THE UNIVERSITY OF MANITOBA

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INFLUENCE OF AMMONIA AND OTHER ABIOTIC FACTORS ON MICROBIAL ACTIVITY AND PATHOGEN INACTIVATION DURING PROCESSING OF HIGH-SOLID RESIDUES

BY

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ABSTRACT

In this research the objective was to achieve a better understanding of the nature of ammonia action that leads to enhancement or inhibition of anaerobic digestion process, and to optimization of alkaline disinfection. These processes are commonly used to treat organic residues. The improvement of anaerobic digestion of substrates with high Carbon-to-Nitrogen (C/N) ratio by means of supplementation of other organics rich in nitrogen can be achieved. Whether in the case with high-solid anaerobic digestion of residues like chicken manure or the organic fraction of municipal solid waste (OF MSW) or with alkaline disinfection of dewatered sludge, microbial injury or death, is the result of combination of the factors, including high free or un-ionized (UAN) ammonia nitrogen concentration. It was attempted to determine if it is ammonia alone, or a combination of factors including free ammonia, that is responsible for the aforementioned outcomes. This work led to the following major advancements:

1. Identification of practical process limitations due to free ammonia inhibition or toxicity during high-solid anaerobic digestion on the examples of (1) chicken manure and of (2) the OF MSW, as substrates for the digestion. The conditions responsible for system upset and technical requirements have been disclosed.

In chicken manure digestion, the highest solids level at which biodegradation of organic matter to methane was feasible was 10 to 12% total solids (TS). Methanogenic populations tolerated free ammonia concentrations of up to 250 mg N/L. The highest biochemical methane potential of 308 mL CH_4/g chemical oxygen demand (COD) was found with fresh manure diluted to 5% TS, confirming that the dilution factor has a

critical effect on digester performance. As the result of digestion at increasing TS level, the initial acclimation to increased organic load and resulting high ammonia was necessary, as observed by reduced activity of anaerobic methanogenic biomass in the first 30 to 40 d following digester start-up.

In digestion of the OF-MSW, supplementation with primary sludge solids was necessary to adjust the C/N ratio of 63 to a level more suitable for methane generation. Co-digestion at C/N ratio of from 51.1 to 39.9 showed improvement in methane yields and conversion of biodegradable volatile solids (BVS). The highest net (from degradation of the OF-MSW alone) specific methane yield of 0.3 to 0.5 L CH_4 g BVS^{-1} d^{-1} was achieved in reactors fed a OF-MSW feed supplemented with highest portion of primary sludge solids of 15% (dry weight). This yield corresponded with BVS conversion efficiency of 50 to 66%. High concentrations of un-ionized ammonia (NH_3) of up to 230 mg N/L were tolerated, but with increasing impact on methane production and BVS reduction.

2. Development of a new, low-dose alkaline disinfection process to treat digested-dewatered sludge or other high-solid (e.g. OF MSW) anaerobic digestion residues. This process utilizes alkaline agents to raise the pH and activate free ammonia disinfection in a closed-system operation. A new bacterial indicator of alkaline disinfection efficiency, the spores of the pathogenic *Clostridium perfringens*, was characterized.

Effective inactivation of non-spore forming bacteria, using fecal coliforms and *Salmonella* species as model organisms, was achieved. The minimum value for the

product of disinfectant concentration and contact time (C×T) for the U.S. EPA Class A biosolids designation, was 100 g h/Kg TS, indicating fast inactivation. The C×T value is valid at lime dose of 30 g/Kg TS (e.g. 9.5 g/Kg wet weight for the sludge at 31.5%TS) and higher. The minimum pH for non-spore formers' inactivation was found to be 9.5, with the corresponding free ammonia concentration of about 1,200 mg N/L. The inactivation of fecal coliform and *Salmonella* sp. bacteria was virtually complete in the entire range of temperatures from 4°C to 22°C in 1 day from start.

Fly ash, when used alone, did not have as high the disinfecting potential as when supplemented with small amount of lime. Alkalinity generation was 2.7 to 13.7 times higher than when each of these alkaline compounds was used alone.

The bacterial spores of pathogenic *C. perfringens* were much harder to disinfect. The C×T>2600 g d/Kg TS was needed to obtain 3-log inactivation (99.9%). Complete inactivation required a dose of 80 g CaO/Kg, pH≥12.0 and 10 to 11 week-long contained storage (C×T>5,600 g d/Kg TS). The corresponding free ammonia concentration in high-solid residues would be more than 2,000 mg N/L, or ~100% of the total ammonia. Spore detection was superior when lysozyme was used to repair alkali-induced injury. Therefore, its use in further research appears to offer advantages.

The spores, under specified conditions, could serve as a surrogate organism in evaluation of inactivation of the parasitic ova of a helminth *Ascaris*. The level of *Ascaris* eggs in sludge is strictly legislated but an inconvenient/impractical indicator. Nevertheless, in experiments at 4-6°C the eggs were inactivated at a lower rate (~1 log in 280 d) than were the spores (>3 logs in 280 d). The common knowledge is that parasitic eggs or cysts have greater resistance than bacterial spores. Apart from structural

differences between these forms, the reasons for the improved resistance are not well explored, but were attempted in the last part of the work.

3. Determination of contribution of free ammonia concentration to the overall pathogen inactivation in alkaline treatment using *Clostridium perfringens* spores and eggs of *Ascaris suum* as model organisms.

