Reduction of indicator and pathogenic microorganisms by psychrophilic anaerobic digestion in swine slurries.

1 Caroline Côté *, 2 Daniel I. Massé and 3 Sylvain Quessy

1 Research and development institute for the agri-environment, 3300 Sicotte, Saint-Hyacinthe, Québec, Canada, J2S 7B8.

2 Dairy and Swine Research and Development Centre, Research Branch, Agriculture and Agri-Food Canada, Lennoxville, Quebec, Canada, J1M 1Z3.

3 Faculty of veterinary medicine, University of Montreal, C.P. 5000, 3200 Sicotte, Saint-Hyacinthe, Québec, Canada, J2S 7C6.

* Corresponding author. Tel.: 450-778-6522; Fax : 450-778-6539; E-mail address: caroline.cote@irda.qc.ca.
ABSTRACT

The objective of this study was to evaluate the efficiency of a low temperature anaerobic treatment to reduce viable populations of indicator microorganisms (total coliforms, Escherichia coli) and selected pathogens (Salmonella, Yersinia enterocolitica, Cryptosporidium and Giardia) in swine slurries. Experiments were carried out in 4 40-L Sequencing Batch Reactors (SBRs). Experimental results indicated that anaerobic digestion of swine manure slurry at 20°C for 20 days in an intermittently fed SBR: 1) reduced indigenous populations of total coliforms by 97.94 to 100%; 2) reduced indigenous populations of Escherichia coli by 99.67 to 100%; 3) resulted in undetectable levels of indigenous strains of Salmonella, Cryptosporidium, and Giardia.

Key words: anaerobic treatment, psychrophilic, swine slurry, methane production, pathogens removal, manure treatment.

INTRODUCTION

Canada is one of the most important pork producing country in the world. Over the past 20 years, the swine industry has evolved from a diversified to a specialized and intensified production system. It has grown more than 400% since 1982. Rapid growth has led to difficulties in the management of swine manure, resulting in serious environmental concerns. The environmental and social issues are presently the greatest challenge faced by Canada's fast growing hog industry. As a result, the industry cannot take advantage of the increasing international market opportunities for pork meat. In some areas, hog producers are forced to limit production due to environmental and social issues.
Environmental problems related to manure management include air, water and soil pollution and health hazards caused by the presence of zoonotic pathogens in the manure. As a matter of consequence, there is an urgent need for cost-effective biotechnologies to address the above environmental issues.

Animal wastes can contain organisms capable of causing infectious disease in humans (Cole et al., 1999). In swine production, microorganisms of interest include members of the Enterobacteriaceae family (Salmonella, and Yersinia enterocolitica) and the protozoan Cryptosporidium and Giardia. In the environment, coliforms and Escherichia coli are used as fecal contamination indicators.

**Coliforms and Escherichia coli**

Coliforms and *Escherichia coli* are natural constituents of the intestinal tract of humans and animals. They are frequently used as a faecal contamination indicators in the environment, *E.coli* being more effective. The majority of intestinal *E. coli* strains are harmless commensals. However, some strains possess virulence factors that can lead to human infection (Nataro et al., 1998). There are at least four categories of recognized diarrheagenic *E. coli*: 1) enterotoxigenic (ETEC), a cause of travelers' and infant diarrhea in developing countries; 2) enteroinvasive (EIEC), cause of watery diarrhea; 3) enteropathogenic (EPEC), responsible for infant diarrhea, and 4) enterohemorrhagic (EHEC), the cause of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (Nataro et al., 1998). *Escherichia coli* O157 is a member of this last group frequently associated with illness in human. It has been isolated from samples of pigs rectal faeces (Chapman et al., 1997). However, isolates were
not typical of strains causing infections in humans. In Quebec, non-O157 pig isolates possessing virulence factors implicated in human infection has been reported (Desrosiers et al., 2001).

**Salmonella**

The genus *Salmonella* comprises more than 2400 serotypes, all potentially pathogenic for humans (Bopp et al., 1999). *Salmonella* usually causes an intestinal infection characterized by diarrhea, fever, and abdominal cramps that often lasts one week or longer. The prevalence of *Salmonella* in Canadian finished swine was estimated at 5.2 % in Quebec (Letellier et al., 1999). Pigs can shed *Salmonella* into the environment without showing any signs of the disease (Gray et al., 1996; Ekperigin et al., 1998).

