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Quantifying the percentage of methane formation via acetoclastic and syntrophic acetate oxidation pathways in anaerobic digesters

Ying Jiang [a,*], Charles Banks [b], Yue Zhang [b], Sonia Heaven [b], Philip Longhurst [a]

[a] Centre for Bioenergy & Resource Management, School of Water, Energy and Environment, Cranfield University, Cranfield MK43 0AL, UK
[b] Faculty of Engineering and the Environment, University of Southampton, Southampton SO17 1BJ, UK

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ABSTRACT

Ammonia concentration is one of the key factors influencing the methanogenic community composition and dominant methanogenic pathway in anaerobic digesters. This study adopted a radiolabelling technique using [2-^{14}C] acetate to investigate the relationship between total ammonia nitrogen (TAN) and the methanogenic pathway. The radiolabelling experiments determined the ratio of $^{14}CO_2$ and $^{14}CH_4$ in the biogas which was used to quantitatively determine the percentage of CH_4 derived from acetoclastic and syntrophic acetate oxidation routes, respectively.

This technique was performed on a selection of mesophilic digesters representing samples of low to high TAN concentrations (0.2–11.1 g kg^{-1} wet weight). In high TAN digesters, the ratio between $^{14}CO_2$ and $^{14}CH_4$ was in the range 2.1–3.0; indicating 68–75% of methane was produced via the hydrogenotrophic route; whereas in low ammonia samples the ratio was 0.1–0.3, indicating 9–23% of methane was produced by the hydrogenotrophic route. These findings have been confirmed further by phylogenetic studies.

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1. Introduction

Anaerobic digestion (AD) has gained popularity over the last decade as a waste management strategy with the added benefit of producing next generation renewable energy in the form of methane (Mata-Alvarez, 2003). Numerous studies have been carried out to optimise the process, increase efficiency and evaluate potential substrates. Food waste in particular has received considerable attention due to its widespread availability and high energy content on dry weight basis. A wide range of prior research shows, however, that substrates including food waste and other farm waste streams which have high nitrogen concentration are prone to unstable digestion. This is due to the inhibitory effect of free ammonia nitrogen (FAN) released during protein hydrolysis and the breakdown of urea in animal manure (Breure et al., 1986; Kayhanian, 1999; McCarty, 1964; Yenigün and Demirel, 2013). It has been reported on a number of occasions and at different scales of operation that a characteristic build-up of volatile fatty acids (VFA) can occur when digesting food waste which can lead to process failure (Banks et al., 2008; Banks and Zhang, 2010; Neiva Correia et al., 2008).

It is now acknowledged that the anaerobes involved in the AD process exhibit different threshold tolerances towards ammonia. Amongst these microorganisms, the methanogens are generally more sensitive than the anaerobic bacterial species (Gallert et al., 1998; McCarty, 1964); within methanogens the acetoclastic methanogens are recognised as being more sensitive to ammonia than hydrogenotrophic methanogens, with the latter reported to have a similar, or in some cases, more tolerance to high ammonia levels compared to syntrophic acetate-oxidizing bacteria (Wang et al., 2015). As a result, ammonia concentration can be one of the most important factors that influence methanogen community structure and could alter the dominant methanogenic pathway in the digester as reported in a number of previous studies (Fotidis et al., 2014; Karakashev et al., 2006; Schnürer et al., 1997; Schnürer and Nordberg, 2008; Westerholm et al., 2012).

The microbial community structure in digesters can be examined using a variety of techniques including targeted DNA amplification and sequencing, and fluorescence *in situ* hybridisation (FISH). These methods are now well established and commonly used to assess the microbial population structure within samples from a wide range of environments and processes. Studies looking at anaerobic digesters (Karakashev et al., 2006, 2005; Nettmann et al., 2010, 2008) have found that the microbial population structure is very dependent on environmental conditions and is

* Corresponding author.
E-mail address: Y.Jiang@cranfield.ac.uk (Y. Jiang).

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influenced by factors such as the concentration of VFA and ammonia as well as temperature. A comprehensive study surveyed methanogen diversity in 15 full-scale biogas plants using the FISH technique (Karakashev et al., 2005). The study concluded that in digesters operating with high ammonia and VFA concentrations the methanogen population was dominated by members of the *Methanosarcinace;* whereas digesters operating at low levels of ammonia and VFA were dominated by members of the *Methanosaetaceae*. Another study used FISH and quantitative polymerase chain reaction (PCR) methods to investigate methanogen community composition in six full–scale commercial digesters treating nitrogen-rich poultry faeces and/or maize silage, and observed that in five of the digesters the hydrogenotrophic *Methanomicrobiales* spp. were dominant (Nettmann et al., 2010). The sixth digester, which had only been operational for a short period, had a significantly lower (1.7 g kg^{-1} wet weight (WW)) concentration of total ammonia nitrogen (TAN) and the acetoclastic *Methanosaetaceae* spp. was found to be dominant. Determining metabolic functionality by analysis of the community structure, however, depends on a clearly known association between a specific metabolic pathway and a particular species or genera. Where such associations exist molecular biology techniques can be an effective tool to monitor change and associate this to particular characteristics, as demonstrated by targeting specific methanogenic spp. It is, however, known (Collins et al., 2003; de Bok et al., 2006; Nettmann et al., 2010, 2008) that a large proportion of methanogens commonly observed in anaerobic digesters belong to the genus *Methanosarcina* which can utilise acetate, methyl compounds, and CO_2/H_2 to produce methane (Galagan et al., 2002). The metabolic pathway to methane production when *Methanosarcina* is the dominant methanogenic species thus remains unclear (Collins et al., 2003). To resolve this uncertainty of methanogenic pathway when there is a large representation of *Methanosarcina*, it is therefore desirable to have a direct method to determine the degree of each methanogenic pathway in anaerobic digesters.

