Available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/watres

Influence of high gas production during thermophilic anaerobic digestion in pilot-scale and lab-scale reactors on survival of the thermotolerant pathogens *Clostridium perfringens* and *Campylobacter jejuni* in piggery wastewater

L.C. Skillman[a,*], O. Bajsa[b], L. Ho[b], B. Santhanam[c], M. Kumar[c], G. Ho[b]

[a]Western Australian State Agricultural Biotechnology Centre (SABC), Lonergan Building, Murdoch University, Perth, WA 6150, Australia
[b]School of Environmental Science, Murdoch University, South Street, Murdoch, Western Australia, Australia
[c]South Australian Research and Development Institute (SARDI), Breakwell Laboratory, Roseworthy Campus, 5371 South Australia, Australia

ARTICLE INFO

Article history:
Received 26 November 2008
Received in revised form
9 March 2009
Accepted 20 April 2009
Published online 3 May 2009

Keywords:
Anaerobic digestion
Campylobacter jejuni
Clostridium perfringens
Piggery effluent
Pathogen
Thermophilic

ABSTRACT

Safe reuse of animal wastes to capture energy and nutrients, through anaerobic digestion processes, is becoming an increasingly desirable solution to environmental pollution. Pathogen decay is the most important safety consideration and is in general, improved at elevated temperatures and longer hydraulic residence times. During routine sampling to assess pathogen decay in thermophilic digestion, an inversely proportional relationship between levels of *Clostridium perfringens* and gas production was observed. Further samples were collected from pilot-scale, bench-scale thermophilic reactors and batch scale vials to assess whether gas production (predominantly methane) could be a useful indicator of decay of the thermotolerant pathogens *C. perfringens* and *Campylobacter jejuni*. Pathogen levels did appear to be lower where gas production and levels of methanogens were higher. This was evident at each operating temperature (50, 57, 65 °C) in the pilot-scale thermophilic digesters, although higher temperatures also reduced the numbers of pathogens detected. When methane production was higher, either when feed rate was increased, or pH was lowered from 8.2 (piggery wastewater) to 6.5, lower numbers of pathogens were detected. Although a number of related factors are known to influence the amount and rate of methane production, it may be a useful indicator of the removal of the pathogens *C. perfringens* and *C. jejuni*.

1. Introduction

Anaerobic digestion is widely used to treat wastewater and organic sludges as it reduces oxygen demand, generating both methane for fuel and rich organic manure. To make reuse efforts safe, it is necessary to remove bacterial pathogens present in the waste to acceptable levels. Thermophilic anaerobic digestion is often preferred as it offers a shorter

* *Corresponding author*. Tel.: +61 (0)8 9360 7407.
E-mail address: l.skillman@murdoch.edu.au (L.C. Skillman).
0043-1354/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.watres.2009.04.031

residence time than ambient temperature (mesophilic) systems.

Several systems have been investigated to digest swine wastewater, some by co-digestion with other waste streams (reviewed by Sakar et al., 2009). These systems include: continuous stirred tank reactors (CSTR) similar to the bench-scale reactors described in this study (Hansen et al., 1998; Kaparaju and Rintala, 2005); upflow anaerobic digesters (UASB) (Sanchez et al., 2005) and hybrid UASB (Lo et al., 1994); baffled anaerobic reactors (Boopathy, 1998); anaerobic sequencing batch reactor (ASBR) (Zhang et al., 1997); and two-stage anaerobic digesters similar to the pilot-scale system described in this study (Zhang et al., 2000). The reactors mentioned above were operated between 25 and 60 °C at volumes between <1 L and 380 L, with an organic loading rate (OLR) of 0.9–15 g VS L^{-1} d^{-1} and hydraulic residence times of <1 to >100 d. Chemical oxygen demand (COD) removal ranged from 57 to 78% and volatile solid (VS) removal from 35 to 61%. Methane yields were reported between 22 and 360 ml CH_4 g^{-1} VS added (Sakar et al., 2009). Many studies on piggery wastewater highlight the inhibitory effects of high levels (1700–2300 mg/L) of ammonia-N on reactor performance (Hansen et al., 1998) but Zhang et al. (1997) reported no adverse effect on reactor performance at levels up to 2470 mg/L in an ASBR. Although the factors which influence solubilisation during thermophilic anaerobic digestion have been well studied (Angelidaki et al., 1999; Batstone et al., 2002), studies on pathogen reduction in the treatment process have been given only secondary consideration (Vanotti et al., 2005).

Pathogen die-off in anaerobic treatment is influenced by many variables including sensitivity of the specific types of pathogens, temperature, retention time (reviewed by Bendixen, 1994), pH, free ammonia (Nielsen and Ahring, 2007), volatile fatty acid (VFA) concentration (Kunte et al., 1997; Salsali et al., 2008; Wilson et al., 2008), moisture content, mixing, pathogen density (Horan et al., 2004), availability of nutrients and the presence of other microorganisms (Sidhu et al., 2001; Wrigley, 2004). In general, increasing digestion temperature reduces pathogen survival in an exponential pattern with concomitant die-off at longer hydraulic residence times. The extent of microbial inactivation also depends on the microorganisms, their age, media, pH and nutrient availability (Smith et al., 2005).

In some cases, increasing thermophilic digestion temperature is not feasible due to the cost of additional energy requirements. In other cases, increasing digestion temperature may be ineffective (Sahlstrom, 2003) or even undesirable against bacterial pathogens which have heat survival mechanisms. Although vegetative cells of *Clostridium perfringens* are susceptible to temperatures above 50 °C, their ability to form spores which can survive over an hour at 100 °C, allows them to resist thermophilic digestion (Olsen and Larsen, 1987; Burtscher et al., 1998). Some non-spore formers have alternate mechanisms to resist heat, which in the case of *Campylobacter jejuni*, involve the expression of heat shock proteins and chaperonins (Stintzi, 2003; Murphy et al., 2005). Both pathogens are known to be present at high levels in piggery wastewater (Chinivasagam et al., 2004). Preliminary studies in our laboratory suggested these two pathogens in particular, survived thermophilic digestion at levels above their infective dose and were selected to monitor die-off during thermophilic digestion. The aim of this study was to investigate solubilisation of piggery wastewater and maximise pathogen destruction. Factors affecting solubilisation and gas production also appeared to influence pathogen destruction.

2. Materials and methods

2.1. *Outline, operation and sampling of pilot-scale thermophilic reactors*

The pilot-scale anaerobic digester system makes use of a two-stage (thermophilic then mesophilic) anaerobic digestion process. The first thermophilic digestion stage utilises two 130 L continuous-flow stirred tank (CFST) reactors running in parallel. The mixing pump operates for 15 min every hour to prevent solid build up and gives thorough mixing of the digester contents. These CFST reactors are fed once-daily with 20 L raw piggery wastewater (0.5–3% total dry solids w/v). They are insulated 175 L ^{16}Cr–^{13}Ni–^{3}Mo 3 mm thick stainless steel vessels, equipped with heating tape, thermocouples and a pressure relief safety valve. They operate on a fixed 7 d hydraulic retention time (HRT), at temperatures between 55 and 70 °C.

