methods and materials used within this project. **Chapter 3** investigates the effect of oxytetracycline addition at different time points on the anaerobic digestion process, specifically looking at two alternative feedstocks, naïve organic cow dung and dairy farm slurry. **Chapter 4** investigates the same effects as described for chapter 3, but replacing oxytetracycline with tylosin. **Chapter 5** considers the effects of oxytetracycline and tylosin addition to anaerobic digesters on the resulting digestate, specifically looking at nutrient content. **Chapter 6** assesses whether biochar can be effectively used to ameliorate the effects of oxytetracycline when added to anaerobic digesters, whilst improving biogas production and quality. **Chapter 7** provides a general discussion of the overall findings from the experimental chapters, whilst trying to tie in and explore the real life effect the findings might have.

CHAPTER 2 - GENERAL METHODS

2.1 COLLECTION OF FEEDSTOCK AND PROCESSING

Freshly deposited cattle dung was collected from fields near the Sutton Bonington Campus of the University of Nottingham, where organically reared animals were grazing. The Lincoln Red beef cattle are permanently maintained outside on grass in spring, summer and autumn and their diet was not supplemented with any other food at the time of dung collection. The organic status of the dung was confirmed with the farmer, making sure no treatments had recently been administered to any of the herd. Multiple fresh dung pats were collected and returned to the laboratory. The dung was thoroughly mixed and then separated into 800g freezer bags and placed into a -80°C freezer for future experimental work. Freezing was employed to allow for storage thereby enabling the same batch of organic dung to be used throughout and to kill any invertebrates that might influence the results. When needed, dung was slowly defrosted at 4^{o}C and then allowed to reach room temperature prior to starting an experiment. Since an active microbial consortium was needed to start the anaerobic process, thawed dung was inoculated with freshly deposited organic cow dung from the same herd, within two hours of deposition. Water was added to achieve an organic matter percentage of 6% prior to each experiment.

Dairy slurry was collected from the slurry tank situated at the University of Nottingham's dairy farm located at the Sutton Bonington Campus (Grid ref., 50°50ˈ21.0''N 1°15ˈ02.0"W) and dispensed into a 1000 L Intermediate Bulk Container (IBC, for a different project). Slurry was taken from the IBC a day before an experiment was set-up. The organic matter content of the slurry was 5.2% and therefore was used 'neat' in the experiments. No start-up inoculum was required because the slurry was visibly producing gas within the IBCs. The dairy farm has 180 Holstein cattle in milk; these cows are zero-grazed and permanently housed.

Dung and urine from the housed cattle is collected, along with floor washing water, disinfectant from footbaths and waste milk and stored in a slurry tank after much of the organic matter has been removed *via* a screw press. The slurry from the slurry tank was use in this investigation. Extracting samples from the bottom the storage IBC meant that the organic matter content was higher than that in the main slurry tank because of a degree of settlement.

Table 2-1 Summary of dung and slurry general characteristics (before any ammendment), values are means of a minimum of 3 replicates ± SE.

Characteristics	Dung	Slurry
pH	7.3-7.9	7.6 – 7.8
Total Solids	13.5% ± 0.06	5.2% ± 0.12
Volatile Solids	10.1% ± 0.04	3.7% ± 0.09

2.2 EXPERIMENTAL SET-UP AND SAMPLING REGIME

2.2.1 Approach

The same experimental design was used throughout and is described here. Chapter-specific modifications are explained throughout the thesis as appropriate. Laboratory scale anaerobic digestion experiments were established to evaluate the effect of the veterinary drugs oxytetracycline (OTC) and tylosin (TYL) using both freshly inoculated organic cow dung and dairy farm slurry as feedstock. Each experiment ran for 35-days. Batch-wise experiments were carried out in 120 ml serum bottles, with a 60 ml aliquot of feedstock (organic dung or dairy slurry) placed in each serum bottle. After adding the feedstock to the bottles, the headspaces were flushed with nitrogen and the bottles sealed with thick butyl stoppers (Rubber B.V, Hilversum, Netherlands) and crimped with an aluminium cap. Serum bottles

were continuously shaken (160 rpm) and heated (35°C) by using reciprocating heated water baths. Water in the baths started at room temperature and after loading with experimental serum bottles, temperature was raised from ambient by 5°C each day (2.5°C in the morning and 2.5°C in afternoon) from day 0 until 35°C was reached (usually within 3 days).

2.2.2 Treatments and Experimental Design

Prior to placing into the water baths, serum bottles containing dung or slurry were spiked with different concentrations of either OTC (oxytetracycline hydrochloride, $C_{22}H_{24}N_2O_9$, 95.9%, CAS No. 2058-46-0, Sigma-Aldrich, UK) or TYL (tylosin tartrate containing 919 µg mg^{-1} tylosin , CAS No. 7461-55-2, Sigma-Aldrich, UK) which was dissolved in deionised water so that 2 ml of the solution contained the required concentration of drug when added to 60 ml of dung or slurry.

OTC and TYL were administered by syringing 2 ml of solution through the butyl stopper and into the dung or slurry. Bottles were then placed into a water bath for incubation; placement was randomised. Three water baths were used in total and these were classed as replicates. Each experiment was established as a randomised block design, with the water baths being the blocks.

Experiments utilising the following treatments were set up for both oxytetracycline and tylosin:

1. Antibiotic (0, 4.33, 21.33 and 86.63 mg L^{-1}) added at start-up (day 0) to organic cow dung.
2. Antibiotic (0, 4.33 and 86.63 mg L^{-1}) added on day 15 to organic cow dung.
3. Antibiotic (0, 4.33 and 86.63 mg L^{-1}) added on day 15 to dairy slurry.

A 'realistic' level of OTC and TYL that might be incorporated into an AD unit was estimated at 4.33 mg L^{-1} after considering OTC/TYL dose rate, estimating the number of animals receiving treatment (based on head of cattle treated at the University farm) and the mixing of dung from treated animals with that of untreated ones. To push the system, 86.63 mg L^{-1} was chosen as a worst-case scenario. A wider range of antibiotic concentrations was used when applied at start-up than in experiments where application was on day 15; the range was modified after seeing the first set of data when it was decided that the additional concentrations did not bring anything new to the results from the 0, 4.33 and 86.63 mg L^{-1} doses.

2.2.3 Sampling Regime

Biogas samples were collected from each bottle and measured by inserting a syringe to allow the headspace pressure to equilibrate. Gas produced pushed up the plunger enabling measurement of biogas in mls at each sampling time whilst ensuring an anaerobic state. Headspace gas was mixed by carefully pushing the plunger back down and repeating twice. Gas samples were collected and stored in Exetainer gas tubes prior to analysis.

Biogas samples were removed from all bottles on days 5, 10, 15, 20, 25, 30 and 35 and dung/slurry digestate samples taken by completely sacrificing a set of 3 replicates per treatment on the same days as gas sampling.

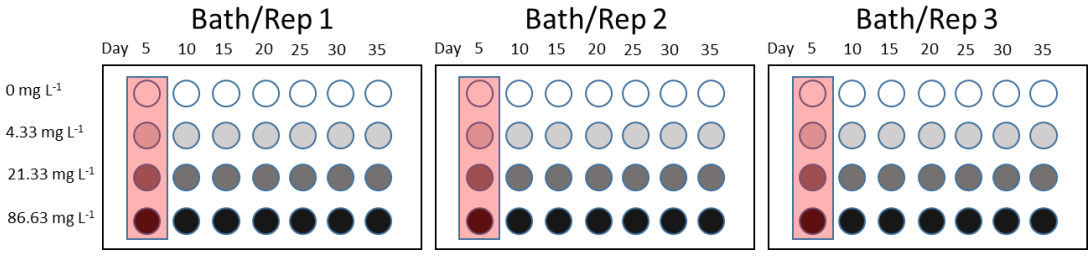

Figure 2-1 Example of an experimental set-up (schematic), indicating serum bottles in 3 replicate waterbaths. Antibiotic concentration on the left indicates control and different

concentrations of pharmaceutical, whilst 'Day' along the top indicates which bottles were sacrificially sampled on that day. All bottles were subject to biogas sampling. The setup illustrated is for clarity and the experimental design was a randomised block design.

2.3 ANALYSES

2.3.1 Biogas Production and Quality

Biogas samples were collected from each serum bottle and measured by inserting a syringe and measuring the volume produced every 5 days as explained above. The collected gas samples were stored in airtight, evacuated 12 ml Exetainers, overfilled to create a positive pressure. A syringe was inserted into the Exetainers allowing the stored gas to equilibrate and push the sample into the syringe. Five mls of the sample were injected into the gas chromatograph equipped with a thermal conductivity detector (TCD) and a flame ionization detector (FID) in parallel (GC-2014, Shimadzu) and analysed for concentration of CO_2 and CH_4. Concentrations of CO_2 and CH_4 were calculated by comparing peak areas against a standard curve prepared from 3 certified gas standards containing: (1) 40.19% CO_2 and 59.81% CH_4, (2) 5.19% CO_2 and 89.67% CH_4 and (3) 48.01% CO_2 and 10.11% CH_4, as follows:

$$Sample\ concentration\ = \frac{GS \times S_{PA}}{GS_{PA}}\ (ppmv)$$

GS = concentration of CO_2 or CH_4 in the gas standard (ppmv)

S_{PA} = sample peak area (individual value)

GS_{PA} = peak area of CO_2 or CH_4 in the gas standard (average value)

Calibration gasses were used to demonstrate linearity and calibration at both high and low CH_4 and CO_2 concentrations.

2.3.2 Total and Volatile Solids

Total solids of digestates were determined by oven drying samples and then calculating the loss on ignition (Rowell, 1994). Digestate samples were placed in weighed crucibles, the total solids of digestate samples was determined by drying at 105°C overnight and using the equation below.

$$\%\, Total\ Solids\ = \frac{Weight\ of\ dry\ sample\ plus\ crucible\ -\ Weight\ of\ dry\ sample}{Weight\ of\ wet\ sample}$$

The oven dried digestates were then heated to 550°C for 8 hours. After allowing crucibles to cool, samples were re-weighed to give the mass of ignited digestate. Total volatile solids were determined using the equation below.

$$\%\, Volatile\ Solids\ = 100 \times \left(\frac{Weight\ of\ wet\ sample\ - Weight\ of\ ignited\ sample}{Weight\ of\ wet\ digestate}\right)$$

2.3.3 pH

A combined electrode was used to measure digestate pH, incorporating reference and pH sensitive glass electrodes into a single electrode stick. The pH meter was calibrated against pH 4.01 and pH 7.00 buffers using the 'buffer' and 'slope' controls, whilst the temperature was set to that of the digestate, in this case room temperature. Digestate pH was measured directly, making sure digestate was fully homogenised. The pH electrode was allowed to stabilise for 2 minutes in digestate before the reading was recorded.

2.3.4 Elemental Analysis

A sub-sample of 0.2 g of digestate was weighed out into labelled digestion tubes (DigiTUBE™; SCP Science, Courtaboeuf, France) and placed into heating blocks situated in a fume cupboard. With the fume cupboard on, 8 ml of concentrated HNO_3 was added to the digestion tubes and left overnight. Then 2 ml of H_2O_2 (30%) were added to the acid and the tops of the tubes covered with specially designed 'watchglasses' and heated at 95°C for 2 hours, allowing 10 minutes for cool down before making up to 50 ml with milli-Q water. Samples were then syringe filtered to remove any possible precipitate into 7 ml vials ready for analysis. Acids were analytical reagent (AR) from Fisher Scientific, UK. Multi-element analysis was undertaken by inductively coupled plasma mass spectrometry (Model X-Series^{II}, Thermo-Fisher Scientific, Bremen, Germany) against appropriate standards.

Other methods are Chapter specific and described elsewhere.

CHAPTER 3 - EFFECTS OF OXYTETRACYCLINE ON BIOGAS PRODUCTION AND QUALITY DURING LABORATORY-SCALE ANAEROBIC DIGESTION OF ORGANIC COW DUNG AND DAIRY CATTLE SLURRY

3.1 INTRODUCTION

Oxytetracycline (OTC) is a broad-spectrum tetracycline-class antibiotic widely used globally to treat livestock for a range of diseases such as respiratory infections, mastitis, keratoconjunctivitis, foot rot, enteritis, septic arthritis, and a host of other bacterial infections (NOAH Compendium; http://www.noah.co.uk/).

OTC is one of three tetracyclines and is effective against Gram-positive and Gram-negative bacteria and once the antibiotic enters the cell it prevents aminoacle tRNA binding with the bacterial ribosome and in effect, inhibits protein synthesis (Tritton, 1977; Chopra *et al*., 1992). According to the US Food and Drug Administration 5,652,855 kg of tetracyclines (active ingredient) were marketed domestically in the USA in 2011 for use in livestock and domestic animal husbandry and 15,321 kg were exported. Of the domestic sales, 59% was used in animal feed (FDA, 2015). Whilst prophylactic use at sub-therapeutic levels (in animal feed) was banned in the EU in 2006, OTC is still widely used therapeutically and administered to cattle, pigs and sheep usually by intramuscular injection (20 mg kg^{-1} body weight, NOAH Compendium).

It is widely accepted that many veterinary pharmaceuticals are not fully metabolised by the treated animal and are excreted in the dung or urine; according to Chee-Sanford *et al*. (2001), 75% of administered antibiotics are excreted in the waste. This was demonstrated to some extent by Ince *et al*. (2013) who injected a cow with 20 mg kg^{-1} OTC and quantified OTC concentrations in the faeces after treating. A total of 20% of the injected OTC was measured in the dung over a 12-day period. Others detected tetracycline concentrations up to 41 mg kg^{-1} several months after manure application to soil (Hamscher et al., 2002; De Liguoro et al.,

2003; Kümmerer, 2004), although Boxall *et al*. (2004) stated that tetracyclines have a half-life of 100 days in manure.

In the UK, there was a 61% reduction in the number of dairy farmers between 1995 and 2014, and a concomitant fall in the dairy herd, although that decline is reversing and the number of cattle increased from 1,796 thousand in 2011 to 1,895 thousand in 2015 (Bate, 2016). This signifies a trend towards fewer, but larger dairy farms. This is significant because large-scale (and primarily housed) dairy herds will require more efficient care methods Love *et al*. (2016) and large open housing was shown by Mason, (2015) to increase respiratory diseases in adult cows. It is likely that expanding herd size will necessitate the need for increasing use of OTC to maintain cattle health. Since 20% of OTC administered is excreted (Ince *et al*., 2013) and the average cow produces approximately 30 kg of faeces per day, it is feasible to foresee significant quantities of slurry being produced that contains residual antibiotics and/or their metabolites. This could have implications for slurry processing and field application.

Over the last decade, deployment of on-farm/farm-fed anaerobic digesters (AD) in the UK has increased and currently there are 254 on-farm AD units (WRAP, 2016). These units are fed with agricultural residues including cattle slurry and produce methane which is sold to the grid (and/or used on-farm) in addition to nutrient-rich digestate which can be used as a fertiliser. There is growing interest in the effects that residual OTC present in the slurry may have on the AD process and on biogas production, although the topic is still under-researched (e.g. Arikan *et al*., 2006; Akyol *et al*., 2016a).

3.2 AIMS

The aims of this current investigation were to quantify the effects of OTC on biogas production and quality (CH_4:CO_2 ratios) when introduced into laboratory-scale digesters containing either dung collected from organically farmed grass-fed cattle, or slurry collected from a conventional dairy farm and to establish when, during the AD process, OTC has the greatest detrimental impact (or otherwise). Since the dairy cattle are treated with antibiotics as required and the organic cattle were free of antibiotics at the time of dung collection, it was anticipated that each feedstock would respond differently to OTC amendments.

3.3 MATERIALS AND METHODS

3.3.1 Experimental Set-up

Laboratory scale anaerobic digestion experiments were established to evaluate the effect of oxytetracycline (OTC) on the anaerobic digestion process when using organic cattle dung and conventional dairy slurry as feedstocks.

Please see sections 2.1 and 2.2 for full details of the dung and slurry collected and the experimental set-up. Briefly, treatments consisted of:

(i) Organic cattle dung spiked with OTC at start-up on day 0, to give final concentrations of 0, 4.33, 21.33 and 86.63 mg L^{-1}.

(ii) Organic cattle dung spiked with OTC 15 days after initiation, to give final concentrations of 0, 4.33, and 86.63 mg L^{-1}.

(iii) Conventional dairy slurry spiked with OTC 15 days after initiation, to give final concentrations of 0, 4.33, and 86.63 mg L^{-1}.

Serum bottles (120 mL capacity, containing 60 mL of organic dung or dairy slurry) were prepared as detailed in section 2.2.1 resulting in an organic matter content of 6% for the organic dung samples and 5.2% for the slurry. There were three replicated bottles per treatment and the experimental design was a randomised block design with three heated reciprocating shaking water baths acting as replicate 'blocks'. One complete set of OTC treatments per feedstock type was set up in each water bath and incubated at 35°C (160 rpm). Treatments (i), (ii) and (iii) were established in sequence because there was insufficient room in the water baths for all the bottles at once.

3.3.2 Sampling Regime

Digestate samples were destructively taken on days 5, 10, 15, 20, 25, 30 and 35 for experiments (ii) and (iii) where OTC was added 15 days after set-up and on days 5, 10, 15, 20, 30 and 35 for experiment (i) where OTC was present from day 0. Digestate was processed as detailed in section 2.3.

Biogas samples were taken on days 5, 10, 15, 20, 25, 30 and 35 from all bottles including those due for destructive sampling on a particular sampling day. For treatments receiving OTC on day 15, biogas and digestate samples were taken prior to adding the antibiotic.

3.3.3 Statistical Analysis

Statistical analyses were performed using Genstat 16th Edition (VSN International, Hemel Hempstead, UK). Two-way analyses of variance were conducted on pH and total volatile solids using antibiotic concentration and time (sacrificial 'harvests') as factors. Biogas yields, methane and carbon dioxide concentrations were analysed by repeated measures ANOVA and repeated measures ANCOVA using pH as the covariate. Normality was tested by plotting

residuals against expected normal quantiles and post-hoc comparisons between means was based on least significant differences at the 0.05 probability level.

3.4 RESULTS

3.4.1 Biogas Production

Addition of OTC to the anaerobic digestion bottles negatively affected biogas production from all three feedstocks. When OTC was applied to organic dung at the start of the experiment (Figure 3.1a) it did not have a marked effect on biogas production until day 20 when the highest OTC concentration reduced gas production, although it recovered thereafter. By the end of the experiment (day 35), biogas produced from bottles spiked with OTC had either reached a plateau (86.63 mg kg^{-1}), or was declining (4.33 and 21.33 mg kg^{-1}). In contrast, the unamended organic dung increased gas production between days 30 and 35 (OTC concentration x time interaction, p=0.005). The total volume of biogas accumulated over the 35 days was greatest for the unamended organic dung (278 ml) and lowest for the 86.63 mg L^{-1} OTC application (236 ml), although the effect of OTC concentration as a single factor on total gas volume was only weakly significant (p=0.064).

In contrast, when OTC was added on day 15, the effects on gas production were sufficiently marked to result in a decreased total volume (0 mg L^{-1}, 261.3 ml biogas; 4.33 mg L^{-1}, 227.0 ml biogas; 86.63 mg L^{-1}, 191.7 ml biogas; OTC concentration as a single factor, p=0.002). Data for day 35 were included in this calculation, although not shown in Figure 3.1b for logistical reasons.

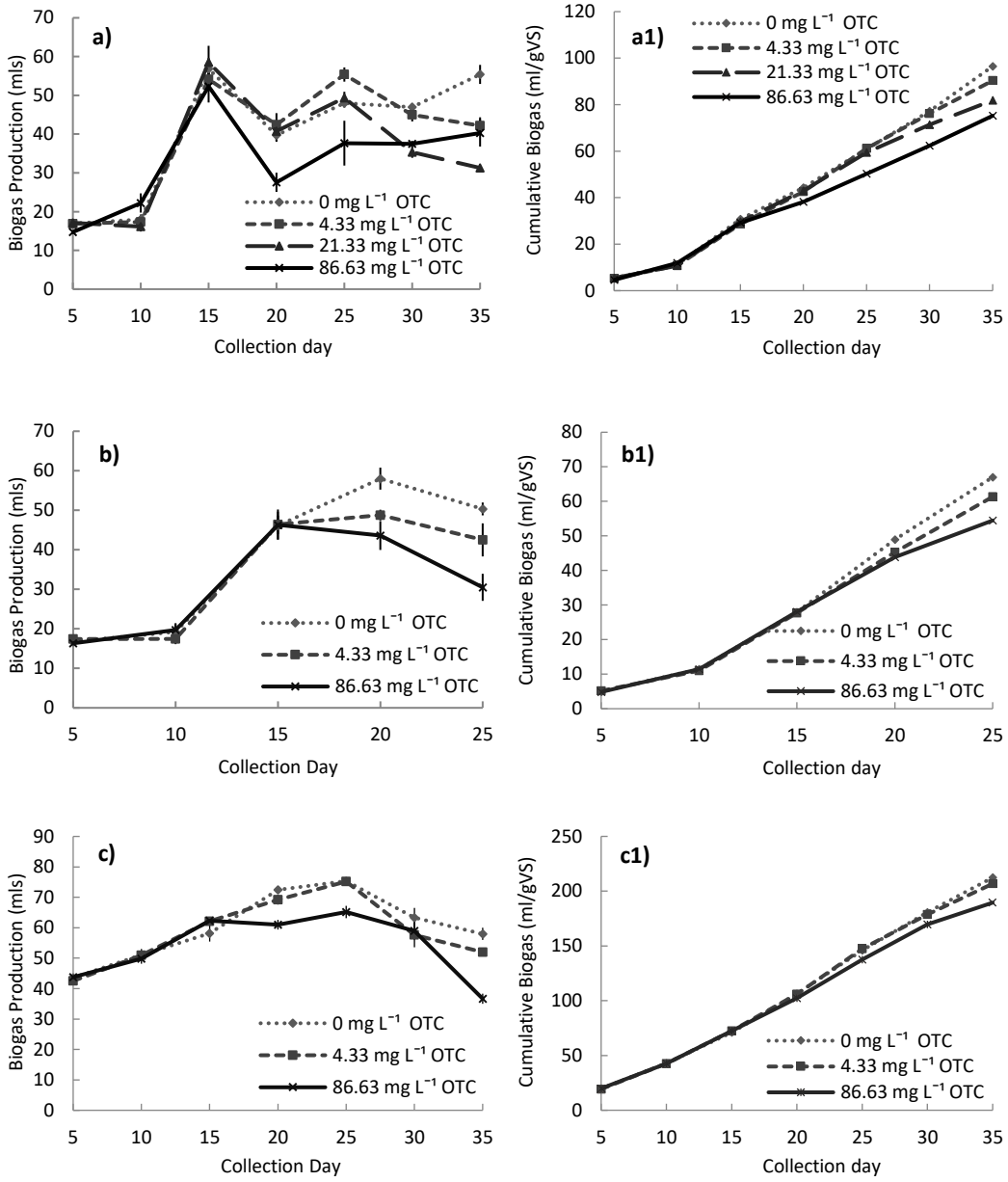

Figure 3-1 Biogas production over a 35-day period. Feedstock OTC concentrations were 0, 4.33, 21.33 and 86.63 mg L^{-1} or 0, 4.33 and 86.63 mg L^{-1}. **(a)** Organic dung with OTC added on day 0; repeated measures ANOVA: OTC x time interaction, p=0.005; LSD=10.46. **(a1)** Cumulative biogas production of organic dung with OTC added on day 0 **(b)** Organic dung with OTC added on day 15; repeated measures ANOVA for data post-OTC amendment: OTC conc. as a single factor, p=0.012; LSD=9.6. **(b1)** Cumulative biogas production of organic dung with OTC added on day 15. **(c)** Dairy cattle slurry with OTC added on day 15; repeated measures ANOVA for data post-OTC amendment: OTC conc. as a single factor, p=0.002; LSD=3.97. **(c1)** Cumulative biogas production of dairy cattle slurry with OTC added on day 15. Values are means of a minimum of 3 replicates ± SE. Samples were taken every 5 days for 35 days in total.

Prior to OTC amendment of organic dung, biogas production was similar in all reactors and increased over time up to day 15 when OTC was added (Figure 3.1b). After day 15, biogas production from the control reactors continued to increase up to day 20 before falling on day 25. In contrast, biogas production from the reactors spiked with the highest OTC concentration (86.63 mg L^{-1}) showed the most marked decrease in biogas production (OTC concentration as a single factor, p=0.012; time as a single factor, p=0.022; OTC x time interaction, NS). The mass removal rate vs gas production rate is a cross check of the validity of data. All the data are comparable because standard feedstock, headspace and experimental design were similar across the whole study.

OTC amendment of the dairy slurry on day 15 resulted in a significant reduction in total biogas produced by the highest concentration (86.63 mg L^{-1}) relative to the control (0 mg L^{-1}, 434.2 ml biogas; 4.33 mg L^{-1}, 412.3 ml biogas; 86.63 mg L^{-1}, 380.2 ml biogas; OTC concentration as a single factor, p=0.007). Five days after the slurry was spiked with OTC, biogas production was reduced in the reactors subjected to the highest concentration (Figure 3.1c). The apparent difference between the control and the 4.33 mg L^{-1} OTC addition over days 30-35 was not significant. From day 25 onwards all reactors followed a similar trend in biogas production, falling significantly up until the end of the experiment (day 35). At the point of OTC addition (day 15), the highest concentration of OTC consistently produced the least biogas compared to the control and low OTC amendment.

Compared to the organic cow dung feedstock (Figure 3.1a and 3.1b), dairy slurry produced higher biogas volumes on start-up, 43.26 ml (±0.35) compared to 16.91ml (±0.14, Figure 3.1a) and 16.83 ml (±0.94, Figure 3.1b); the final total volume across all OTC treatments was 409 ml of biogas produced by the slurry, compared with 227 ml and 262 ml generated from the organic dung amended on day 15 and day 0 respectively.