The independence of *C. perfringens* spore inactivation from the presence of free ammonia at a level of up to ~1,300 mg N/L (pH 12.0) was demonstrated. *Ascaris* egg inactivation was, in turn, dependent on free ammonia concentration, and progressed linearly with UAN ranging from 57% to 100% of the total ammonia (from 663 mg N/L at pH 9.5 to 1,237 mg N/L at pH 12.0). The share of the free ammonia in overall inactivation was 54 to 67%. Similar to the findings from sludge disinfection, the extent of permanent spore inactivation over time was much higher as compared with the eggs of *Ascaris suum*. Irreversible spore inactivation was complete after 2 d of storage, whereas the resulting decrease in viability of *Ascaris* ova was only 0.1 log. The extent of inactivation for both pathogenic forms was different, but when spores of *C. perfringens* were reduced by 99.8%, a corresponding reduction of about 80% in viable *Ascaris suum* egg population was noted. The ova, although affected by high pH and corresponding high free ammonia concentration, were much more resistant than the spores of *C. perfringens*.

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NOMENCLATURE AND ABBREVIATIONS

ADS – anaerobically digested sludge
BMP – biochemical methane potential
CaO – lime or quick lime
COD – chemical oxygen demand
CFU – colony forming units
dil. - diluted
FA – fly ash
FM – fresh manure
HAc – acetic acid
HPr – propionic acid
N_{org} – organic nitrogen
NH_3 – dissolved ammonia (free ammonia, de-ionized ammonia)
NH_4^+ - ammonium ion
MPN – most probable number
OF-MSW – organic fraction of municipal solid waste
ORP – oxidation-reduction potential
PFU – plaque forming units
PM – pit manure
PS – primary sludge
STP – standard temperature and pressure
TAN – total ammonia nitrogen
TKN – total Kjeldahl nitrogen
TS – total solids
TSS – total suspended solids
UAN – un-ionized ammonia nitrogen
undil. - undiluted
U-VFA – un-ionized volatile fatty acids
v/v – volume to volume
VFA – volatile fatty acids
VS – volatile solids
VSS – volatile suspended solids

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DEDICATION

This thesis is dedicated to:

My parents, Irena and Bolesław, for their wisdom and long-distance support from Poland

and

My wife Maja for her love and companionship

Chapter 1.

INTRODUCTION AND SCOPE

Ammonia is one of the products of anaerobic digestion - a process most commonly used to treat wastes of animal and human origin. It is generated through ammonification during anaerobic breakdown of organic compounds rich in nitrogen. Depending on ammonia concentration and alkalinity, it can serve as beneficial nutrient or toxic agent to the microbial community. The toxic, un-ionized or so called free, form of ammonia increases with increasing pH and starts to prevail at a pH higher than 8.

The presence of ammonia at a proper concentration ensures microbial stability of the anaerobic digestion process. In high-solid digestion however, as opposed to conventional slurry-mode digestion, the organic and therefore nitrogen load is much higher and more challenging to the microbial population responsible for the degradation. The knowledge of operational conditions promoting high-solid digestion allows proper control over such factors like pH and resulting free ammonia concentration, solids level, and temperature.

On the other hand, the acute effect of free ammonia when at high concentration (i.e. alkaline pH range) can be effectively utilized during alkaline disinfection of the treated solids following anaerobic digestion. Optimization of the process through activation and confinement of the inherent ammonia in the system brings new prospects for its use.

This research evaluated the role of ammonia and other abiotic factors in high-solid anaerobic digestion. The lack of knowledge on process mechanisms and limitations

has limited the use of high-solid anaerobic biotechnology. The aim was to bring more light into the understanding of these interactions, and resulting improved process performance.

In the residuals disinfection part, the aim was to develop a process of low-dose alkaline disinfection utilizing dosages of alkali(s) smaller than commonly used to partially disinfect sludge. The improved pathogen inactivation was achieved due to contained "anoxic" storage of treated solids, which increased partial pressure of the free ammonia and allowed better penetration of the matrix. The demonstration of disinfection efficiency of the process was made based on inactivation patterns of few microbial indicators including fecal coliforms, *Salmonella* sp., spores of *C. perfringens*, and eggs of a helminth, *Ascaris*.

Chapter 2.

2 LITERATURE REVIEW

2.1 AMMONIA CHARACTERIZATION

Nitrogen can exist naturally in the form of ammonia, nitrate, nitrite, and molecular nitrogen, and in a variety of organic compounds such as proteins, nucleic acids, or lipids. Ammonia (here the sum of NH_3 and NH_4^+) is a key intermediate in the nitrogen cycle (Fig. 2-1). It is apparently the most universal and preferred nitrogen source in microbial metabolism [Gottschalk, 1979], and a nutrient for microbial cell growth [McCarty, 1964 a]. Proteins are hydrolyzed to amino acids that are further fermented to carbon dioxide, ammonia and volatile fatty acids such as valeric and butyric acid [Barker, 1981]. In humans, ammonia does not accumulate in the body. It is removed from the body in sweat or converted to ammonium salts or urea and passed out of the system in the urine [CCOHS, 1988]. Excretion of ammonia by the bacteria proceeds either through de-amination or ammonification.

The naturally occurring ammonia is well managed in the environment and usually does not pose a concern. However, when found at high levels, its origin is usually linked either directly or indirectly to man. Apart from industrial production, ammonia originates from organic wastes. The conversion of organic nitrogen found in wastewater, sludge, manure, food waste and other organic wastes, to ammonia in the process of mineralization is very efficient.