Piggery wastewater was collected from the on-site holding sump from a 500 head pig farm (0–2 year olds), which are housed in roofed pens and fed commercial pig feed. Sufficient wastewater for each trial was collected from the sump and stored at 4 °C. Solids' analyses and pH measurements were performed according to standard methods (American Public Health Association, 1989). Gas production was measured using positive displacement type gas meters with on-line logging of gas flow. The primary component of this device was a U-tube containing silicone oil (Dow Corning Pty. Ltd.), a three-way solenoid valve, float switch and a timer. The biogas produced accumulated at the top of one side of the U-tube and displaced the liquid so that the liquid level on the other side increased. When the liquid on the other side reached a certain level, a float above the liquid activated a switch. This triggered three events simultaneously: 1) a signal sent to the counter to record the number of clicks and displayed it; 2) accumulated biogas was exhausted to atmosphere through the solenoid valve, which resets the liquid level; and 3) a timer was activated to allow biogas to escape and to allow liquid to reach a steady level. The timer was manually set at 3 s after which, the solenoid valve returned to its original position. During the vent cycle, the three-way solenoid valve isolated the reactor from the meter. Gas production rate was estimated by the volume of gas required to initiate one click multiplied by the number of clicks. Calibration was checked before and after each experimental run. All the tubes used in the experiment were Masterflex tygon tubes (No. 18) (Cole Parmer).

A Varian Star 3400 Model Gas Chromatograph equipped with a TCD was used for biogas analysis. High purity nitrogen gas was used as the carrier gas with detector and column temperature at 120 °C. Porapak Q 80/100 column of length 6 inch and tubing 1/8 inch SS were used. Gas chromatograms were recorded and processed. Retention times of hydrogen, carbon dioxide and methane were used for the detection of peaks. The

operating conditions for the anaerobic system were chosen based on chemical oxygen demand (COD) reduction and nutrient removal capacity of the system (Lettinga and Hulshoff Pol, 1991; Guerrero et al., 1999; Yu and Fang, 2001). Samples were collected for chemical analyses during process optimisation trials from the thermophilic reactors at different operating temperatures (55 and 70 °C). Determination of total (TS) and volatile (VS) solids, total (TCOD) and soluble (SCOD) chemical oxidation demand, total kjeldahl nitrogen (TKN), total ammonia-nitrogen (TAN), soluble (SP) and total phosphorus (TP) were carried out according to the standard methods (American Public Health Association, 1989). Volatile fatty acids (VFA) in the samples were analysed by a gas chromatograph with a flame ionisation detector and capillary column.

2.1.1. *Sampling period 1*

After allowing the system to operate through a minimum of four hydraulic retention time periods to achieve a hydraulic steady state, 30 ml samples were collected from one thermophilic reactor, running between 56 and 58 °C at 7 d HRT. Samples included the raw effluent on days 1 and 7 to check pathogen populations during effluent storage at 4 °C. The initial reactor sample was collected just after the effluent had been introduced into the thermophilic reactor and mixed thoroughly (T0). Remaining samples were collected at 24 hourly intervals for 7 further days (T1–T7) and on day 12 (T8) but collected just before effluent addition (20 L) to the thermophilic reactor (130 L). All samples except T8 were frozen immediately after collection for real-time PCR quantitation and sample T8 was sent to Murdoch University on ice by overnight delivery for most probable number (MPN) enumeration.

2.1.2. *Sampling period 2*

As above, the system was operated for 4 HRT periods to achieve a hydraulic steady state. Thirty millilitre samples were collected from one thermophilic reactor, running at 65 °C, and 7 d HRT. Samples were collected at weekly intervals over four weeks before and after effluent addition (20 L) to the thermophilic reactor (130 L). Samples included the raw effluent each week. All samples were frozen immediately after collection for real-time PCR quantitation.

2.1.3. *Sampling period 3*

As above, the system was operated for 4 HRT periods to achieve a hydraulic steady state. Thirty millilitre samples were collected from both thermophilic reactors on the same day, running at 50 °C and 7 d HRT over an eight-month period. Samples were collected immediately before effluent addition (20 L) to the thermophilic reactor (130 L). The samples were sent fresh to Murdoch University on ice by overnight delivery for MPN enumeration and quantitation by real-time PCR.

2.2. *Outline, operation and sampling of lab-scale thermophilic reactors (CSTR)*

Two continuous-flow, stirred thermophilic reactors (CSTR) were set up using computer control (Labview, National Instruments) of the feed and effluent pumping as well as real-time data logging of temperature and gas production. Total volumes of 1.2 L were seeded with working volumes of 0.9 L of activated sludge, acclimatised to anaerobic conditions for four weeks, and fed with effluent, diluted with distilled water to a final COD feed concentration of 4.4, 7.2 and 12.9 g/L. The reactors were drained and fed intermittently (10 times in 48 h) at 2 d HRT. The lab-scale reactors were run at a short, 2 d HRT, to encourage acidification, although this was achieved with synthetic wastewater in preliminary studies, acidification was not achieved with piggery wastewater due to its large buffering capacity. The short HRT was used to investigate the degree of solubilisation and acidification, rather than maximise methane production.

Four volumes of HRT turnover were allowed before active sampling of gas production and effluents began for 8 consecutive days. The volume of biogas produced daily was measured as previously described. The biogas composition was analysed for hydrogen, methane and carbon dioxide using a Varian gas chromatograph as described above. The effluent samples were analysed for pH, alkalinity, total and soluble COD, total and volatile suspended solids, C2–C6 volatile fatty acids, ammonia-nitrogen, soluble phosphorus and pathogen levels by real-time PCR. Student's *t*-tests were used to statistically compare the reactors' performance for solubilisation, acidification and methanogenesis.

2.3. *Batch vials*

Piggery wastewater collected from the lab-scale thermophilic reactor at 10 d HRT was used to inoculate a mixed microbial community. To ensure there were adequate degradable volatile fatty acids as food source in the reactor effluent, 10% v/v chilled raw piggery wastewater was added to the reactor effluent and divided into five 50 ml portions in 120 ml serum bottles capped with butyl rubber stoppers and crimped tightly with aluminium seals: control vial without pH adjustment; pH adjusted to 8.2 with concentrated hydrochloric acid; and pH 7.5; pH 7.0 and pH 6.5. The headspace was purged with nitrogen and degassed after equilibrating to 55 °C, before incubating in a shaking water-bath at 55 °C for 10 d. Gas volume was measured by plunger displacement and composition was analysed daily using a Varian gas chromatograph as above. From the gas volume and methane concentration, cumulative methane production was calculated for each sample vial. At the conclusion of the experiment, samples were taken for pH, total and soluble COD, VFA and ammonia-nitrogen and real-time PCR analysis.

2.4. *Organisms, growth conditions and most probable number (MPN) enumeration*

Two isolates were used as positive controls in MPN estimations and to prepare enumerated cultures for real-time PCR standards. Firstly, *C. jejuni* subsp. *jejuni* strain NCTC 11351 was maintained at 4 °C on Skirrow agar (Skirrow, 1977) and subcultured every two weeks. Plates were grown for 48 h in sealed jars at 42 °C under microaerophilic conditions using a gas generating system (Oxoid, Hampshire, UK). For liquid culture and MPN enumeration, Preston broth was prepared as described previously by Bolton and Robertson (1982) according to the Australian Standards™ (AS 5013.6-2004), with the addition of FBP supplement (George et al., 1978). Duplicate

dilution series were prepared for each sample in nutrient broth and added to test tubes containing Preston broth as per 3 test tube MPN methods described in the Australian standards™ (AS4276.1:2007). They were incubated for 4 h at 37 °C before the addition of selective antibiotic supplement (Amyl) (0.1 ml). The tubes were then incubated at 42 °C for a further 44 h. An aliquot from each tube was streaked onto Skirrow agar plates and incubated in a microaerophilic atmosphere for 48 h at 42 °C. The number of positive enrichments was used to calculate MPN per 100 ml. Confirmation of the presence of *C. jejuni* was based on Gram stain, motility, catalase and oxidase tests, antibiotic sensitivity and hippurate test to distinguish from *Campylobacter coli*.