3.4.2 Biogas Composition

3.4.2.1 CH_4 and CO_2 concentrations when OTC was added at start-up (organic dung)

The quality of biogas produced was determined by the ratio of methane to carbon dioxide present in the gas at each sampling point (Figure 3.2a). Both are present in biogas; methane concentrations generally range from 50-75% and carbon dioxide from 25-50% (Bywater, 2011). Irrespective of antibiotic amendment, organic cow dung with OTC added on day 0 produced higher concentrations of CO_2 than of CH_4 during the first stages of the AD process, but from day 15 onwards, the ratio of CH_4:CO_2 was adversely affected by the OTC applied at the start of the experiment (Figure 3.2a; OTC concentration x time interaction, p=0.010). The highest OTC concentration (86.63 mg L^{-1}) resulted in the greatest decrease in CH_4:CO_2 but some recovery was observed after day 20. The lower OTC concentrations affected the CH_4:CO_2 ratio later on from day 25. By the end of the experiment (day 35) the CH_4:CO_2 ratio was similar for all OTC amendments. In contrast, the unamended reactors continued to produce higher methane concentrations over the course of the trial, resulting in a significantly greater CH_4:CO_2 ratio on days 30 and 35. The general pattern observed for the CH_4:CO_2 ratio was similar to that of the biogas yield overall (Figure 3.2a).

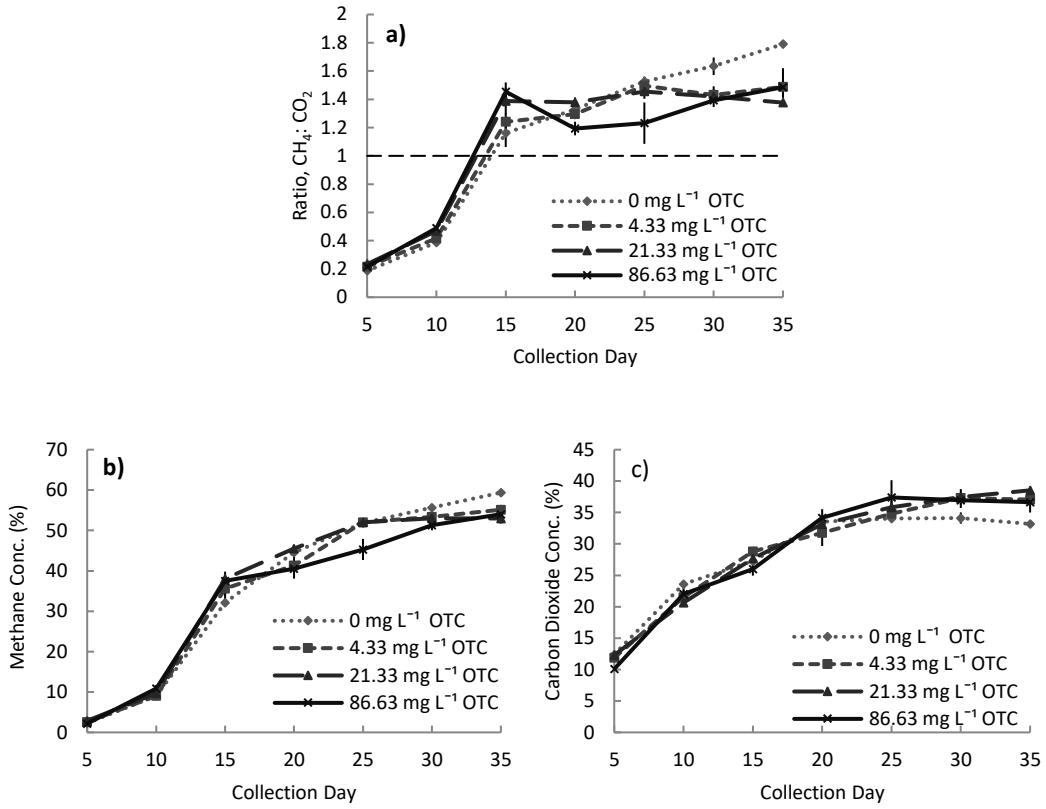

Figure 3-2 **(a)** CH_4:CO_2 ratio within biogas collected from reactors containing organic cow dung ± OTC applied on day 0; repeated measures ANOVA: OTC x time interaction, p=0.010; LSD=0.21. **(b)** CH_4 concentration (percentage); repeated measures ANOVA: OTC conc. as a single factor, p=0.053, LSD=2.16. **(c)** CO_2 concentration (percentage); repeated measures ANOVA: OTC x time interaction, p=0.041; LSD=4.59. Values are means of a minimum of 3 replicates ± SE. Samples were taken every 5 days for 35 days in total.

The CH_4:CO_2 ratio is a product of the concentrations of each within the biogas. The most notable observations are that within reactors not receiving OTC, the CH_4 concentration continued to increase whilst that of the CO_2 plateaued and then began to decrease (Figure 3.2b and 3.2c), resulting in a difference between OTC and control treatments at the end of the incubation period for both gases (OTC concentration as a single factor, p=0.053 and p=0.041 for CH_4 and CO_2 respectively).

3.4.2.2 CH_4 and CO_2 concentrations when OTC was added on day 15 (organic dung)

Amending the reactors with OTC on day 15 resulted in apparent OTC-related differences in the CH_4:CO_2 ratio from day 20 onwards, although these were not significant (OTC

concentration as a single factor, p=0.077). The increased CH_4:CO_2 ratio observed following application of 4.33 mg L^{-1} OTC relative to the other two treatments was primarily due to a non-significant reduction in the CO_2 concentration. However, the high OTC amendment significantly lowered the concentration of methane relative to that of the control (Figure 3.3b; OTC as a single factor, p=0.017). OTC treatment had no significant effect on biogas CO_2 concentration (Figure 3.3c).

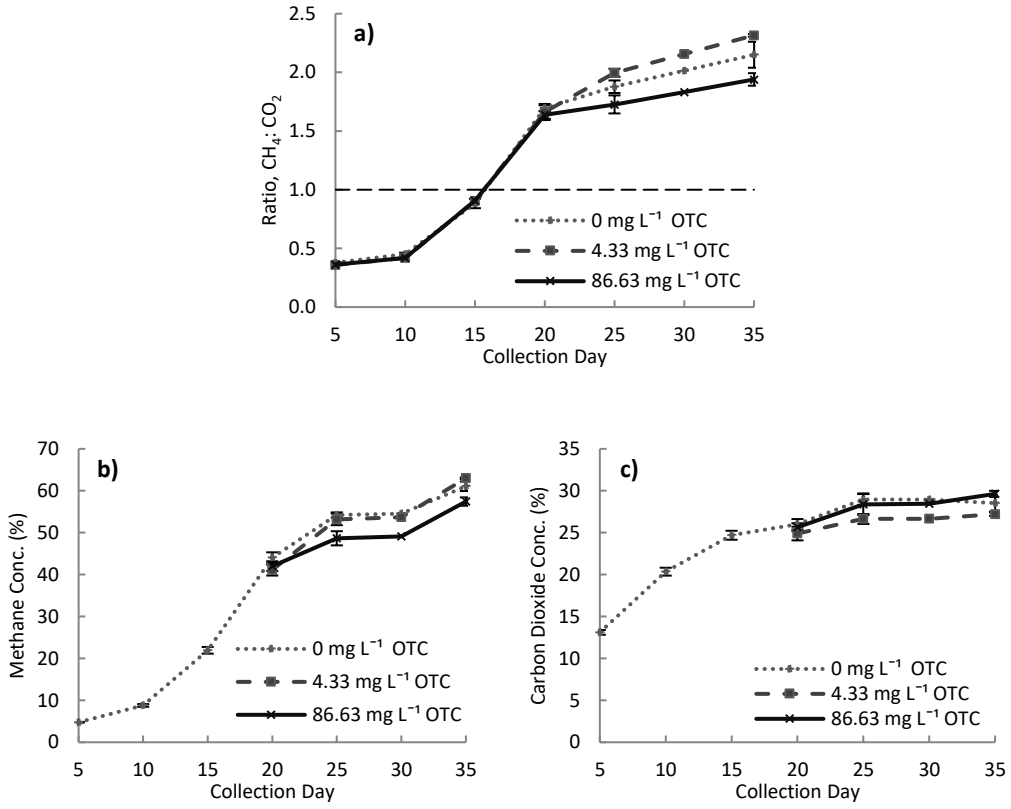

Figure 3-3 (a) CH_4:CO_2 ratio within biogas collected from reactors containing organic cow dung ± OTC applied on day 15; only time as a single factor was significant, p=0.004. **(b)** CH_4 concentration (percentage); repeated measures ANOVA: OTC conc. as a single factor, p=0.017; LSD=3.02. **(c)** CO_2 concentration (percentage); only time as a single factor was significant, p=0.036. Values are means of a minimum of 3 replicates ± SE. Samples were taken every 5 days for 35 days in total.

3.4.2.3 CH_4 and CO_2 concentrations when OTC was added on day 15 (dairy slurry)

No significant effects of OTC were observed for CH_4 (p=0.455) or CO_2 (p=0.510) concentrations or for the CH_4:CO_2 ratio (p=0.245) when dairy slurry was amended with the antibiotic on day 15. Time was a significant factor for all the treatments.

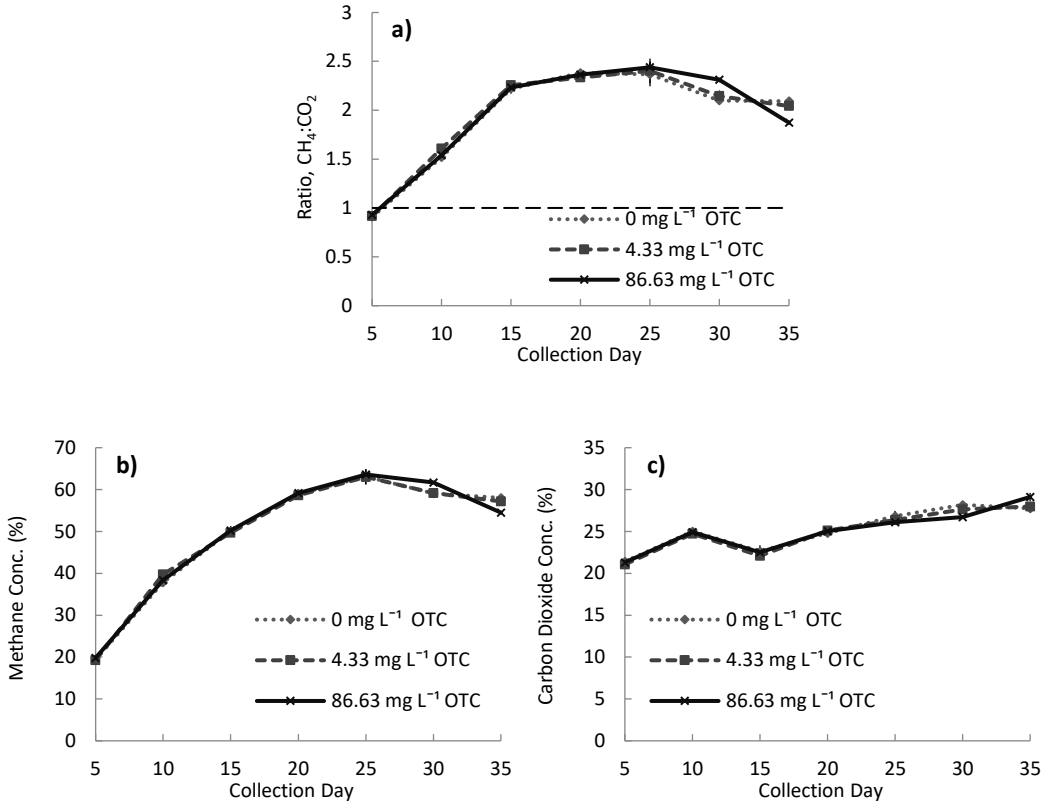

Figure 3-4 (a) CH_4:CO_2 ratio within biogas collected from reactors containing dairy slurry ± OTC applied on day 15. **(b)** CH_4 concentration (percentage). **(c)** CO_2 concentration (percentage). Values are means of a minimum of 3 replicates ± SE. Samples were taken every 5 days for 35 days in total. Only time was a significant factor.

3.4.3 pH

pH of the organic cattle dung digestate was significantly affected by an OTC x time interaction (p=0.003) when OTC was added to the system on day 0 - pH of the unamended digestate decreased by more than that of the other treatments (day 10) and increased again by a greater degree as time progressed (days 30 and 35). OTC was not significant as a single factor and it should be noted that the maximum treatment difference observed was 0.28 pH unit,

with most between-treatment differences on any particular sampling day being less than that (~0.1 pH unit). OTC was significant as a single factor (p=0.024) in post-amendment organic dung digestates with a small increase in pH resulting from OTC addition (control, pH 7.04; OTC [86.63 mg L^{-1}], pH 7.13). Similar pH changes were observed in the dairy slurry treatments but these were not significant.

Digestate pH changed with time in all three systems tested; organic dung, OTC amendment on day 0, p<0.001; organic dung, OTC amendment on day 15, p<0.001 for pre- and post-OTC addition; dairy slurry, pre-OTC, NS and post-OTC, p=0.022. Despite the significant time effect, the pH of the slurry digestates did not markedly alter over the duration of the incubation (Figure 3.5c). In contrast, pH of both organic dung systems dropped during the first ten days and then increased again. The differences in pH from days 5 to 10 (Figure 3.5a and 3.5b) which are pre-OTC amendment, demonstrate the variability within the system, but also the buffering capacity (days 10 to 20).

To test the biological relevance of these changes in the context of this study, pH was included as the covariate in an analysis of covariance when gas production was tested against OTC concentration. Since it was not a significant covariate, this suggests that changes in gas yield were independent of pH.

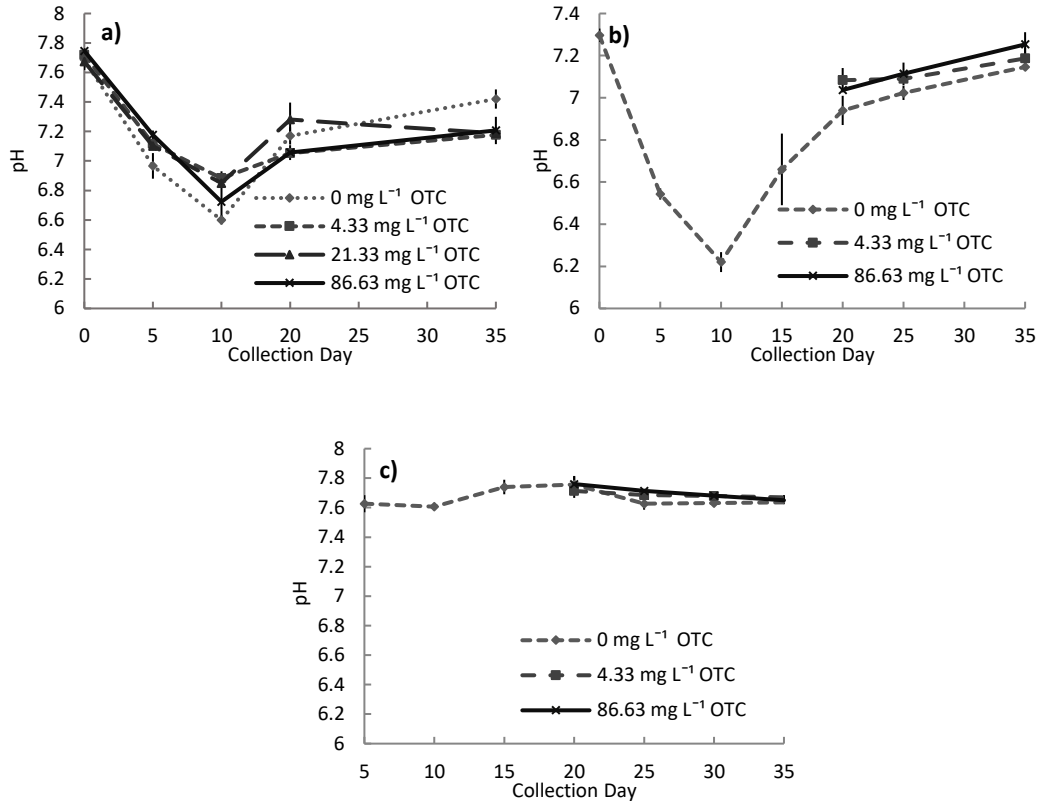

Figure 3-5 pH of digestates collected over 35 days of anaerobic digestion of: **(a)** Organic cow dung ± OTC applied on day 0. **(b)** Organic cow dung ± OTC applied on day 15. **(c)** Dairy slurry ± OTC applied on day 15. Values are means of a minimum of 3 replicates ± SE.

3.4.4 Total and Volatile Solids

Total and volatile solid content of the organic cow dung reduced over the 35-day incubation period (time as a single factor, $p<0.001$ for both parameters) and whilst the loss of total solids appeared greater from the control treatment than from digestate supplemented with OTC, this trend was not significant ($p=0.094$, Figure 3.6a). However, OTC-related inhibition of volatile solids (Figure 3.6b) from organic cow dung digestates was significant (OTC as a single factor, $p=0.006$, LSD= 0.065), with the control treatment being significantly different from all the OTC treatments when data across the incubation times were pooled.

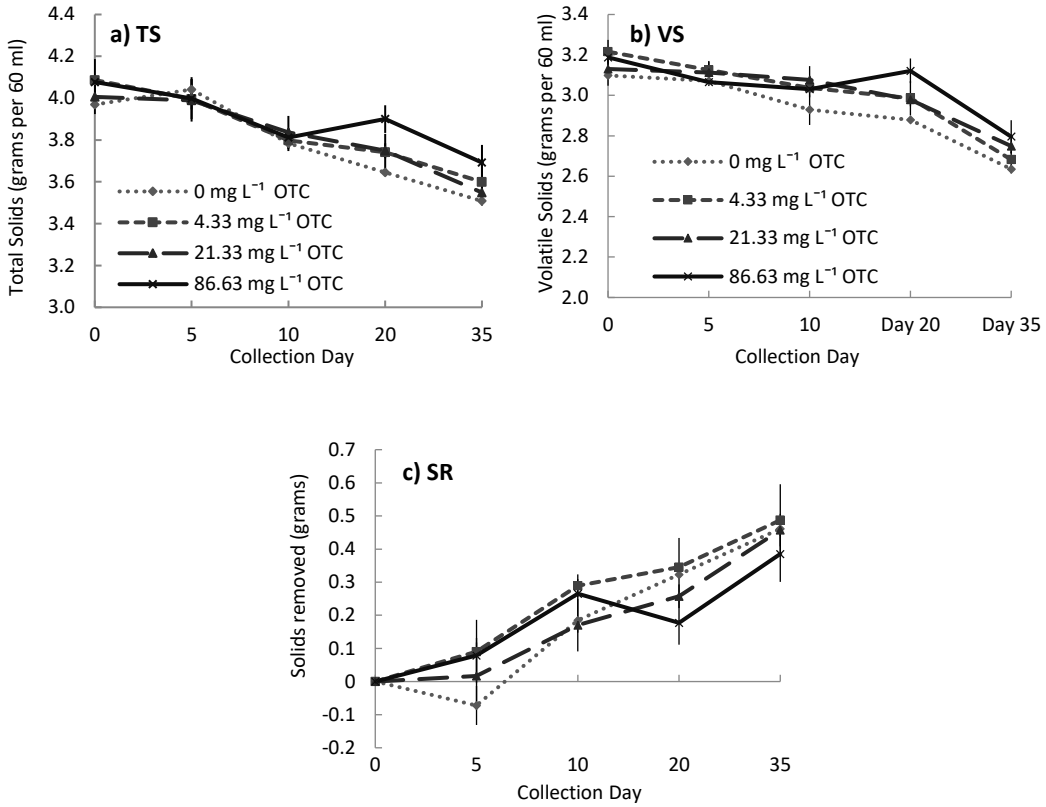

Figure 3-6 (a) Total solids, **(b)** volatile solids and **(c)** solids removed content of anaerobically digested organic cow dung ± OTC applied on day 0 over 35 days. Data are means of 3 replicates ± SE.

Neither incubation time nor OTC treatment significantly affected the total or volatile solid content of the digestates in the experiment with organic dung where OTC was added on day 15 (Figure 3.7a and 3.7b respectively). The starting values and finishing values were like those found in the previous experiment using the same feedstock (Figure 3.6), however, the same pattern was not observed and the variation in the system is evident.

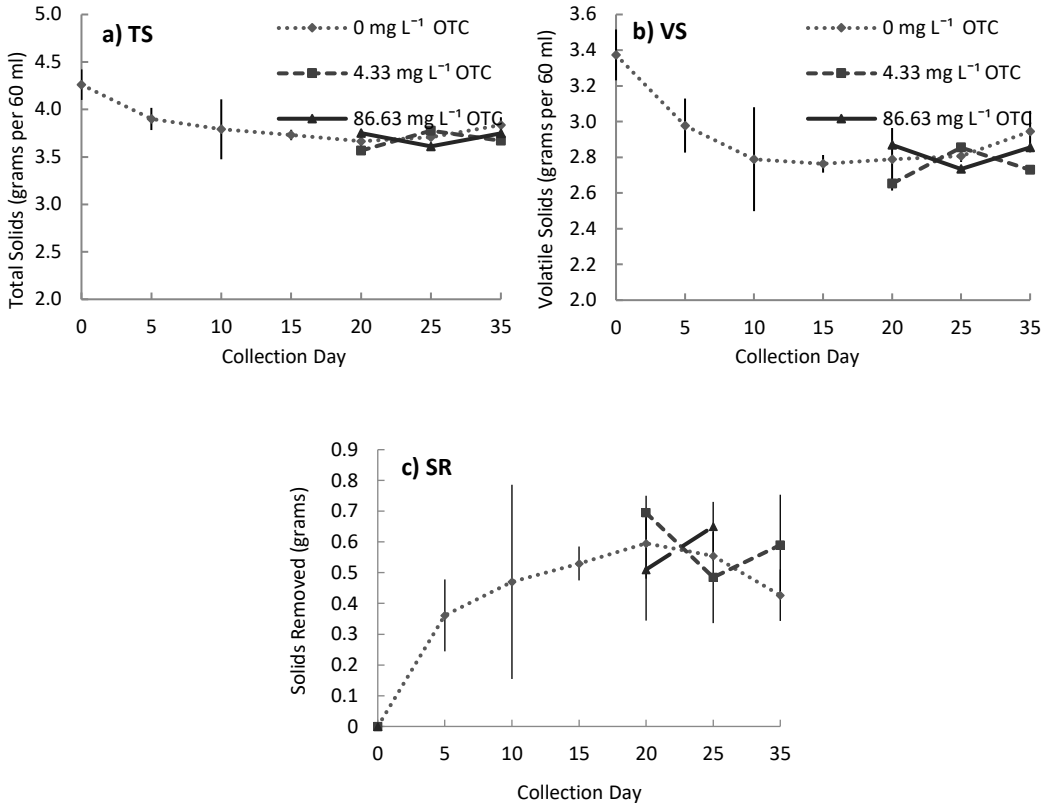

Figure 3-7 (a) Total solids, **(b)** volatile solids and **(c)** solids removed content of anaerobically digested organic cow dung ± OTC applied on day 15 over 35 days. Data are means of 3 replicates ± SE.

The reduction in total solids from the dairy slurry digestates was slow for the first 15 days (day 5, 51.98 g L^{-1}; day 15, 49.51 g L^{-1}). From day 20, the decrease became significant (OTC x time interaction, p=0.005) with solids in the control slurry reduced from 46.7 g L^{-1} on day 20 to 42.4 g L^{-1} on day 35. In contrast, the solid content of both OTC treatments remained similar from days 20 to 35 at around 45 g L^{-1}.

Volatile solid content significantly declined from days 5 to 15 (p=0.009) and then more rapidly post-OCT amendment on day 15 (time as a single factor, P<0.001) with the largest reduction between days 25 and 35. OTC amendment had no effect on removal of the volatile solids (Figure 3.8).

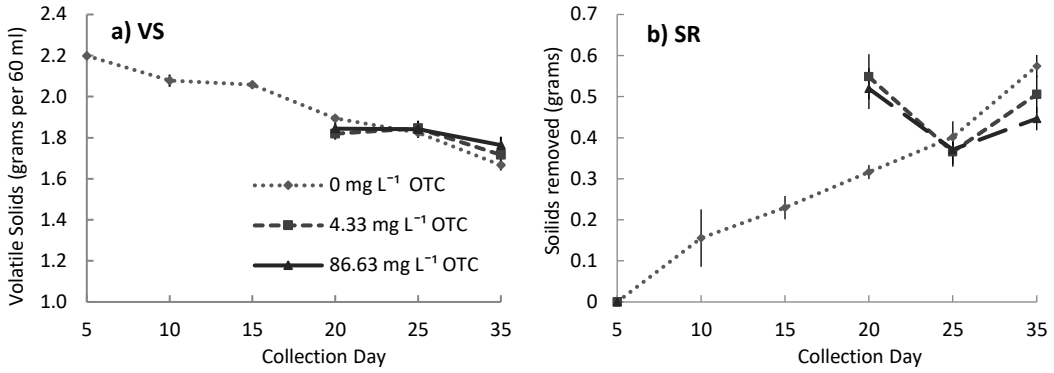

Figure 3-8 (a) Volatile solids and **(b)** soilids removed content and of dairy slurry digestates ± OTC applied on day 15. Data are means of 3 replicates ± SE.