A radiolabelling method was established using ^{14}C labelled sodium acetate ($^{14}CH_3COONa$) and has been applied successfully in a number of studies carried out by Schnürer et al. (1999), Schnürer and Nordberg (2008), Karakashev et al. (2006, 2005) and Fotidis et al. (2013). The method determines the ratio of radioactive ^{14}C in CO_2 and CH_4 in the biogas, following the addition of $^{14}CH_3COONa$ into a digestate sample. This $^{14}CO_2/^{14}CH_4$ ratio provides a rapid indication of the degree of syntrophic methanogenesis occurring in the anaerobic microbial consortium. It also avoids the high analysis cost and lengthy sample preparation commonly associated with molecular biology methods. To the best of the authors' knowledge, however, in the previous literature $^{14}CO_2/^{14}CH_4$ ratio was used as a qualitative indicator and an $^{14}CO_2/^{14}CH_4$ ratio of 1 was accepted as the threshold to distinguish the dominant methanogen pathway (Fotidis et al., 2013). Based on this previous radiolabelling work, the current study uses stoichiometric calculation to explore the correlation between $^{14}CO_2/^{14}CH_4$ ratio and the percentage of acetoclastic and hydrogenotrophic methanogenic pathways that contribute to the final methane formation from acetate as substrate in a mixed culture AD system.

To study the influence of ammonia concentration to $^{14}CO_2/^{14}CH_4$ ratio, ^{14}C studies were carried out on digestate samples from food waste digesters operating at various ammonia nitrogen concentrations and from a digester treating municipal wastewater biosolids (MWB) with a low ammonia nitrogen concentration. This MWB digester had initially been used to inoculate all of the food waste digesters. To complement the ^{14}C study a methanogenic community analysis was also performed on one high nitrogen food waste digestate samples and on the MWB digestate sample. This used a pyrosequencing technique using a fragment of the methyl Co-A reductase gene (*mcrA*) which is common to all known methanogens.

2. Materials & methods

2.1. Digester operation and sampling

Digestate samples were collected from eight digesters (F1-8) which had been operating for over two years on source segregated domestic food waste. The full operational history of these digesters and analytical methods for key operational parameters including pH, VFA, TAN and daily biogas production has been described in detail elsewhere (Banks et al., 2012). Samples were taken between days 760–780, when total ammonia nitrogen (TAN) concentrations were in the range 3.5–5.3 g N kg^{-1} WW. Digestate samples were also collected from three pairs of digesters (R1-6) which had been operating for over 1 year. R1 and 2 were fed on food waste similar to that used for digester F1-8: however the total Kjeldahl nitrogen (TKN) level was artificially increased from ~7 g N kg^{-1} WW to 12 g kg^{-1} WW with urea (Reagent grade, Sigma Aldrich, UK). R3-R6 were fed with low nitrogen food waste (TKN = 2.08 g N kg^{-1} WW) which was specially prepared following a recipe described in Yirong (2014). All the digesters described above were operated at mesophilic temperature (36 °C).

Additionally, a low nitrogen digestate sample was collected from a mesophilic digester treating municipal wastewater biosolids with digestate TAN at 1.58 g N kg^{-1} WW (Millbrook Wastewater Treatment plant, Southampton, UK).

A total of nine digestate samples were prepared for dosing with isotope labelled acetate as the feed. A summary of the key operational parameters at the time of sampling associated with the digesters from which the samples were taken is given in Table 1: these include TAN and VFA concentration and pH.

2.2. ^{14}C radiolabelling experiment

2.2.1. Sample preparation

The experimental method for radiolabelling tests was adopted and adapted from Schnürer et al. (1999) and Fotidis et al. (2013). 50 g of each digestate sample was transferred into a 250 mL conical flask containing 100 mL of the non-selective culture medium, and thoroughly mixed. The medium recipe was as described in (Zinder and Koch, 1984), and consisted of the following (g L^{-1}): NH_4Cl, 1; NaCl, 0.1; $MgCl_2{\cdot}6H_2O$, 0.1; $CaCl_2$ $2H_2O$, 0.05; $K_2HPO_4{\cdot}3H_2O$, 0.4, and sodium acetate, 0.25. Five trace element solutions of the following concentrations (g L^{-1}):$CoCl_2{\cdot}6H_2O$, 4.03; $(NH_4)_6$ $Mo_7O_{24}{\cdot}4H_2O$, 0.184, $NiCl_2{\cdot}2H_2O$, 4.11; $Na_2WO_4{\cdot}2H_2O$, 0.18 and Na_2SeO_3, 0.219; were prepared individually and 0.1 mL of each solution was added into 1 L of the medium. The medium solution was boiled under N_2 and autoclaved (15 min, 120 °C) before use.