Secondly, *C. perfringens* ATCC 13124 (Oxoid) was maintained at 4 °C on RCM broth, which was boiled just before inoculation to remove oxygen, and subcultured monthly. Liquid cultures were grown for 24 h in sealed jars at 37 °C under anoxic conditions. For liquid culture and MPN enumeration, differential reinforced clostridial medium (DRCM; Amyl) was prepared as per manufacturer's instructions. After collection, each sample was heated at 70 °C for 20 min to select for spore-forming bacteria and serially diluted as per MPN methods described in the Australian standards™ (AS4276.17.2:2000). All MPN tests were performed in duplicate. Confirmation of the presence of *C. perfringens* was based on sulphite reduction, gelatin liquefaction and acid production. The number of positive enrichments for each dilution was used to calculate the MPN per 100 ml as per Australian standards™ (AS4276.1:2007).

Pure cultures were enumerated microscopically using a Neubauer haemocytometer and used to prepare standards for real-time PCR. Digester samples with pathogens enumerated by MPN techniques were also used as external standards.

2.5. DNA extraction

DNA extracted from environmental samples often contains high levels of PCR inhibitors such as humic acids. Although DNA extraction kits are faster than conventional DNA extraction methods and provide clean, pure DNA, yields can be lower. Freeze-thawing improved the yield of DNA from pure cultures of both pathogens and from digester samples (personal observation).

Therefore, total DNA was extracted from freeze-thawed thermophilic digester samples and enumerated bacterial cultures, using a Mo Bio (Solana Beach, CA) UltraClean Soil DNA kit. The procedure was slightly modified from the manufacturer's instructions in the following ways: bead solution was removed and replaced with 0.5 ml buffer equilibrated phenol pH 8.0, chloroform, isoamyl-alcohol (25:24:1) (Sigma–Aldrich, St. Louis, MO); mixed with 0.5 ml digester samples and physically disrupted; the upper layer was then mixed with buffer S1 and applied to the spin column; the amount of buffer S3 was doubled followed by an extra wash of the column with buffer S4. A final elution volume of 30 μl was used to elute purified DNA from the spin column.

2.6. Real-time PCR enumeration

Target specificity is particularly important when measuring pathogens in mixed communities and several previously described real-time PCR primers were tested using DNA purified from the pathogens above and from *Escherichia coli* (WACC 4, PathWest Culture Collection, Perth), *Salmonella typhi* (ATCC 14028), *Enterococcus faecalis* (WACC 28, PathWest, Perth), *Methanoculleus* spp. (bioreactor isolate). Of these, the most promising primer sets were used to quantify different groups (Table 1).

Primer pairs selected to quantify all bacteria, *C. jejuni* or methanogens (Table 1), were included in 20-μl real-time PCR mixtures containing 10 μl SYBR Green mix (IQ SYBR Green Supermix; Bio-Rad), 7 μl distilled H_2O, 1 μl forward primer (10 μM concentration), 1 μl reverse primer (10 μM concentration), and 1 μl DNA. Real-time PCR amplification was conducted on a Rotorgene 3000 (Corbett Life Science, Sydney, Australia) according to the manufacturer's instruction. Amplification was initiated by denaturation at 95 °C for 10 min and was followed by up to 40 cycles of denaturation at 95 °C for 15 s and annealing at 55 °C (50 °C in the case of methanogens) for 30 s, and then by extension at 72 °C for 30 s. Fluorescence was acquired during extension using an excitation wavelength of 470 nm and emission detection at 530 nm. A final melting-curve analysis was carried out by continuously monitoring fluorescence between 55 °C and 95 °C with 0.5 °C increments every 10 s. Threshold cycles were calculated automatically by the Rotorgene software (Version 6), standardized amounts of DNA extracted from enumerated cultures were included in each run to monitor and correct any between-run variability.

Fisher Biotech PCR reagents (Wembley, Western Australia) were used for Taqman real-time PCR for *C. perfringens*. Each 20 μl reaction contained: 1 μl hybridisation probe (2 μM), 2 μl forward primer (10 μM), 2 μl reverse primer, 2 μl PCR buffer, 2 μl dNTPs (2 mM), 2 μl $MgCl_2$ (25 mM), 0.2 μl Hotstart Taq, 7.8 μl dH_2O and 1 μl DNA. The cycling conditions were as follows: an initial 10-min step at 94 °C for Taq activation, followed by 35 cycles of denaturation at 94 °C for 10 s, annealing at 55 °C for 20 s, and extension at 70 °C for 10 s. Fluorescence was acquired following the annealing step.

As the levels of PCR inhibitors may vary in each digester sample, it is crucial to estimate PCR efficiency, as this can have a large influence on resulting quantitation according to the equation $N = N_0\varepsilon^n$ (N_0 is the number of cells/amount of DNA present initially, before the PCR; ε is the efficiency of the PCR; and n is the number of cycles). PCR efficiency (ε) was calculated using the Rotorgene software (Version 6). The number of cells in the digester samples (N_0) was calculated from duplicate DNA extractions, calculation of PCR efficiency, and substitution of the constant (N) calculated from the standards into the following equation: $N_0 = N/\varepsilon^n$ (Tichopad et al., 2003).

3. Results and discussion

3.1. Chemical properties of raw piggery wastewater and pilot-scale thermophilic digester samples

Chemical analyses conducted during initial optimisation trials are presented in Table 2. Although the piggery wastewater strength varied seasonally and due to washing

Table 1 – PCR primers selected for quantitation of microbial populations in thermophilic digester samples. Presented according to the oligonucleotide probe nomenclature (Alm et al., 1996).