3.5 DISCUSSION

Contradictory results have been reported where anaerobic digesters have been amended with OTC ; for example, Lallai *et al*. (2002) did not observe any inhibition of biogas production from OTC-spiked swine manure, using 125 and 250 mg L^{-1} of OTC in a mesophilic set-up. It was concluded that acid-forming and methane-forming bacteria were not affected by the presence of OTC in this case, as backed up by Gamel-El-Din, (1984), who hypothesised the biogas inhibition found in their study was likely to be due to OTC inhibiting microorganisms involved in the degradation of volatile solids (VS). In the current experiment, however, every condition caused a decreased in biogas production whether drugs were added at the start of the experiment or on day 15. It could be hypothesised that OTC added on day 0 to organic cow dung, inhibited microorganisms that breakdown VS as stated by Gamel-El-Din, (1984), but whilst this effect was observed in the organic cattle dung, it was not in the slurry digestions. Changes in volatile solids over anaerobic digestion are a good indicator of digester performance; upon degradation of volatile solids, volatile fatty acids are produced which then in turn are utilized by methanogens to produce CH_4 and CO_2 (Beneragama *et al*., 2013). In contrast to the findings of Lallai *et al*. (2002), Álvarez *et al*. (2010) reported that swine manure never exposed to OTC and spiked with 10, 50 and 100 mg L^{-1} OTC and

chlorotetracycline from the start of their experiment decreased biogas production by 42.5, 56.5 and 64.1% respectively, which was a higher reduction than that observed in this current investigation where OTC concentrations of 4.33, 21.33 and 86.63 mg L^{-1} resulted in a 4.0, 12.8 and 18.4% reduction in biogas production respectively. The differences may be explained by the dual 'contamination' by two products in the study by Álvarez *et al*. (2010). Adding OTC on day 15 was designed to determine the effects of the antibiotic on the anaerobic digestion process at the point where the system is producing CH_4 near its maximum potential to try and establish whether OTC has a greater effect at the start of the process or at the methanogenesis stage. In this instance, it was found that OTC had a greater effect when added on day 15, with 4.33 and 86.63 mg L^{-1} resulting in a 17.5 and 36.6% decrease respectively in biogas production relative to the control, a greater decrease than when added on day 0. This result is also comparable to a previous study which found a 32, 40 and 49% biogas decrease in production in a mesophilic batch digestion of cow dung spiked with 12.5, 37.5 and 75 mg L^{-1} of OTC respectively (Gamel-El-Din, 1984). Furthermore, OTC amendment through residues in calf/cow dung following feeding or intramuscular injection have been found to decrease biogas production (Arikan *et al*., 2006; Ince *et al*., 2013). It should be noted that different methods of introducing OTC into an anaerobic digestion system might potentially alter results. Cow dung from cattle given OTC might respond differently from spiked samples because the drug may undergo some metabolic changes prior to elimination in dung, but diet and husbandry methods may modify physical and microbial properties of the animal dung. Dung from cattle fed with OTC supplemented food has been shown to have fewer VS, more fungi and a higher concentration of K than waste from control, untreated treated animals (Patten *et al*., 1980). It is evident that there are a large number of factors that play a role in the fate and effect of pharmaceutical drugs in anaerobic digestion systems, some which include feedstock and inoculum source, inoculum/feedstock ratio, drug concentration, experimental design and reactor operating

conditions (Álvarez *et al*., 2010). As hypothesised by Alvarez *et al*. (2010), the absence of inhibition found by Arikan *et al*. (2006) could be as a result of using swine slurry which had previously been exposed to OTC, even though they stated the slurry came from pigs that had not been treated in a considerable time. Depending on the turnaround and usage of the slurry, OTC may still have been present in small quantities, resulting in acclimation of the faecal microorganisms to OTC.

The variability of the half-life of OTC depending on environmental conditions will influence its activity and longevity within an AD unit. OTC has been reported to have a half-life of just 0.26 ± 0.11 days at 43°C in deionised water compared to 14.01 ± 5.41 days at 25°C, a 54 fold reduction indicating high sensitivity to temperature (Doi and Stoskopf 2000). These values however seem to change dramatically depending on the substrate involved, along with other factors. For instance, the half-life of OTC in cattle manure and swine manure was reported to be 55-57 days in two separate papers, but at two distinct temperatures, 35°C and 8°C (Winckler and Grafe, 2001; Arikan *et al*., 2006) illustrating the overall variability in the published data which could be due to a host of reasons, including analytical inconsistencies due to difficulties in extracting OTC from the organic matrix. Winckler *et al*. (2003) furthermore reported an OTC half-life of 105 days in spiked outdoor samples, in complete contrast to Kühne *et al*. (2000) who recorded a much shorter half-life of only 4.5 and 9 days respectively in aerated and unaerated pig manure for tetracycline at ambient temperature. pH plays a large part in drug degradation; an alkaline pH value (pH >7.5) may lead to faster degradation of tetracyclines and in alkaline solutions tetracyclines metabolize to iso-tetracyclines with almost no *in vitro* activity compared to their parent compound (Kühne *et al*., 2000), which in turn are rapidly decomposed into smaller fragments (Hlavka and Boothe, 1985). If the OTC in the dairy slurry digestate was metabolised more rapidly than that in the organic dung, then this could explain the much higher total biogas yields from the slurry treatments (409 ml total from the slurry and 227 ml from the comparable organic dung

treatment). pH of the slurry digestate was consistently above pH 7.5, whilst that of the organic cow dung was <7.5.

There have been few studies conducted on the effects of antibiotics on anaerobic digestion, particularly in systems where substrates have previously been exposed to antibiotics. Given the prevalence of microbial resistance to OTC and other tetracyclines (resistance mechanisms have been described for 39 Gram-negative and 23 Gram-positive genera (Chopra and Roberts, 2001)) it is likely that pre-exposure of animal dung to OTC will enhance the likelihood of resistance genes in the AD feedstock. Therefore, differences observed in biogas yield and quality between the slurry and organic dung may be due to the presence of resistant microbes in the dairy slurry, but not in the naïve organic dung. The current study shows the complexity of the system since there are many factors that control the production of biogas, including quality of the organic matter and microbial consortia. OTC had a less detrimental effect on biogas quality from dairy slurry with likely historic use of OTC when compared to that of naïve, organic dung. It has been reported that mesophilic anaerobic digestion is unable to eliminate antibiotic resistant genes, with some genes increasing due to the process (Ghosh *et al*., 2009). The lack of OTC-driven inhibition in the dairy slurry could therefore be explained by the potential existence of specific antibiotic resistance genes, which may continue to function in the presence of OTC.

A common reason for reduced biogas production is a build-up of ammonium within a reactor; levels above 1.7 g L^{-1} can inhibit methanogenesis, although the ammonium and bicarbonate contents of cattle dung may buffer the system against pH decreases (Franke-Whittle *et al*., 2014 and references therein). Whilst digestate ammonium was not measured, the organic dung exhibited signs of a pH buffering mechanism since the drop in pH observed on day 10 increased again, although not to starting levels. The drop in pH in the organic dung samples

suggests production of volatile fatty acids and H^+ during the acidogenesis phase with methanogenesis occurring at around day 15, which would correspond with the increased pH observed on day 20, since methanogens utilise the volatile fatty acids. Whilst the drop in pH was relatively small, it has been argued that this may nevertheless underestimate high volatile acid concentrations in buffered systems (Murto *et al*., 2004). Normally the different steps in the AD process would overlap and it is possible that this was the case in the slurry samples since those samples produced methane immediately and did not exhibit a drop in pH, possibly because of an overlap between the functional groups within the slurry preventing the build-up of acidic components.

The data here support the hypothesis that each feedstock would respond differently, but negatively to OTC amendment. It was anticipated that gas production and quality would be more detrimentally affected by OTC amendment when added to the organic (naïve) dung than to the dairy slurry which has been shown to contain OTC (Dave Barrett, pers comm., Appendix I). The dairy slurry produced more biogas overall than the organic dung, partly because the lag phase was not as long in the slurry reactors as it was in the dung samples when the experiment began. This might be due to differences in quality of organic matter, or more likely due to the slurry already containing a functioning consortium of methanogens. The organic dung was inoculated at the start, whilst the dairy slurry was not because it was already producing gas. Whilst differences in gas yield may be partly explained by a faster start to the process in the slurry samples, it does not explain why OTC did not adversely affect the CH_4:CO_2 ratio in the digesters with dairy slurry feedstock, but did so in the reactors with organic dung. This infers that microbes involved in metabolic processes prior to methanogenesis may be more susceptible to OTC than the methanogens. The dairy slurry contained slightly less organic matter than the organic dung (5.2% vs 6% respectively) and it has been shown that reactors with a higher solids content are more effective (Yi *et al*., 2014).

The current experiment does not support that finding and therefore the most likely explanation for the differences in biogas yield between the slurry and the dung is due to differences in the microbial consortia within each feedstock.

CHAPTER 4 - EFFECTS OF TYLOSIN ON BIOGAS PRODUCTION AND QUALITY DURING LABORATORY-SCALE ANAEROBIC DIGESTION ON ORGANIC COW DUNG AND DAIRY CATTLE SLURRY

4.1 INTRODUCTION

Antibiotics have been used substantially in the last 70 years as a response to bacterial infections, these therapeutic responses however quickly moved to prophylactic, metaphylactic and growth promoter use. Antibiotics are defined as naturally occurring, semi-synthetic and synthetic compounds intended to be used for anti-microbial purposes, that can be applied orally, parentally or topically (Kemper, 2008). Growth promoters were first discovered in the 1940s, after it was observed that *Streptomyces aureofaciens* containing chlorotetracycline residues improved animal growth (Castanon, 2007). Dibner and Richards, (2005) and Niewold, (2007) suggested that most of the benefits derive from the effects on the intestinal microflora, most likely caused by the inhibition of production and excretion of catabolic mediators by intestinal inflammatory cells. Although the use of antibiotics as growth promoters has been banned in the UK since 2006, antibiotics are often used prophylactically in large quantities irrespective of animal health (De Liguoro *et al*., 2003), although pressure is building to decrease the overall use of antibiotics in both human and animal healthcare. As a result of extensive pharmaceutical use, antimicrobial-resistant microbes are ever more abundant and the rise in their numbers has become a health concern (Mellon *et al*., 2001), sparking calls by politicians to reduce antibiotic use on farms as recommended by a recent review (O'Neill, 2016).

Tylosin is a 16-membered macrolide that acts by inhibiting bacterial protein synthesis by interacting with the 50S ribosomal subunit (Mazzei *et al*., 1993). It is more effective against Gram-positive than Gram-negative bacteria and is used for medicating pig and chicken feed (administered at 50-200 mg kg^{-1} body weight) and as an intramuscular injection for cattle (at

4-10 mg kg^{-1} body weight) (NOAH Compendium; http://www.noah.co.uk/). In the USA in 2011, 582,836 kg of macrolides (active ingredients) were marketed in contrast to the 5,652,855 kg of tetracyclines, although that is not an insubstantial quantity of active ingredient. The internal USA market share of the tetracyclines was 41% and for macrolides it was 4% (FDA, 2015). Nevertheless, tylosin is widely administered to farm animals to control intestinal and respiratory infections (De Liguoro *et al*., 2003) and is frequently used to treat bovine mastitis (Entorf *et al*., 2014). Mastitis is a concern of many farmers that house their cattle since it can result from contagion or from environmental factors (EFSA, 2009). Tylosin has been used to treat dairy cattle on the University of Nottingham farm since these cows tend to suffer from environmental mastitis. However, tylosin residues were not detected in the slurry (Dave Barrett, pers comm., Appendix I) when tested.

Previous studies on the effects of tylosin on anaerobic digestion systems appear contradictory (Poels *et al*., 1984; Sanz *et al*., 1996; Massé *et al*., 2000; De Liguoro *et al*., 2003; Loftin and Henny, 2005; Angenent *et al*., 2008; Shimada *et al*., 2008; Mitchell *et al*., 2013). Given this information, the use of tylosin in dairy herds and the different mode of action from oxytetracycline, experiments were established to test its effect on anaerobic digestion using laboratory-scale batch reactors.

4.2 AIMS

The aims of this experiment were to quantify the effects of tylosin on biogas production and quality (CH_4:CO_2 ratios) when introduced into laboratory-scale digesters containing either dung collected from organically farmed grass-fed cattle, or slurry collected from a conventional dairy farm and to establish when, during the AD process, tylosin has the

greatest detrimental impact (or otherwise). The experiments mirror those conducted with oxytetracycline for comparison.

4.3 MATERIALS AND METHODS

The methodology used for the current experiments was the same as that described in Chapter 2 and in section 3.3 of Chapter 3.

Laboratory scale anaerobic digestion experiments were established to evaluate the effect of tylosin (TYL) on the anaerobic digestion process when using organic cattle dung and conventional dairy slurry as feedstocks. Please see sections 2.1 and 2.2 for full details of the dung and slurry collected and the experimental set-up. Briefly, treatments consisted of:

(i) Organic cattle dung spiked with TYL at start-up on day 0, to give final concentrations of 0, 4.33, 21.33 and 86.63 mg L^{-1}.

(ii) Organic cattle dung spiked with TYL 15 days after initiation, to give final concentrations of 0, 4.33, and 86.63 mg L^{-1}.

(iii) Conventional dairy slurry spiked with TYL 15 days after initiation, to give final concentrations of 0, 4.33, and 86.63 mg L^{-1}.

Serum bottles (120 mL capacity, containing 60 mL of organic dung or dairy slurry) were prepared as detailed in section 2.2.1 resulting in an organic matter content of 6% for the organic dung samples and 5.2% for the slurry. There were three replicated bottles per treatment and the experimental design was a randomised block design with three heated reciprocating shaking water baths acting as replicate 'blocks'. One complete set of TYL treatments per feedstock type was set up in each water bath and incubated at 35°C (160 rpm). Treatments (i), (ii) and (iii) were established in sequence because there was insufficient room in the water baths for all the bottles at once.

4.3.1 Sampling Regime

Digestate samples were destructively taken on days 5, 10, 15, 20, 25, 30 and 35 for experiments (ii) and (iii) where TYL was added 15 days after set-up and on days 5, 10, 15, 20, 30 and 35 for experiment (i) where TYL was present from day 0. Digestate was processed as detailed in section 2.3.

Biogas samples were taken on days 5, 10, 15, 20, 25, 30 and 35 from all bottles including those due for destructive sampling on a particular sampling day. For treatments receiving TYL on day 15, biogas and digestate samples were taken prior to adding the antibiotic.

4.3.2 Statistical Analysis

Statistical analyses were performed using Genstat 16th Edition (VSN International, Hemel Hempstead, UK). Two-way analyses of variance were conducted on pH and total volatile solids using antibiotic concentration and time (sacrificial 'harvests') as factors. Biogas yields, methane and carbon dioxide concentrations were analysed by repeated measures ANOVA and repeated measures ANCOVA using pH as the covariate. Normality was tested by plotting residuals against expected normal quantiles and post-hoc comparisons between means was based on least significant differences at the 0.05 probability level.

4.3 RESULTS

4.3.1 Biogas Production

After 35 days, biogas production was reduced by 40% when dung was amended with the highest concentration (86.63 mg L^{-1}) of TYL on day 0 compared to the control (Figure 4.1a). Less biogas was produced from day 20 onwards from reactors spiked with the highest TYL

concentrations and by day 35 the control dung produced more biogas than the amended reactors, with the 86.63 mg L^{-1} treatment performing least well. There was no significant TYL concentration x time interaction, but TYL concentration as a single factor was significant ($p<0.001$) with consecutively lower biogas yields with increasing TYL concentration; every treatment was significantly different from each other. The average biogas yields from reactors amended with different TYL concentrations across all time points were: 0 mg L^{-1} TYL = 37.75 ml biogas; 4.33 mg L^{-1} TYL = 33.26 ml; 21.33 mg L^{-1} TYL = 27.58 ml; 86.63 mg L^{-1} = 22.63 ml. LSD = 4.026.

A similar trend was observed when the total (cumulative) biogas yield over the 35 days was calculated ($p=0.002$) although in this case, yields from the two lowest TYL concentrations (0 and 4.33 mg L^{-1}) were not significantly different from one another and nor were the yields from the two highest concentrations. The data are below - similar superscripted letters (calculated from a Tukey's multiple comparison test following ANOVA) indicate no significant difference: 0 mg L^{-1} TYL = ^{c}265.0 ml biogas in total; 4.33 mg L^{-1} TYL = ^{bc}233.3 ml; 21.33 mg L^{-1} TYL = ^{ab}184.9 ml; 86.63 mg L^{-1} = ^{a}158.4 ml.

When TYL was added to the reactors containing organic dung on day 15, a marked response was evident. Prior to TYL amendment (days 5-15) biogas production was similar in all reactors and increased over time ($p<0.001$) up to day 15 when TYL was added. (TYL was added on day 15 after gas samples had been taken.) In the control reactors from day 15 onwards, biogas production continued to increase up to day 25 when it plateaued and remained constant until the end of the experiment on day 35 (data for day 35 not shown in Figure 4.1b for logistical reasons). In contrast, biogas yields dropped markedly on addition of TYL, irrespective of the concentration used (post-amendment ANOVA: TYL concentration x time interaction, $p=0.008$, LSD=12.74; Figure 4.1b). The similar effect of each concentration when

TYL was added on day 15 was different from the drug response when added at start-up (Figure 4.1a).

Biogas production from the control (unamended) reactors containing dairy slurry (Figure 4.1c) followed a different pattern from that seen in the previous experiment (Chapter 3, Figure 3.1c) indicating a degree of (microbial) variability within the farm slurry tank. Rather than immediately increasing biogas production over time as in the previous experiment (Chapter 3, dairy slurry), biogas production in the current trial initially fell before increasing again from day 10 onwards. The drop from an overall average of 18.30 ml on day 5 to 12.74 ml on day 10 and 13.41 ml on day 15 was significant ($p<0.001$). The system had started to recover when TYL was added on day 15 and treatment-related differences in biogas yield slowly appeared over time with maximum effect on day 30; the highest concentration resulted in the greatest drop in biogas production, whilst the control continued to increase production. However, on day 35, biogas production from unamended reactors collapsed (post-amendment ANOVA: TYL concentration x time interaction, $p<0.001$, LSD=6.56; Figure 4.1c). The cumulative biogas production over the 35 days was significantly different ($p<0.001$) between each TYL concentration: 0 mg L^{-1} TYL = ^{c}242.7 ml biogas in total; 4.33 mg L^{-1} TYL = ^{b}225.0 ml; 86.63 mg L^{-1} = ^{a}196.7 ml. Similar superscripted letters were calculated from a Tukey's multiple comparison test following ANOVA.

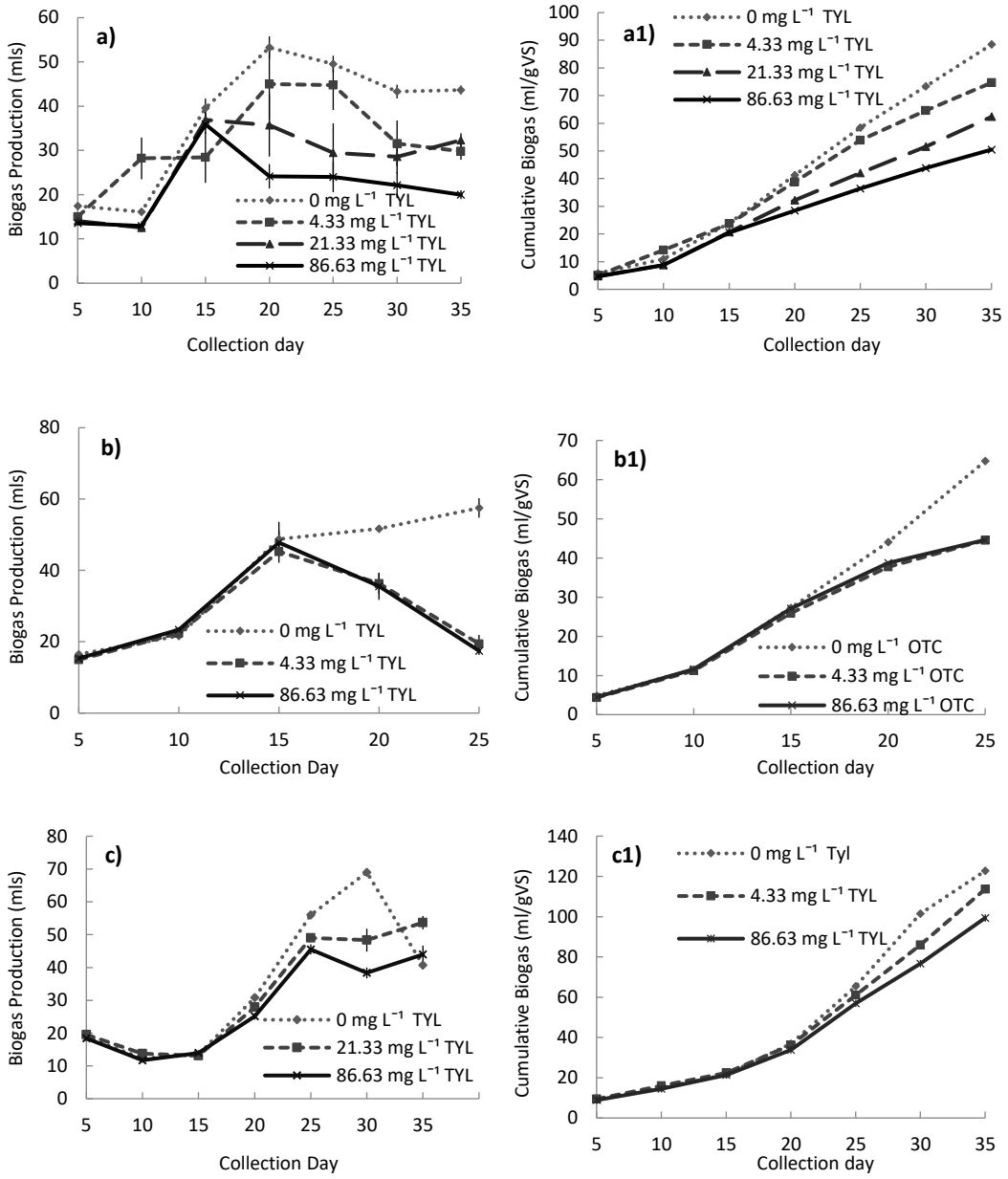

Figure 4-1 Biogas production over a 35-day period. Feedstock TYL concentrations were 4.33, 21.33 and 86.63 mg L^{-1}. **(a)** Organic dung with TYL added on day 0. **(a1)** Cumulative biogas production of organic dung with TYL added on day 0. **(b)** Organic dung with TYL added on day 15. **(b1)** Cumulative biogas production of organic dung with TYL added on day 15. **(c)** Dairy cattle slurry with TYL added on day 15. **(c1)** Cumulative biogas production of dairy cattle slurry with TYL added on day 15. Values are means of a minimum of 3 replicates ± SE.

4.3.2 Biogas Composition

4.3.2.1 CH_4 and CO_2 concentrations when TYL was added at start-up (organic dung)

The CH_4:CO_2 ratio changed with time (time as a single factor, $p<0.001$, LSD=0.18), but the effects of TYL amendment were not significant (TYL as a single factor, p=0.065; TYL concentration x time, p=0.082). The CH_4:CO_2 ratio is a product of the CH_4 and CO_2 concentrations and values over 1 indicate a higher concentration of CH_4 than of CO_2. There appears to be a non-significant trend of higher methane production in the reactors with the two highest concentrations of TYL, but this is not the case (see Figures 4.2b and 4.2c). Methane production in all the reactors was greater than CO_2 output by day 15 (Figure 4.2a).

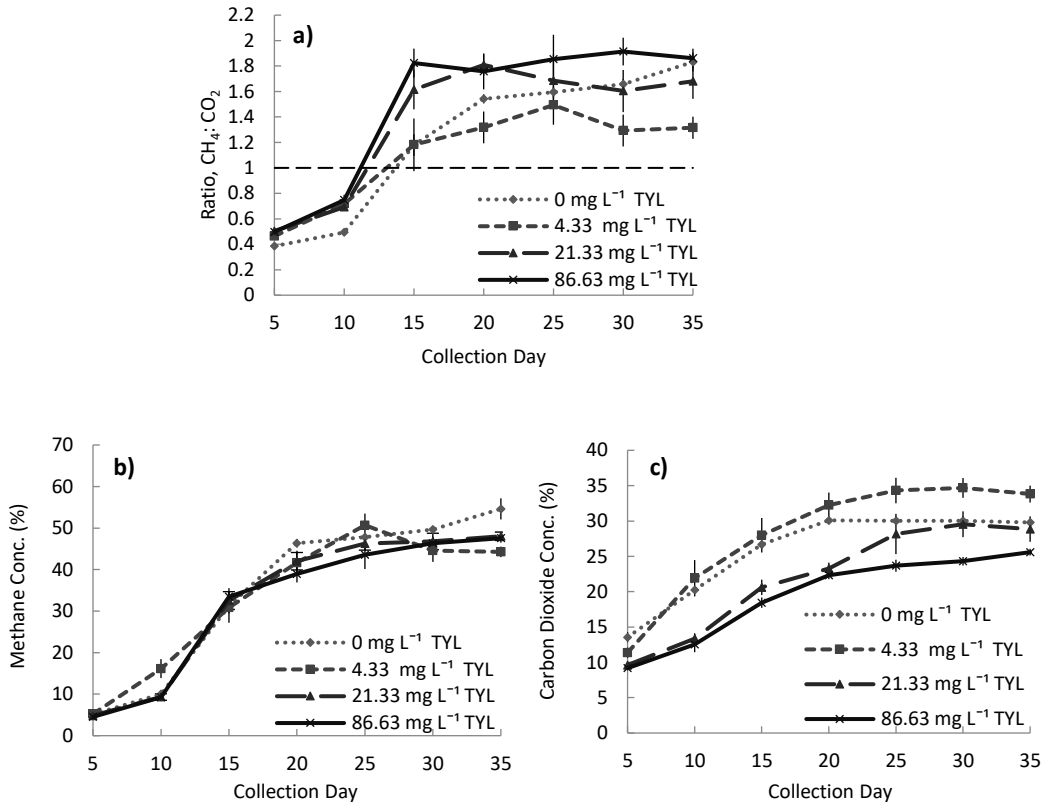

Figure 4-2 (a) CH_4:CO_2 ratio within biogas collected from reactors containing organic cow dung ± TYL applied on day 0. **(b)** CH_4 concentration (percentage). **(c)** CO_2 concentration (percentage). Values are means of a minimum of 3 replicates ± SE. Samples were taken every 5 days for 35 days in total.