45 mL aliquots of the sample/culture medium mix were dispensed into crimp top serum bottles with a capacity of 119 mL. The specified quantity (see Table 2) of the $^{14}CH_3COONa$ (MP biomedical, Solon, OH, United States) solution was added into each mix. The headspace of the serum bottle was flushed with N_2/CO_2 (80:20) (BOC, UK) before sealing with a crimp cap with PTFE coated silicon septum. The serum bottles were then placed in an incubator (Hybaid Maxi 14, Thermo Scientific, UK) at 37 °C with orbital shaking at ~50 RPM for ~12 h to allow biogas to be produced. The test was carried out in duplicate for each of the digestate samples.

2.2.2. Scintillation counting

After incubation, a 10% mix of oxygen in nitrogen was sparged into the liquid and headspace of the serum bottle at a flow rate of 30 mL min^{-1} for 45 min. The sparge gas from the serum bottle

Table 1
Summary of key parameters associated with digestate samples.

ID	Sampling digester	OLR (g VS L^{-1} day^{-1})	Specific CH_4 production (L g^{-1} VS day^{-1})	Sample TAN (g N L^{-1})	Total VFA (mg L^{-1})	pH	Note
MWB	Municipal wastewater biosolids digester	NA*	NA	1.6	<100	7.1	
F1	F1	1.8	0.39	6.1	14,000	7.6	Sample was not mixed from duplicated digester pair, as one of the paired digesters failed at the time of sampling
F2-3	Evenly mixed from duplicate digester F2 and F3	5.5	0.43	5.1	280	7.7	
F4-5	Evenly mixed from duplicate digester F4 and F5	5.5	0.43	4.6	110	7.6	
F6	F6	4.0	0.41	5.6	59	7.6	Same as noted for F1
F7-8	Evenly mixed from duplicate digester F7 and F8	5.5	0.43	4.7	120	7.7	
R1-2	Evenly mixed from duplicate digester R1 and R2	2	0.39	11.1	40,000	8.0	
R3-4	Evenly mixed from duplicate digester R3 and R4	2	0.39	0.2	<100	7.1	
R5-6	Evenly mixed from duplicate digester R5 and R6	2	0.39	0.3	<100	7.2	

* Note: NA = not available.

Table 2
Results from ^{14}C acetate labelling experiment.

Sample	Free ammonia (mg N L^{-1})**	Subsample	Radioactivity in subsample (KBq)	Total 14C recovered (KBq)	Total ^{14}C added in sample (KBq)	^{14}C Recovery (%)	$^{14}CO_2$:$^{14}CH_4$
MWB (Low N control)	22.5	Sludge supernatant	0.4 (±0.06)*	9.9	10.0	99	0.1
		CO_2	0.5(±0.05)				
		CH_4	9.0(±0.42)				
F1	299.2	Sludge supernatant	8.9(±0.24)	9.4	10.0	95	3.0
		CO_2	0.4(±0.01)				
		CH_4	0.1				
F2 + 3	316.9	Sludge supernatant	0.3(±0.06)	4.9	5.0	98	2.4
		CO_2	3.2(±0.10)				
		CH_4	1.3(±0.01)				
F4 + 5	228.1	Sludge supernatant	1.0(±0.15)	5.1	5.0	103	2.6
		CO_2	3.0(±0.10)				
		CH_4	1.2				
F6	277.4	Sludge supernatant	1.2(±0.01)	5.7	5.0	114	2.5
		CO_2	3.3(±0.06)				
		CH_4	1.3(±0.01)				
F7 + 8	244.0	Sludge supernatant	4.8(±0.08)	11.1	10.0	112	2.1
		CO_2	4.3(±0.41)				
		CH_4	2.0(±0.01)				
R1 + 2	1214.5	Sludge supernatant	2.22(±0.02)	2.3	2.5	94	3.0
		CO_2	0.09(±0.005)				
		CH_4	0.03(±0.001)				
R3 + 4	3.4	Sludge supernatant	0.63(±0.03)	2.1	2.5	85	0.3
		CO_2	0.31(±0.001)				
		CH_4	1.17(±0.002)				
R5 + 6	5.35	Sludge supernatant	0.86(±0.02)	2.3	2.5	92	0.3
		CO_2	0.33(±0.01)				
		CH_4	1.11(±0.06)				

* Note: Number in parentheses shows the range of the duplicated results.
** Free ammonia was calculated using the following formula: $NH_3\ (Free) = TAN \times \left(1 + \frac{10^{-pH}}{10^{-(0.09018 + \frac{2729.92}{T(K)})}}^{-1}\right)$ (Koster, 2007).