Primer	Target group	Position on 16S	Sequence	Reference
Fq	16S gene, all bacteria and methanogens	1097	CGGCAACGAGCGCAACCC	Christophersen et al. (personal communication)
Rq	16S gene, all bacteria and methanogens	1221	CCATTGTAGCACGTGTGTAGCC	Christophersen et al. (personal communication)
MET630F	16S gene, methanogens	630	GGATTAGATACCCSGGTAGT	Christophersen et al. (personal communication)
MET803R	16S gene, methanogens	803	GTTGARTCCAATTAAACCGCA	Christophersen et al. (personal communication)
CampF	DNA gyrase (GyrA)	NA	TGGGTGCTGTTATAGGTCGT	Fukushima et al. (2003)
CampR	DNA gyrase (GyrA)	NA	GCTCATGAGAAAGTTTACTC	Fukushima et al. (2003)
CperfF	16S gene, *Clostridium perfringens*	176	CGCATAACGTTGAAAGATGG	Wise and Siragosa (2005)
CperfR	16S gene, *Clostridium perfringens*	258	CCTTGGTAGGCCGTTACCC	Wise and Siragosa (2005)
Cperf probe		190	5′-[FAM]TCATCATTCAACC AAAGGAGCAATCC[TAMRA]-3′	Wise and Siragosa (2005)

procedures, the percentage solids were generally between 0.5 and 3%; total chemical oxygen demand (TCOD/L) was between 20 and 30 g/L and total ammonia concentration was between 1.8 and 2.4 g/L. Assessment of the initial soluble COD/total COD ratio of the raw piggery wastewater revealed that more than 30% of the initial organic matter had already been solubilized almost entirely to volatile fatty acids by the indigenous microbial populations in the effluent holding sump under ambient conditions. During thermophilic digestion, pH increased from 7.2 to 7.8 and the total COD, soluble COD and total VFAs reduced as they were used up during conversions by the resident microbial communities. Solubilisation of the piggery wastewater was higher in the 55 °C reactor (28% TCOD removed) than the 70 °C (12% TCOD removed). The predicted biogas production based on mass balance of total COD removed in the 55 °C thermophilic digester of 49.7 L/d, is higher than the gas production measured (32.4), but could be explained due to the high variability of the influent total COD (SD 9.5 g/L) (Table 2). The predicted biogas production based on total COD removed in the 70 °C thermophilic digester of 21.7 L/d, is higher than the gas production measured (5.4), but could be explained by the high variability of both the influent and effluent TCOD (9.5 and 5.3 g/L respectively). The methane production rates in the 55 °C and 70 °C thermophilic digesters were calculated at 1.02 and 0.12 L/L/d and methane yield 0.14 and 0.04 m^3 methane/kg TCOD removed respectively.

At the higher digestion temperature of 70 °C, COD reduction was lower and total VFAs remaining were higher; with associated gas production much lower (5 vs 34 L/d at 55 °C, Table 2) with a lower proportion of methane (45 vs 64%) in the biogas.

Table 2 – Chemical analysis of piggery wastewater and pilot-plant thermophilic digesters running at 55 and 70 °C, over a three-week period, standard deviation indicated.

Parameter	Unit	Piggery wastewater (influent)	Thermophilic digester (effluent)	Thermophilic digester (effluent)
Temperature			55	70
HRT	d		7	7
pH		7.2 (0.2)	7.8	7.8
Loading rate	g/L/d		3.73 (0.9)	3.73 (0.9)
Total chemical oxygen demand	g TCOD/L	25.0 (9.5)	17.9 (1.5)	21.9 (5.3)
Soluble chemical oxygen demand	g SCOD/L	10.3 (0.5)	5.7 (1.2)	9.5 (2.6)
Total volatile fatty acids	g VFA COD/L	1.65 (0.01)	0.46 (0.12)	0.67 (0.5)
Acetate	mg/L	736 (118)	233 (75)	278 (185)
Propionate	mg/L	250 (18)	114 (16)	131 (103)
Ammonium-nitrogen	mg/L	1361 (319)	1728 (596)	1611 (346)
Gas production	L/d		32.4 (15.4)	5.4 (3.8)
Carbon dioxide	%		35	50
Methane	%		63.5	45
Methane production rate	L/L/d		1.02 (0.49)	0.12 (0.09)
Methane yield	m^3/kg TCOD removed		0.15 (8%)	0.04 (24%)

3.2. Pathogen levels in pilot-scale reactors at different operating temperatures

Samples were collected from pilot-plant thermophilic reactors running at 50, 57 and 65 °C and *C. perfringens* and *C. jejuni* levels measured by MPN techniques. Both pathogens were detected at high levels in raw piggery wastewater (10^5 and 10^3 per gram, respectively). As expected, the number of pathogens surviving thermophilic digestion declined with increasing temperature (Table 3), but some remained, even at a digestion temperature of 65 °C. The statistical significance was examined using ANOVA and Tukey's pairwise comparison following log transformation of the data to stabilise variance, the levels of *C. jejuni* were significantly different between 50 and 65 °C ($p = 0.009$) (Table 3). As high temperatures were not sufficient to remove all pathogens, further samples were collected to investigate other chemical parameters associated with pathogen removal.

Samples were collected daily from one thermophilic reactor in the pilot plant and pathogens measured by real-time PCR. Viable counts of the pathogens were also measured in the final sample (day 12). Variation in the *C. perfringens* populations, increasing and declining over time, was evident following enumeration by real-time PCR (Fig. 1). This trend was not apparent in the total microbial population, which was monitored to allow for potential differences in consistency between samples. The total microbial population remained relatively constant (between 6 and 6.7×10^9 per ml, SD 0.2), so the observed variation was unlikely to reflect sample inconsistency. Low levels (20–80 per 100 ml) of *C. jejuni* were detected by culturing and real-time PCR in the raw effluent sample and reactor samples on days 2 and 12 where gas production rate was lowest (1.8 L/h).

Gas production was measured at hourly intervals and averaged over the 24 h period immediately preceding sample collection and plotted alongside the calculated *C. perfringens* populations (Fig. 1). There was a striking inversely proportional relationship between gas production and number of *C. perfringens*. Regression analysis of gas production and number of *C. perfringens* showed a significant correlation ($p = 0.0017$, $r^2 = 0.81$). As Clostridia produce hydrogen during anaerobic breakdown of organic waste, a positive correlation might be expected. If *C. perfringens* cells were competing with other Clostridia for substrates, the observed negative correlation may be the result.

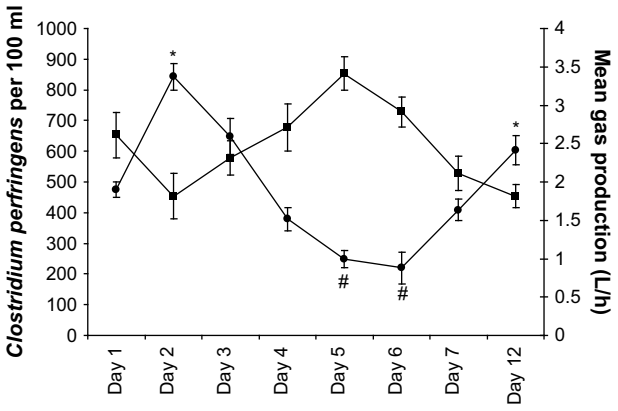

Fig. 1 – Levels of *Clostridium perfringens* (●) in samples collected daily from a pilot-scale thermophilic reactors (57 °C, 7 d HRT), measured by real-time PCR and corrected to total microbial population. Mean gas production in the 24 h period preceding sampling is presented on the secondary axis (■). Standard errors are indicated. Methanogen levels were compared in samples marked # and *.

Table 3 – Levels of pathogens measured by MPN culture methods in pilot-scale thermophilic reactors at different temperatures, 7 d HRT. Standard errors indicated, numbers with different superscripts are significantly different and $p = 0.05$.