Methane concentration within the biogas increased over time (time as a single factor, $p<0.001$; Figure 4.2b), but was unaffected by TYL amendment (TYL as a single factor, $p=0.506$). In contrast, TYL amendment significantly affected CO_2 production (TYL concentration as a single factor, $p=0.007$, LSD=3.75; Figure 4.3c). Since the TYL treatment trend remained constant from the outset, there was no TYL concentration x time interaction.

From start up, the two highest TYL concentrations (21.33 and 86.63 mg L^{-1}) consistently produced a lower concentration of CH_4 compared to the control and the 4.33 mg L^{-1} treatment up until day 25. When considering the pooled data for TYL concentration over all sampling times, there was no significant difference between the zero amendment and the 4.33 mg L^{-1} treatment, or between the two highest TYL concentrations. There was a significant difference between the zero and the highest TYL amendments and between the lowest (4.33 mg L^{-1}) and the 21.33 mg L^{-1} amendment.

4.3.2.2 CH_4 and CO_2 concentrations when TYL was added on day 15 (organic dung)

In contrast to the effects observed when tylosin was added at start-up, when organic dung was amended on day 15, the CH_4:CO_2 ratio was significantly reduced relative to the control treatment from day 25 onwards (post-amendment ANOVA: TYL concentration x time interaction, $p=0.019$, LSD=0.349). On day 20, the difference between the zero amendment and the highest TYL concentration was significant, but the difference between the lowest TYL addition and the zero amendment was not, but became significant by day 25. The apparent increase in the biogas CH_4:CO_2 ratio from the TYL amended reactors from day 25 to day 35 was not significant (based on appropriate LSD values).

Tylosin added on day 15 to organic cow dung significantly reduced the concentration of CH_4 in the biogas until day 25 when there was an apparent recovery; however, the difference in

CH_4 concentration between days 25 and 35 is not significant for the 86.63 mg L^{-1} TYL treatment, but it is for the 4.33 mg L^{-1} amendment (post-amendment ANOVA: TYL concentration x time interaction, p=0.003, LSD=7.39; Figure 4.3b). This reflects the variability of the data and the relatively large standard errors.

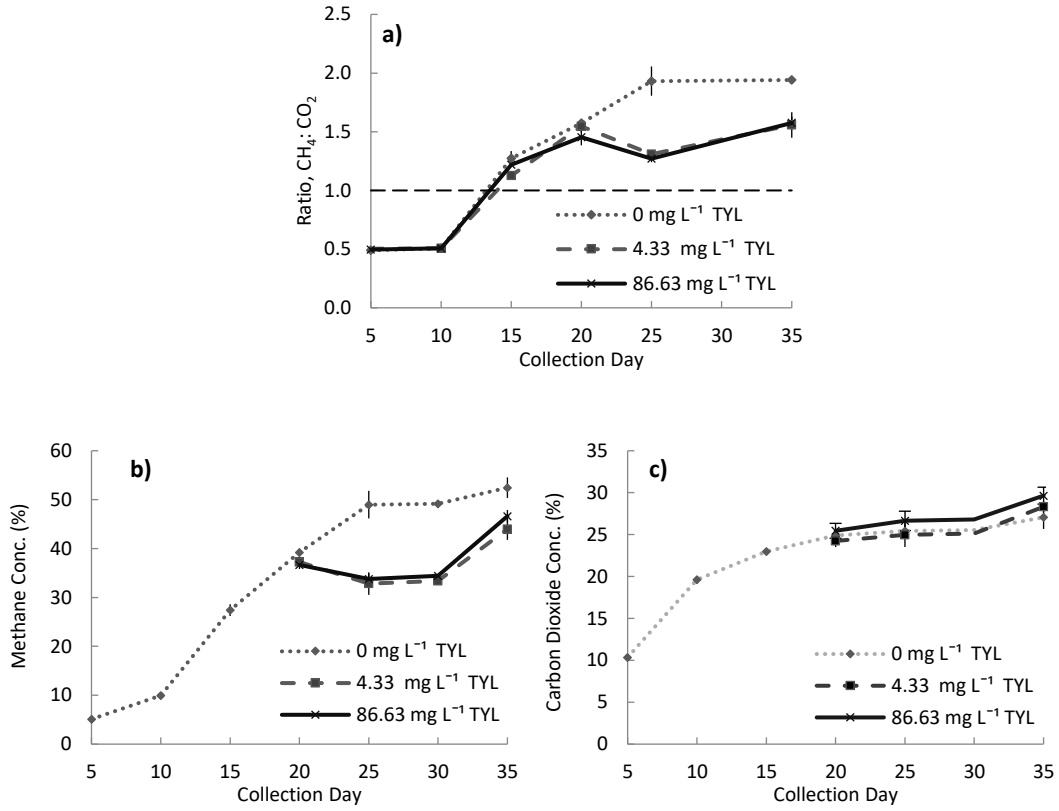

Figure 4-3 (a) CH_4:CO_2 ratio within biogas collected from reactors containing organic cow dung ± TYL applied on day 15. **(b)** CH_4 concentration (percentage). **(c)** CO_2 concentration (percentage). Values are means of a minimum of 3 replicates ± SE. Samples were taken every 5 days for 35 days in total.

However, TYL concentration was significant as a single factor (p=0.018, LSD=6.88) and concentration data pooled over all time points post-amendment corroborated the reduction in biogas methane concentration resulting from TYL addition (means: TYL (0 mg L^{-1}) = 456.1 ppt; TYL (4.33 mg L^{-1}) = 345.7 ppt; TYL (86.63 mg L^{-1}) = 347.6 ppt). Whilst TYL was detrimental to methane generation, it has no significant effect on the biogas CO_2 concentrations (Figure

4.3c). Therefore, the ratio of CH_4:CO_2 (Figure 4.3a) mirrors that of CH_4 production (Figure 4.3b). Tylosin, when added to organic cow dung on day 15 caused a similar response in both biogas production (Figure 4.1b) and CH_4 production (Figure 4.3b), with both high and low tylosin concentration causing an identical negative effect.

4.3.2.3 CH_4 and CO_2 concentrations when TYL was added on day 15 (dairy slurry)

Adding tylosin on day 15 did not significantly affect concentrations of either CH_4 or CO_2 in the biogas produced from dairy slurry (Figures 4.4b and 4.4c). However, when CH_4 concentration is considered as a single factor and data are pooled across the sampling points, there is a trend towards a reduction in CH_4 concentrations with increasing TYL concentration, with the biggest difference being between the zero amendment and the highest TYL concentration (post-amendment ANOVA: TYL concentration as a single factor, p=0.068). Whilst this trend is not significant, it is amplified when considered in light of the CO_2 concentration. The result is that the CH_4:CO_2 ratio was affected by TYL concentration (post-amendment ANOVA: TYL concentration as a single factor, p=0.013, LSD= 0.15). Specifically, the pooled average ratio for the control samples (2.84) was significantly higher than that for both the TYL amendments (low TYL = 2.65 and high TYL = 2.54), but the difference between the two TYL concentrations was not significant.

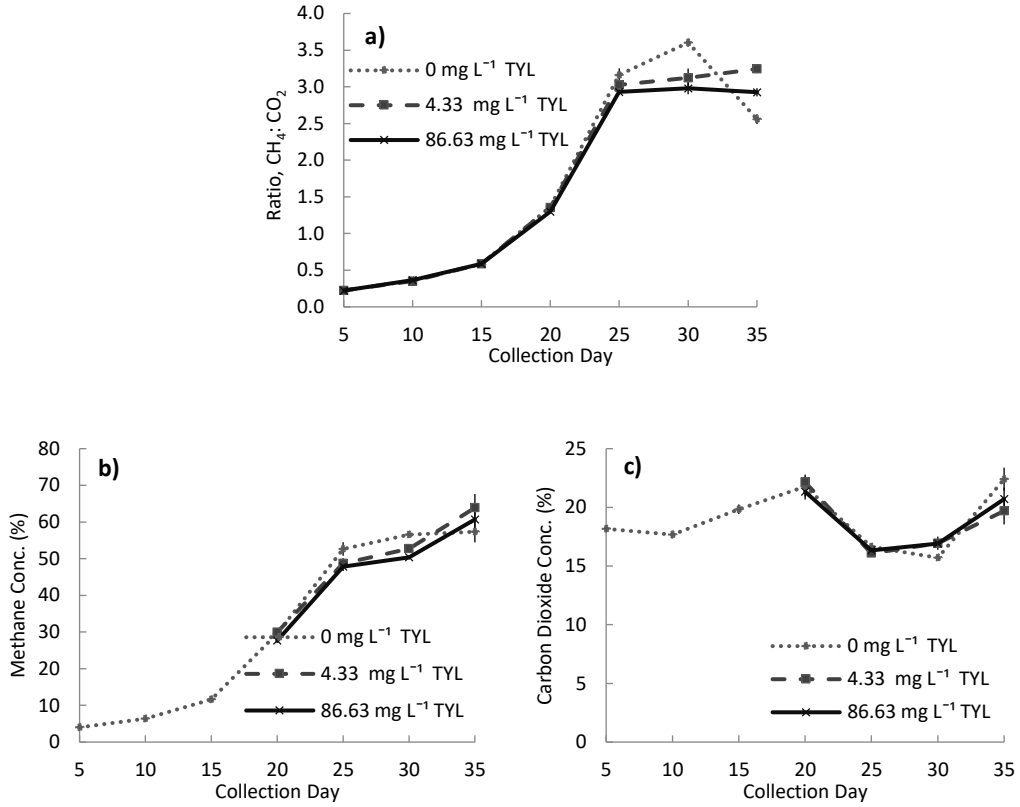

Figure 4-4 (a) CH_4:CO_2 ratio within biogas collected from reactors containing dairy slurry ± TYL applied on day 15. **(b)** CH_4 concentration (percentage). **(c)** CO_2 concentration (percentage). Values are means of a minimum of 3 replicates ± SE. Samples were taken every 5 days for 35 days in total.

4.3.4 pH

Tylosin amendment of organic dung at the start of the experiment resulted in a divergence in digestate pH almost for the duration of the experiment (Figure 4.5a). Digestate pH from the reactors with zero TYL amendment and 4.33 mg L^{-1} TYL was similar; however, the pH values were different from those for digestate from the reactors amended with the two highest TYL concentrations, which in turn were not different from each other (post-amendment ANOVA: TYL concentration as a single factor, $p<0.001$, LSD = 0.234). Time was also significant as a single factor ($p<0.001$) and this is reflected by the drop in pH observed on day 10 and the subsequent increase again across all treatments. However, whilst the TYL-

induced pH change was statistically significant, the maximum difference between the pooled means was only 0.39 of a pH unit, and the importance of this could be questioned. Nevertheless, at discrete points during the incubation period, the difference in pH between TYL treatments was greater than that and may reflect more important changes in volatile fatty acid profiles which were not measured here. The effect of time and the significant decrease in pH leading up to day 10 and the subsequent increase (both of which were less pronounced in digestates with the high TYL amendments) may reflect changes in microbial functioning in the system.

In contrast to the changes in pH observed when TYL was added at start-up, no significant TYL-related effects were observed when the antibiotic was added on day 15 (Figure 4.5b) to organic dung. However, the same time-dependent dip in pH was also observed on day 10. No time effect was observed for pH in the slurry treatments, although both concentrations of TYL amendment significantly increased digestate pH to virtually the same pH value (post-amendment ANOVA: TYL concentration as a single factor, $p=0.010$, LSD = 0.057).

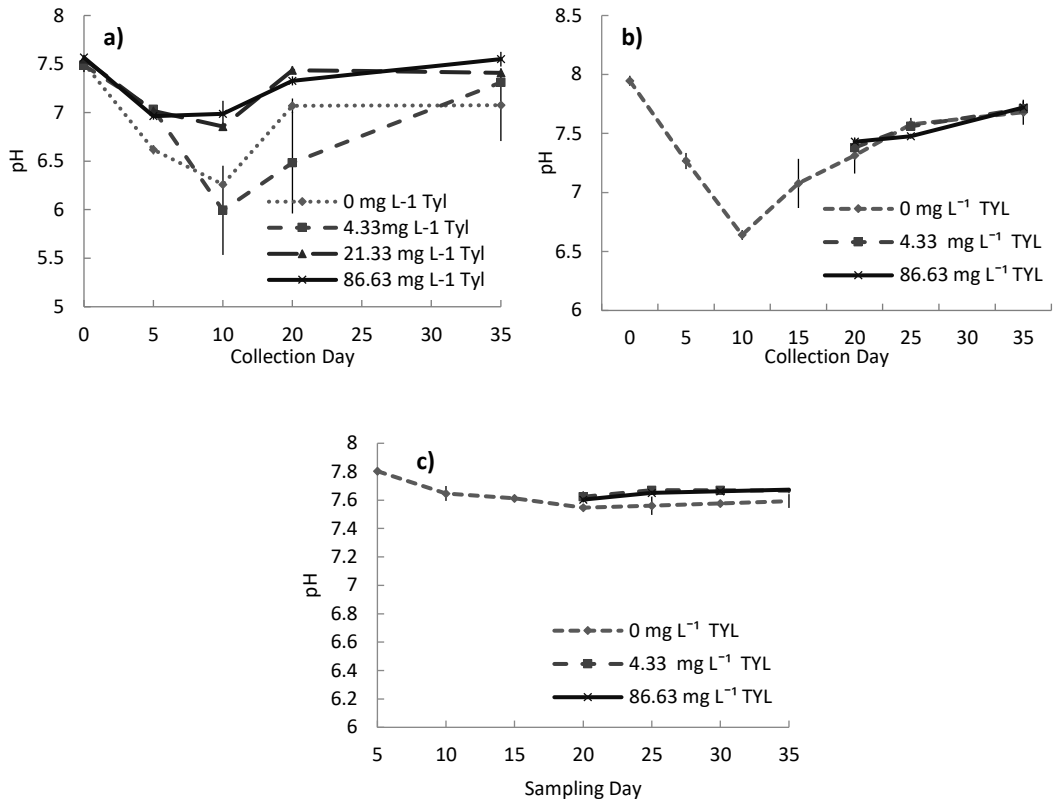

Figure 4-5 pH of digestates collected over 35 days of anaerobic digestion of: **(a)** Organic cow dung ± TYL applied on day 0. **(b)** Organic cow dung ± TYL applied on day 15. **(c)** Dairy slurry ± TYL applied on day 15. Values are means of a minimum of 3 replicates ± SE.

4.3.4 Total and Volatile Solids

Tylosin addition to organic cow dung on day 0 did not cause any significant difference in either TS or VS within the digestates. TS and VS both decreased over the incubation period (time as a single factor, $p<0.001$; LSD = 0.098 for TS; LSD = 0.087 for VS).

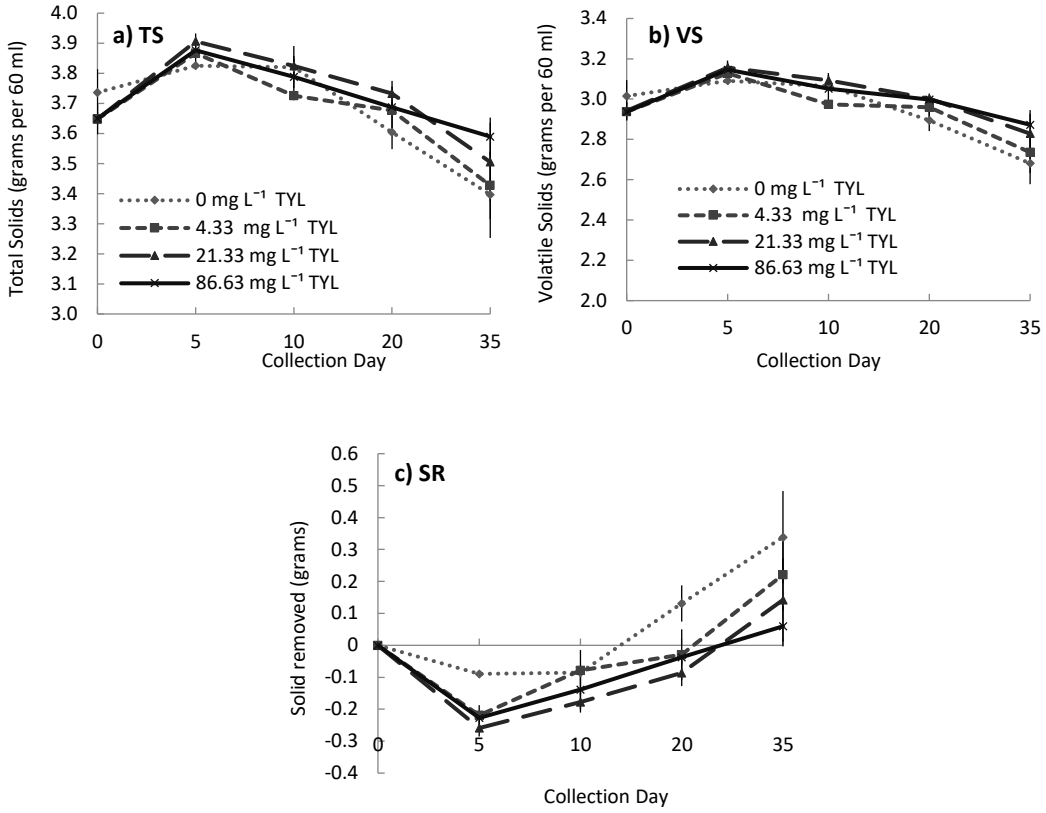

Figure 4-6 (a) Total solids, **(b)** volatile solids and **(c)** solids removed content of anaerobically digested organic cow dung ± TYL applied on day 0 over 35 days. Data are means of 3 replicates ± SE.

Total solids were unaffected by TYL amendment on day 15 and not even time altered the digestate TS content from day 20 onwards. However, digestate total solids decreased significantly from day 0 to day 15 (pre-amendment ANOVA: time as a single factor, p=0.013; Figure 4.7a) and only stopped decreasing after day 20. In contrast, removal of volatile solids was reduced by both TYL concentrations compared to the unamended samples (post-amendment ANOVA: TYL concentration as a single factor, p=0.027, LSD = 0.108).

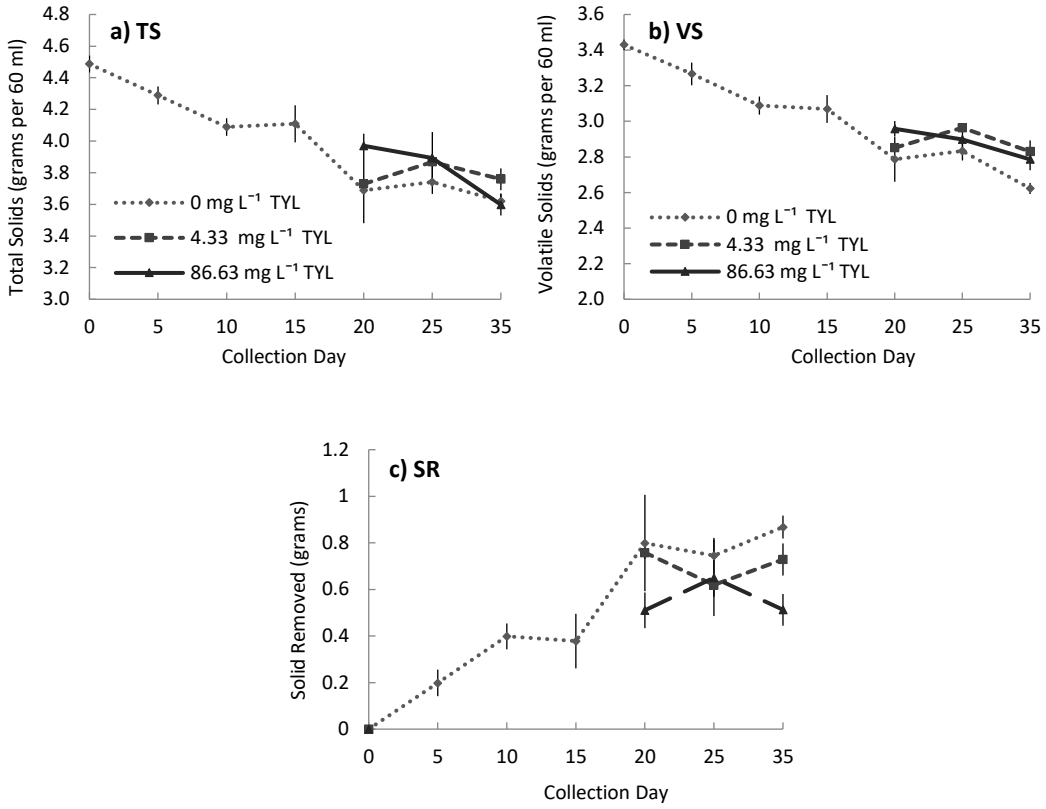

Figure 4-7 (a) Total solids, **(b)** volatile solids content and **(c)** solids removed content of anaerobically digested organic cow dung ± TYL applied on day 15. Data are means of 3 replicates ± SE.

Neither total nor volatile solids in the dairy slurry digestates were affected by TYL addition on day 15 of the incubations. Both TS and VS decreased with time ($p=0.001$ for TS and $p<0.001$ for VS).

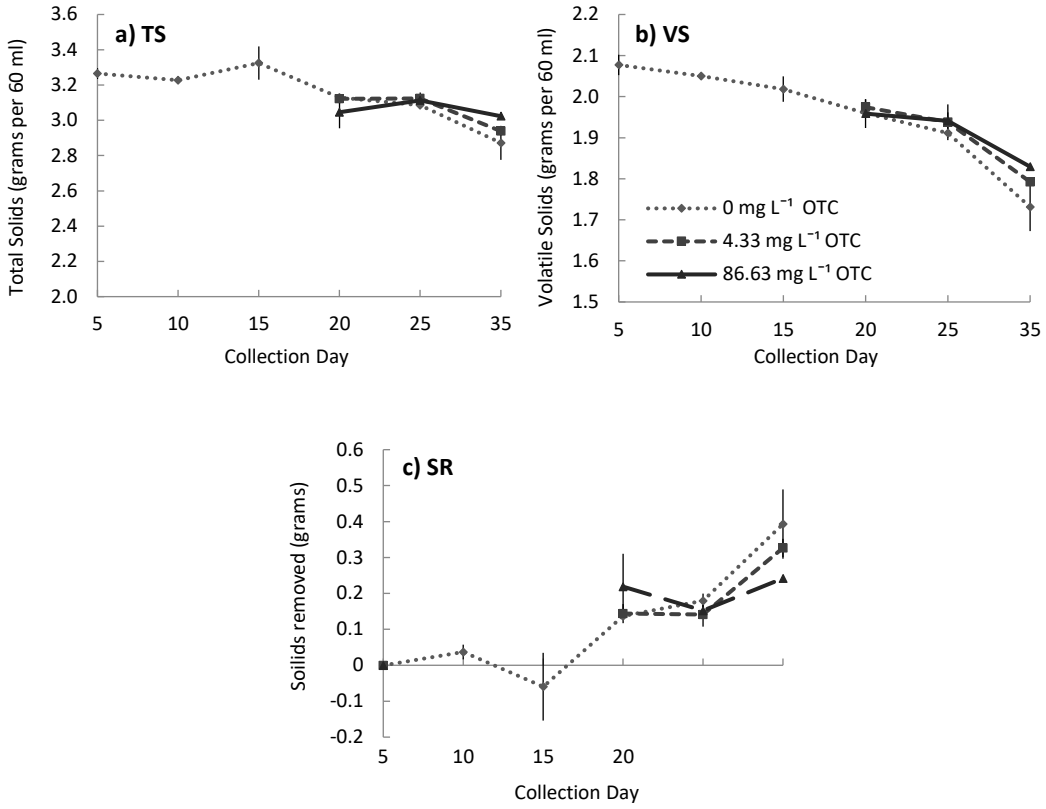

Figure 4-8 (a) Total solids, **(b)** volatile solids and **(c)** solids removed content of anaerobically digested organic cow dung ± TYL applied on day 15. Data are means of 3 replicates ± SE.

4.4 DISCUSSION

Biogas production, CH_4:CO_2 ratio, pH, TS and VS all had the potential to be affected by the addition of tylosin, however with each different experiment and hence different factors such as feedstock and time of tylosin addition the results varied widely, and were even contradictory at times. Tylosin addition greatly affected biogas production (Figure 4.1), however the extent of the effect changed depending on feedstock (organic cow dung or dairy slurry) and at which point during the incubation period tylosin was added. When tylosin was added on day 15 to organic cow dung, both the 4.33 and 86.63 mg L^{-1} treatment had the same detrimental effect on biogas production (Figure 4.1b), whilst also having the same detrimental effect on CH_4 production (Figure 4.3b). The resulting decrease in biogas production and CH_4 concentration was not step-wise, the more tylosin added did not

produce greater negative effects, nor did it change pH of the AD unit (Figure 4.5b). In this system both a low and high concentration of tylosin had immediate negative effects on the overall efficiently of the AD units. Tylosin in this instance reduced CH_4 concentrations rapidly, however, in a previous experiment, when tylosin was added at the start-up on day 0 to the same feedstock, tylosin had no effect on CH_4 production, but rather, reduced CO_2 production in a step wise manner instead (Figure 4.2c, the more tylosin added, the greater the CO_2 decrease, except for the lowest concentration – 4.33 mg L^{-1}). These results show that with the same conditions, with just the time of tylosin addition changing, tylosin has the potential to affect both CO_2 and CH_4 negatively. Tylosin also affected biogas production in a step-wise manner, with more tylosin resulting in a greater effect on biogas production (Figure 4.1a and 4.1c). Tylosin addition on day 15 to dairy slurry (Figure 4.4) resulted in a different change of CH_4 and CO_2 concentration when compared to the same experiment, but with an organic cow dung feedstock (Figure 4.3). There were subtle changes in both CH_4 and CO_2 concentration due to the addition of tylosin on dairy slurry, resulting in an overall decrease in the CH_4:CO_2 ratio.