first passed through 20 mL of 5 M NaOH to trap $^{14}CO_2$. The exit gas from the first trap was then passed to a tube furnace heated to 800 °C and filled with CuO as an oxidiser, which instantaneously converted any CH_4 to CO_2. The exit gas from the furnace was then passed to a second NaOH CO_2 trap (20 mL, 5 M) where CO_2 converted from methane was absorbed. A specified known volume (between 1 and 2 mL) of the NaOH was taken from each of the traps and mixed with 15 mL Hionic-Fluor™ scintillation cocktail (PerkinElmer Inc., Buckinghamshire, UK). In addition the culture/medium mix in the serum bottle was centrifuged at 12,400g (Beckman Microfuge 12, Beckman Coulter Inc. UK) and 0.5 mL of the supernatant was mixed with 15 mL scintillation cocktail to determine residual radiolabelled acetate. A standard 20 mL scintillation vial made of high density polyethylene and a polypropylene, linerless screw cap was used for the scintillation samples. All counting was carried out on a LS6500 scintillation counter (Beckman Coulter Inc., UK) with the automatic quench compensation function applied. Each sample was counted for 2 min to ensure accuracy.

2.3. Phylogenetic studies of food waste digestate and sludge digestate

Phylogenetic studies were carried out on digestate samples taken from F7&8 and MWB, as they were considered to contain representative adapted microbial communities from high and low ammonia origins, respectively. DNA was extracted from a 250 mg sample using the Power Soil extraction kit (MO BIO Laboratories, Carlsbad, Germany) according to the manufacturer's instructions. This method included a bead-beating step, which was performed for 5 min. All DNA extracts were eluted with 60 mL of Tris buffer (10 mM) and stored at −20 °C until analysed. Methanogen community analyses were performed based on PCR amplification of the methyl Co-A reductase gene (*mcrA*) unique to, and ubiquitous in, all known methanogens (Luton et al., 2002).

Two primers as described by Luton et al. (2002) were used in this study (underlined): mcrAf (5′-*CCATCTCATCCCTGCGTGTCTC CGACTCAG*NNNNGGTGGTGTMGGATTCACACARTAYGCWACAGC-3′) and mcrAr (5′-*CCTATCCCCTGTGTGCCTTGGCAGTCTCAG*TTCATTGCRTAGTTWGGRTAGTT-3′) in combination with Roche 454 pyrosequencing adaptors (in italics). The proof-reading polymerase Phusion (New England Biolabs, USA) was used for the amplification of all targets. Next generation sequencing (NGS) of all amplicons was completed using the GS FLX + System (Roche, UK). Emulsion PCR was carried out according to the manufacturer's instructions (Roche, UK). The obtained sequence data were processed using the Galaxy platform to remove low quality reads and short sequences (<100 bp). Sequences obtained from mcrA amplicons were clustered based on nucleotide identity using the BLASTclust algorithm (Altschul, 1997) with parameters set to cluster sequences of >97% similarity over 90% of their length. Representative sequences of each cluster were aligned with *mcrA* sequences from the Genbank database and a phylogenetic tree was constructed to indicate the taxonomic identity of each cluster.

3. Results & discussion

3.1. ^{14}C radiolabelling study

The determination of the dominant acetate oxidation pathway is based on measuring the production of $^{14}CH_4$ and $^{14}CO_2$ from acetate radiolabelled on the methyl group (C-2 labelled). When converted by the acetoclastic route the methyl group on the acetate will be converted to methane (Ferry, 1993), in which case if only acetoclastic methanogenesis occurred all ^{14}C labels would be found in methane but not in CO_2. When syntrophic acetate oxidation feeds hydrogenotrophic methanogenesis, both of the carbon atoms of acetate are converted to carbon dioxide, and a proportion of the carbon dioxide produced is subsequently reduced to methane by H_2 (Schnürer et al., 1999). Therefore, when [2-^{14}C] acetate is provided to a syntrophic acetate oxidation and hydrogenotrophic methanogenic system, a significant quantity of the ^{14}C will be present in carbon dioxide. The degree of acetate oxidation (measured as the $^{14}CO_2/^{14}CH_4$ ratio) is thus an indicator of the dominant methanogenic route. A low $^{14}CO_2/^{14}CH_4$ ratio suggests an acetoclastic-dominated route whereas a high ratio suggests a high level of syntrophic acetate oxidation (Fotidis et al., 2013; Karakashev et al., 2006).

The results from the ^{14}C acetate labelling experiment are summarised in Table 2. For the MWB digestate sample, out of the total 10 KBq acetate added, 9.5 KBq radioactivity was later found in gaseous products (CO_2 and CH_4). However, only 0.5 KBq out of the 9.5KBq ^{14}C was found in CO_2 and the majority of the ^{14}C was found in CH_4, with a $^{14}CO_2/^{14}CH_4$ ratio of 0.06. A different result was observed for the food waste digestate samples tested. In those samples from digesters with high nitrogen content, 66–74% of the total ^{14}C was found in CO_2 with $^{14}CO_2/^{14}CH_4$ ratios in the range of 2.1–3.0. These results confirm that acetate oxidation is the primary metabolic route with methane formed by CO_2 reduction. Although it is widely acknowledged when $^{14}CO_2/^{14}CH_4$ ratio is >1 the system is believed to have a strong reliance on hydrogenotrophic methanogenesis route (Fotidis et al., 2013; Karakashev et al., 2006; Schnürer and Nordberg, 2008) to the authors' best knowledge no previous work has related the $^{14}CO_2/^{14}CH_4$ ratio to the exact percentage of methane formation contributed by each methanogenic pathway.