Digestion temperature	*Clostridium perfringens* per 100 ml	*Campylobacter jejuni* per 100 ml
Raw effluent	>11 000	~1000
65 °C, 7 d HRT	424 (94)	5 (3)[a]
57 °C, 7 d HRT	615 (248)	50 (26)[ab]
50 °C, 7 d HRT	1670 (719)	393 (207)[b]

To investigate this correlation further, a second set of samples was collected weekly, both before and after effluent addition, to one thermophilic digester over a longer four weekly period (Fig. 2). The reactor was running at a higher temperature of 65 °C. Gas production declined over the course of the experiment from 0.8 to 0.47 L/h by the end, perhaps reflecting declining substrate availability. Levels of both bacterial pathogens were high at the start of the trial (Fig. 2A and B). This may have resulted from higher initial substrate levels. Following the high reading in the first samples, *C. perfringens* levels peaked when gas production was lowest (Fig. 2A), supporting previous findings, although the correlation using regression analysis was not significant. Despite the low number of samples, *C. jejuni* levels were significantly higher where gas production was lowest (Fig. 2B) ($p = 0.057$, $r^2 = 0.89$).

Levels of both pathogens were slightly lower during this trial, likely to reflect the higher operating temperature (65 vs 58 °C). However, at both temperatures, gas production appeared to be inversely proportional to pathogen number. Gas production also varied between the two pilot-scale thermophilic digesters which were set up in parallel, probably as a result of the differences in resident bacterial communities.

Viable counts and real-time PCR of *C. perfringens* were determined in both thermophilic digesters during a third sampling period with digesters running at 50 °C over a three-month period. On one occasion, the levels of pathogens in the two reactors varied considerably (June 2007), despite running at the same temperature using the same raw effluent (Table 4). Reactor two had a mean gas production more than double that of thermophilic reactor one (1.13 vs 0.45 L/h). This correlated with considerably lower numbers of pathogens surviving (Table 4). Regression analysis showed that there was a significant correlation between gas production and level of *C. perfringens* with a *p*-value of 0.007 and $r^2 = 0.86$ (Fig. 3).

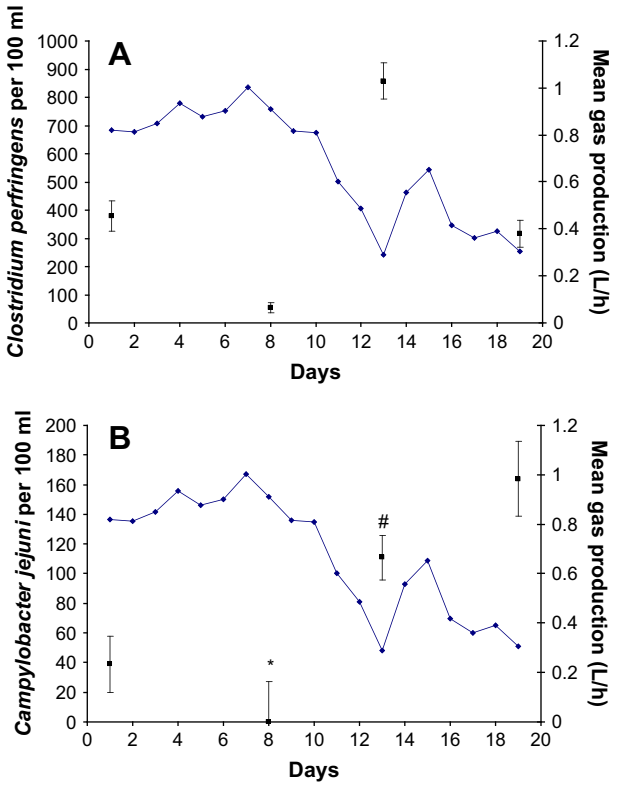

Fig. 2 – Gas production (mean L/h) (SE between 0.03 and 0.05) and pathogen counts measured by real-time PCR during a four-week trial with samples collected on days 7, 14, 21 and 28. Thermophilic digesters were running at 65 °C (±2), 7 d HRT. Pathogen counts, corrected to the total microbial population, are presented on secondary axis in graph A (*Clostridium perfringens*) and graph B (*Campylobacter jejuni*) with standard errors indicated. Methanogen levels were compared in samples marked # and *.

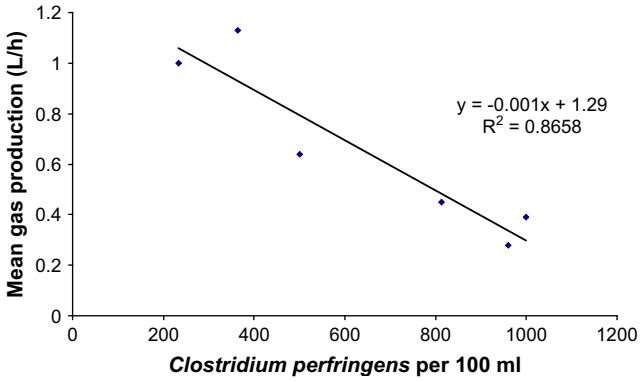

Fig. 3 – Correlation (*p*-value 0.007) between gas production and *Clostridium perfringens* populations measured by real-time PCR in two parallel pilot-scale thermophilic anaerobic digesters running at 50 °C and 7 d HRT.

3.3. *Methanogen levels in pilot-scale reactors at different operating temperatures*

The terminal and rate-limiting step of anaerobic digestion is methanogenesis, with methanogens converting hydrogen and carbon dioxide to methane. It would be expected that the number or activity of methanogens would be higher in samples with higher levels of gas production and therefore, methanogens may also be an important indicator of pathogen removal. Only trace levels of hydrogen were present in the thermophilic reactors running at 55 °C with the gas consisting partly CO_2 (30–40%) and the remainder methane (Table 2).

The numbers of methanogens in samples with high pathogen numbers and low gas production (* Figs. 1 and 2) and low pathogen numbers and high gas production (# Figs. 1 and 2) were compared. The microbial population was almost twice as high when gas production was high (4.3×10^{10} SE 0.7 vs 2.5×10^{10} SE 0.4), with methanogen levels almost three-times as high (5.50×10^{8} SE 1.2 vs 1.90×10^{8} SE 0.6). Although piggery wastewater contained predominantly hydrogen-utilising methanogens, aceticlastic methanogens related to *Methanosarcina thermophila* appeared to rapidly predominate in the reactors during anaerobic digestion at the relatively high pH found in piggery wastewater (data not shown).

3.4. *Pathogen levels in laboratory-scale reactors using different strength piggery wastewater*

The correlation between gas production and pathogen levels was investigated further in bench-scale continuously stirred tank reactors (CSTR) which allowed closer control of temperature and monitoring of chemical properties. The lab-scale reactors were run at a short 2 d HRT to investigate solubilisation, rather than maximise methane production. The first

Table 4 – Viable counts and real-time PCR of pathogens in samples collected from both thermophilic reactors running at 50 °C, 7 d HRT.

Sample	Gas production (mean L/h)	*Clostridium perfringens* (MPN) per 100 ml	*Clostridium perfringens* (real-time PCR) per 100 ml	*Campylobacter jejuni* (real-time PCR) per 100 ml
Raw effluent	N/A	11 000	>11 000	~5000
T1 April 2007	1	ND	233	0.65
T2 April 2007	0.64	ND	501	0.9
T1 June 2007	0.45	3100	813	780
T2 June 2007	1.13	240	364	6.8
T1 Nov 2007	0.39	11 000	10 000	15
T2 Nov 2007	0.28	7800	9600	23.4

trial investigated the effect of feed strength (low, medium and high) on methane production, pathogen levels and chemical properties. With less available substrates, methane production would be expected to be lower and this may influence pathogen removal. Loading rates were comparable between the bench-scale reactors (2.2 low, 3.5 mid and 6.4 high g TCOD/L/d) and pilot-scale digesters (3.7 g TCOD/L/d). Maximum TCOD removal was achieved using mid-strength feed (29%), with high-strength and low-strength feed lower (19 and 1% respectively). Methane yield was almost twice as high in the mid-strength compared to the high-strength (0.27 vs 0.15). The methane yield in the low strength appeared to be much higher (1.9), but this was likely a result of inaccuracy of measuring very low amounts of TCOD removal and large standard deviation. Methane yield was similar between the pilot-scale reactors operating at 55 °C and bench-scale reactors using full-strength feed water as feed, despite the different HRT of 7 d and 2 d HRT respectively.