These findings corroborate those of Loftin and Henny (2005) and Sanz *et al.* (1996) who reported similar results regardless of dosage. Loftin and Henny (2005) investigated the effects of tylosin on anaerobic activity in microcosms prepared from freshly collected lagoon slurries amended with tylosin at 1, 5 and 25 mg L^{-1} for a dose-response study. They observed that the addition of tylosin (added at the start) at concentrations of 1, 5 and 25 mg L^{-1} resulted in methane inhibition of 32, 30 and 29% respectively (Loftin and Henny, 2005). Their findings corroborate those reported here (Figure 4.3b), where a decrease in CH_4 concentration as a result of tylosin addition was observed, but with the exact amount of tylosin present not affecting the percentage decrease in CH_4 concentration. Sanz *et al*. (1996) found TYL could inhibit methane production by 35% at 25 mg L^{-1}, with a higher concentration of tylosin (250 mg L^{-1}) producing only a slight increase in inhibitory effect (45%). This was due

to the specific inhibition of C-degrading bacteria, while acetoclastic bacteria suffered no inhibitory effects (Sanz *et al*., 1996). Adversely, 9.2 mg L^{-1} of tylosin had no significant effect on biogas production, however, 130, 260, 520 and 912 mg L^{-1} of tylosin inhibited biogas production by 10, 20, 30 and 38% respectively (Mitchell *et al*., 2013). This dose-response, even though with much higher concentrations of tylosin, reflects the findings here (Figure 4.1a and 4.1c) of a dose dependant reduction in biogas production. Similar results were published by Shimada *et al*. (2008), with 1.67 mg L^{-1} showing negligible effects on overall performance, while 167 mg L^{-1} resulted in a gradual decrease in CH_4 production. Overall, tylosin addition did not result in a large change in the digestate pH suggesting that pH change is not the driver behind the antibiotic-induced changes in biogas and CH_4 production (Figure 4.5). The majority of literature on tylosin reports no inhibition due to tylosin addition to AD units (Poels *et al*., 1984; Massé *et al*., 2000; Angenent *et al*., 2008; Stone *et al*., 2009). With the findings of this current study and findings of others often contradicting each other, the exact cause of biogas production reduction and a drop in CH_4 concentration is hard to conclude. It is, however, evident that tylosin can affect microorganisms such as acetogens and methanogens and potentially those involved in the degradation of volatile solids at a range of concentrations, both low and high depending on the circumstances. Mitchell *et al*. (2013) hypothesised a few possible explanations; (1) limited spectrum of activity against Gram negative bacteria, (2) the bactericidal characteristics of macrolide antibiotics at different concentrations, (3) the amount of inoculum used, and (4) the different availabilities of certain tylosin components. It is evident that there are many factors that play a role in the fate and effect of pharmaceutical drugs in anaerobic digestion systems, from feedstock used, day of drug addition to experimental design. The data in this chapter support the hypothesis that each feedstock would respond differently, but negatively to TYL amendment.

CHAPTER 5 - ELEMENTAL COMPOSITION OF ANAEROBIC DIGESTATES MADE FROM ORGANIC COW DUNG AND DAIRY CATTLE SLURRY

5.1 INTRODUCTION

Anaerobic digestates generally have increased nitrogen availability compared to the original feedstock, making them potentially valuable fertilisers. This enhanced short-term N-fertiliser effect enhances crop nitrogen use efficiency and minimises N losses if it is applied to the soil at the optimum time for plant growth (Weiland, 2010). Digestates can therefore be integrated into an agronomic fertiliser plan enabling reduced reliance on chemical fertilisers and simultaneously improving the organic matter status of the soil.

Since anaerobic digestate is the effluent resulting from the digestion process, digestate quality is dependent on the chemistry and composition of the original feedstock. Few nutrients are lost during anaerobic digestion and furthermore, the process inactivates parasites, fungi, viruses, bacteria (e.g. *Salmonella* spp., *Escherichia* spp., *Listeria* spp.) and weed seeds, depending on digester efficiency (i.e. running temperature, retention time and mixing) (Sahlström, 2003). Anaerobic digestion is carried out by a consortium of microorganisms, converting organic material into CO_2, CH_4 and other trace gases, thereby altering the physico-chemical nature of the feedstock (Massé *et al*., 2007). Currently there are few reports on the impact of anaerobic digestion on digestate quality, with most just focusing on the fate of nitrogen and phosphorus (Massé *et al*., 2007). Digestates originating from feedstocks with high degradability such as cereals, poultry and pig manure with a highly concentrated diet are generally characterised by a high NH_4^+:total N ratio and low C:N ratio, whilst feedstock low in N results in a digestate with a low NH_4^+:total N ratio (de Boer, 2007; Emmerling and Barton, 2007; Möller and Stinner, 2010; Möller and Müller, 2012). Although

digestates are generally thought to be safe, some contradictory results have been published regarding phytotoxicity. Whilst authors have reported no phytotoxic effects of digestate (Sánchez *et al*., 2008; Gell *et al*., 2011), others have found phytotoxic effects (Poggi-Varaldo *et al*., 1999; Salminen and Rintala, 2002; Abdullahi *et al*., 2008), possibly related to high NH_4^+-N and organic acid concentrations. However, any negative effects due to phytotoxicity are usually short-term, and decrease within a short period of time after application (Möller and Müller, 2012). Total nitrogen and phosphorus of dung is generally conserved during anaerobic digestion, however, the mineralised fraction of the two nutrients increases, allowing increase nutrient availability for plant uptake (Field *et al*., 1984; Messner and Amberger, 1987; Boxall *et al*., 2006). As one of the main aims of anaerobic digestion is to produce biogas, carbon is lost *via* CH_4 and CO_2 production, thereby reducing the C/N ratio relative to the feedstock and resulting in less biological degradation of the remaining carbon (Chaussod *et al*., 1986; Messner and Amberger, 1987; Plaixats *et al*., 1988). Reports indicate that total nutrients are conserved within anaerobic digestion, with Field *et al*. (1984) demonstrating nearly 100% conservation of total phosphorus, potassium, calcium and magnesium, but extractable fractions of P, Ca and Mg were decreased, possibly due to sorption or precipitation as struvite or calcium phosphate (Field *et al*., 1984; Massé *et al*., 2007). Advantages of using digestate as a fertiliser include improved flow properties, allowing faster penetration into soil resulting in potential reduced nitrogen loss by ammonia emissions. Furthermore, anaerobically digested material can positively change the composition of odours, reducing them relative to the original feedstock by up to 80% (Weiland, 2010). Using anaerobic digestate as a fertiliser is thought to be the most sustainable use for it, with benefits to society in general and to the environment by helping to recycle natural resources; nonetheless, for use as a fertiliser it is imperative that the digest be of high quality, free of pathogens, chemical and physical impurities and pollutants (Al Seadi *et al*., 2013). Digestate can also be re-fed to the anaerobic digester if using wet

digestion processes as a means of achieving a desirable organic matter content for the feedstock mixture. The anaerobic digestion process does not change heavy metals, which can originate from feedstock and eventually be applied to soil when digestate is used as fertiliser (Al Seadi and Lukehurst, 2012). Micro-nutrients can be utilised when digestate is applied to land whilst heavy metals and other persistent contaminants cannot, therefore these contaminants can cause problem and must be monitored (Lukehurst *et al*., 2010).

The aim of this Chapter is to report the nutritional analyses of digestates produced during anaerobic digestion of organic cattle dung and dairy slurry when supplemented with either oxytetracycline or tylosin. These data may be used as a guide to their quality as a fertiliser and to help interpret differences in anaerobic digestion efficiencies between the organic dung and dairy slurry.

5.2 MATERIALS AND METHODS

Digestates utilised here were produced during laboratory-scale anaerobic digestion of organic cattle dung and dairy slurry derived from the experiments detailed in Chapter 3 (*Effect of oxytetracycline on biogas production and quality during laboratory-scale anaerobic digestion of organic cattle dung and dairy cattle slurry*) and from Chapter 4 (*Effect of tylosin on biogas production and quality during laboratory-scale anaerobic digestion of organic cattle dung and dairy cattle slurry*).

Digestates were acid digested and macro- and micro-nutrients analysed by ICP-MS as described in Chapter 2, section 2.3.4.

5.2.2 Statistics

All data were analysed in Genstat 17th Edition (VSN International) using analyses of variance, with element as the variate and sample day and antibiotic concentration as factors. Block structure was incorporated because of use of replicate water baths and least significant differences between means at the 0.05 probability level were used for post-hoc comparisons. Residuals were plotted against expected normal quantiles to test for normality.

5.3 RESULTS

5.3.1 Nutrient Composition of the Anaerobic Digestates

Tables 5.1 and 5.2 show the main total macro- and micro-nutrient characteristics of digestate from organic cow dung spiked with oxytetracycline (OTC) and tylosin (TYL) respectively, at start-up. Elemental composition of the digestates was unaffected by time of either antibiotic amendment, therefore data relating to digestates produced from experiments where OTC and TYL were added on day 15 are not shown.

Table 5-1 Macro- and micro-nutrients in anaerobically digested organic cow dung over a digestion period of 35 days. These samples were derived from the OTC-experiment (Chapter 3). Sampling was destructively carried out at each time-point, therefore a separate set of replicates was analysed on each day. The * symbol indicates a higher concentration than when compared to previous days at the p = 0.05 level of significance, values are means of a minimum of 3 replicates ± SE.

Nutrient (mg kg^{-1} DM)	Day 5	Day 10	Day 20	Day 35
P	11025 ± 386.7	10478 ± 485.0	10732 ± 605.0	12253* ± 120.0
K	12762 ± 428.3	12012 ± 520.0	12337 ± 666.7	13938* ± 71.7
Ca	15890 ± 598.3	14775 ± 696.7	15255 ± 823.3	17256.* ± 208.3
Mg	5775 ± 203.3	5425 ± 243.3	5592 ± 313.3	6436* ± 65.0
S	7542 ± 388.3	6095 ± 480.0	6867 ± 860.0	5947 ± 598.3
Mo	4.8 ± 0.17	4.7 ± 0.33	4.8 ± 0.17	5.5 ± 0.17
Fe	2300.0 ± 93.3	2168 ± 100.0	2248 ± 135.0	2721* ± 40.0
Mn	173.3 ± 6.7	165.0 ± 6.7	166.7 ± 8.3	193.3* ± 1.7
Cu	18.3 ± 1.7	15.0 ± 1.7	16.7 ± 1.7	18.3 ± 0.00
Na	2333 ± 73.3	2188 ± 88.3	2110 ± 100.0	2158 ± 21.7
Zn	96.7 ± 8.3	106.7 ± 16.7	83.3 ± 5.0	105.0 ± 5.0
Ni	4.8 ± 0.33	4.0 ± 0.17	4.3 ± 0.33	4.0 ± 0.17
Al	1457 ± 136.7	1232 ± 93.3	1418 ± 141.7	1718* ± 58.3

Neither oxytetracycline nor tylosin amendment affected total elemental composition of the digestates. Nutrient concentrations differed between the organic dung and dairy slurry (Tables 5.1 – 5.4).

Total nutrient content in digestate derived from dairy slurry generally remained consistent throughout the sampling period. Some apparently significant differences in elemental concentrations appeared to change across sampling days in digestates derived from organic dung. Specifically, P, K, Ca, Mg, Fe, Mn and Al concentrations were greater on day 35 of sampling ($p<0.05$, Table 5.1). It must be noted however, that even though these changes were significant, the % change was relatively small.

In contrast, P, K, Ca, Mg, S, Na and Al concentrations appeared to change over incubation time in digestates originating from organic dung used in the tylosin-amendment experiment

(p<0.05, Table 5.2). Again, however, it must be noted that these changes (except for those relating to Sulfur (S)) are relatively small and are more likely to reflect the variability of the dung between sampling vessels than a consequence of the digestion process in this instance. In contrast to the other elements, the marked fall in S content during incubation was clearly a real and significant decline of 92% from day 5 to day 35 (p<0.05, Table 5.2).

Table 5-2 Macro- and micro-nutrients in anaerobically digested organic cow dung over a digestion period of 35 days. These samples were derived from the TYL-experiment (Chapter 4). Sampling was destructively carried out at each time-point, therefore a separate set of replicates was analysed on each day. The * symbol indicates a higher concentration than when compared to previous days at the p = 0.05 level of significance, values are means of a minimum of 3 replicates ± SE.

Nutrient (mg kg^{-1} DM)	Day 5	Day 10	Day 20	Day 35
P	11835 ± 126.7	11730 ± 148.3	11116* ± 345.0	10895* ± 230.0
K	13355 ± 140.0	13428 ± 116.7	12488* ± 345.0	12221* ± 275.0
Ca	16442 ± 185.0	16587 ± 173.3	15601* ± 511.7	15208* ± 350.0
Mg	6202 ± 65.0	6147 ± 70.0	5833* ± 175.0	5725* ± 125.0
S	5043 ± 586.7	5602 ± 328.3	2013* ± 553.3	386.7 ± 50.0
Mo	5.5 ± 0.00	5.5 ± 0.33	5.0 ± 0.17	4.8 ± 0.17
Fe	2370 ± 38.3	2390 ± 36.7	2270 ± 90.0	2238 ± 53.3
Mn	183.3 ± 1.7	185.0 ± 1.7	173.3 ± 5.0	171.7 ± 3.3
Cu	18.3 ± 0.00	21.7 ± 0.00	20.0 ± 0.0	20.0 ± 1.7
Na	2172 ± 53.3	2205 ± 15.0	1990 ± 68.3	1913* ± 51.7
Zn	103.3 ± 3.3	113.3 ± 3.3	113.3 ± 11.7	101.7 ± 3.3
Ni	4.2 ± 0.17	5.2 ± 0.50	4.0 ± 0.17	4.0 ± 0.17
Al	1445 ± 33.3	1425 ± 28.3	1420 ± 76.7	1291* ± 26.7

Table 5-3 Macro- and micro-nutrients in anaerobically digested dairy slurry over a digestion period of 35 days. These samples were derived from the OTC-experiment (Chapter 3). Sampling was destructively carried out at each time-point, therefore a separate set of replicates was analysed on each day. The * symbol indicates a higher concentration than when compared to previous days at the p = 0.05 level of significance, values are means of a minimum of 3 replicates ± SE.

Nutrient (mg kg^{-1} DM)	Day 5			Day 15			Day 20			Day 25		
P	18954	±	675.0	19040	±	34.6	18212	±	119.2	18669	±	200.0
K	26273	±	775.0	25525	±	313.5	248558*	±	144.2	25744	±	151.9
Ca	57642	±	1663	54708	±	1192	48888*	±	1112	55517	±	467.3
Mg	7900	±	228.8	7621	±	38.5	7065*	±	128.8	7663	±	51.9
S	21463	±	1188	14990*	±	1406	15196*	±	540.4	19688	±	663.5
Mo	7.1	±	0.19	6.5*	±	0.19	6.3*	±	0.00	6.3*	±	0.00
Fe	2290	±	65.4	2235	±	23.1	2036.5	±	71.2	2273	±	23.1
Mn	484.6	±	13.5	473.1	±	0.0	446.2	±	5.8	469.2	±	3.8
Cu	840.4	±	23.1	867.3	±	0.0	821.2	±	9.6	830.8	±	9.6
Na	6508	±	163.5	6540	±	67.3	6758	±	338.5	6425	±	42.3
Zn	1556	±	44.2	1483	±	9.6	1398*	±	17.3	1452	±	11.5
Ni	5.8	±	0.96	5.4	±	0.19	5.0	±	0.19	4.8	±	0.19
Al	936.5	±	55.8	798.1	±	7.7	686.5*	±	46.2	921.2	±	48.1

Table 5-4 Macro- and micro-nutrients in anaerobically digested slurry over a digestion period of 35 days. These samples were derived from the TYL-experiment (Chapter 4). Sampling was destructively carried out at each time-point, therefore a separate set of replicates was analysed on each day. The * symbol indicates a higher concentration than when compared to previous days at the p = 0.05 level of significance, values are means of a minimum of 3 replicates ± SE.

Nutrient (mg kg^{-1} DM)	Day 5			Day 15			Day 20			Day 25		
P	16200*	±	1231	18362	±	734.6	18033	±	251.9	18863	±	423.1
K	23329	±	1817	25810	±	1019.2	26008	±	459.6	26663	±	596.2
Ca	71362*	±	5271	80598	±	2926.9	77298	±	1437	83060	±	2142
Mg	6723*	±	507.7	7573	±	309.6	7504	±	101.9	7917	±	192.3
S	11313	±	1171	11290	±	484.6	10133	±	544.2	9621	±	1592
Mo	4.8	±	0.19	9.4	±	3.5	5.8	±	0.19	6.0	±	0.19
Fe	6112	±	623.1	6594	±	296.2	6431	±	92.3	6798	±	159.6
Mn	303.8*	±	23.1	344.2	±	11.5	336.5	±	3.8	365.4	±	13.5
Cu	336.5	±	23.1	375.0	±	13.5	373.1	±	3.8	388.5	±	9.6
Na	6131*	±	498.1	7579	±	175.0	7258	±	144.2	7433	±	180.8
Zn	717.3*	±	50.0	821.2	±	11.5	800.0	±	5.8	842.3	±	15.4
Ni	2.9	±	0.19	4.4	±	0.96	3.7	±	0.00	4.0	±	0.19
Al	1296*	±	98.1	1508	±	19.2	1421	±	26.9	1513	±	34.6

5.4 DISCUSSION

Animal manures often have relatively high proportions of N and P, typically due to digestion inefficiencies (Van Horn *et al*., 1996; Lukehurst *et al*., 2010). Manure nutrient composition can vary significantly and influence the anaerobic digestion process when used as a feedstock, thereby also affecting the nutrient content of the digestate. Furthermore solid manure (such as poultry manure) can have as much as 5 times the N concentration of liquid manure (Crolla *et al*., 2013). Liquid dairy slurry, poultry broiler manure, swine slurry and horse dung have vastly different total P concentrations, with values of 700±300 mg kg^{-1}, 1200±400 mg kg^{-1}, 1300 mg kg^{-1} and 400±100 mg kg^{-1} respectively (OMAFRA, 2011; Crolla *et al*., 2013). Total P in anaerobic digestate samples in this study ranged from 662 – 986 mg kg^{-1} (fresh weight), falling within range found by Bochmann and Montgomery, 2013b and others (Kleinman *et al*., 2002; Allen and Mallarino, 2008). Total S decreased by 92% over a 30-day period in an experiment using organic cow dung (Table 5.2), dropping from 5043 ± 586.7 mg kg^{-1} (DM) on day-5 to 3867 ± 50 mg kg^{-1} (DM) on day-35. A drop in S (in a closed system) is often an indication of increased H_2S production, as the only way S could decrease in a closed system is through biogas which is expelled on a regular basis (Further discussed in Chapter 6) (Massé *et al*., 2007; Marcato *et al*., 2008). Anaerobic digestates destined to be used as fertiliser fall within soil protection legislation, fertiliser or waste legislation or a combination thereof (Al Seadi *et al*., 2013). As part of the EU, the United Kingdom falls under both EU regulations and national regulations, and even more specific national regulations for digestate use as a fertiliser (PAS110, 2010). PAS110 sets limits on heavy metals, organic pollutants and pathogens in digestates, that are often stricter than the corresponding EU regulations (Al Seadi and Lukehurst, 2012). The aims of such regulations are to ensure high quality, safe fertiliser. Organic cow dung digestate falls well within the PAS110 (2010) limits for potentially toxic elements in whole digestate, when applied as fertiliser (Table 5.5). Dairy

farm slurry exceeded the PAS110 (2010) limits for some heavy metals in whole digestate (Table 5.5).

Table 5-5 Potentially toxic elements (mg kg^{-1} DW) in digestate derived from organic cow dung and dairy farm slurry, compared to PAS110 (2010) limits when applied as fertilisers. Data correspond to the day 5 sampling.

Digestate type	Cd	Pb	Ni	Zn	Cu	Cr
Organic Cow Dung (5.1)	0.15	6.8	4.8	96.7	18.3	3.0
Organic Cow Dung (5.2)	0.35	7.2	4.2	103.3	18.3	3.0
Dairy Farm Slurry (5.3)	0.60	6.3	5.8	1555.8*	840.4*	4.0
Dairy Farm Slurry (5.4)	1.56*	8.0	2.9	717.3*	336.5*	6.7
Pas110 (2010) limits - UK	1.5	200	50	400	200	100

Bracketed numbers refer to the Table from which the data were calculated as means on a dry matter basis.

In the dairy farm slurry digestate calculated from data in Table 5.3, Zn and Cu concentrations were 289% and 320% respectively above the PAS110 (2010) limits for potentially toxic elements if applied as fertiliser; Cd, Pb, Ni and Cr were all within the limits (Table 5.5). Additionally, dairy slurry digestate originating from a different experiment and therefore a different slurry collection time (data from Table 5.4), had concentrations of Zn, Cu and Cd that were 79%, 68% and 4% respectively above the PAS110 (2010) limits for potentially toxic elements (PTEs) if applied as fertiliser. Pb, Ni and Cr were all within acceptable limits (Table 5.5). It should be noted that even though the dairy farm slurry was collected from the same source, collection took place at different times. The differences in nutrient content (Table 5.3 and 5.4) and potentially toxic elements (Table 5.5) between the two sets of dairy slurry samples highlights the variability within feedstock and hence digestate quality, even from an apparently consistent source. The composition and safely of digestate is therefore highly dependent on the quality of feedstock used, but it is also influenced by a stable anaerobic digestion process, controlling parameters such as pH, temperature, concentrations of volatile fatty acids, retention time of feedstock, feedstock loading and many others. The PAS110 (2010) limits are designed to reduce possible risks to both plant and human life. Cd

toxicity symptoms in plants are easily identifiable, the most general symptoms include stunting and chlorosis (loss of green colouration of leaves), with Cd shown to interfere with the uptake, transport and use of several nutrients (Ca, Mg, P, K and Fe) and water (Das *et al*., 1997). Cd toxicity in humans can lead to kidney, bone and pulmonary damages, with uptake through the human gastrointestinal approximately 5% of total digested Cd (Jin *et al*., 2002; Godt *et al*., 2006). Zn toxicity in plants can lead to reduced yields and stunted growth and often occurs in agricultural soils treated with sewage sludge, however, Zn toxicity is far less of an issue than zinc deficiency (Broadley *et al*., 2007). Cu toxicity can often occur in plants through release of heavy metals into the environmental through mining, smelting, manufacturing and agricultural processes, with plant symptoms including reduced biomass and chlorosis (Yruela, 2005). In humans, Zn and Cu are inherently interlinked, with excessive absorption of zinc causing a suppression in Cu absorption along with nausea, vomiting and other such issues, Cu toxicity may cause long-term damage of the liver and kidneys (Valko *et al*., 2005). It must be pointed out, that at this time the dairy farm slurry is not used on a commercial scale for anaerobic digestion, although it is applied to the fields in an undigested form.

The nutrient and PTE content of the organic cow dung digestates between the two experiments (Tables 5.1, 5.2 and 5.5) was relatively consistent which is not surprising since the same batch of dung was used throughout. Nevertheless, variations in elemental content are evident, both between the two experiments and within digestion batches in each experiment. The anaerobic digestions carried out in these experiments were batch digestions and no new material was added. Apparent between 'digestion time' differences in elemental concentrations are likely to be due to the inherent variability of the organic material used, since total nutrients were measured after acid digestion. Anaerobic digestion can however act as a concentrator of micronutrients and heavy metals due to mass reduction during

anaerobic digestion (Govasmark *et al*., 2011), this is potentially the reason for some elements increasing in concentration over the sampling period. The exception to this is the decrease in S that occurred during digestion of organic cow dung (Table 5.2) because it is feasible that S would be lost as H_2S. That the same effect was not observed during anaerobic digestion of the organic dung during an experiment carried out at a different time is interesting. The most likely explanation for the difference is that the starting inoculum used for the different organic cow dung digestions contained different microbial consortia. Freshly voided dung was collected immediately before establishing the experiments and differences in the microbial composition of the voided dung inoculum are more likely than substrate differences in dung feedstock, because that originated from the same batch.

5.5 CONCLUSION/SUMMARY

- The elemental composition of the organic cow dung digestate in terms of nutrient content is comparable with reported values, and is within EU and UK regulations for use as a fertiliser. Heavy metals were within guidelines and as such deemed safe and at an acceptable level. There were slight differences in nutrient content between the two digestates throughout the sampling periods, indicating the variability in the feedstock.

- Sulphur loss during anaerobic digestion suggests the anaerobic digestion system is not functioning efficiency for purpose, that of producing CH_4. A biogas with higher than standard levels of H_2S can reduce the quality of biogas, as well as indicate a potential inhibition problem within the anaerobic digestion process.