Ammonia exists in aqueous solutions in two principal forms depending on the protonation state, ammonium ion or NH_4^+ and dissolved ammonia gas or NH_3. The term ammonia throughout this document is used to refer to the sum of NH_3 and NH_4^+ species. The equilibrium can be described by the following equation:

$$NH_4^+ \leftrightarrow NH_3 + H^+ \qquad (2\text{-}1)$$

The relative concentrations of the two forms are pH-dependent and can be described as follows:

$$K_a = [H^+][NH_3]/[NH_4^+] \qquad (2\text{-}2)$$

where K_a is the dissociation constant of ammonia.

The relative concentrations of ammonia are temperature dependent, as described by the following equation [Emerson *et al.*, 1977]:

$$pK_a = 0.09018+2729.92/(273.2+T) \qquad (2\text{-}3)$$

where K_a is the dissociation constant of ammonia and T is temperature in °C. The dissociation constant of aqueous ammonia at a given temperature can be obtained from Weast [1987]. The first numeric term in the equation is very low and, therefore, may be omitted.

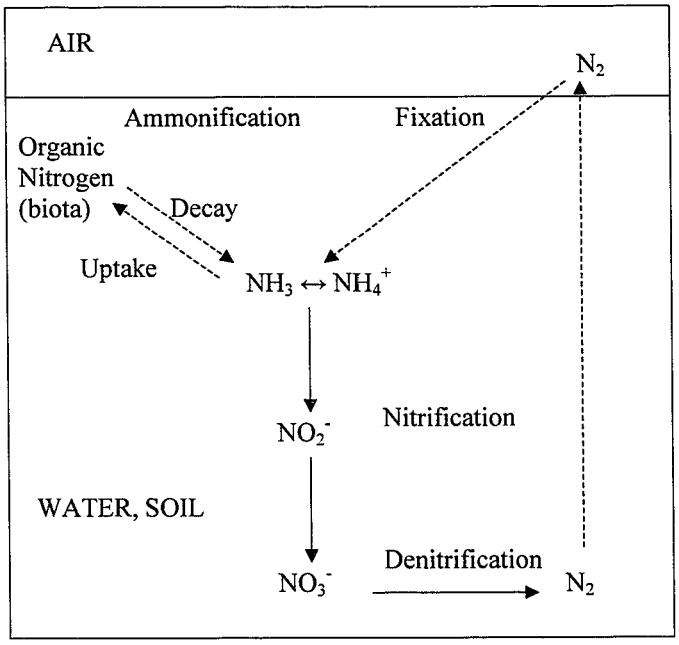

Figure 2-1. Simplified schematic for the microbial processes of the nitrogen cycle (adapted from the U.S. Department of Health and Human Services, 1990)

The graphical dependence of ammonia on temperature and pH is depicted in Fig. 2-2.

2.2 AMMONIA IN ORGANIC WASTES

Some 80% of the man-made share of ammonia is used as fertilizer in agriculture [U.S. Dept. Health & Human Services, 1990]. Ammonia is a slow-uptake nutrient, the characteristic being beneficial for crop production. The use of treated municipal sludge or manures as fertilizers reduces the market demand for synthetic fertilizers and is more sustainable. Because of application of wastewater biosolids on agricultural land, fertilizer costs can be reduced roughly by more than $100 per acre [Crittenden, 2001].

A considerable load of organic nitrogen reaching the treatment plant is distributed among liquid and sludge streams of treatment. Almost complete removal of organic nitrogen from the liquid stream by the end of treatment is accomplished by several means. Settling of the particulate organic matter in primary clarifiers and of the excess biomass in secondary clarifiers, and mineralization of dissolved organic nitrogen through ammonification-nitrification-denitrification sequence to gaseous nitrogen in activated sludge process, are the common pathways. Typical composition of untreated municipal wastewater, and sludge at different stages of processing, with regard to various forms of Nitrogen, is shown in Table 2-1.

Table 2-1. Characterization of different forms of Nitrogen in untreated municipal sewage and in sewage sludge at different stages of processing

Stream type	Forms of Nitrogen					
	Total		Organic		Ammonia	
	typical	Range	typical	range	typical	range
	as N, mg/L					
Untreated WW	40	20-85	15	8-35	25	12-50
Untreated PS [A]	1250	300-3200	-	-	-	-
Untreated WAS [A]	-	125-700	-	-	-	-
Digested PS [A]	3000	960-7200	-	-	-	-
Digested PS+WAS		800-6000 [A]				400-1000 [B]

WW: wastewater, PS: primary sludge, WAS: waste activated sludge; Digested: refers to anaerobically treated sludge

[A]: values for sludges were re-calculated by converting reported N concentration from originally given units as % TS to the unit of mg/L, for a direct comparison with the values for untreated wastewater;

[B]: values in the supernatant from anaerobic digester treating PS+WAS;

Data in table quoted after Metcalf and Eddy [1991], and Qasim [1994].