Mid-strength feed produced a higher methane yield (0.27) in the bench-scale reactors than the pilot-scale reactors, likely due to dilution of inhibitors present in high-strength feed (Table 5) and improved mixing. In another study with fixed-bed reactors treating piggery wastewater, Sanchez et al. (2006) also found optimal removal efficiency at 4–8 g TCOD/L, with decreasing efficiency at higher concentrations.

The highest level of methanogens (8×10^7) was found using the mid-strength piggery wastewater feed, which also showed the highest pathogen kill (30%) (Table 5). Two-way ANOVA showed the methane yield from all three feed strengths was significantly different ($p < 0.001$). However, there was no significant difference between methanogen and *C. perfringens* levels, largely due to variations between replicates. The number of methanogens was >10 fold higher in the bench-scale thermophilic reactors than in the pilot-scale thermophilic reactors, irrespective of feed strength, which could reflect better mixing and substrate availability.

In other studies (L. Ho, 2008, unpublished), artificially lowering the pH of piggery wastewater from 8.2 to 5.5 inhibited methanogenesis but enhanced solubilisation and acidification. By contrast, lowering the pH to 6.5 enhanced substrate utilisation and methane production (data not shown). The effect of lowering pH on pathogen survival was therefore investigated (Fig. 4). Levels of *C. perfringens* and methanogens were measured by real-time PCR in vials after 10 d incubation. Methane production rate increased as pH was lowered, with cumulative methane production 33 ml at pH 6.5, 22.5 ml at pH 7, 16 ml at pH 7.5 and 7 ml at pH 8.3. Methanogen numbers were correspondingly higher (1×10^4 at pH 8.2 and 8×10^5 at pH 6.5) and *C. perfringens* were lower (2.5×10^4 at pH 8.2 and 2×10^3 at pH 6.5) (Fig. 4).

Under conditions which increased gas production such as increasing feed strength or decreasing pH from 8.2 to 6.5, *C. perfringens* survival was correspondingly lower.

4. Discussion

Optimal thermophilic digestion temperature in anaerobic digesters is often a compromise between the best temperature for solubilisation and the best for pathogen destruction. Both the pilot-plant thermophilic digesters and bench-scale digesters were operated in the same way by draw and fill. They also used the same wastewater as influent. The data presented here from each reactor differ in hydraulic residence time (HRT) and feed strength. Initially both systems were operated on a 4 d HRT but 7 d was selected for the pilot-scale

Table 5 – Chemical composition and analyses of lab-scale thermophilic digesters, running at 55 °C, 2 d HRT with three feed strengths. Standard deviations indicated, methane yields with different capital letter superscripts are significantly different ($p = 0.001$).

Parameter	Unit	Low strength	Mid-strength	High-strength
Temperature		55	55	55
HRT	d	2	2	2
Loading rate	g TCOD/L/d	2.2	3.5	6.4
Influent TCOD	g TCOD/L	4.44 (0.40)	7.12 (0.49)	12.87 (0.77)
Influent SCOD	g SCOD/L	2.70 (0.12)	5.18 (0.29)	7.62 (0.75)
Total alkalinity	g $CaCO_3$	2.19 (0.17)	3.60 (0)	5.60 (0.49)
Effluent TCOD	g TCOD/L	4.38 (0.40)	5.05 (0.45)	10.37 (0.77)
Effluent SCOD	g SCOD/L	2.75 (0.12)	3.65 (0.23)	7.12 (0.42)
Total alkalinity	g $CaCO_3$	2.51 (0.07)	4.01 (0.12)	6.50 (0.17)
Total volatile fatty acids	g VFA COD/L	2.22 (0.24)	2.55 (0.19)	6.01 (0.31)
Acetate (C2)	mg COD/L	761 (97)	1003 (113)	2652 (141)
Propionate (C3)	mg COD/L	474 (59)	1074 (91)	1964 (169)
Ammonium-nitrogen	mg/L	690 (61)	968 (28)	2150 (191)
Carbon dioxide	%	24 (2)	20 (1)	30 (2)
Methane	%	65 (3)	84 (2)	72 (1)
Biogas production rate	L/L/d	0.093 (0.01)	0.329 (0.016)	0.249 (0.019)
Methane production rate	L/L/d	0.06 (0.006)	0.28 (0.013)	0.19 (0.015)
Methane yield	m^3/kg TCOD removed	1.9 (9%)[aA]	0.27 (9%)[B]	0.15 (6%)[C]
Methanogens per ml	$\times 10^7$	4.65 (0.4)	8.17 (0.9)	7.7 (0.8)
Clostridium perfringens per 100 ml		229 (180 in)	279 (400 in)	350 (425 in)
% Reduction		N/A	30%	18%

a Methane yield is likely to reflect inaccuracy in measuring the low TCOD removal using the low-strength feed.

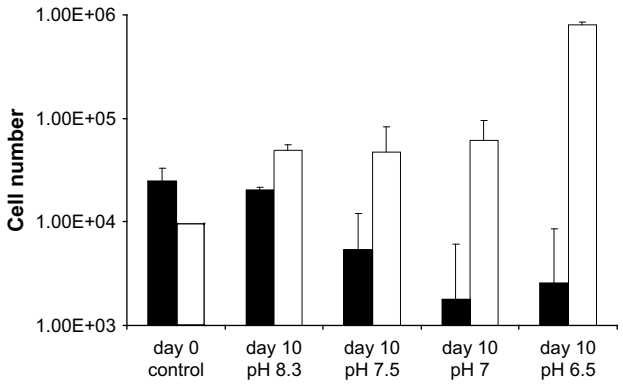

Fig. 4 – Methanogens per ml (□) and *Clostridium perfringens* per 100 ml (■) levels in samples collected from batch vial experiments with pH levels between 6.5 and 8.3, measured by real-time PCR and corrected to total microbial population. SE indicated.

system as optimal for digestion of solids, to produce nutrients and increase the availability of inorganic substrates. The lab-scale reactors were run at a short, 2 d HRT, to encourage acidification, although this was achieved with synthetic wastewater in prior studies, acidification was not achieved with piggery wastewater due to its large buffering capacity. Other studies have shown up to 78% removal of TCOD in some similar systems (Sakar et al., 2009). The 55 °C pilot-scale thermophilic reactor was operating at 28% TCOD removal with a methane yield of 0.14 m^3/kg TCOD removed. The TCOD removal and methane yield were lower when operated at 70 °C (12.4% and 0.04 respectively). The TCOD removal and methane yield were slightly higher in the bench-scale reactors (29% and 0.27 respectively with the mid-strength feed), likely to result from better mixing, despite the sub-optimal 2 d HRT.