- Dairy farm slurry produced digestate high in Cd, Zn and Cu, above limits imposed by PAS110 (2010) for digestates for use as fertiliser. These findings indicate the

importance of knowing, and controlling the input of feedstock to a farm slurry tank, but more importantly the inputs into an anaerobic digestion system if digestate is to be used as a fertiliser, as potential loss of digestate fertiliser use could induce large financial losses.

CHAPTER 6 - INVESTIGATING THE POTENTIAL OF BIOCHAR TO ENHANCE EFFICIENCY OF ANAEROBIC DIGESTION AND AMELIORATE THE NEGATIVE EFFECTS OF OXYTETRACYCLINE ON BIOGAS PRODUCTION

6.1 INTRODUCTION

Biochar, a carbon rich product, is the result of thermal degradation of biomass such as wood, manure or leaves in the absence of oxygen at comparatively low temperatures (<700°C), distinguished from charcoal only by the intended use (Lehmann and Joseph, 2009). Biochar has been reported to improve soil fertility, water retention, crop yields and reduce greenhouse gas emissions (Lehmann *et al*., 2009). Fertility improvement has been linked to both pH increase in acidic soils (van Zwieten *et al*., 2010) and by improved nutrient retention through more efficient cation exchange capacity and retention (Liang *et al*., 2006). Biogas production through anaerobic digestion often operates below optimal performance due to factors such as stability (pH for example), reactor design (suboptimal mixing) and other inhibitors, therefore optimisation of the overall process is still needed to achieve higher methane production (Ward *et al*., 2008). Low methane yields and process instability are often encountered within anaerobic digestion, often due to the instability and vulnerability of micro-organisms (Chen *et al*., 2008). In particular, when it comes to acid-forming and methane forming microorganism their nutritional needs, growth kinetics, physiology and sensitivity to environmental factors differ widely (Pohland and Ghosh, 1971), causing potential reactor instability if the balance between these needs is not met (Demirel and Yenigün 2002). Numerous researchers have aimed to enrich and strengthen microbes within anaerobic digestion (Kato *et al*., 2012; Yang *et al*., 2015), with the addition of supporting media having been shown as an effective immobilization method (Show and Tay, 1999). Biochar is a porous, carbonaceous and biostable material (Luo *et al*., 2015) that has been shown to have benefits when used as a soil amendment (Schulz and Glaser, 2012). However, is must be noted that even though biochar amendment has resulted in observed positive

effects for mycorrhizae and total microbial biomass, biochar is not necessarily always beneficial to soil biota abundance(Warnock *et al*., 2007) Additionally, negative plant responses due to biochar application have been reported (Mikan and Abrams, 1995), however the exact reasons of these responses need further research. Therefore it should be noted that although reports are mainly positive in regards to biochar application, biochar can also have limitations in its use. Luo *et al*. (2015) stated that the residual biochar in anaerobic digestates can be used to improve soil properties and that there should be no environmental risk to using biochar in anaerobic digestion and it could even be beneficial. It has been hypothesised that biochar would provide a high surface area for adhesion and growth of methanogenic consortia whilst reducing inhibition through absorbing chemical inhibitors, as has been shown with activated carbon (Wang and Han, 2012). Biochar as an organic and inorganic sorbent has been investigated a large amount, to reduce availability of contaminants and therefore reduce environmental risk (Beesley *et al*., 2011). Biochar as a sorbent has been shown to be effective, due to a negative large surface area and a high charge density, producing a greater sorption ability when compared to most natural soil organic matter (Zheng *et al*., 2010). The sorption capacity of biochar has been shown to include pharmaceuticals, including simazine, metsulfuron-methyl, sulfamethoxazole, tylosin and tetracycline (Zhang *et al*., 2013; Zheng *et al*., 2013; Guo *et al*., 2016). It must be noted however that competition for absorption sites can exist, Zhang *et al*. (2012) described competition on straw biochars between metsulfuron-methyl (a herbicide) and tetracycline (an antibiotic). Overall, research suggests biochar addition in an anaerobic digestion system may be beneficial, potentially increasing the performance and adding value as a soil amendment. The aim of this investigation was to quantify the effects of OTC and biochar addition on biogas production, biogas quality and digestate quality when introduced into a laboratory-scale digester *via* organic cattle dung.

Research question: Due to the known absorptive capacity of biochar and the increased surface area of relatively non-degradable particles acting as sites for biofilm formation, it was hypothesised that amendment of the feedstock with biochar would reduce the detrimental effect of the antibiotics. Therefore, the key objective of this chapter was to determine whether biochar could be used to ameliorate the negative consequences of contaminants present within an AD system.

6.2 MATERIALS AND METHODS

6.2.1 Experimental Design

Laboratory scale anaerobic digestion experiments were set up to evaluate the effect of the veterinary drug oxytetracycline (OTC) and biochar using organic cow dung as feedstock over a 35-day period at 35°C. Batch-wise experiments were set up using 120 ml serum bottles, only filled half way to 60 ml with dung (mixed with deionised water to give a 'slurry' containing 6% organic matter) to allow for headspace. Biochar was added to serum bottles to achieve 0%, 3%, 12% or 24% biochar on a dry weight basis. Headspace was flushed with nitrogen before being closed using thick butyl stoppers and aluminium crimps. Samples were distributed in a randomised block design between 3 water baths as previously described in Chapter 2. Temperature increased from ambient on day 0 by 5°C each day (2.5°C in the morning and 2.5°C in the afternoon) until desired temperature was reached. On day-15 of the experiment, OTC was dissolved in water and a 2-ml aliquot was injected into the serum bottles, to achieve the desired levels of OTC, of either 0 mg L^{-1}, 4.33 mg L^{-1} or 86.63 mg L^{-1}. Serum bottles of each treatment type were replicated 3 times, to achieve a total of 36 bottles, containing all variations of both OTC concentration and biochar percentage amendment.

6.2.2 Biochar Collection and Processing

Biochar was commercially sourced from Bioregional HomeGrown® (BioRegional Charcoal Company Ltd., Wallington, Surrey, UK), composed of mechanically chipped trunks and branches of *Fraxinus excelsior* L., *Fagus sylvatica* L. and *Quercus robur* L. and pyrolysed at 450°C for 48 h (Jones *et al*., 2011). The biochar was manually ground with a hammer and then a pestle and mortar, before going through various sieves to achieve a final working amount of biochar of particle size 0.1 - 1mm. A small particle size was chosen due to the laboratory scale of the anaerobic digestion units, making sure mixing of the digestates was still possible.

6.2.3 Feedstock and Inoculation

Organic cow dung was collected from a known organic herd of cattle as described in Chapter 2 (section 2.1).

6.2.4 Biogas Collection

Biogas samples were collected from each bottle and measured as described in Chapter 2 (section 2.2.3). Gas data are only shown up to day 25 although the experiment was extended to 35 days because yields were low towards the end.

6.2.5 Digestate Analysis

Digestate chemistry was analysed on day 35 when the experiment was terminated. Total carbon and total nitrogen were determined by placing 10 ml of digestate sample into foil trays and placing them in a 60°C oven for 48 hours. Once dried, samples were finely ground by mortar and pestle and 0.025 g weighed into individual tin capsules along with vanadium pentoxide (V_2O_5) to help fully oxidise the samples. Samples were analysed in a CNS Elemental Analyzer (Flash EA1112, CE Instruments, UK) along with cysteine, an amino acid standard.

Water soluble carbon and nitrogen were determined by making 10 ml of digestate sample up to 50 ml using deionised water; samples were then filtered through Whatman No.1 filter paper (Whatman, Maidstone, England). The resulting filtered liquid was diluted 1:10 (2 ml + 18 ml) and then further syringe filtered. Samples were run though a TOC-V/TN analyser (Shimadzu Corporation, Koyoto, Japan).

Total elemental concentrations were analysed following acid digestion as described in Chapter 2 (section 2.3.4).

6.2.6 Statistics

Statistical analyses were performed using Genstat 16th Edition (VSN International, Hemel Hempstead, UK). Two-way analyses of variance were conducted on pH, total solids, CN, and elemental concentrations, using antibiotic concentration and percentage biochar as factors, whilst block was the replicate shaking water bath in which samples were placed. Biogas yields, methane and carbon dioxide concentrations were analysed by repeated measures ANOVA and repeated measures ANCOVA using pH as the covariate. Normality was tested by plotting residuals against expected normal quantiles and post-hoc comparisons between means was based on least significant differences at the 0.05 probability level.

6.3 RESULTS

6.3.1 Biogas Production

Biogas production had a slow start-up period with low yields on days 5 and 10. By day 15, biogas production had increased considerably and a biochar-related enhancement of yield was evident for both the 12% and 24% amendments (time x biochar interaction, $p<0.001$, LSD = 4.26; for days 5 to 15). However, by day 20, this advantage was no longer evident, suggesting that any benefits of biochar in the reactor are transient (Figure 6.1), although the presence of char was not detrimental to gas yields later in the incubation (when data were

analysed without including results from OTC + biochar treatments). When the whole data set for days 20 and 25 was analysed, char was significant as a single factor and the pooled means indicate that higher concentrations of char negatively affected gas production (p=0.024). This contradicts the conclusions of the analysis when OTC was omitted from it. A larger data set will have more statistical power, but nevertheless it can be observed in Figure 6.1 (days 20 and 25) that there appears to be a trend towards lower gas yields in reactors where there is biochar together with the highest concentration of OTC (although not exclusively the highest concentration, see Figure 6.1). When these data were analysed separately across the range of OTC concentrations, but with just 0% and 24% biochar an interaction was evident in which gas production was significantly lower in the presence of both OTC concentrations and the highest biochar amendment (Table 6.1).

Table 6-1 OTC/biochar interaction in which the highest biochar treatment inhibited biogas production even at low OTC concentrations.

	Char (0%)	**Char (24%)**
No OTC	50.5	50.7
OTC (4.33 mg L^{-1})	45.0	39.2
OTC (86.63 mg L^{-1})	36.7	26.8
OTC x biochar interaction: p=0.021 (LSD=4.66)		

Overall, during the first 15 days of the experiment, 12% and 24% biochar additions led to a 17% and 13% increase in biogas production respectively, whilst OTC over days 20 - 25 caused an average decrease in biogas with each increase in antibiotic concentration (OTC as a single factor, p<0.001, LSD = 3.06; Figure 6.1).

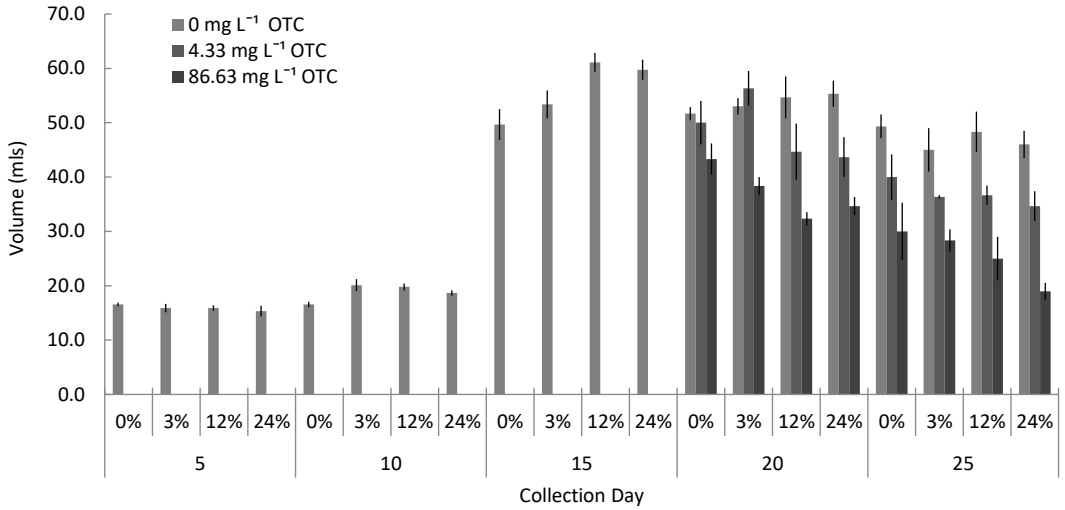

Figure 6-1 Biogas production over a 25 day period. OTC was added to organic cow dung ± biochar on day 15 to achieve OTC concentrations of 0, 4.33, 21.33 and 86.63 mg L^{-1}. Biochar concentrations were 0, 3, 12 or 24 %. Values are means of a minimum of 3 replicates ± SE.

Methane and carbon dioxide concentrations in the biogas were both negatively affected by biochar as a single factor from day 20 to day 25 ($p<0.001$ for both gases), although biochar did not alter CH_4 or CO_2 concentrations in comparison to the control from day 5 to day 20, prior to OTC amendment (Figures 6.2 and 6.3).

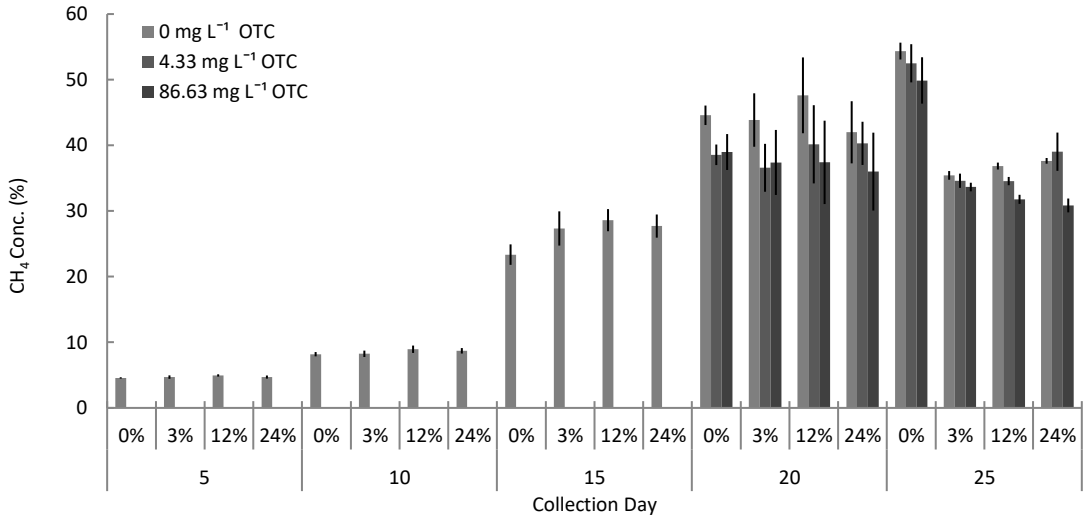

Figure 6-2 Methane concentration (percentage) of biogas over a 25-day period. OTC was added to organic cow dung ± biochar on day 15 to achieve OTC concentrations of 0, 4.33, 21.33 and 86.63 mg L^{-1}. Biochar concentrations were 0, 3, 12 or 24 %. Values are means of a minimum of 3 replicates ± SE.

OTC amendment on day 15 decreased both CH_4 ($p<0.004$, OTC as a single factor) and CO_2 ($p=0.040$, OTC as a single factor) concentration of the biogas with 4.33 mg L^{-1} and 86.63 mg L^{-1} OTC additions leading to an average decrease of 8% and 6% CH_4 respectively and a decrease of 13% and 6% for CO_2 respectively over days 20 and 25 (Figures 6.2 and 6.3).

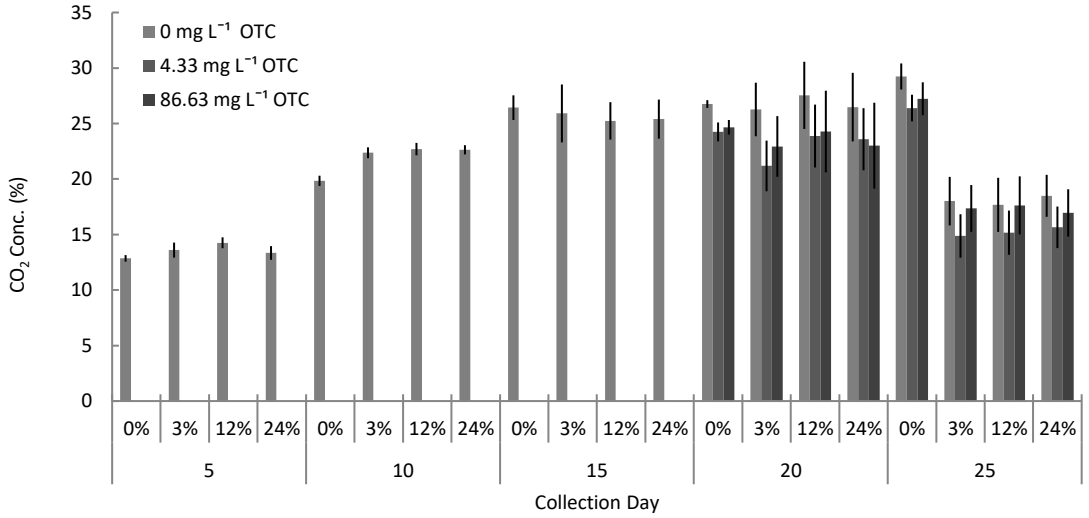

Figure 6-3 Carbon dioxide concentration (percentage) of biogas over a 25-day period. OTC was added to organic cow dung ± biochar on day 15 to achieve OTC concentrations of 0, 4.33, 21.33 and 86.63 mg L^{-1}. Biochar concentrations were 0, 3, 12 or 24 %. Values are means of a minimum of 3 replicates ± SE.

6.3.2 Total Solids and pH

pH was significantly affected by biochar as a single factor ($p=0.014$, LSD= 0.077); the digestates derived from the two highest biochar treatments had a significantly lower pH than the digestates from the controls. Whilst statistically significant, the difference in pH was around 0.1 of a pH unit and as such is unlikely to be biologically significant considering the within-char-treatment variation present (Figure 6.4a).

Biochar amendment and OTC addition as single factors both resulted in an increase in the total solids content of the digestates at the end of the experiment. OTC addition resulted in higher total solids in all samples apart from those containing 24% biochar when compared

to the zero-OTC treatments (p=0.050). Total solids were generally significantly higher with increasing biochar concentration (p<0.001, Figure 6.4b), although the digestates without biochar did not follow the pattern. The digestate had an average TS content of 6.8% ± 0.01 and a pH of 7.2 ± 0.01 (Figure 6.4).

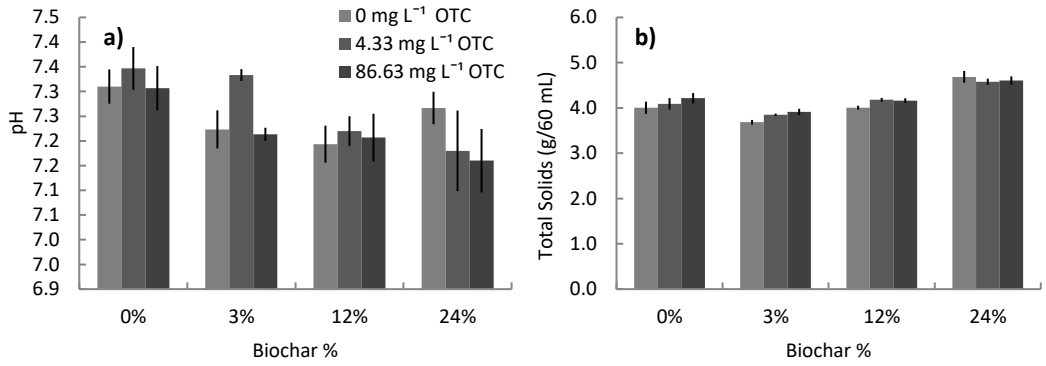

Figure 6-4 (a) pH values of anaerobic digestate derived from organic cow dung ± biochar (0%, 3%, 12% or 24%) and ± oxytetracycline (0 mg L^{-1}, 4.33 mg L^{-1} or 86.63 mg L^{-1}) and incubated for 35 days at 35°C. **(b)** Total solids content of the anaerobic digestates. Values are means of a minimum of 3 replicates ± SE.

6.3.3 Digestate Quality

Total organic carbon (TOC) and total nitrogen (TN) were calculated from the liquid fraction of the digestates. At the end of the incubation period, average TOC and TN concentrations of the digestates without added char were increased by the presence of OTC, particularly by the highest OTC concentration (86.63 mg L^{-1}), but this trend was not as obvious in the presence of biochar (Figures 6.5a and 6.5b). Nevertheless, for TOC data, the highest OTC amendment significantly increased the TOC content of the digestates (OTC as a single factor, p<0.001, LSD = 199.7). Pooled averages across the biochar concentrations for TOC were: OTC (0 mg L^{-1}) = 1594 mg L^{-1}; OTC (4.33 mg L^{-1}) = 1579 mg L^{-1}; OTC (86.63 mg L^{-1}) = 2002 mg L^{-1} TOC. The TOC concentration recorded in digestates amended with the highest OTC concentration was significantly higher than that of the other treatments.

TN data were interesting because a different pattern of response to OTC amendment was observed in the absence of biochar than in the presence of biochar, with the shifting response becoming more marked with increasing biochar concentration (OTC x biochar interaction, p=0.026, LSD = 71.55; Figure 6.5b). In the zero char samples, OTC increased digestate TN; the TN of the digestate with the lowest char amendment responded similarly; TN was unaffected by OTC when digestates contained 12% biochar; TN flipped in the 24% biochar samples and was higher in the absence of OTC.

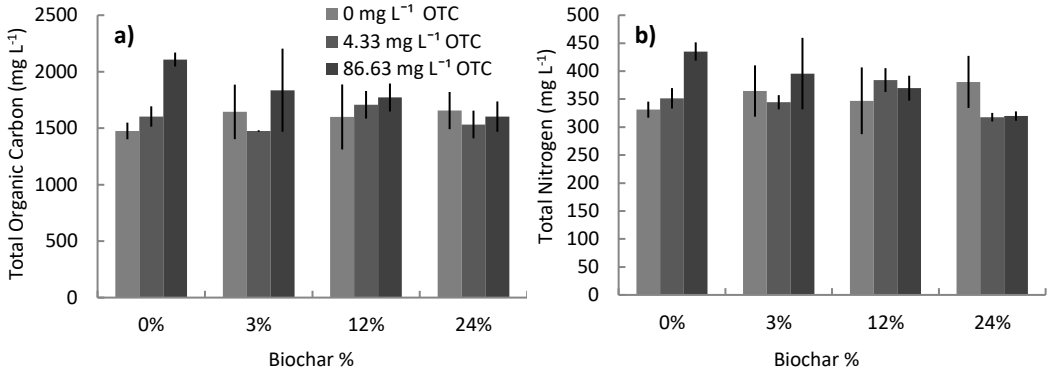

Figure 6-5 (a) TOC concentrations in experimental anaerobic digestate derived from organic cow dung ± biochar (0%, 3%, 12% or 24%) and ± oxytetracycline (0 mg L^{-1}, 4.33 mg L^{-1} or 86.63 mg L^{-1}) and incubated for 35 days at 35°C. **(b)** TN concentration of the anaerobic digestates. Values are means of a minimum of 3 replicates ± SE.

Total percent carbon and percent nitrogen based upon the dry matter of samples sacrificed at the end of the experiment (day 35) were both affected by biochar amendment. Digestate carbon (%) significantly increased with increasing biochar concentration (biochar as a single factor, p=0.022, LSD = 5.01). In contrast, biochar amendment adversely affected the percent nitrogen which decreased with increasing biochar concentration (biochar as a single factor, p=0.007, LSD = 0.269; Figures 6.6a and 6.6b).

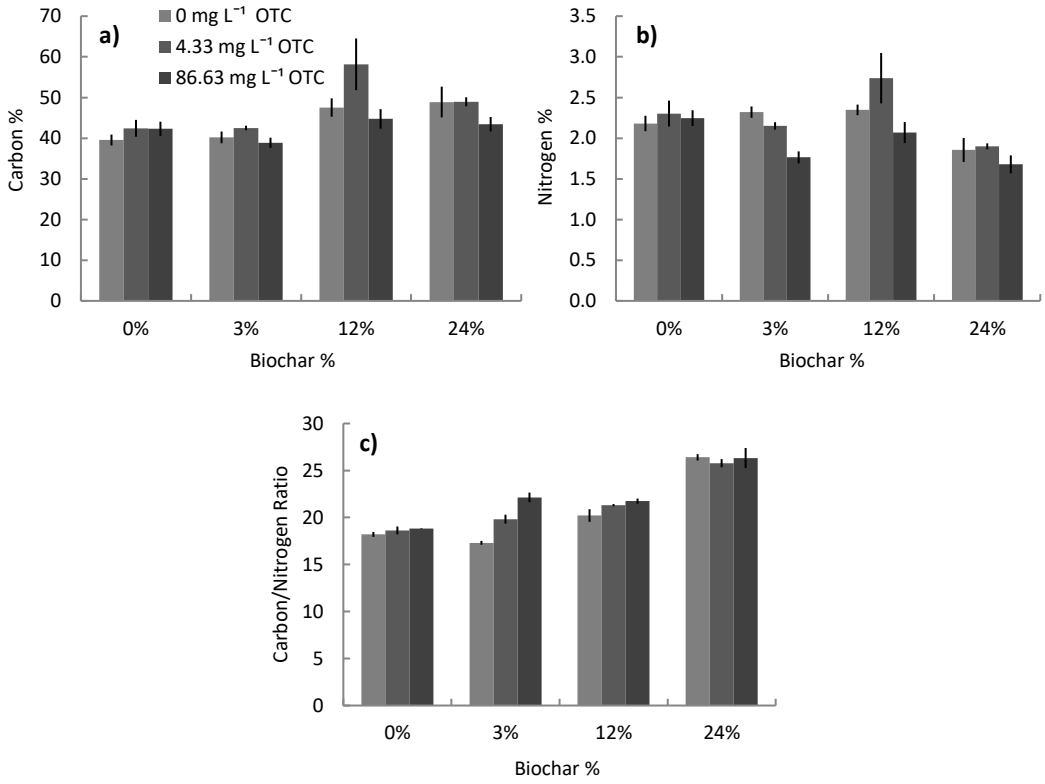

Figure 6-6 (a) Percentage C in experimental anaerobic digestate derived from organic cow dung ± biochar (0%, 3%, 12% or 24%) and ± oxytetracycline (0 mg L^{-1}, 4.33 mg L^{-1} or 86.63 mg L^{-1}) and incubated for 35 days at 35°C. **(b)** Percentage N **(c)** C/N ratio. Values are means of a minimum of 3 replicates ± SE.