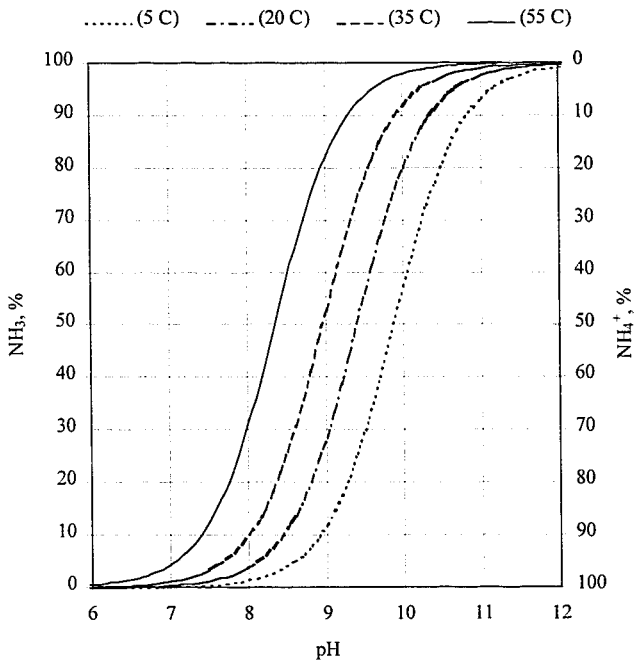

Figure 2-2. Influence of pH and temperature on distribution of ammonia gas and ammonium ion in solution

Overall, some 20% of the total nitrogen reaching the treatment plant is settled in primary and secondary clarification, and is further treated in a sludge stream [Oleszkiewicz *et al.*, 2000]. Most common mode of sludge stabilization is through anaerobic digestion. The majority of mostly particulate organic nitrogen at this stage is eventually converted to ammonia; only a small fraction in refractory organics remains intact.

2.2.1 Impact of ammonia on anaerobic digestion

2.2.1.1 Description of the anaerobic digestion process

Anaerobic digestion is a biological process relying on synergistic actions between many classes of bacteria to breakdown organic matter to two ultimate products, methane and carbon dioxide. It is commonly recognized that the degradation proceeds in at least a four-stage pattern [McCarty and Smith, 1986].

In the first stage, the solubilization and hydrolysis of complex organic materials (e.g. nucleic acids, proteins, lipids, polysaccharides) is catalyzed by extracellular enzymes of hydrolyzing bacteria. This stage is relatively slow and may be limiting in anaerobic digestion of the organics recalcitrant to biodegradation, such as lignin. In the following stage, the resulting soluble monomer molecules such as amino acids, nucleotides, sugars, fatty acids and glycerol, are converted to organic acids (e.g. formic, acetic, propionic, butyric, valeric, lactic, or succinic), alcohols and ketones (e.g. ethanol, methanol, acetone), carbon dioxide, dihydrogen (H_2), and ammonia. The fermentative acidogenic bacteria are responsible for carrying out this phase.

Acetogenic bacteria of the third stage digestion (syntrophic stage) convert the remaining soluble organics (organic acids, alcohols, ketones) into acetate and H_2. This stage has to be well balanced with the last stage of anaerobic breakdown, methane formation. The syntrophy, or interdependence of one group on another, is related to the concentration of H_2. Degradation of short-chain organic acids to acetic acid takes place only at low hydrogen tension, otherwise causing incomplete conversion to propionate and ethanol. Methanogenic bacteria utilizing H_2 (hydrogenotrophs) are responsible for the maintenance of hydrogen concentration at a level enabling acetogenic bacteria to conduct efficient production of acetic acid, the substrate for acetoclastic methanogens. This synergistic relationship is called *interspecies hydrogen transfer*, and plays an important role in the overall substrate-product balance. The complexity of this phenomenon is compounded by the fact that the specific growth rates of most methanogenic bacteria are smaller than those of acidogenic bacteria by one to two orders of magnitude [Speece, 1996]. A non-equilibrium state between methane producing bacteria and acidogenic bacteria is the most common cause of pH decline, characterized by fast VFA production and slow conversion to CH_4 [Koster, 1986].

2.2.1.2 Sources of ammonia

Ammonia is a metabolic product of anaerobic digestion of organic compounds containing nitrogen [Gottschalk, 1979]. Hence, it is commonly found in anaerobic digester effluents such as municipal sludge [Melbinger and Donnellon, 1971].

During anaerobic breakdown, organic compounds rich in nitrogen (urea, proteins, lipids) contribute to the production of VFA and ammonia through hydrolysis and acidogenesis. In a well-balanced anaerobic digestion environment, simultaneous

production of VFA and CO_2 during acidogenic stage offsets this pH increase. The increase in pH is usually associated with generation of alkalinity from degradation of protein and other nitrogen-containing organic matter. If the organic nitrogen content is high, the ultimate degradation to ammonia leads to an increase in pH due to consumption of protons by NH_3 to form NH_4^+. Alkalinity generated during organic nitrogen breakdown to ammonia is estimated to account for some 23% of the total alkalinity [Melbinger and Donnellon, 1971]. Decline in CH_4 production at increased pH is therefore indicative of inhibition by ammonia. Ammonia induces a series of inhibitory events that eventually lead to an end of methane formation. In this case, an increase in VFA is the result of UAN inhibition. There are no nitrogen sinks during anaerobic digestion and the TKN found in the effluent is usually equal to that in the influent to a reactor [Melbinger and Donnellon, 1971].

Proper Carbon-to-Nitrogen (C/N) ratio warrants optimum performance of an anaerobic digestion process. The proper C/N is the one accounting for the bio-available, or biodegradable, carbon and nitrogen. In anaerobic digestion, alkalinity generated because of organic nitrogen breakdown influences the overall stability of the process.

2.2.1.3 Toxicity of ammonia to different groups of anaerobic bacteria

As already mentioned, ammonia under anaerobic regime cannot be bio-transformed, however acclimation to its presence is possible. After passing an inhibitory threshold, any further increase in ammonia concentration causes toxicity. The nature of this toxicity is uncompetitive [Speece, 1996], as an abrupt failure results following even slight further increase.