**Yersinia enterocolitica**

*Yersinia enterocolitica* causes illness characterized by fever, abdominal pain, and diarrhea in humans. In Quebec, 80% of swine herds were found positive to *Yersinia enterocolitica* (Pilon et al., 2000). Serogroup O:3, the most common strain associated with disease in people, was found in 93.5 % of the isolates recovered in this study. Pigs are considered to be the most common source of human infection (Cole et al., 1999).

**Cryptosporidium spp. and Giardia spp**
Cryptosporidium spp. and Giardia spp. are protozoan pathogens causing diarrhoeal illness in humans and a wide range of vertebrates. Cryptosporidiosis is self-limiting in healthy subjects, but can be life-threatening in highly immunocompromised patients. There is no effective treatment for cryptosporidiosis.

The majority of individuals infected with Giardia are asymptomatic. Symptomatic individuals can experience nausea and watery diarrhea. Humans, dogs, cats and beaver are recognized as the principal reservoirs of Giardia, while Cryptosporidium is common in humans and calves. However, pigs are now recognized as an important reservoir (Xiao et al., 1994; Morgan, 1999). In Canada, Giardia and Cryptosporidium were identified in four out of six hog operations with an overall prevalence of 9% for Giardia and 11% for Cryptosporidium (Olson et al., 1997).

**Anaerobic digestion**

Few studies have been carried out to assess the efficiency of anaerobic digestion to remove pathogens from organic wastes. Bendixen (1994) indicated that thermophilic temperature destroyed pathogens, while mesophilic temperature had no effect on reduction of pathogens. Kumar et al. (1999) investigated the survival of some pathogens in anaerobic batch reactors. Escherichia coli and Salmonella survived up to 20 and 10 days at temperatures of 20 and 35°C respectively. The survival of Salmonella Typhi increased from 20 to 35 days when the solid contents was increased form 9% to 15%. Duarte et al. (1992) have been successful in using AD at 37 and 54.9°C to remove Salmonella, Streptococci and coliforms from swine slurry. Kearny et al. 1993 investigated the
efficiency of full scale AD reactor operated at 28 °C to remove pathogenic bacteria. *Escherichia coli*, *Salmonella Typhimurium*, *Yersinia enterocolitica*, *Listeria monocytogenes* and *Campylobacter jejuni* were only partially removed during the treatment period. There is no information in the literature on the efficiency of AD process for the removal of *Cryptosporidium* and *Gardia*.

Massé et al. (1996; 1997a; 1997b) evaluated the feasibility of using psychrophilic anaerobic digestion (PAD) at 20 °C in non-mixed and intermittently fed SBRs to stabilize and deodorize swine manure slurry while recovering biogas for energy. Experimental results indicated that PAD of swine manure slurry in SBRs was technically feasible to stabilize and deodorize swine manure slurry. The process was very stable and not affected by high concentration of volatile acids (VA) and ammonia nitrogen. It produced high quality biogas and provided excellent settling conditions to retain high concentrations of anaerobic bacteria in the system.

There is few information in the literature on the efficiency of low temperature anaerobic process (15 – 20 °C) to remove pathogens. Therefore the objective of this study was to evaluate the efficiency of the psychrophilic anaerobic digestion process in a sequencing batch reactor to reduce viable populations of indicator microorganisms (total coliforms, *Escherichia Coli*) and indigenous pathogens (*Salmonella*, *Yersinia enterocolitica*, *Cryptosporidium* and *Gardia*) in swine slurries from different sources.
EXPERIMENTAL PROCEDURE

Figure 1 is a schematic of the bench scale SBRs used in this study. Four 42 L plexiglas digesters were located in a temperature controlled room (20°C). The sludge volume at the beginning of a cycle was 21 L. SBRs were mixed 5 minutes every morning by recirculating the biogas. Wet tip gas meters were used to measure the daily biogas production. Manure slurries were obtained from manure transfer tanks and long term storages located on commercial growing-finishing, nursery and maternity hog operations. The samples of manure collected on commercial farms, were send to the Agriculture and Agri-food Canada laboratory and used to feed to the laboratory scale digestors within 48 hours period. Feed and react period length of 2 weeks each (total treatment cycle length of 4 weeks) had been used in this study.