Although increasing temperature and retention time improves pathogen kill, in this system, optimal solubilisation was achieved at 55 °C. At a temperature of 65 °C, quite high levels of viable *C. perfringens* remained (424 per 100 ml) and some *C. jejuni* survived (5 per 100 ml). Even at temperatures above 65 °C, some viable *C. perfringens* and *C. jejuni* remained. This may have resulted from insufficient mixing in the pilot-scale digesters (130 L), reducing the effective hydraulic residence time (HRT). Experiments were therefore also carried out in bench-scale reactors and batch vials, with better mixing and less heterogeneity.

Although pathogen survival in thermophilic digestion is undoubtedly temperature related (Bendixen, 1994), in both pilot- and laboratory-scale reactors, increased gas production in addition to temperature, appeared to correlate with lower levels of *C. perfringens* and *C. jejuni*. The correlation was less pronounced with *C. jejuni* which may have reflected their lower density (6550 vs >11 000 per 100 ml) or different mechanisms of heat resistance. On some occasions, *C. jejuni* levels were below the limit of detection by real-time PCR (approximately 10 cells/ml) and could not be detected reliably in samples from bench-scale reactors. *C. jejuni* does not sporulate and reportedly loses viability rapidly at temperatures above 50 °C (ICMSF, 1996), however above 56 °C the cells demonstrated a non-logarithmic reduction in numbers, resulting in a tailing effect which could be significant. It has also been shown that a stress response can be induced in *C. jejuni* at the alkaline pH found in piggery wastewater (Wu et al., 1994). Other species of bacterial pathogens were investigated but appeared to be removed effectively by thermophilic digestion at these temperatures, so it is not clear whether the correlation between methane production and pathogen kill is confined to *C. perfringens* and *C. jejuni*.

C. perfringens, as a spore-forming hydrogen producer, might be expected to increase in number as methane production increases, with the hydrogen produced converted to methane by hydrogenotrophic methanogenic groups, but the opposite trend was observed. The observed negative correlation may reflect inhibition of hydrogen-consuming groups and/or competition with other spore formers for limited substrates. For example, the growth of homoacetogenic Clostridia (e.g. *Clostridium thermoaceticum*) could outcompete *C. perfringens* for available substrates, increasing the amount of acetate produced with more available for conversion to methane by the aceticlastic methanogens. This explanation would be supported by the observation that aceticlastic groups of methanogens such as *M. thermophila* appeared to predominate over hydrogen-utilising groups in the thermophilic reactors (personal observation). The growth of the pathogens may also be inhibited by indigenous microorganisms (Sidhu et al., 2001) or reflect reduced activity (and hydrogen production) during conditions which favour sporulation. Although neither method of bacterial quantitation, real-time PCR or MPN, could distinguish between spores and vegetative cells, it would be of interest to know the proportion of each.

The growth or activity of bacteria in the digester communities is likely to influence pathogen survival through competition for available substrates or the production of inhibitory compounds. In the case of *C. perfringens*, sporulation and subsequent survival have been shown to be influenced by the presence of *Bacteroides fragilis* and short-chain fatty acids (Wrigley, 2004). Under conditions which favour hydrogen producing spore formers, such as increasing digestion temperatures, hydrogen-consuming groups may decline, with more hydrogen available for methanogenesis. An increase in number and proportion of hydrogen-consuming methanogens has been reported during thermophilic digestion at 65 °C compared to 55 °C (Ahring et al., 2001). We also found that increased gas production correlated with higher levels of methanogens. However, a number of other factors are known to influence methane production. High concentrations of ammonia are inhibitory, particularly to hydrogen-consuming methanogens. The total ammonia concentration in the undiluted piggery wastewater used in both pilot- and laboratory-scale reactor experiments was between 1.8 and 2.4 g/L which is reportedly inhibitory to methanogens at pH above 7.6 (Hashimoto, 1986). Artificially lowering the pH in bench-scale experiments, increased methane production and reduced pathogen survival, and coincided with the lowest levels of free ammonia.

A correlation between the survival of these two pathogens has been previously reported (Skanseng et al., 2006), although it is not clear whether this occurred because of direct interactions or indirectly with similar conditions regulating their heat resistance mechanisms. Work is continuing on comparing the bacterial and archaeal communities in these digester samples

to examine changes which may be associated with increased methane production and improved pathogen removal.

5. Conclusions

- During thermophilic anaerobic digestion, to capture energy and nutrients from piggery effluent, higher temperatures reduced the survival of the bacterial pathogens *C. jejuni* and *C. perfringens* as expected. However, higher levels of gas production also correlated with lower pathogen levels, irrespective of temperature.
- This correlation was confirmed under different operating temperatures over periods of a week, several weeks and months. It did not appear to result from variation in the size of the microbial digestion communities although the types of bacteria will undoubtedly influence digestion and subsequent hydrogen production.
- The terminal step in anaerobic digestion is the conversion of hydrogen and carbon dioxide and acetate to methane by methanogens. Methane can be captured and used as an energy source. The number of methanogens in reactor samples correlated with increased methane production and enhanced pathogen kill.
- Under conditions which enhanced gas production in lab-scale reactors, such as increased organic loading rate or reduced pH (6.5), pathogen removal was also enhanced.

Acknowledgements

This work was supported by the Australian Government through the Environmental Biotechnology Cooperative Research Centre (EBCRC) and the South Australian Research and Development Institute (SARDI). Assistance from the team at Roseworthy including Paul Harris, Sandy Wyatt and Belinda Rodda is gratefully acknowledged.

REFERENCES

Ahring, B.K., Ibrahim, A.A., Mladenovska, Z., 2001. Effect of temperature increase from 55 °C to 65 °C on performance and microbial population dynamics of an anaerobic reactor treating cattle manure. Water Research 35, 2446–2452.

Alm, E.W., Oerther, D.B., Larsen, N., Stahl, D.A., Raskin, L., 1996. The oligonucleotide probe database. Applied and Environmental Microbiology 62, 3557–3559.

American Public Health Association, American Water Works Association, Water Pollution Control Federation, 1989. Standard Methods for the Examination of Water and Wastewater, 17th ed. American Public Health Association, Washington, DC, USA, p. 40.

Angelidaki, I., Ellegaard, L., Ahring, B.K., 1999. A comprehensive model of anaerobic bioconversion of complex substrates to biogas. Biotechnology and Bioengineering 63, 363–372.

Batstone, D.J., Keller, J., Angelidaki, I., Kalyuzhny, S.V., Pavlostathis, S.G., Rozzi, A., Sanders, W.T.M., Siegrist, H., Vavilin, V.A., 2002. Anaerobic Digestion Model No. 1 (ADM1). IWA Publishing, London, UK (Scientific and Technical Report 13).

Bendixen, H.J., 1994. Safeguards against pathogens in Danish biogas plants. Water Science and Technology 30, 171–180.

Bolton, F.J., Robertson, L., 1982. A selective medium for isolation of *Campylobacter jejuni/coli*. Journal of Clinical Pathology 35, 462–467.

Boopathy, R., 1998. Biological treatment of swine waste using anaerobic baffled reactors. Bioresource Technology 64, 1–6.

Burtscher, C., Fall, P.A., Christ, O., Wilderer, P.A., Wuertz, S., 1998. Detection and survival of pathogens during two stage thermophilic/mesophilic anaerobic treatment of suspended organic waste. Water Science and Technology 38, 123–126.