Ammonia can be toxic to a variety of organisms ranging from viruses and bacteria to mammals [Ward and Ashley, 1977; Albertson, 1961; Taylor *et* al., 1978; Borgmann and Borgmann, 1997; Kleiner *et* al., 1998]. Dissolved ammonia (NH_3) denatures proteins and membranes and readily diffuses across cellular membranes, whereas ammonium ion (NH_4^+) competes with other cations for active transport by means of carrier proteins. Although the physical-chemical action of ammonia is known, proposed cellular mechanisms of ammonia toxicity are contradictory [Kleiner *et* al., 1998; Schnaeider *et* al., 1996]. In general, the free ammonia (NH_3) acts as a un-coupler destroying the membrane potential.

In an anaerobic consortium, methanogens are the group that is most susceptible to adverse conditions. However, other groups of bacteria, especially the non-spore forming bacteria, are affected by ammonia in the same or similar manner. Wastewater sludges are considered hostile to the majority of enteric, pathogenic organisms. However, they can often retain the ability to grow once under conditions satisfactory for their growth, even in the state of bacterial injury. Enteric bacteria, among them salmonellae or other facultative anaerobic pathogens (e.g. *E. coli*), are found in water and soil and as normal intestinal flora in man and animals [Bailey and Scott, 1966]. Some of them are among those contributing to the acidogenic and acetogenic stages of anaerobic digestion. Vegetative cells of spore forming anaerobic bacteria, e.g. those belonging to the genera *Clostridium*, are also vulnerable to ammonia but their spores can survive for long times in such an environment. Clostridia are responsible for soil putrefaction processes and their contribution to the process of microbial degradation is well known.

Although the majority of pathogenic bacteria differ in their characteristics from methanogenic populations, they all experience the lack of a protection mechanism that would let them survive under adverse conditions of high pH and ammonia. In addition, hostile conditions within sludge have an impact on some bacterial pathogens such as *Salmonella* spp. too [Yanko *et* al., 1995]. The major factor that would allow microorganisms to survive is the shielding effect of the bacterial cell and organic matter (solids) clusters that are formed in attached growth wastewater treatment systems and in wastewater sludges. Digested-dewatered sludge provides limitations in influx of the external species to such clusters. This makes certain disinfection methods unsuitable for the use in sludge treatment. High pH and ammonia treatment using, in part, original ammonia content of the sludge and of the chicken manure is believed to provide efficient means of pathogen kill.

According to Sprott *et al.* [1984], combined effect of both ammonia gas and ammonium ion takes place when ammonia gas enters the cell. NH_3 drives some protons off the cell to create the same type of equilibrium with NH_4^+ as on the outside. Consequently, in order to maintain its optimum internal pH the cell takes up some protons from the outside but, at the same time, it sacrifices the potassium ion (K^+)- another essential nutrient- and dies. Another model, linked to the previous description, predicts that ammonia displaces divalent ions (Ca^{2+}, Mg^{2+} or Mn^{2+}) necessary for CH_4 synthesis from the cytoplasmic membrane [Sprott *et al.*, 1985]. When the lost ions were supplied to the cells of the tested organism, *Methanospirillum hungatei*, CH_4 production was reinstated. The extent of ammonia toxicity is dependent on the presence and

concentration of other cations having synergistic and antagonistic effects [Kugelman and Chin, 1971].

The definitions of terms describing effect of ammonia on microbial populations in this work were adopted from Speece [1996], and include: toxicity (an adverse effect, not necessarily lethal, on bacterial metabolism), and inhibition (an impairment of microbial function).

Toxicity of ammonia in methanogenic systems is an example of substrate/product toxicity. This particular case involves generation of ammonia as the result of biodegradation of nitrogenous organic compounds at high concentrations, like in chicken manure.

The issue of ammonia toxicity discussed further in this section pertains mainly to the toxicity to bacteria. This is because while several types of microorganisms are implicated in aerobic, activated sludge treatment process, anaerobic digestion is driven mostly by bacteria.

When methanogens are concerned, the combination of low biogas production, low CH_4 content in the biogas, and a high VFA concentration are the symptoms of ammonia toxicity [Van Nelsen, 1979; Poggi-Varaldo *et al.*, 1991].

The impact of ammonia on different trophic groups of methanogens is not the same. Aceticlastic, or acetate-utilizing, methanogens are more sensitive to ammonia compared to hydrogenotrophic methanogens. The specific methanogenic activity of the acetate-utilizing populations decreased by 72% as compared to hydrogen-utilizing methanogens in UASB (upflow anaerobic sludge blanket) reactors inhibited by 7 g N/L ammonia [Borja, 1996 a]. Moreover, the specific growth rate of the first group was

reduced by 50% at TAN concentration of 4 g N/L while much higher concentration (7.5 g N/L) was needed to observe a similar reduction in the hydrogenotrophs.

Methanogenesis from H_2/CO_2 (80:20) during thermophilic digestion of swine manure was possible at free ammonia concentrations significantly higher than for a similar experiment with swine manure as sole substrate [Hansen *et al.*, 1998]. The relative apparent specific growth rate (μ_r) of the H_2–utilizing methanogens (H_2/CO_2, manure) remained unchanged when free ammonia level was increased from 1,100 to 1,200 mg N/L UAN while a 31% reduction was seen for the overall system (manure only). An increase to 1,300 mg N/L UAN caused 23% reduction in hydrogenotrophic activity versus 33% reduction in overall activity. This suggests that acetate-utilizing methanogens are a rate-limiting factor, even after accounting for lower maximum specific growth rates of these bacteria when compared with H_2-utilizing methanogens.