The cycle time is defined by

\[ t_c = t_f + t_r + t_{sd} \]

where \( t_c \) represent the total treatment cycle time, \( t_f \) represent the duration of the feed period, \( t_r \) represents the duration of the react period and \( t_{sd} \) the duration of the settle and draw periods. Final reaction and settling occurred at the end of the react period. Draw times were less than one-half hour.

**Physico-chemical analysis**

A mixed liquor sample was withdrawn from each SBR at the beginning of the experiment and once a week during each experimental procedure. At the end of each experiment, after the sedimentation
period, additional samples were withdrawn from the supernatant. The samples were analysed for pH, alkalinity, solids, VFAs, total Kjeldahl nitrogen (TKN), ammonia nitrogen, total chemical oxygen demand (TCOD), and Soluble COD (SCOD). Biogas production was monitored daily and its composition was analyzed weekly. SCOD was determined by analyzing the supernatant of centrifuged slurry. The pH, alkalinity, and solids were determined using standard methods (APHA, 1992). TKN and ammonia nitrogen were determined using an auto-analyzer. VFAs and biogas composition were determined by gas chromatography.

**Microbiological analysis**

Sludge samples were aseptically taken from each SBRs before feeding to verify the presence of coliforms, *Escherichia coli*, *Salmonella*, *Yersinia enterocolitica*, *Cryptosporidium*, and *Giardia*. Microbiological analysis of raw manure slurry was made within 24 hours before feeding the SBRs. At the end of each treatment cycle, after the sedimentation period, additional samples were taken from the supernatant for microbiological analysis.

Coliforms and *Escherichia coli* were counted by plating 3M Petrifilms with 1 ml of intact or diluted liquid hog manure. Dilutions were made with phosphate-buffered saline. Pink colonies producing gas were counted as coliforms, and blue ones with gas were counted as *Escherichia coli*.

In order to verify the presence of *Escherichia coli* O:157, 25 g of the sample were incubated in 225 ml of modified Tryptic soy broth with novobiocin for 24 h at 42°C. One loopful of the culture was
inoculated onto modified sorbitol MacConkey agar containing tellurite, cefixime, and cefsulodin. Suspect (colorless) colonies were used to inoculate purple broth base with cellobiose. Biochemical assays (indole, MR, VP, citrate) were used to confirm the identification of cellobiose negative colonies.

*Salmonella* was detected by incubating 25 g of the samples in 225 ml of nutrient broth (Difco laboratories, Detroit, Mich.) overnight at 37°C. Following this pre-enrichment step, one ml of nutrient broth was incubated into 9 ml of Tetrathionate Brilliant Green broth (BBL Microbiology Systems, Cockeysville, Md) overnight at 37°C. One loopful of the TBG culture were inoculated onto a Brilliant Green Sulfa agar (Difco) containing 20 ìg/ml of novobiocine (Sigma chemicals Co., St. Louis, Mo.) and incubated for 24 to 48 hours at 37°C. Lactose negative colonies were tested biochemically (triple sugar iron and urea hydrolysis) and identification was confirmed by API processing (Biomerieux, Ville St-Laurent, Quebec).

For the detection of *Yersinia enterocolitica*, ten grams of samples were incubated in 90 ml of phosphate-buffered saline containing sorbitol (2 %) and biliary salts (0.15 %) at 4°C for 21 days. Isolation was carried out on *Yersinia* agar base (cefsulodin-irgasan-novobiocin agar, Oxoid) with an incubation time of 24 to 48 h at 28°C. Typical colonies were biochemically tested (Triple Sugar Iron and urea hydrolysis). API testing (Biomerieux) was done in order to complete the identification.

The detection of *Cryptosporidium* and *Giardia* was done using Enzyme-linked immunosorbent assays (ELISA). Prospect *Cryptosporidium* microplate assay and Prospect *Giardia* microplate assays
(Alexon-Trend, Inc., Ramsey, MN) were performed according to manufacturer's instructions.