Chinivasagam, H.N., Thomas, R.J., Casey, K., McGaham, E., Gardner, E.A., Rafiee, M., 2004. Microbiological status of piggery wastewater from 13 piggeries in the south east Queensland region of Australia. Journal of Applied Microbiology 97, 883–891.

Fukushima, H., Tsunomori, Y., Seki, R., 2003. Duplex real-time SYBR green PCR assays for detection of 17 species of food- or waterborne pathogens in stools. Journal of Clinical Microbiology 41, 5134–5146.

George, H.A., Hoffman, P.S., Smibert, S.M., Krieg, N.R., 1978. Improved media for growth and aerotolerance of *Campylobacter fetus*. Journal of Clinical Microbiology 8, 36–41.

Guerrero, L., Omil, F., Mendez, R., Lema, J.M., 1999. Anaerobic hydrolysis and acidogenesis of wastewaters of food industries with high content of organic solids and protein. Water Research 33, 3281–3290.

Hansen, K.H., Angelidaki, I., Ahring, B.K., 1998. Anaerobic digestion of swine manure: inhibition by ammonia. Water Research 32, 5–12.

Hashimoto, A.G., 1986. Ammonia inhibition of methanogenesis from cattle wastes. Agricultural Wastes 17, 241–261.

Horan, N.J., Fletcher, L., Betmal, S.M., Wilks, S.A., Keevil, C.W., 2004. Die-off of enteric bacterial pathogens during mesophilic anaerobic digestion. Water Research 38, 1113–1120.

ICMSF (International Commission on Microbiological Specifications for Foods), 1996. Micro-organisms in Foods 5. Microbiological Specifications of Food Pathogens. Blackie Academic and Professional, London.

Kaparaju, P., Rintala, J., 2005. Anaerobic co-digestion of potato tuber and its industrial by-products with pig manure. Resources, Conservation and Recycling 43, 175–188.

Kunte, D.P.M., Yeole, T.Y., Chiplonkar, S.A., Ranade, D.R., 1997. Inactivation of *Salmonella typhi* by high levels of volatile fatty acids during anaerobic digestion. Journal of Applied Microbiology 84, 138–142.

Lettinga, G., Hulshoff Pol, L.W., 1991. UASB-process design for various types of wastewaters. Water Science and Technology 24, 87–107.

Lo, K.V., Liao, P.H., Gao, Y.C., 1994. Anaerobic treatment of swine wastewater using hybrid UASB reactors. Bioresource Technology 47, 153–157.

Murphy, C., Carroll, C., Jordan, K.N., 2005. Environmental survival mechanisms of the foodborne pathogen *Campylobacter jejuni*. Journal of Applied Microbiology 100, 623–632.

Nielsen, H.B., Ahring, B.K., 2007. Effect of tryptone and ammonia on the biogas process in continuously stirred tank reactors treating cattle manure. Environmental Technology 28, 905–914.

Olsen, J.E., Larsen, H.E., 1987. Bacterial decimation times in anaerobic digestions of animals slurries. Biological Wastes 21, 153–168.

Sahlstrom, L., 2003. A review of survival of pathogenic bacteria in organic waste used in biogas plants. Bioresource Technology 87, 161–166.

Sakar, S., Yetilmezsoy, K., Kocak, E., 2009. Anaerobic digestion technology in poultry and livestock waste treatment: a literature review. Waste Management Research 27, 3.

Salsali, H., Parker, W.J., Sattar, S.A., 2008. The effect of volatile fatty acids on the inactivation of *Clostridium perfringens* in anaerobic digestion. World Journal of Microbiology and Biotechnology 24, 659–665.

Sanchez, E., Borja, R., Travieso, L., Martin, A., Colmenarejo, M.F., 2005. Effect of organic loading rate on the stability, operational parameters and performance of a secondary upflow anaerobic sludge bed reactor treating piggery waste. Bioresource Technology 96, 335–344.

Sanchez, E., Borja, R., Travieso, L., Martin, A., Colmenarejo, M.F., Nikolaeva, S., 2006. An evaluation of variables affecting the stability and performance of down-flow anaerobic fixed bed reactors treating piggery. Environmental Technology 27, 85–93.

Sidhu, J., Gibbs, R.A., Ho, G.E., Unkovich, I., 2001. Selection of *Salmonella typhimurium* as an indicator for pathogen regrowth potential in composted biosolids. Letters in Applied Microbiology 29, 303–307.

Skanseng, B., Kaldhusdal, M., Rudi, K., 2006. Comparison of chicken gut colonisation by the pathogens *Campylobacter jejuni* and *Clostridium perfringens* by real-time quantitative PCR. Molecular and Cellular Probes 20, 269–279.

Skirrow, M.B., 1977. Campylobacter enteritis: a ''new'' disease. British Medical Journal 2, 9–11.

Smith, S.R., Lang, N.L., Cheung, K.M., Spanoudaki, K., 2005. Factors controlling pathogen destruction during anaerobic digestion of biowastes. Waste Management 25, 417–425.

Stintzi, A., 2003. Gene expression profile of *Campylobacter jejuni* in response to growth temperature variation. Journal of Bacteriology 185, 2009–2016.

Tichopad, A., Dilger, M., Schwarz, G., Pfaffl, M.W., 2003. Standardized determination of real-time PCR efficiency from a single reaction set-up. Nucleic Acids Research 31, 122.

Vanotti, M.B., Millner, P.D., Hunt, P.G., Ellison, E.Q., 2005. Removal of pathogen and indicator microorganisms from liquid swine manure in multi-step biological and chemical treatment. Bioresource Technology 96, 209–214.

Wilson, C.A., Murthy, S.M., Fang, Y., 2008. The effect of temperature on the performance and stability of thermophilic anaerobic digestion. Water Science and Technology 57, 297–304.

Wise, M.G., Siragosa, G.R., 2005. Quantitative detection of *Clostridium perfringens* in the broiler fowl gastrointestinal tract by real-time PCR. Applied and Environmental Microbiology 71, 3911–3916.

Wrigley, D.M., 2004. Inhibition of *Clostridium perfringens* sporulation by *Bacteroides fragilis* and short-chain fatty acids. Anaerobe 10, 295–300.

Wu, C., Clos, J., Giorgi, G., Haroun, R.I., Kim, S.-J., Rabindran, S.K., Westwood, J.T., Wisniewski, J., Yim, G., 1994. Structure and regulation of heat shock transcription factor. In: Morimoto, R.I., Tissieres, A., Georgopoulos, C. (Eds.), The Biology of Heat Shock Proteins and Molecular Chaperones. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 395–416.

Yu, H.Q., Fang, H.H.P., 2001. Acidification of lactose in wastewater. Journal of Environmental Engineering 127, 825–831.

Zhang, R.H., Yin, Y., Sung, S., Dague, R.R., 1997. Anaerobic treatment of swine waste by the anaerobic sequencing batch reactor. Transactions of the American Society of Agricultural Engineering 40, 761–767.

Zhang, R.H., Tao, J., Dugba, P.N., 2000. Evaluation of two-stage anaerobic sequencing batch reactor systems for animal wastewater treatment. Transactions of the American Society of Agricultural Engineering 43, 1795–1801.