Similar conclusions were derived by Heinrichs *et al.* [1990] who showed that mixed aceticlastic methanogenic cultures were more prone to ammonia toxicity, as compared to propionate utilizing acetogenic bacteria.

Aceticlastic methanogens produce approximately 70% of methane (Kugelman and McCarty, 1965; Jeris and McCarty, 1965; Smith and Mah, 1966) from the breakdown of organic matter. The remainder, i.e. 30%, is produced from reduction of CO_2 by H_2, and to some extent by other intermediate electron donors [Hashimoto *et al.*, 1980]. The hypothesis that acetate-utilizing methanogens are more responsible for limited digestion at high ammonia levels is, therefore, justified.

Methanogens are not the only bacterial group affected by high ammonia levels. However, the response of different trophic groups within microbial population of a

digester to high ammonia concentration is not same. In one study, acidogenesis was almost not affected by high ammonia concentrations in the range from 4051 to 5734 mg N/L (7.39<pH<7.81), while methanogenic activity decreased by 56.5% [Koster and Lettinga, 1988].

The efficiency of hydrolysis and acidification was examined during swine manure digestion at 6,000 mg N/L TAN and temperature ranging from 37 to 60°C [Hansen *et al.*, 1998]. A potential CH_4 yield from the VFA produced was similar at all temperatures tested, which demonstrated that increasing UAN concentrations (from 750 to 2,600 mg N/L) had no significant influence on both fermentative stages. Nevertheless, this potential yield was only 73% of the actual CH_4 yield, which shows that the same inhibition will occur on hydrolysis and acidification.

One of the dramatic effects of ammonia (pH 8.0) on a methanogen, *Methanospirillum hungatei*, was induction of an efflux of up to 98% of the cytoplasmic K^+ through an NH_4^+/K^+ exchange reaction [Sprott *et al.*, 1984], which leads to depletion of K^+ and cell death. The quoted research showed a similar effect in cells of *Escherichia coli* and *Bacillus polymyxa*, proving that this phenomenon is likely to occur in other bacteria.

2.2.1.4 pH dependence of ammonia toxicity

Free ammonia is recognized as more toxic of the two species to bacterial metabolism [Koster and Koomen, 1988; Sprott *et al.*, 1984; Kroeker *et* al. 1979; McCarty and McKinney, 1961]. With increasing either pH or temperature, or both, the fraction of dissolved ammonia gas (NH_3 or UAN) in the liquid fraction of the treated sludge

increases (Fig. 2-2). Free ammonia molecules inhibit microorganisms by diffusing into their cells and altering the chemical equilibrium between the cells and the solution. Certain enzymes are no longer capable of functioning properly and the cell dies. The range of un-ionized ammonia concentrations reported to be toxic to methanogens starts from 150 mg/L as N [Braun *et al.*, 1981; McCarty and McKinney, 1961], but successful digestion operations were conducted with UAN up to 345 mg/L (Ripley *et al.*, 1985), or even twice as much (Lapp *et al.*, 1975) when a very long acclimation period was used.

Ionized form of ammonia is also toxic, however at much higher concentrations, as it hinders methane formation and growth rates of the methanogenic bacteria. The mechanism relies on displacement of the magnesium ion (Mg^{++}) and calcium ion (Ca^{++}) from the plasma membrane of the cell, which affects the membrane protein responsible for CH_4 synthesis from CO_2 [Sprott and Patel, 1986]. In the research by Wiegant and Zeeman [1986], during thermophilic (55°C) anaerobic digestion at pH 6.7-6.9, total ammonia concentrations above 3500 mg/L caused failure of H_2/CO_2 methanogenesis resulting in H_2 increase and propionate accumulation. McCarty and McKinney [1961] showed that at higher volatile fatty acid (VFA) salt concentrations (9 and 16 g NH_4Ac /L, as acetic acid) any toxicity to acetate utilizing methanogens is associated with salt toxicity. It is mainly dependent on the cations of the salts and ammonium ion was recognized as the most toxic of tested salt cations. It reacts with the bicarbonate ion, which results in significant increase in buffering capacity of the system. This, in turn, leads to an increased pH and increased fraction of NH_3 in the digester over the limits acceptable by bacteria. McCarty [1964a] suggested that ammonia inhibition of anaerobic digestion occurs at TAN concentration of more than 3,000 mg N/L, regardless of the pH.

The mathematical description of inhibition by ammonia was shown to better fit a model that utilized the total ammonia concentration and the pH when compared to a model using a calculated free ammonia concentration alone [Hunik *et al.*, 1990]. Nevertheless, the fact that un-ionized ammonia is more toxic of the two species is credible.

Comparison of 50% toxicity threshold for UAN as compared to other toxicants such as sulphide, phenol, formaldehyde, ortho-xylene, revealed the free ammonia impact of the same order of magnitude [Heinrichs *et al.*, 1990]. This confirms the highly toxic potential of this species.

2.2.1.5 Temperature effects in ammonia inhibition

Hashimoto [1986] investigated the effects of total and free ammonia on methane generation at mesophilic (35 and 37°C) and thermophilic (55°C) temperatures, with the emphasis on feasibility of ammonia acclimation at thermophilic conditions. The thermophilic digesters were capable of adaptation after 43 days of acclimation at a lower ammonia concentration. Initial inhibition was seen in unacclimated reactors at both temperature conditions at about same ammonia concentration: 2,640 mg N/L at 37°C (pH 7.04, UAN 32 mg N/L) and 2,380 mg N/L at 55°C (pH 7.33, UAN 209 mg N/L). Acclimated thermophilic biomass was not inhibited at TAN concentrations up to 4,380 mg N/L (pH 7.33, UAN 384 mg N/L), even though the UAN was very high.