Consider an AD system in which both acetoclastic methanogenesis (reaction (1)) and syntrophic acetate oxidation (reaction (2)) followed by hydrogenotrophic methanogenesis (reaction (3)) occur, and assume 1 mol of [2-^{14}C] acetic acid is fed to the system and then entirely converted to methane and carbon dioxide. If P_a is the proportion of methane produced via acetoclastic methanogenesis from the unit mole of [2-^{14}C] acetic acid in this system ($0 \le P_a \le 1$), then $1 - P_a$ is the proportion of methane produced via reactions (2) and (3).

$$^{14}CH_3{}^{12}COOH = {}^{14}CH_4 + {}^{12}CO_2 \quad (1)$$

$$^{14}CH_3{}^{12}COOH + 2H_2O = {}^{14}CO_2 + {}^{12}CO_2 + 4H_2 \quad (2)$$

$$^{14}CO_2 + 4H_2 = {}^{14}CH_4 + 2H_2O \quad (3)$$

Via reaction (1) (acetoclastic methanogenesis), P_a mol $^{14}CH_4$ is produced along with P_a mol of $^{12}CO_2$. Via syntrophic acetate oxidation in reaction (2), the remainder of the [2-^{14}C] acetic acid ($1 - P_a$ mol) is oxidised into $(1 - P_a)$ mol of $^{14}CO_2$ and $(1 - P_a)$ mol of $^{12}CO_2$ along with the production of $4(1 - P_a)$ mol of hydrogen. This amount of hydrogen can only reduce $(1 - P_a)$ mol of CO_2 in reaction (3). The total CO_2 produced from the previous reaction (1) and (2) is $P_a + 2(1 - P_a)$, therefore in excess. If hydrogenotrophic methanogens have no preference between $^{14}CO_2$ and $^{12}CO_2$, or in other words if every CO_2 molecule regardless of its isotopic form shares the same probability of being utilised by hydrogenotrophic methanogens, the $^{14}CH_4$ produced from hydrogenotrophic route (reaction (3)) can be expressed as:

$$\text{Hydrogenotrophic } {}^{14}CH_4 = ({}^{14}CO_2/Total\ CO_2) \times H_2 = \frac{1 - P_a}{P_a + 2(1 - P_a)} \times (1 - P_a) \quad (4)$$

As it is generally acknowledged, however, that microorganisms prefer naturally 'lighter' isotopes (Fotidis et al., 2013), another variable f ($0 < f < 1$) must therefore be introduced to provide a mechanism to express the affinity of $^{14}CO_2$ to hydrogenotrophic methanogens carrying out reaction (3). $f = 1$ indicates no preference between the two isotopes, while a smaller value for f indicates it is less probable that $^{14}CO_2$ will be utilised in reaction (3). With the extra variable f, Eq. (4) becomes:

$$\text{Hydrogenotrophic } {}^{14}CH_4 = \frac{f(1 - P_a)}{P_a + 2(1 - P_a)} \times (1 - P_a) \quad (5)$$

Combining this with the $^{14}CH_4$ produced from reaction (1), the total $^{14}CH_4$ produced from the system is:

$$\text{Total } {}^{14}CH_4 = P_a + \frac{f(1 - P_a)}{P_a + 2(1 - P_a)} \times (1 - P_a) \quad (6)$$

In reaction (3), to produce $\frac{f(1-P_a)}{P_a+2(1-P_a)} \times (1 - P_a)$ mol of $^{14}CH_4$, an equal amount of $^{14}CO_2$ will be consumed, therefore the total $^{14}CO_2$ in the final biogas produced after complete conversion of 1 mol of [2-^{14}C] acetic acid in this system will be:

$$\text{Total } {}^{14}CO_2 = (1 - P_a) - \frac{f(1 - P_a)}{P_a + 2(1 - P_a)} \times (1 - P_a) \quad (7)$$

and by combining this with Eq. (6), the $^{14}CO_2/^{14}CH_4$ ratio is expressed as follows:

$$^{14}CO_2/^{14}CH_4 = \frac{(1-P_a) - \frac{f(1-P_a)^2}{P_a+2(1-P_a)}}{P_a + \frac{f(1-P_a)^2}{P_a+2(1-P_a)}} \quad (8)$$

In many naturally occurring anaerobic environments, e.g. paddy soil, the f value has a profound effect on the correlation between $^{14}CO_2/^{14}CH_4$ ratio and percentage of acetoclastic methanogenesis calculated, especially in a system where acetoclastic methanogen activity is low. It is therefore difficult to accurately link the $^{14}CO_2/^{14}CH_4$ ratio to the split between methanogenic pathways without a detailed knowledge of the ability of hydrogenotrophic methanogens to discriminate between $^{14}CO_2$ and the naturally lighter form. Information on the uptake of ^{14}C is limited, although a number of studies have reported a preference for the lighter ^{12}C compared to the stable isotope ^{13}C in methanogens (Conrad et al., 2010; Lv et al., 2014; Nikolausz et al., 2013; Penning et al., 2006, 2005).