**Organic Loading Rates and Cycle Operation**

Organic loading rates that are given in Table 1 are based on the amount of COD fed to the volume of sludge present at the start of a cycle (21L). The loading equation is as follow:

\[ L_{S,f} = \frac{V_f C}{V_s t_f} \]

where \( L_{S,f} \) is loading rate based on initial sludge volume and feed time; \( V_f \) is the volume of feed; \( C \) is the COD concentration in the feed; \( V_s \) is the volume of sludge at the beginning of a cycle; and \( t_f \) is the fill time.

Table 1 gives the experimental design used in this study. It identifies for each treatment cycle the source of manure sample, the organic loading rate and the volume of manure slurry fed to each bioreactors. Digestion of swine manure slurry no. 40 was repeated two times simultaneously in two SBRs.

The reactors were fed at different organic loading rates due to physical constrain. Some of the
manure slurry were so diluted that the bioreactor were not large enough to receive sufficient volume to reach the design organic loading rate of 2.00 g COD / L-d.
RESULTS AND DISCUSSION

Table 2 gives the physico-chemical characteristics of the swine manure slurries collected on 20 commercial swine farms. The characteristics of the manure slurry fed to the bioreactors were highly variable. Total solids, total COD and total VFAs contents varied from 1.1 to 15.2% (weight basis), 12.9 to 96.3 mg/L and 0.4 to 34 mg/L respectively.

Sludge samples taken in SBRs before the beginning of the experiment were free of coliforms, Escherichia coli, Salmonella, Yersinia enterocolitica, Cryptosporidium, and Giardia.

*Escherichia coli* O:157 and *Yersinia enterocolitica* were not found in any sample of raw manure slurries. Table 3a shows the types and concentrations of indicator microorganisms and pathogens found in manure slurries before the treatment. Total coliforms counts varied from 0 to 3.3 x 10^6 CFU/g. Initial *Escherichia coli* populations were also highly variable, ranging from 0 to 2.6 x 10^6 CFU/g. *Salmonella, Cryptosporidium* and *Giardia* were detected in 7, 4, and 2 samples respectively.

The treatment resulted in undetectable levels of coliforms in 9 out of 20 manure samples. One liquid swine manure didn't contain coliforms before the treatment. In the remaining 10 samples, a reduction of 1.62 - 4.23 log CFU/ml was observed (97.94 - 99.99 % reduction). Olsen (1988) observed the impacts of mesophilic (35°C) anaerobic filter treatment on indigenous coliforms populations in liquid pig manure. He reported an average reduction of 1.1 and 1.0 log CFU/ml for hydraulic retention times of 4.2 and 0.8 days respectively.
Undetectable levels of *Escherichia coli* were observed in 15 out of 20 samples of swine manure slurries after anaerobic digestion, including one raw liquid swine manure free of this bacteria and sample no. 40 used two times. In the five remaining samples, a reduction of 2.48 - 4.16 log CFU/ml was observed (99.67 - 99.99 % reduction). Juris et al. (1996) observed similar results at higher temperature. He reported a complete elimination of *Escherichia coli* EC 5 strain after a 18 days anaerobic mesophilic (35-37°C) digestion of pig slurry in a 800 l fermenter. Kumar et al. (1999) used an ampicillin-resistant strain of *Escherichia coli* to study the persistence of this bacteria in cattle dung slurry during anaerobic digestion. The survival was 25 days at room temperature (18-25°C) and 15 days at 35°C.

In the present study, psychrophilic anaerobic digestion resulted in undetectable levels of *Salmonella* in the seven swine manure slurries positive for this bacteria. Kumar et al. (1999) reported a longer survival of artificially added streptomycin-resistant strain of *Salmonella Typhi* in cattle dung slurry during anaerobic digestion. He observed a complete elimination of this bacteria on the fifteenth day at 35°C and on the twentyfifth day at room temperature. The survival time of *Salmonella Typhi* increased when the solid contents of the digester were elevated from 9 % to 15 %. The mean decimal reduction time ($T_{90}$) of *Salmonella* during a full scale mesophilic anaerobic digestion was 34.5 days according to Kearny et al. (1993).