The profound effect of temperature was shown during anaerobic digestion of swine manure in step-fed reactors with 6,000 mg N/L TAN and at a wide range of temperatures- 37, 45, 55 and 60°C [Hansen *et al.*, 1998]. The starting methanogenic

biomass was acclimated to 3,000 mg N/L TAN (pH 7.9-8.1, UAN 275-413) and exposure to twice as high the level resulted in increased inhibition at increasing temperature. A steady-state operation at 37°C was possible only after 52 days from start, with specific methane yield of 188 mL g VS^{-1} (pH 8.06, UAN 750 mg N/L), similar to the steady-state after 45 days at 45°C (141 mL CH_4 g VS^{-1}, pH 8.15, UAN 1,400 mg N/L). Stable digestion at 55°C followed from day 63 on (67 mL CH_4 g VS^{-1}, pH 7.97, UAN 1,600 mg N/L), whereas the 60°C reactor failed after some 45-54 days from start (22 mL CH_4 g VS^{-1}, pH 8.15, UAN 2,600 mg N/L). The potential CH_4 yield from manure degradation was 300 mL/g VS.

Decreasing the temperature of cattle manure digestion to below 55°C was found stimulatory with respect to biogas yield and process stability [Angelidaki and Ahring, 1994].

2.2.1.6 Evidence of ammonia being the toxic agent

Comparison of methanogenic tolerance to a shock or adaptation, caused by different concentrations of NaCl and NH_4Cl, showed a distinct difference [De Baere *et al.*, 1984]. The initial inhibition threshold in experiments using the sodium salt was twice as high for adapted biomass as for the shocked one. In contrast, regardless of the operational regime, ammonia-fed biomass was initially inhibited at very similar level of TAN corresponding to free ammonia concentration of 80 to 94 mg N/L. This phenomenon points at limited initial tolerance of the UAN in methanogenic cultures.

The progress of ammonia inhibition was described during gradual increase in TAN from 3,100 (pH 8.0, UAN 961 mg N/L) to 8,100 mg N/L (pH 7.9, UAN 2,130 mg N/L) in thermophilic swine manure digestion [Hansen *et al.*, 1998]. The process was uninhibited (stage 1) up to the threshold of 1,100 mg N/L UAN (TAN 4,100 mg N/L). The initial inhibition (stage 2) occurred between this and 1,200 mg N/L UAN level with the decrease in relative specific growth rate (μ_r) of 31%, followed by a plateau (stage 3). This plateau ended when UAN level reached about 1,400 mg N/L, and μ_r continued to decrease (stage 4) to the ultimate level (at highest tested UAN of 1,900 mg N/L) of 21% of the original value from prior inhibition.

Similar findings on staging of ammonia inhibition were obtained as assessed by observations of decreasing maximum specific growth rate (μ_{max}) of aceticlastic methanogenic bacteria [Poggi-Varaldo *et al.*, 1991]. An initial inhibition in the UAN range of 0 to 113 mg N/L led to over 50% reduction of μ_{max}, followed by a plateau extending up to 540 mg N/L UAN, and another rapid drop to roughly 10% of the original μ_{max} in cultures with 700 mg N/L free ammonia.

This staged inhibition of methanogenic biomass by ammonia suggests that there is more than just one mechanism of inhibition, or that the onset of inhibition among different groups of methanogens is concentration-dependent. In a study by Angelidaki and Ahring [1993], inhibitory patterns for aceticlastic populations and hydrogenotrophic populations were different, sigmoidal and linear, respectively. This supports the latter hypothesis of concentration dependence rather than of staged mechanism.

2.2.1.7 Sudden shock versus gradual acclimation to high ammonia levels

Often ammonia toxicity is the result of a rather sudden organic overload of a digester. In such case, the need to adapt methanogens to an increased ammonia load is inevitable.

Ammonia inhibition of thermophilic anaerobic digestion of cattle manure was reduced by gradual increase in TAN concentration from 3 to 7 g N/L, as oppose to a shock, one-step increase [Borja *et al.*, 1996 a]. Similar results were obtained by Hashimoto (1986), where acclimated thermophilic biomass was not inhibited at TAN concentrations up to 4,380 mg N/L (pH 7.33, UAN 384 mg N/L), as oppose to the inhibitory 2,380 mg N/L (pH 7.33, UAN 209 mg N/L) and 2,640 mg N/L (pH 7.04, UAN 32 mg N/L) at thermophilic and mesophilic conditions, respectively.

The importance of acclimation was shown by Van Velsen [1979]. Two methanogenic biomass types, digested sewage sludge acclimated to 815 mg N/L TAN and digested piggery manure already acclimated to 2,420 mg N/L ammonia reacted differently to increasing ammonia concentrations. Considerable time of 10 to 40 days was needed for the sewage sludge biomass to adapt to increasing TAN levels of 2,360 to 4,990 mg N/L (max. pH 7.4, max. UAN 45 to 95 mg N/L), whereas the manure biomass showed no lag in methane production up to 3,075 mg N/L (max. pH 7.6, UAN 92 mg N/L), the highest level tested.