Although the value of f has an important influence on the $^{14}CO_2/^{14}CH_4$ ratio, it is also worth noting that Eq. (8) was obtained based on the assumption that there were no external H_2 or CO_2 sources that contributed to reactions (1), (2) and (3). Under the experimental conditions applied in this study, large quantities of non-radiolabelled acetate are present in the sample as a result of acid fermentation by both acidogenic and acetogenic pathways, as well as that present in the culture medium. It is therefore the case that the quantity of non-radiolabelled acetate is orders of

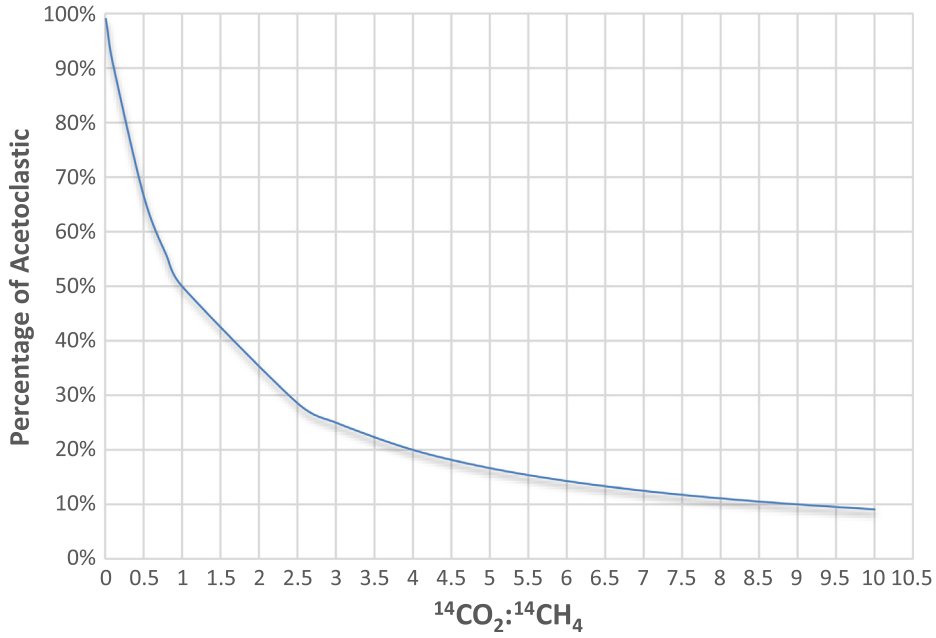


Fig. 1. Plot of correlation between percentage of acetoclastic methanogenesis and $^{14}CO_2$:$^{14}CH_4$ based on the simplified Eq. (12).

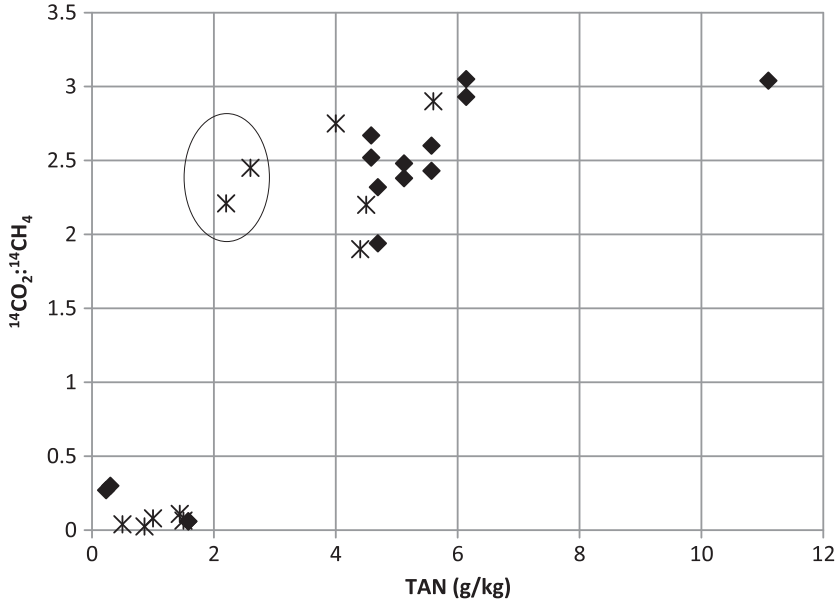


Fig. 2. A plot of $^{14}CO_2$:$^{14}CH_4$ ratio as a function of TAN. Note: Diamonds '◆' represent experimental data obtained in this study; asterisks '*' are plotted using data from Karakashev et al. (2006). Highlighted in circle are two thermophilic digesters reported in Karakashev et al. (2006).