OTC addition did not change either the percent carbon or percent nitrogen within the digestates. However, increasing concentrations of OTC significantly enhanced the C/N ratio in the 3% biochar treatments ($p<0.05$), caused predominantly by a drop in nitrogen rather than an increase in carbon (Figure 6.6c). Furthermore, OTC was significant as a single factor ($p=0.003$, LSD = 1.14) meaning that when data are pooled across all biochar concentrations, a trend towards higher C/N ratios in response to increasing OTC is observed.

Table 6.2 shows the average values (mg L^{-1} DM) of P, K, Ca, Mg, S, Mo, Fe, Mn, Cu, Na, Zn, Ni, Al and Co after 35 days of anaerobic digestion ± biochar at 0%, 12% or 24%. The biochar treatments appeared to have significantly reduced sulfur concentrations within the

digestates, with the 12% and 24% biochar amendment lowering digestate sulfur concentrations by 79% and 84% respectively ($p<0.001$; Table 6.2). Biochar at the 24% digestate amendment was associated with lower iron concentrations, whilst manganese and zinc increased. It must be noted however that these changes were in no way as substantial as the changes that occurred with sulfur. OTC addition did not alter any macro- or micro-nutrients concentrations, regardless of whether biochar was added or not.

Table 6-2 Macro- and micro-nutrients in anaerobically digested organic cow dung, with the addition of different concentrations of biochar. Values are means of a minimum of 3 replicates ± SE.

	Biochar (mg kg^{-1} DM)	0% Biochar (mg kg^{-1} DM)	12% Biochar (mg kg^{-1} DM)	24% Biochar (mg kg^{-1} DM)
P	257.2 ± 0.48	12178 ± 389.4	11026 ± 302.9	9634 ± 222.5
K	2367 ± 14.6	12125 ± 207.7	11411 ± 288.7	10546 ± 146.8
Ca	5031 ± 63.1	17936 ± 529.0	16248 ± 486.2	1426 ± 373.1
Mg	335 ± 2.2	6289 ± 130.4	5627 ± 92.5	4938 ± 96.7
S		7599 ± 861.3	1633 ± 167.3	1184 ± 260.2
Mo	0.06 ± 0.04	6.5 ± 0.32	6.0 ± 0.15	4.5 ± 0.64
Fe	95.4 ± 36	2392 ± 75.9	2092 ± 26.8	1736 ± 57.2
Mn	374.2 ± 2.9	194.6 ± 5.7	209.2 ± 8.2	211.6 ± 9.3
Cu	5.1 ± 0.23	20.0 ± 0.63	20.2 ± 0.44	17.3 ± 4.9
Na	40.3 ± 2.5	2335 ± 35.3	2356 ± 51.7	2010 ± 120.6
Zn	45.8 ± 0.05	93.0 ± 4.1	107.3 ± 11.2	107.5 ± 6.6
Ni	0.2 ± 0.02	4.6 ± 0.16	4.4 ± 0.15	3.9 ± 0.13
Al	8.24 ± 0.68	1233 ± 29.5	1148 ± 81.3	1034 ± 29.5
Co	0.16 ± 0	0.60 ± 0.02	0.58 ± 0.01	0.49 ± 0.04

6.4 DISCUSSION

6.4.1 Biogas Volume and Concentration

Throughout the 25-day experiment, all laboratory scale anaerobic digestion units produced biogas containing CO_2 and more importantly, CH_4. The volume of biogas and concentrations of CH_4 and CO_2 differed depending on both OTC and biochar concentration within the digestates. Biochar amendment encouraged biogas production during the first 15 days of the experiment (Figure 6.1) which corroborates reports that addition of charcoal or activated carbon to swine manure or cattle dung increased biogas yields during anaerobic digestion (Geeta *et al*. 1986; Kumar *et al*. 1987; Desai and Madamwar 1994). These authors hypothesised that the increase in biogas yields was due to the higher available surface area which could enable biofilm formation. This hypothesis has also been forwarded more recently by others (e.g. Wang and Han, 2012). Biochar and products such as activated carbon and charcoal are not the only additions to anaerobic digestion that have been thought to promote microorganisms. Zeolites are inorganic particles (Mumme *et al*., 2014) that can remove ammonia and ammonium ions through absorption and ion exchange (Milán *et al*., 2003; Ho and Ho, 2012), as well as being suitable for supporting microbial growth because of their high surface area and micro-porous structure (Fernández *et al*., 2007). The increase in biogas production (Figure 6.1) as a result of biochar addition, specifically observed on day 15 could have been the result of ammonia inhibition, or of biofilm formation on the surface of the biochar particles, or perhaps biochar stimulated degradation of the organic matter by exerting a priming effect (Kuzyakov, 2010) and therefore greater microbial activity. Since biochar is known to alter C/N ratios, thereby encouraging bacteria to sequester nitrogen (Martin *et al*., 2015) and since biochar amendment altered the C/N ratio of the digestates, it is feasible to hypothesise that initial sequestration of ammonium or ammonia inhibition could have occurred, thereby enabling a flush of growth and biogas production that was

observed on days 15 to 20. Ammonia inhibition by zeolites has previously been observed (Ho and Ho, 2012), hence in principle, ammonia inhibition could have occurred here, as shown by Mumme *et al*. (2014). Furthermore, biochar-fixed beds from anaerobically digested slurry have been demonstrated to be an efficient material for absorbing ammonium, with an effective slurry treatment time of 10-100 h with over 75% ammonium absorption happening within the first 10 h (Kizito *et al*., 2016). Ammonia has been widely attributed to the failings of biogas production (Hansen *et al*. 1998; Bujoczek *et al*. 2000; Sung and Liu 2003), possibly because of the 4 main groups of microorganisms associated with AD, methanogens are the least tolerant to ammonia and hence the most likely to cease growth due to its presence (Kayhanian, 1994).

OTC addition on day 15 caused a significant drop in biogas production for both concentrations of OTC on days 20 and 25 (Figure 6.1). Although biochar caused a decrease in biogas production on day 25, interactions between biochar amendment and OTC were tentative, indicating that biochar may only influence the actions of OTC under certain conditions. In a previous study, OTC and chlorotetracycline reduced biogas production by 42.5%, 56.5% and 64.1% as a result of spiking 10 mg L^{-1}, 50 mg L^{-1} and 100 mg L^{-1} respectively. Additionally, OTC has been shown to decrease biogas production in anaerobic digestion systems when fed with dung from cattle treated with OTC either as an intramuscular injection or as a food additive (Arikan *et al*., 2006; Ince *et al*., 2013). Interestingly, when mixed with swine manure, OTC did not inhibit anaerobic digestion in a similar experimental setup (Lallai *et al*., 2002). In the current experiment, OTC probably inhibited degradation of the solids within the digestate (Figure 6.6b). There was a stark decrease in both CH_4 and CO_2 production, of 33% and 39% respectively on day 25 in the biochar amended bottles (Figure 6.3 and 6.4). In a previous report investigating pyrochar, a 16% lower CH_4 yield was recorded from pyrochar amended units when compared to unamended controls, despite the pyrochar

initially producing more (Mumme *et al*., 2014). It was stated pyrochar as an additive showed early and strong signs of exhaustion (Mumme *et al*., 2014).

6.4.2 Total Solids and pH

Biochar amendment resulted in generally higher total solids by the end of the experiment (Figure 6.4b) compared to treatments with lower biochar additions. There are two possible reasons for this: The first is that because biochar amendment slowed down gas production towards the end of the experiment, this would in turn reduce substrate utilisation in the digestate. The second reason is the volume of additional recalcitrant material that was added to each bottle containing biochar. In anaerobic digestion, degradation occurs *via* microbial action, the high aromatic and molecular compounds found in biochar (pyrochar) (Lehmann *et al*., 2009) are resistant to anaerobic microbial degradation and are similar to lignin (Op den Camp *et al*., 1988). Both hydrochar and pyrochar are considered to be completely degradable under aerobic conditions in soil, however, it should be noted that hydrochar degrades one order of magnitude faster than pyrochar (Bai *et al*., 2013). In anaerobic conditions complete degradation does not occur, due to inert and non-labile fractions (Mumme *et al*., 2014). These factors may explain the high total solids content found with increased biochar addition, anaerobic digestion did not significantly degrade biochar.

6.4.3 Digestate Quality

In the context of anaerobic digestion, the effects of biochar on nutrients, either by desorption from the char, or by adsorption or precipitation are little understood. However, chars are known to adsorb metals from solution and successfully remove ammonium from aqueous solutions (Yao *et al*., 2012). However, behaviour of biochar is contingent with its composition and in turn will depend on the feedstock with which it is made. It is perhaps not surprising that Yao *et al*. (2012), using biochar, successfully reduced nitrate, ammonium and

phosphate leaching by 34.0%, 34.7% and 20.6% respectively, yet when they used a different char, phosphate leaching was enhanced.

The amount of sulfur present within the test biochar is unknown, however, values from oak biochars are in the range between 160-170 mg kg^{-1} (Cheah *et al*., 2014). At the end of the experiment, total sulfur concentrations within the digestates amended with 12% and 24% biochar were significantly lower (1633 mg kg^{-1} DW and 1184 mg kg^{-1} DW respectively) than those of the unamended controls (7599 mg kg^{-1} DW). Since the system was a closed one, the only means of removal was *via* the biogas. It may therefore be hypothesised that sulfate reduction was enhanced in the presence of biochar, maybe because of sorption of sulfate onto biochar, allowing increased access by sulfate reducing bacteria/archaea and producing hydrogen sulfide (H_2S) which was removed *via* biogas discharge. The reduction in CO_2 and CH_4 concentrations on day 25 (Figures 6.2 and 6.3), coupled with no obvious reduction in biogas volume (Figure 6.1) indicates a replacement of the 'missing' gas fraction which could be H_2S and further supports this hypothesis. There is currently a lack of research into sulfur losses during anaerobic digestion, with Möller and Müller (2012) stating that further studies are necessary to improve our understanding of sulfur losses during anaerobic digestion and subsequent fertiliser value of the digestate. Sulfur losses in anaerobic digestion have previously been reported, these have mostly been attributed to the presence of H_2S in biogas (Massé *et al*. 2007; Marcato *et al*. 2008). Sulfur most commonly occurs in nature in combined forms as sulfide and sulfate. In anaerobic digestion, sulfate is readily reduced to sulfide by sulfate reducing bacteria (Koster *et al*., 1986). Sulfide can inhibit methane production, primarily by way of competition for common substrates by sulfide reducing bacteria (Harada, 1994), but also possible inhibition can occur via toxicity of the sulfide to other bacteria groups (Oude Elferink *et al*., 1994). Furthermore H_2S can be toxic due to possible diffusion in cell membranes (Tursman and Cork, 1988; O'Flaherty *et al*., 1999). Sulfur is however a required

nutrient for methanogens (O'Flaherty *et al*., 1999). Sorption of copper to biochar has been proven, whilst sulfate sorption to biochar was negligible in the same study. There is clearly much scope to understand these dynamics, particularly given that behaviour of different chars will vary (Borchard *et al*., 2012).

CHAPTER 7 - GERERAL DISCUSSION

7.1 APPROACH AND GENERAL FINDINGS

The key findings for each experimental chapter are listed below in the summary table.

Table 7-1 Key findings of Chapters 3, 4, 5 and 6.

Chapter	Key Findings
Chapter 3 *OTC/biogas*	• Oxytetracycline addition to anaerobic digestion feedstock caused a significant drop in biogas production for all experiments, with addition on day 15 causing the most significant drop. • Generally, higher concentrations of oxytetracycline caused a greater reduction in biogas production. • Oxytetracycline amendment generally negatively altered the concentration of CH_4 but not of CO_2 in the biogas. • pH dropped significantly in organic cow dung before recovering, whilst dairy farm slurry pH remained consistent.
Chapter 4 *TYL/biogas*	• Tylosin addition to anaerobic digestion feedstock caused a significant decrease in biogas production (greater reduction than oxytetracycline) in all experiments, with addition to naive organic cow dung causing the greatest reduction in biogas. • Addition of tylosin on day 15 to organic cow dung caused the same level of biogas reduction, regardless of tylosin concentration, whilst for other tylosin based experiments higher concentrations resulted in a further inhibition of biogas. • Tylosin addition on day 0 to organic cow dung mainly caused the CO_2 concentration to drop, whilst when added on day 15, CO_2 concentration was unaffected, but CH_4 concentration dropped. Tylosin addition to dairy slurry caused a variety of effects to both CH_4 and CO_2 concentrations. • Again, pH dropped significantly in organic cow dung before recovering, whilst dairy farm slurry pH remained consistent.
Chapter 5	• Total nutrients of organic cow dung (with added water) fall within PAS110 (2010) limits and are in comparison with results found elsewhere for similar feedstock.

Digestate Nutrients	• Sulphur reduced over the experimental period in dairy farm slurry digestate, thought to be converted to H_2S by sulphate reducing bacteria, indicating an inefficient system for proposed purpose. • Dairy farm slurry had high levels of CH, Zn and Cu, above limits imposed by PAS110 (2010) for digestate use of fertiliser.
Chapter 6 *Biochar*	• Biochar addition increased biogas production by a small amount within the first 15 days of the experiment (specifically on day 15). • Oxytetracycline addition on day 15 caused a significant drop in biogas production, with higher concentrations producing a greater biogas reduction. • Biochar addition caused a significant drop in both CH_4 and CO_2 concentration on day 25, hypothesised to be caused by an increase in hydrogen sulphide (H_2S). Biochar addition led to substantial decreases in sulphur, thought to be caused by the accumulation of sulphur through sorption, which was then targeted by sulphate reducing bacteria producing H_2S.

7.1.1 Effects of Oxytetracycline and Tylosin

Pharmaceuticals found in the environment mostly result from human origin, however veterinary pharmaceuticals also occur in water as a result of aquaculture or agricultural run-off (Boxall *et al*., 2003; Lissemore *et al*., 2006), with the most likely pathways to the environment being the application of slurry or manure from intensive agriculture, and from direct application in urine and dung from grazing animals (Kools and Boxall, 2008). For example, pharmaceuticals derived from sewage sludge are able to transfer to crops (Wu *et al*., 2010), whilst repeated application of manure to soil accumulates quinolones and tetracyclines (Hamscher *et al*., 2005). However, soil amended with swine manure containing chlorotetracycline and tylosin resulted in accumulation of chlorotetracylines in plant tissue, but not of tylosin (Kumar *et al*., 2005). This highlights that pharmaceutical drugs can be detected and may be present in both amended soils and in plants, and one of the likely pathways is through animal wastes. Topsoil (30 cm) collected from a crop field directly after

application of cow manure (from calves previously administered oral doses of oxytetracycline and tylosin) harboured 6-7 μg kg^{-1} of oxytetracycline, but no detectable tylosin (De Liguoro *et al*., 2003). In a sandy soil fertilized annually with manure and slurry for recurrent years, 43 - 199 μg kg^{-1} of tetracycline and 3.7 - 7.3 μg kg^{-1} of chlortetracycline were found, but no oxytetracycline or tylosin were detected (Hamscher *et al*., 2002). It should be noted that Hamscher *et al*. (2002) reported no detectable tetracyclines or tylosin in soil or ground water at the same sampling site. Concentrations of tylosin and oxytetracycline in cattle manure left outside for 135 days fell from 32.8 to <0.01 mg kg^{-1} and from 366.8 to 2.1 mg kg^{-1} respectively (De Liguoro *et al*., 2003), with the degradation of pharmaceuticals in animal manure mainly a result of microbial activity (Song and Guo, 2014). The concentrations of tylosin and oxytetracycline found within farm-yard manure adds weight to the antibiotic concentrations chosen within this thesis (Chapters 3, 4 and 6). However, in this regard it should be stated that chapters within this thesis only utilized single antibiotics in each experiment, whilst in a commercial anaerobic digestion unit a consortium of pharmaceuticals is far more likely. To overcome this and make the work more relevant to the 'real world', dairy slurry was used as a non-naïve feedstock in addition to the 'clean' organic dung.

Tylosin added to autoclaved soil reportedly had a half-life of 7-8 days, whilst when added to water its half-life was 200 days (Hu and Coats, 2007); but the half-life of tylosin in the aqueous phase in manure may be <2 days (Loke *et al*., 2000). It is thought that such a short half-life of tylosin within manure might be due to sorption onto solid particles rendering it less detectable, or to biodegradation (Hu and Coats, 2007). Within anaerobic digestion specifically, the half-life of oxytetracycline was calculated at 56 days in a mesophilic anaerobic digester with a retention time of 35 days (Arikan *et al*., 2006). In contrast the half-life of tylosin was estimated at <2.5 hours (Angenent *et al*., 2008), which is in agreement with the findings of Zilles *et al*. (2005) who were unable to detect any tylosin in treated swine

waste despite known sub-therapeutic use. Finally, in stored swine manure, bacteria, *Enterococcus fecalis* and *E. coli* were all found to be resistant to at least one of the following pharmaceuticals: tylosin, tetracycline, ampicillin, chloramphenicol, kanamycin, streptomycin or sulphonamides, with evidence of enterococci being able transfer the tetracycline resistance phenotype to *Bacillus thuringiensis* (Haack and Andrews Jr., 2000; Cotta *et al*., 2003). These reports highlight the possibility of accumulation of pharmaceuticals within the environment, specifically in the soil and manure, but also highlight the potential risk of uptake by plants destined for consumption. However, it is also apparent that the persistence and accumulation of pharmaceuticals in the environment are varied depending on a large number of environmental and agricultural factors, and therefore it is difficult to accurately predict concentrations and behaviour of pharmaceuticals in different matrices and under different conditions. Even before the addition of slurry/feedstock to anaerobic digesters, there is clear evidence that both oxytetracycline and tylosin are subject to degradation, however tylosin seems much more susceptible to degradation with a much shorter half-life, degrading to a non-detectable limit from almost immediately to <2 days. These findings question whether tylosin would ever reach an anaerobic digester in any great quantity, but with added evidence of tylosin resistant micro-organisms found within manure the effects of tylosin in anaerobic digestion may further be minimalised. Oxytetracycline however, persists in the environment far longer than tylosin, with reports indicating a half-life of >50 days in an anaerobic digester and examples of oxytetracycline accumulation within soil from repeated manure applications. Oxytetracycline therefore is far more likely to reach an anaerobic digestion unit, and furthermore has the potential to withstand the anaerobic digestion process. It should be noted however that oxytetracycline would still significantly degrade before use in an anaerobic digestion system, with concentrations likely to drastically reduce as manure is collected and stored.

In the current organic cow dung (naïve) experiments, both oxytetracycline and tylosin had a large negative impact on biogas production and biogas quality. This was to be expected, as theoretically the naïve feedstock would have never interacted with these drugs before, and therefore pose no resistance to the action of them, as seen by a similar experiment conducted by Coban *et al*. (2016). This was demonstrated by the immediate and uniform negative response of biogas production and biogas quality when both oxytetracycline and tylosin were added on day 15. In reality however, the probability of a completely naïve fed digester is highly unlikely, with feedstocks often ranging in sources to ensure anaerobic digester efficiency/productivity throughout the year, hence a completely organic, pharmaceutical free digester is very improbable.

A more likely scenario would be feedstock similar to the dairy farm slurry used within the experiments described in this thesis. The dairy farm slurry originated from cattle permanently housed indoors; oxytetracycline had previously been detected in the farm slurry, whilst tylosin was under the detection limit and hence not found (Dave Barrett, pers comm., Appendix I). Furthermore, in the current study Cd, Zn and Cu were measured in high concentrations within the dairy slurry (thought to be from the addition of the contents of foot baths), furthering the possibility of antibiotic resistance due to additional selection pressures exerted by the metals (Ji *et al*., 2012). Dairy slurry with oxytetracycline or tylosin added on day 15 provided a more realistic approach to determining whether pharmaceuticals can affect the anaerobic digestion process. Even though this approach is more realistic, the evidence of rapidly degrading oxytetracycline and tylosin in the environment as well as the mixing of feedstocks to produce a high yield of biogas indicates that even the low concentrations of drugs tested (4.33 mg L^{-1}) probably exceeds real life expectations. However, the data on the effects of oxytetracycline at 4.33 mg L^{-1} in non-naïve

feedstock on anaerobic digestion could be seen as a worst case scenario likely to affect a commercial anaerobic digestion unit.

A review of anaerobic digestion units on UK farms reported no failures or problems caused by veterinary pharmaceuticals, but rather reported barriers to the technology such as reliability issues, increased digester complexity, capital costs and access to enough quality feedstock. With these factors in mind, it is highly unlikely that veterinary pharmaceuticals in feedstocks would put off a farmer feeding them into an anaerobic digestion system, even considering the negative impacts. In terms of a commercial anaerobic digester, experimental results obtained here suggest an antibiotic-driven decrease in biogas yield of 5.6% with neither CO_2 nor CH_4 being largely affected. Crudely scaling that up from a 120 ml bottle to a hypothetical 265m^3 digester, the drop in total volume of biogas would be approximately 33.25m^3 over a 15-day period. If the biogas was fed into a combined heat and power unit, it is doubtful whether the drop in biogas production would affect run-time of the unit, a likely effect however would be a drop in its potential capacity. There may be other costs associated running with a less efficient anaerobic digester, such as processing inefficiently digested slurry and lost fertiliser quality.

7.1.2 Amelioration

Inhibition of anaerobic digestion on a commercial scale can lead to a shut-down of the digester, or cause process inefficiencies, possibly causing more severe monetary losses. Veterinary pharmaceuticals have been shown to have the potential to severely decrease anaerobic digestion efficiency through decreased biogas production and biogas quality. One potential amelioration method under consideration is that of adding biochar to the anaerobic digester in order to enhance methane production. Biochar has the potential to be highly sorbent due to a large negative surface area and high charge density, with the sorption

capacity being demonstrated to include the pharmaceuticals simazine, sulfamethoxazole and importantly oxytetracycline and tylosin (Zheng *et al*., 2010; Zheng *et al*., 2013; Zhang *et al*., 2013; Guo *et al*., 2016). Overall, biochar did not appear to ameliorate the negative effects of oxytetracycline within the experiment. Biochar addition in the absence of oxytetracycline led to increased biogas production early on in the experiment, but this was not maintained throughout. Once oxytetracycline was added to the setup, biochar did not remedy the detrimental effects of the oxytetracycline; at best there was no interaction and at worst, there was some evidence that a high concentration of biochar enhanced the negative effect of the oxytetracycline, but this needs further consideration.

Overall, veterinary pharmaceuticals in the anaerobic digestion process can cause a large negative impact as shown in previous chapters, the real life likelihood of such an event occurring due to various factors such as drug degradation, antibiotic resistant genes, heavy metal contaminants, non-naïve feedstocks and dilution of pharmaceuticals remains low, with the cheapest and easiest amelioration to possible negative effects in anaerobic digestion being the safe storage of contaminated feedstock to allow natural degradation of the drug to occur.

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APPENDICES

Appendix I

Appendix I is a preliminary analytical study to demonstrate expertise and capacity, for success was required in order to make future funding applications credible and successful. The aims of the feasibility study were:

- Determine which antibiotics had been historically used at the University of Nottingham's dairy farm.
- Optimise equipment operating condition for the antibiotics found.
- Develop a suitable detection method.
- Develop a simple extraction method to recover antibiotics from milk, faeces and slurry samples.
- Undertake initial screening of samples.

Author Contribution

Dave Barret and Catharine Orori in collaboration with Dov Stekel, Christine Dodd, Stephen Ramsden, Richard Emes and Rachel Gomes.

Quantifying the emergence of antimicrobial resistant human pathogens in livestock farming: initial feasibility study on the detection of antibiotics in dairy farm slurry

Pharmacy School Research Committee funded project

Dave Barrett, Catharine Ortori

Collaboration with Dov Stekel, Christine Dodd, Stephen Ramsden (Biosciences), Richard Emes (SVMS); Rachel Gomes (Engineering)

Summary

Information has been gathered on antibiotic use at the University Farm and a comprehensive list of antibacterial agents has been generated which would potentially require monitoring in samples from the dairy herd, specifically in samples of slurry. LC-MS/MS methodology has been established for the simultaneous profiling of sixteen antibiotics in farm slurry, faeces and milk. A simple extraction procedure using methanol (4:1 with sample) has proved suitable to give an acceptable recovery of all the antibiotics. The initial screening of samples showed the presence in slurry and faeces of amoxicillin and oxytetracycline, and in milk of penicillin G and trimethoprim.

This preliminary work has established the feasibility of monitoring antibiotics in farm slurry. The next stage of the project will optimise the analytical procedures so that quantitative monitoring can be established.

Introduction

The aim was to create analytical methodology to assess the feasibility of analysing six classes of antibiotics (aminoglycosides, tetracycline, cephalosporins, macrolides, sulphonamides and β-lactams) in cow slurry. This bid is linked to matched funding from the School of Biosciences for genome analysis of multi-drug resistant bacterial strains isolated from slurry and for mathematical modelling/prediction of the spread of antimicrobial resistance in cattle populations.

The wide range of compound chemical structures creates an analytical challenge both in terms of sample extraction and chromatographically. The majority of antibiotic studies target a small selection of antibiotics and are usually confined to one group of chemically similar compounds. A few workers have addressed the multiclass issue using either volatile ion-pairing agents or by using multiple sequential LC-MS methods (Parthasarathy, in preparation). An ion-pairing agent, heptafluorobutyric acid (HFBA), has been commonly used (for example, Gbylik-Sikorska, 2015) during the extraction and analysis of poorly retained antibiotic compounds, such as the aminoglycosides hence the feasibility of using HFBA is investigated in this preliminary work. Despite the separation advantages of ion-pair chromatography, the addition of the ion-pairing agents such

as HFBA is known to be detrimental to MS electrospray signal detection with a resulting loss of sensitivity.