Psychrophilic anaerobic digestion destroyed *Cryptosporidium* and *Giardia* present in 4 and 2 samples of liquid swine manure respectively. This is the first report on the impact of psychrophilic anaerobic digestion on those parasites.
Temperature and retention time are decisive factors for indicator organisms and pathogens survival during anaerobic digestion of effluents. According to Olsen et al. (1987) hygienization similar to anaerobic thermophilic treatment is obtained at mesophilic temperatures by increasing retention time. Kumar et al. (1999) observed a faster elimination of Escherichia coli and Salmonella at 35°C than at room temperature during anaerobic digestion of cattle slurry. Salmonella Typhimurium, Yersinia enterocolitica and Listeria monocytogenes also declined more rapidly at 17°C than at 4°C during anaerobic digestion of cattle slurry (Kearny et al., 1993). It appears that under the conditions of this experiment, retention time of 28 days at 20°C was sufficient to ensure a stabilization of the liquid swine manure.

It is important to note that in most reported experiments, only one source of manure slurry was used. According to Olsen et al. (1987) decimation times of pathogens and indicator microorganisms were not influenced by the type of slurry (cattle or pig). However, no data were available with different sources of slurry for a specific animal specie. Our results have shown the effectiveness of the psychrophilic anaerobic digestion with swine manure slurry from different sources.

Many experiments concerning the impact of anaerobic digestion on indicator and pathogenic microorganisms have been made by inoculating manure slurries with laboratory or antibiotic-resistant strains. This last approach is useful since the strains can be selected on agar containing antibiotics after the treatment. According to Olsen et al. (1987) laboratory strains can be less resistant than coliforms indigenous to the slurry. On the other hand, Abdul et al. (1985) observed a longer persistence of antibiotic-resistant strains of Escherichia coli compared to sensitive isolates during
anaerobic digestion of pig slurry at 37°C. The use of natural liquid swine manure slurries permitted us to confirm the effectiveness of the psychrophilic anaerobic digestion on indigenous microorganisms.

Tappouni (1984) demonstrated in laboratory studies that the maximum biogas production during semi-continuous digestion at a hydraulic retention time of 7.5 days corresponded to an increase effect in reducing the numbers of *Salmonella* spp. This decline was correlated with increased volatils fatty acids and a decrease in pH. However, concentrations in the range of 2000 mg/l can inhibit biomethagenesis (Winter, 1984). It is then important to reach volatils fatty acids levels permitting the destruction of pathogens without affecting biomethagenesis.

Figures 2 and 3 give the acetic acid and total volatile fatty acid (TVFA) profile for each treatment cycle. Acetic and TVFA accumulated in the SBR during the fill period and the early stage of the react period. Similar profiles were obtained for propionic and butyric acids. For each treatment cycle the volatile fatty acids were completely utilized at the end of the react period. From these results it can be concluded that the SBRs were very stable at these loading rates and operating conditions.

**Overall Performance of the digesters**

The biogas produced in test runs 1 to 5 was of high quality with a methane concentration that generally exceeded 70%. Table 5 gives the effluent pH for each treatment cycle. All SBRs maintained pH between 7.6 and 8.1. The pH decreased slightly during the feed period due to TVFA accumulation and increased slightly during the react period due to VA utilization. The pH range was
appropriate for microorganisms growth and process stability.

CONCLUSIONS

Psychrophilic anaerobic digestion in sequencing batch reactors successfully treated raw swine manure slurries from different sources. It removed the indigenous populations of *Salmonella*, *Cryptosporidium*, and *Giardia*. Natural populations of indicator microorganisms (*Escherichia coli* and coliforms) were reduced by 97.94 to 100 %. It can be considered as an effective method for eliminating indigenous pathogens and reducing indicator organisms populations in liquid swine manure slurries varying in their physico-chemical and microbiological properties.
ACKNOWLEDGEMENTS

This project was financially supported by the Livestock environmental initiative and the Fédération des producteurs de porcs du Québec. The technical support by Katline Guay, Louise Beausoleil, Louise Lessard, L. Masse, and D. Deslauriers are appreciated.
REFERENCES