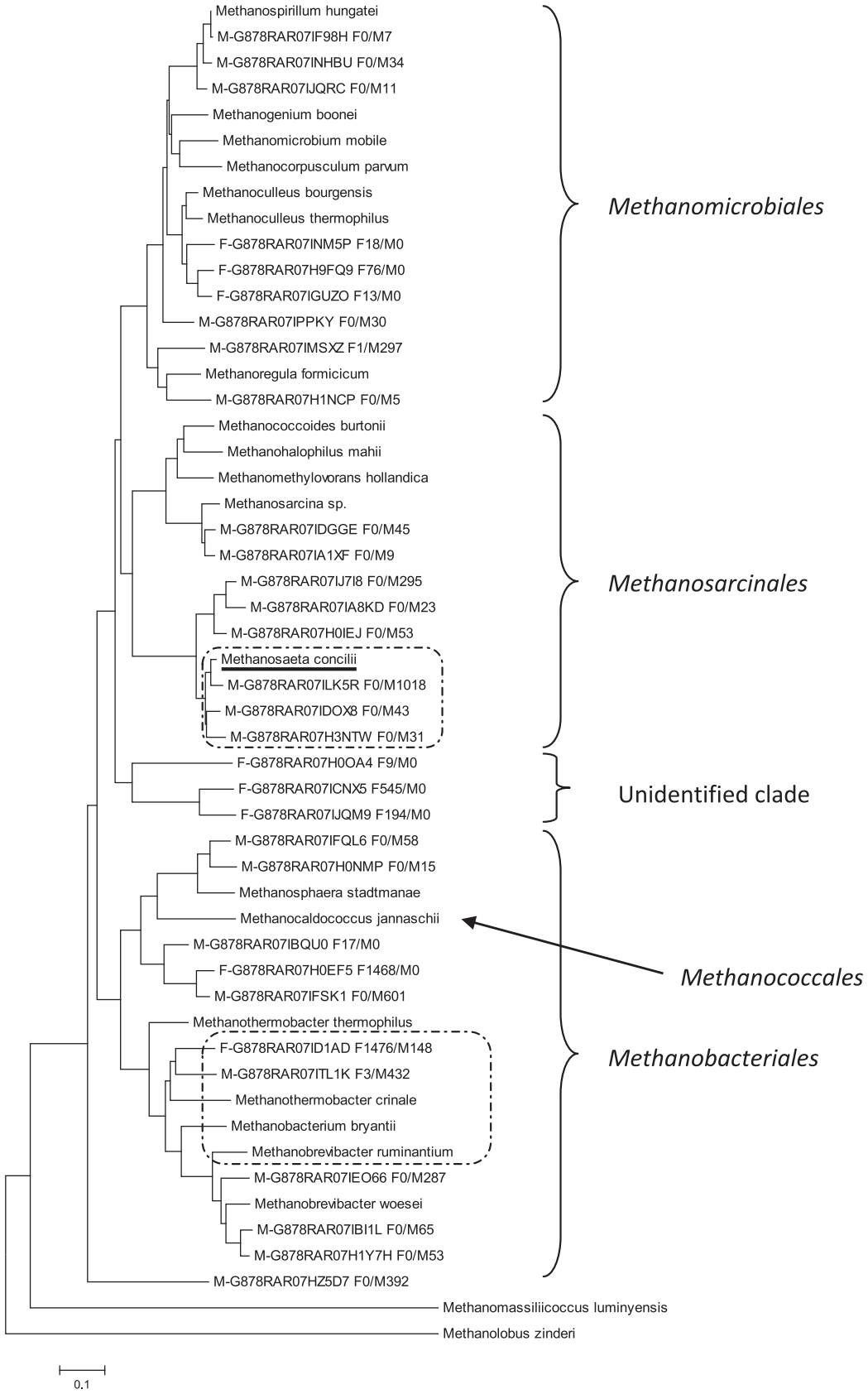


Fig. 3. Neighbour-joining tree of partial mcrA sequences showing representatives from clusters of sequences sharing 97% similarity. Numbers of sequences derived from each sample (F (Food waste digestate) or M (MWB)) in each cluster are given.

magnitude greater than radiolabelled acetate: in this study 9×10^{-5} mol non-radiolabelled sodium acetate was present in the sample of culture medium compared to 2.16×10^{-9} mol radiolabelled acetate when 5KBq activity was added. Under these circumstances the amount of $^{14}CO_2$ produced via syntrophic acetate oxidation (reaction (2)) is negligible compared to a much larger pool of $^{12}CO_2$. As a result, the amount of $^{14}CO_2$ taking part in reaction (3) and hence the $^{14}CH_4$ produced through reaction (3) can be ignored. Therefore Eqs. (6), (7) and (8) can be simplified as follows:

$$\text{Total } {}^{14}CH_4 = P_a \tag{9}$$

$$\text{Total } {}^{14}CO_2 = (1 - P_a) \tag{10}$$

$${}^{14}CO_2/{}^{14}CH_4 = \frac{1 - P_a}{P_a} \tag{11}$$

$$\text{Therefore, } P_a = 1/({}^{14}CO_2/{}^{14}CH_4 + 1). \tag{12}$$

Fig. 1 shows a plot of the above function. Based on Eq. (12), the percentage of hydrogenotrophic methanogenesis can be estimated as being in the range of 68–75% in the high nitrogen food waste digesters (F1-8) tested in this study, whereas for low nitrogen samples hydrogenotrophic methanogenesis accounts for about 9–23% of the total CH_4 production.