Aims for this feasibility study:

1. Determine which antibiotics have been in use at the University's dairy farm, situated at Sutton Bonnington over the last 2 years.
2. Optimise LC-MS operating conditions for each antibiotic, following procurement of standards.
3. Develop a suitable LC-MS method and investigate the advantages and disadvantages of using the volatile ion-pairing agent, heptafluorobutyric acid (HFBA) including ion suppression effects
4. Develop a simple extraction method to recover antibiotics from the milk, faeces and slurry samples to detect the presence of antibiotics (at this stage non-quantitatively).
5. Undertake initial screening of samples of slurry, faeces and milk obtained from cows from the University's dairy farm

Strong preliminary analytical data to demonstrate expertise and capacity for success is required in order to make the planned future funding application credible and successful.

Experimental

Samples provided: Milk from dairy cows with/without mastitis, faecal matter and slurry pit contents from the School of Veterinary Studies at Sutton Bonnington. These samples were stored at 4°C locally until transfer to the School of Pharmacy Boots Building, where they were briefly stored at -20°C.

The extracted samples were stored at -80ºC prior to analysis.

Type of analysis: Liquid chromatography-tandem electrospray mass spectrometry (LC-MS/MS)

Analyst: Dr Catharine Ortori, Centre for Analytical Bioscience, School of Pharmacy, University of Nottingham

Equipment:

- LC: A modular HPLC system from Agilent, 1100 comprising vacuum degasser, binary pump, cooled autosampler , column oven
- MS: Waters Quattro Ultima
- Centrifuges, ThermoFisher Heraus Multifuge IS and Harrier 18/08
- Jouan centrifugal evaporator.

Sample Extraction Methods:
Milk. Aliquots (2ml) were mixed with 8ml cold methanol, vortexed for 5min, centrifuged and the supernatant dried and reconstituted in 0.2ml 80%methanol.

Faecal Samples. Approximately 4g aliquots were dispersed and washed by vortex mixing with 8ml methanol for 20minutes. The samples were then prepared as above.

Slurry samples. 7ml slurry aliquots were washed in 21ml methanol and treated as above.

Analytical details: Two types of LC mobile phases were tested, either with 0.1% formic acid or 0.025% HFBA ion-pairing agent. The column was a Phenomenex Gemini 150x2.1mm, which by the use of 100% aqueous mobile phase, should improve retention of more polar compounds.

The LC gradient was as follows:

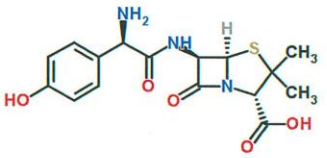
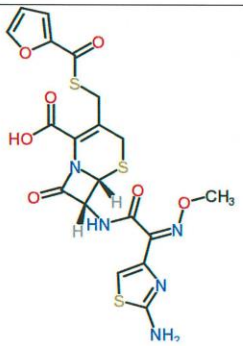
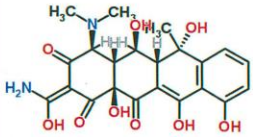
Time (min)	A% Aqueous	B% Acetonitrile	Flow (µl/min)
0	100	0	600
5	66.5	33.5	300
15	0	100	600
16	100	0	600
18	100	0	600

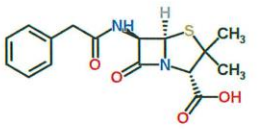
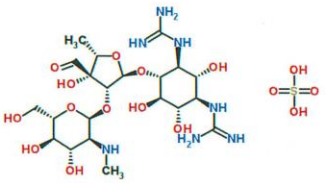
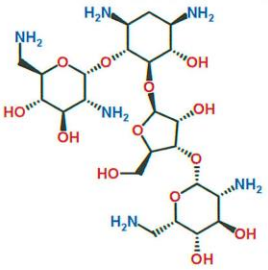
RESULTS

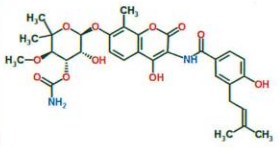
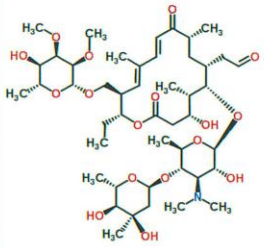
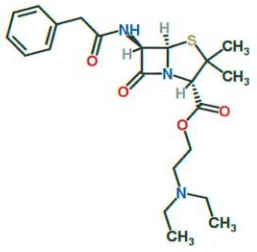
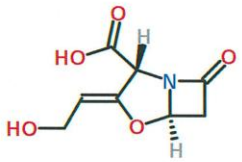
1. List of antibiotics that have been in use at the University's dairy farm during the 2-year period 2012-2013

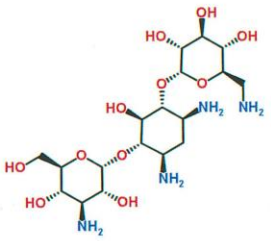
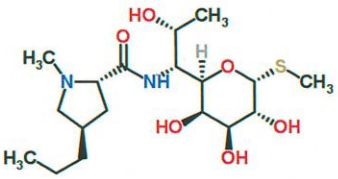
With the help of Dr Chris Hudson and Prof Jon Hobman the following list of antibiotics in use has been compiled. It is not clear that all of the listed antibiotics have been used over the last two years, but for completeness all antibiotics which potentially could have been used have been included in the list. These are shown in Table 1 below with associated chemical structure and physicochemical properties.

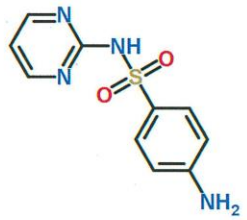
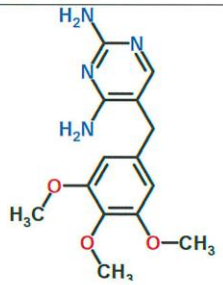
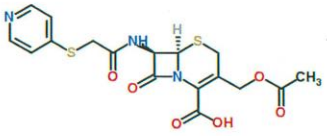
Table 1: List of antibiotics

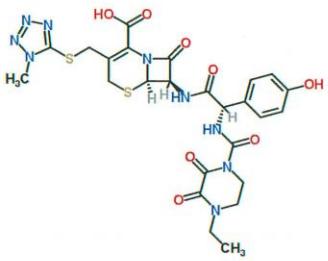
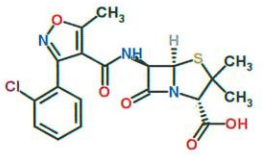
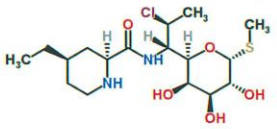
Name	formula	MW	pK_a	Structure
Cefquinome	$C_{23}H_{24}N_6O_5S_2$	528.6		
Amoxicillin	$C_{16}H_{19}N_3O_5S$	365.4	2.3	
Ceftiofur	$C_{19}H_{17}N_5O_7S_3$	523.56	3.7?	
Oxytetracycline	$C_{22}H_{24}N_2O_9$	460.434	3.3,7.3. 9.1	

Name	formula	MW	pK_a	Structure
Penicillin G	$C_{16}H_{18}N_2O_4S$	334.390	2.8	
Streptomycin sulphate	$C_{21}H_{41}N_7O_{16}S$	679.653 (581.574)	10.9, 11.9	
Neomycin sulphate (Framycentin)	$C_{23}H_{46}N_6O_{13}$	614.644	10, 12.9	

Name	formula	MW	pK_a	Structure
Novobiovin (read as Novobiocin)	$C_{31}H_{36}N_2O_{11}$	612.6243	4.3, 9.1	
Tylosin	$C_{46}H_{77}NO_{17}$	916.100	7.73	
Penethamate Penicillin G 2-Diethylaminoethyl Ester	$C_{22}H_{31}N_3O_4S$	433	8.2	
Clavulanic acid	$C_8H_9NO_5$	199.161	3.32, -2.6	

Name	formula	MW	pK_a	Structure
Cephalexin	$C_{16}H_{17}N_3O_4S$	347.389	3.45, 7.44	
Kanamycin	$C_{18}H_{36}N_4O_{11}$	484.5	9.75, 12.11	
Lincomycin	$C_{18}H_{34}N_2O_6S$	406.537	8, 12.4	

Name	formula	MW	pK_a	Structure
Sulphadiazine	$C_{10}H_{10}N_4O_2S$	250.277	6.99	
Timethoprim	$C_{14}H_{18}N_4O_3$	290.3177	7.1	
Cefapirin	$C_{17}H_{17}N_3O_6S_2$	423.463	3.5	

Name	formula	MW	pK_a	Structure
Cefperazone	$C_{25}H_{27}N_9O_8S_2$	645.667	3.38	
Cloxacillin (Oxacillin)	$C_{19}H_{18}ClN_3O_5S$	435.881	3.75	
Pirlamycin	$C_{17}H_{31}ClN_2O_5S$	410.956	8.5	

RESULTS (continued)

2. Optimise LC-MS operating conditions for each antibiotic following procurement of standards.

Antibiotic analytical details including optimised mass spectrometry settings are summarised in Table_2. The aminoglycosides, being hydrophilic, were unretained by the reversed phase method. All other antibiotics eluted in a useful time, the entire set eluting within 12minutes. When necessary these preliminary parameters can be transferred to a premium instrument for greater sensitivity with minimal method development.

3. Develop a suitable LC-MS method and investigate the advantages and disadvantages of using the volatile ion-pairing agent, heptafluorobutyric acid (HFBA) including ion suppression effects

The addition of the ion pairing agent improved retention of two of the three aminoglycosides but the signal of most of the analytes was reduced to 25% compared to the reversed phase method. Because these antibiotics are of uncertain interest to the project, the signals were monitored, recognising that further work would be required if the group was of importance. The rest of the experiment was continued using rp-chromatography, using the acidic mobile phase.

4. Use a simple extraction method to recover antibiotics from the milk, faeces and slurry samples to detect the presence of antibiotics.

All of the antibiotics are soluble in either water, methanol or acetonitrile mixes. Therefore as an initial method it seemed reasonable to wash the solids and slurry with methanol and to precipitate any protein in the milk samples. All sample types contained water although of unknown proportion. Recovery experiments are an important component of this type of method development. However, no attempt to measure extraction efficiency was made at this stage, due to time constraints. Larger sample volumes than typically were used at this stage of crude extraction.

5. Undertake initial screening of samples of slurry, faeces and milk obtained from cows from the University's dairy farm

The samples were analysed against a dilution series of standards. Table 4 highlights those samples in which the antibiotics were detected. Many of the chromatographic traces showed peaks at elution times elsewhere in the chromatogram, reflecting the complexity and size of the sample and the lack of sample clean-up. In this series of experiments peaks were identified by matching with the standard. Even so, the sample complexity raises issues regarding suppression of ionisation. No solid-phase extraction was attempted but would be a useful route to explore. ScEME houses an large scale extraction facility to which access would be essential for further method development.

The following are comments on the detection of antibiotics. Trimethoprim is noted as ambiguous although significant signals observed against a high background signal, which was due to this compound was resident in the LC-MS system from another current application. PenecillinG was clearly detected in some of the milk samples as expected as the preparation is applied locally. The corresponding faeces samples showed a small signal against a very noisy chromatogram baseline.

Oxytetracycline and amoxycillin were detected in faeces samples and slurry. The chromatogram due to cefiotur showed a large distinct peak in some of the milk samples, at an elution time shorter than the standard. It could be speculated that this represents a more hydrophilic metabolite and warrants further investigation.

Conclusions and plans for future work

The aim of the work was to investigate the feasibility of simultaneously measuring a comprehensive range of antibiotics in cow slurry, faeces and milk samples. There are many published methods to assay antibiotics by LC-MS, most of which focus on a small number of antibiotic classes due to the diversity of structures and hence are not suitable for our purpose. The comprehensive profiling method proposed here opens up the possibility to screen for multiple antibiotic groups but has highlighted some of the analytical difficulties involved. The diversity of structures is a factor that influences several aspects of the analysis and will need to be addressed. Were the aminoglycosides to be proved necessary for the study, further LC method development would be required. One approach would be to create two methods analogous to that of Parthasarathy's; this however would require higher through-put than during previous projects. A second approach would be to revisit and optimise the use of ion paring agents or alternative LC chemistries, resorting to normal or HILIC type methods. The Centre for Analytical Biosciences hosts several alternative LC-MS systems offering greater sensitivity than that used for this preliminary study.

The study aim did not include the search for metabolites or degradation products but it seems reasonable to expand the analysis to search and include any positively identified compounds in the absence of the native antibiotic.

The current method has proved feasible for the β-lactam antibiotics have been highlighted as major interest. Initially it was proposed that larger sample volumes could be prepared by large scale SPE. This was successful as used for a similar study by one of our post-graduate students Shridharan Parthasarathy from the School of Engineering. For the sake of speed, this yet has to be attempted for this different set of diverse compounds but would enable more efficient sample clean-up and greater concentration of the samples.

While method development can be performed on the current instrument, use of our AB Qtrap would offer greater sensitivity for sample analysis allowing the use of HFBA. The speed of this instrument also would enable a larger number of analytes to be monitored. This will be of some use where antibiotic metabolites and degradants were to be monitored.

Table 2: The LC-MS details of the 16 antibiotics used during this study.

Name	formula	Salt?	MW	Q1>Q2	Dwell	CV	CE	Retention Time (min)
Sulphadiazine	C10H10N4O2S		250.277	251.10>156.10	0.1	35	18	7.0
Trimethoprim	$C_{14}H_{18}N_4O_3$		290.3177	291.30>230.20	0.1	35	23	6.2
Penicillin G	C16H18N2O4S	K	334.390	335.10>160.00	0.1	35	18	9.5
Cephalexin	C16H17N3O4S		347.389	348.20>158.10	0.1	35	11	6.3
Amoxycillin	$C_{16}H_{19}N_3O_5S$		365.4 g	366.20>208.20	0.1	35	12	5.5
Lincomycin	$C_{18}H_{34}N_2O_6S$	HCl	406.537	407.30>126.20	0.1	35	18	5.7
Cloxacillin	C19H18ClN3O5S	Na	435.881	436.00>277.00	0.1	35	17	10.4
Oxytetracycline	C22H24N2O9	HCl	460.434	461.10>426.30	0.1	35	22	6.5
Kanamycin	C18H36N4O11	SO_4	484.5	485.10>162.80	0.1	65	22	1*
Cephtiofur	$C_{19}H_{17}N_5O_7S_3$		523.56	524.20>197.10	0.1	35	30	9
Cephquinome	$C_{23}H_{24}N_6O_5S_2$	Na	528.6	529.20>134.10	0.1	35	25	6.6
Streptomycin	C21H39N7O12	SO_4	581.574	582.30>263.30	0.1	50	35	1*
Novobiocin	$C_{31}H_{36}N_2O_{11}$	Na	612.6243	613.30>189.20	0.1	50	31	10.5
Neomycin	C23H46N6O13	SO_4	614.644	615.30>163.20	0.1	50	35	1*
Cephoperazone	$C_{25}H_{27}N_9O_8S_2$	Na	645.667	646.00>142.80	0.1	35	41	9.2
Tylosin	C46H77NO17	Tartrate C4H4O6	916.100	916.20>174.40	0.1	35	36	8

*Aminoglycosides are unretained

Table 3: Samples showing the presence of antibiotics in the samples provided. Note that the method is as yet not quantitative.

Sample Text	SLURRY	SOLID 295	SOLID 362	MILK 1 509RF	MILK 253 LR	MILK 14T	MILK 362	MILK 6 295
Trimethroprim	-	-	-	*	*	*	-	-
Sulphadiazine								
PenG						*****	*****	*****
Cephalexin	?							
Amoxycillin	*	**	**					
Lincomycin								
Oxytetracycline	*	**	**					
Kanamycin								
Cephtiofur								
Cephquinome								
Streptomycin								
Novobiocin								
Cephoperazone								
Tylosin								
Cloxacillin								
Neomycin								

Reference:

Malgorzata Gbylik-Sikorska, Andrzej Posyniak, Tomasz Sniegocki, Jan Zmudzki
Liquid chromatography–tandem mass spectrometry multiclass method for the determination of antibiotics residues in water samples from water supply systems in food-producing animal farms
Chemosphere, Volume 119, January 2015, Pages 8-15

Appendix II

What follows is a summary of the microbial analyses carried out to date on the anaerobic digestates produced in each of the experiments described in the main body of the thesis. It was intended that the data would be included in a chapter within the thesis, but most of the samples are still being analysed and thesis submission cannot be delayed. What follows is an example of some of the data obtained immediately prior to submitting the thesis. The data are therefore in a crude form. The data will be analysed fully and published.

Metagenomic analysis of anaerobic digestates derived from organic cattle dung and conventional dairy slurry and the effects of antibiotic contaminants

Introduction

Several methods have been utilized in order to examine microbial communities within the anaerobic digestion process, including fluorescence in situ hybridisation (Braguglia *et al*., 2012), denaturing gradient gel electrophoresis analysis (Palatsi et al., 2010, 2011), and clone library of 16S rRNA genes (Rincón *et al*., 2008), however, compared to metagenomic approaches based on high-throughput sequencing these methods only give limited data (Yang *et al.*, 2014). Information on metagenomic analysis for full-scale on farm anaerobic digesters is very limited, but costs of performing such analyses are becoming more affordable. The microbial community of an anaerobic digester mainly consists of hydrolytic, acidogenic and acetogenic bacteria, and hydrogenotrophic and acetoclastic archaea (methanogenesis). Within an anaerobic digester methanogenesis occurs via two pathways: (i) hydrogenotrophic from the utilization of CH_2 and CO_2, and (ii) acetoclastic from acetate (Coban *et al*., 2016), however, the methane-producing microorganism that usually dominates with anaerobic digesters are the acetoclastic mathanogens (Zinder, 1993). Fate and inhibitory effects of oxytetracycline on the anaerobic digestion system have been commonly studied (Loftin and Henny, 2005; Álvarez *et al*., 2010) and although some contradictory results have been reported, adverse effects of oxytetracycline on CH_4 yield in anaerobic digestion units have been reported (Fedler and Day, 1985; Lallai *et al*., 2002; Arikan *et al*., 2006; Stone *et al*., 2009; Álvarez *et al*., 2010; Ince *et al*., 2013). These studies are not uniform in their findings, nor do most of these studies find a complete failure of the

system, but rather just a decrease in yield. These reports highlight how much difference environmental and operational conditions can have on the inhibition of an anaerobic digestion system. With this in mind, investigations of microbial communities and structure within an anaerobic digester in the presence of oxytetracycline are limited (Loftin and Henny, 2005; Arikan *et al*., 2006; Álvarez *et al*., 2010; Akyol *et al*., 2016a), with further, in-depth investigation needed.

Materials and Methods

DNA Extraction

DNA was extracted from digestates using a Powersoil DNA kit (Mo-Bio Laboratories Inc., Carlsbad, California, USA) since this kit had previously been used in the past for similar digestate and yielded good results, furthermore upon testing with other similar kits this particular kit proved to yield a better concentration of DNA. In brief, 0.3 g of digestate was added to a Powerbead tube and vortexed for a short period, Solution C1* was then added to the tube before mixing on a fat bed vortex for 10 minutes to allow cell lysis, tubes were then centrifuged at 8,500 rpm (10,000 x Gravity (g)) for 30 seconds. Supernatant was transferred to a clean Eppendorf and solution C2* added to precipitate out non-DNA components during incubation for 5 minutes at 4°C before centrifuging at 8,500 rpm for 1 minute. Supernatant was transferred into a new clean Eppendorf, ensuing to avoid the pellet and solution C3*added to precipitate out non-DNA material during incubation at 4°C for 5 minute. Avoiding the pellet, supernatant was transferred to a new clean Eppendorf along with solution C4 to allow binding of DNA and vortexed briefly. Supernatant was loaded into spin filters in 3 stages, before centrifuging the spin filter at 8,500 rpm for 1 minute with all flow through discarded. After 3 stages of spin filter loading, the collected DNA was washed and cleaned using solution C5 and centrifuged for 30 seconds at 8,500 rpm. The spin filter was then placed in a new clean Eppendorf and DNA elusion solution C6 was added, which removes the DNA from the spin filter in solution, the sample was then centrifuged at 8,500 rpm. The sample of DNA was frozen at -80°C prior to downstream use. Prior to DNA samples being sent for analyses, samples were quantified by NanoDrop 1000 (Thermo Scientific) and a Qubit fluorometer (Invitrogen) to allow for correct concentration (50 ng/µl) and volume (20 µl) of DNA.

DNA samples were sent to Oxford Genomics Centre (Wellcome Trust Centre for Human Genetics, Roosevelt drive, Oxford, UK)

Analyses Pipeline Method

In brief, for each sample Deep Seq (Nottingham University, UK) concatenated the two raw fastq files into a single file (Trimmomatic). This generated one trimmed, paired fastq file and a second unpaired fastq file (per sample, per direction). Quality control analyses were performed via the FastQC (Babraham Bioinformatics,) read analysis tool, with the number and quality of the reads assessed. Overlapping read-pairs in the trimmed, paired data were then merged into a single fragment using FLASH (Fast Length Adjustment of SHort reads, https://ccb.jhu.edu/software/FLASH/), with standard parameters expect for the maximum overlap parameter, which was set to 150, as the samples had longer reads than FLASh assumes for default. Reads were then assembled *de novo* using CLC assembler. The reads that were successfully paired but not combined by FLASh were flagged to be treat as pairs of known distance and orientation in the assembly. The two unpaired files and the combined fragments were also imported. For Metagenomin analyses, the fastq assembly files were uploaded to OneCodex (https://app.onecodex.com) where they could be explored and exported in various ways.

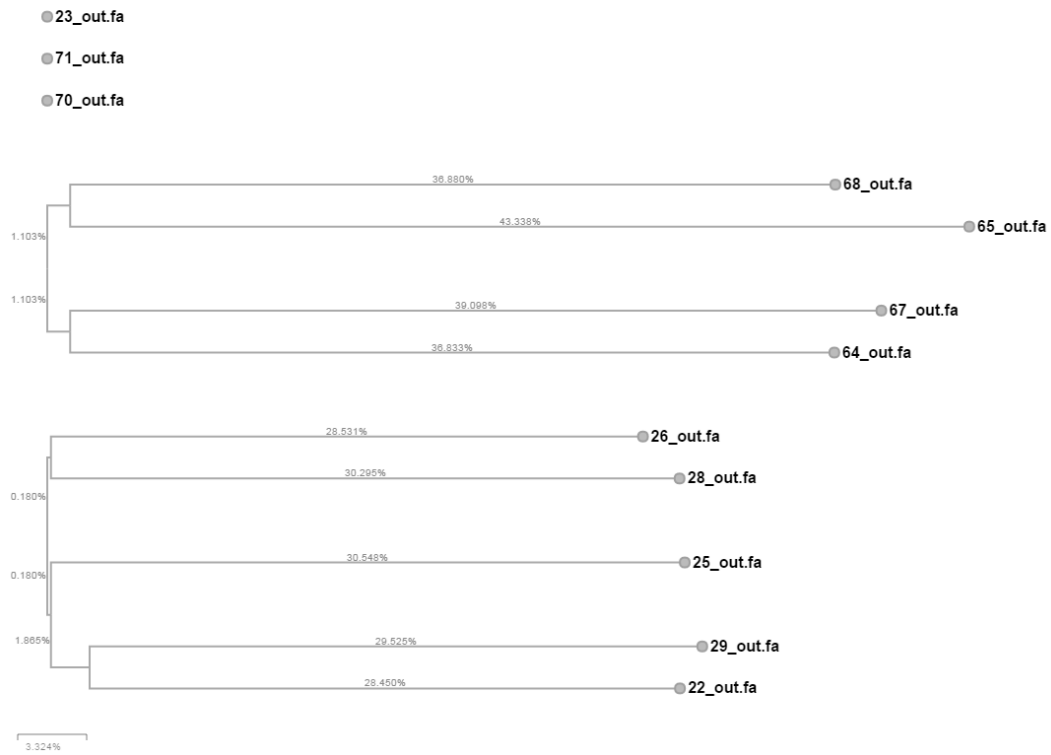
Results to Date

Data returned to date relate to digestates made from organic cow dung ± oxytetracycline (4.3 mg L^{-1}) and conventional dairy slurry ± oxytetracycline (4.3 mg L^{-1}). As with all metagenomic analyses the data need careful analysis so what follows are just some interesting points generated from screen dumps from the One Codex data base that is currently being generated for the samples.

From the data generated it appears that oxytetracycline amendment has a larger effect on the microbial consortia in the organic dung than in the slurry. This might be expected since the microbes in the slurry are subjected to inputs of various antibiotics, one of which is tetracycline. This needs further investigation but the cluster diagram (utilising data for bacteria and Archae) below suggests similarities between samples 65 and 68 (both organic dung +OTC) and samples 64 and 67 (both organic dung -OTC). Two dung samples appear to be outliers (70 and 71).

In contrast, there is no OTC-related reason for the clustering of the slurry samples since the paired samples in the diagram cover both OTC treatments. There is one slurry outlier (sample

23). It is clear that there are no similarities between the microbial components of the slurry and the organic dung.



Sample key:
Samples 64, 67, and 70 = Organic dung, no OTC
Samples 65, 68 and 71 = Organic dung, plus OTC

Samples 22, 25 and 28 = Dairy slurry, no OTC
Samples 23, 26 and 29 = Dairy slurry, plus OTC

Full analyses of these data are being undertaken – the aim here is to highlight the work underway.

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