The quantitative determination for the partitioning of methanogenic pathways responsible for acetate to methane conversion based on $^{14}CO_2/^{14}CH_4$ ratio described in this study allows more accurate interpretation of the flow of carbon and electrons from acetate towards CH_4, therefore a significant advance compared with using the empirical ratio of 1 for a qualitative estimation of the dominant pathway. In practise, an improved understanding of the methanogenic pathways partitioning in an AD system can significantly increase the reliability and accuracy of the anaerobic degradation process modelling.

However, it should be noted that there are many factors not considered in this study can potentially affect the accuracy of the calculated methanogenic pathway partitioning percentage. One factor is the dissolved CO_2 in digestate samples. Although acidification of the samples significantly improve the accuracy of the result, there is still an optional of reduced recovery of $^{14}CO_2$ which leads to a lower experimental $^{14}CO_2/^{14}CH_4$ ratio than its actual value. To accurately quantify the trace amount of dissolved CO_2, more detailed study of the pH, CO_2 partial pressure required. Additionally, due to the complex composition of the sample solution, ionic strength and dissolved organic matters in the solution must be considered as they also influence the values of Henry's law constants. Other uncertainties including the isotope fractionation effects in methanogenic pathways are not considered in this study. To further improve the accuracy of the quantification of the methanogenic pathways partitioning, significant experimental efforts are required for future studies.

The results obtained from this study are in strong agreement with existing literature (Karakashev et al., 2006), where methanogenesis in full-scale digesters fed on high ammonia manure and low ammonia wastewater sewage sludge has been assessed using a similar radioactive isotope method. To illustrate the relationship between TAN concentration in the digestate sample and the degree of acetate oxidation (i.e. $^{14}CO_2/^{14}CH_4$ ratio), Fig. 2 was plotted using results obtained from this study and previous literature data (Karakashev et al., 2006). A clear bimodal distribution is shown with a sudden shift of $^{14}CO_2/^{14}CH_4$ ratio from below 0.1 to above 2.0 as soon as the TAN concentration exceeds 2 g N L^{-1}. Interestingly, all digester samples shown in Fig. 2, with the exception of the two thermophilic digesters reported in Karakashev et al. (2006) (circled in Fig. 2), were collected from mesophilic digesters. These two thermophilic samples have TAN concentrations of ~2.1–2.4 g N L^{-1} which is considered to be at the borderline of inhibition for acetoclastic methanogens. However, a radiolabelling experiment revealed a high acetate oxidation level in these samples, indicating inhibition of the acetoclastic route. This is likely due to the higher proportion of FAN present at thermophilic temperatures, and this observation provides further evidence that the ammonia inhibition is caused by FAN rather than ammonium ions.

3.2. Phylogenetic study

The phylogenetic study revealed a high diversity of methanogens in both samples analysed; however a strong divergence in methanogen community composition was observed between digestate F7 + F8 and the low nitrogen MWB sludge sample. In the MWB sample, methanogen species were found belonging to both acetoclastic, e.g. *Methanosaeta concilii* (~1000 sequences were found in MWB but none in foodwaste digestate sample), and hydrogenotrophic families, e.g. *Methnospirillum hungatei* (52 were found in MSW sample, none in foodwaste digestate sample), and *Methanobrevibacter woesei* (~400 were found in MSW sample, none in foodwaste digestate sample). As indicated by the number of analysed sequences, the predominant phylotypes, however, were affiliated to the strictly acetoclastic *Methanosaeta concilii* (highlighted in Fig. 3). In the food waste digestate sample (F7 + 8), hydrogenotrophic methanogens were predominantly represented by phylotypes affiliated to *Methanobacteriales* (Fig. 3) and the acetoclastic *Methanosaeta* family was not observed.

The phylogenetic results are in agreement with, and further confirm, the FISH study published previously (Banks et al., 2012), where the family *Methanosaetaceae,* consisting mainly of acetoclastic *Methanosaeta* spp., appeared as the dominant methanogen in the sewage sludge digestate sample, whilst in high N food waste digesters methanogenic groups were members of the order *Methanoimicrobiales* and no acetoclastic methanogens were found.

Additionally, this phylogenetic study supported the conclusion drawn from the ^{14}C tracer experiment. It therefore appears conclusive that, under a high ammonia concentration, food waste digestion adopts a syntrophic acetate oxidation coupled to hydrogenotrophic methanogenesis pathway as the major route for methane production.

4. Conclusions

In this study, a radioactive ^{14}C-acetate tracer technique was applied to determine the methanogenesis pathway in anaerobic digesters based on ratio of $^{14}CO_2$ and $^{14}CH_4$ in biogas. The stoichiometric calculation successfully established a quantitative correlation between $^{14}CO_2/^{14}CH_4$ ratio and the percentage of methane formed via acetoclastic and syntrophic methanogenesis.

In high ammonia digestate samples, the dominant metabolic pathway for methane formation was confirmed to be via the syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis, responsible for 68–75% of the CH_4 formation; whereas in low ammonia samples, the hydrogenotrophic pathway only accounted for 9–23% of the total CH_4 production. A phylogenetic study confirmed the radiolabelling study.

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Jiang, Ying; Banks, Charles J.; Zhang, Yue; Heaven, Sonia; Longhurst, Philip J.

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