Mixed cultures from sewage sludge digester, comprising mostly of *Methanosarcina* species (>99% of the methanogenic biomass), were subjected to ammonia load either at shock concentrations or gradually increasing levels [De Baere, 1984]. The initial inhibition during shock treatment occurred at TAN concentration of

8,635 mg N/L (pH 7.00, UAN 94 mg N/L), which led to a 40% drop in biogas production. Gradual acclimation to increasing levels of ammonia showed similar initial inhibitory threshold of 7,850 mg N/L. A 50% drop was observed at TAN concentration of 11,776 mg N/L (pH 6.80, UAN 81 mg N/L) in the shocked biomass, and at TAN of 9,159 mg N/L (pH 6.80, UAN 63 mg N/L) in the adapted methanogenic population.

McCarty and McKinney [1961] studied the effect of various chloride and acetate salts fed at slug quantities to batch reactors with acetate-utilizing methanogenic biomass. The salts involved were those of divalent cations, calcium and magnesium, of the monovalent sodium and potassium, and of ammonium. The most toxic salts were the ammonium salts, regardless of the salt used. Chloride salt contributed 2,172 mg N/L TAN (pH 7.40, UAN 59 mg N/L, assuming the temperature of 35°C as it was not reported in the paper), resulting in longest time needed to utilize acetate substrate. The acetate salt was added to produce ammonia levels of 2,100 mg N/L (pH 7.85, UAN 152 mg N/L), and 3733 mg N/L (pH 7.80, UAN 243 mg N/L). Half of the acetate fed was utilized in the first case and only 19% at the other level, before complete inhibition of methane production. Shock tolerance due to cations other than ammonium was much better indicating a different type of toxicity exerted by the ammonia. During gradual adaptation to increasing levels of ammonia using acetate salt, the initial inhibitory effect was visible at TAN concentration as low as 933 mg N/L. Significant inhibition was evident at TAN level similar to the one observed at the lowest NH_4Cl level in shocked biomass, i.e. around 2,333 mg N/L.

Because of more than doubling the original TAN concentration of 1,000 mg N/L, acclimation of methanogenic sludge at three pH levels of 7.0, 7.25 and 7.6 and the

corresponding maximum UAN of 18, 33, and 68 mg N/L, took some 60 days [Koster, 1986]. After that the maximum specific methanogenic activity was 64.8, 62.0 and 31.2 percent of the initial activity, respectively, and was clearly affected by the level of free ammonia during acclimation period. Further biomass adaptation to TAN concentrations up to 11,831 mg N/L (pH 7.38 to 7.57, UAN 216 to 332 mg N/L), six times higher than the initial toxicity threshold, was still possible but impractical. The resulting methanogenic activity at the highest TAN concentration was only 5.3% of the initial activity at 1,000 mg N/L, or 8.7% of the activity following initial acclimation to 2,300 mg N/L.

2.2.1.8 Reversible nature of ammonia toxicity

Ammonia toxicity in methanogenic granular sludge mostly comprising the species of *Methanothrix* (99%) exposed to TAN of 9139 g N/L (pH 7.40 to 7.68, UAN 175 to 327 mg N/L) for three weeks was found reversible after reactor contents was diluted [Koster and Lettinga, 1988]. The maximum specific methanogenic activity recorded at the resulting TAN concentration of 5229 mg N/L (pH 7.40 to 7.60) was 0.12 g COD g VS^{-1} day^{-1}, same as the activity of biomass from another reactor operated at a similar TAN load but without prior shock.

A complete recovery of methanogenic population from a shock load of from 8,636 mg N/L (pH 7.0, UAN 94 mg N/L) to 20,935 mg N/L (pH 6.4, UAN 58 mg N/L) was possible within one day after each shock. The biomass was immobilized on a porous medium and, therefore, lowering of the TAN concentration to recover could be easily accomplished by feeding a low ammonia substrate (TAN concentration as prior to the

shock) and washing out the concentrated waste. A longer time of four days was needed to completely recover methane production by a biomass that was gradually adapted to high ammonia concentrations, but eventually reached a toxic level of 18,318 mg N/L (pH 6.6, UAN 80 mg N/L) and failed [De Baere *et al.*, 1984].

2.2.1.9 Inhibition by ammonia in pure cultures

Inhibition by ammonia was studied in pure cultures of methanogenic *Methanospirillum hungatei*, *Methanobacterium formicicum*, *Methanosarcina barkeri*, and *Methanobacterium thermoautotrophicum* [Jarrell and Saulnier, 1987]. The first three organisms were grown at 37°C and the last one at 60°C. A 50% reduction in the rate of methane production by respective cultures occurred at TAN concentrations of 4,200, 10,500, 11,900, and 19,800 mg N/L. The pH was 6.5 and the corresponding UAN concentrations were 17, 42, 48, and 392 mg N/L. Astonishing performance of the hardiest methanogen, *Methanobacterium thermoautotrophicum*, at such high UAN is worth noting, since 5 times higher fraction of TAN exists at 60°C as compared with 37°C. In addition, methane production at half of the original rate under these conditions compares favourably to the values observed in mixed cultures of *Methanothrix* species, an obligate acetoclastic methanogen, by Koster [1986]. Even though, following initial acclimation, a reduction of only 35% at TAN of 4563 mg N/L (pH 7.40 to 7.70, UAN 87 to 171 mg N/L) was observed, further ammonia increase to 4,992 mg N/L and higher resulted in at least 65% reduction.

In another study [Sprott *et al.*, 1986], several strains of methanogenic bacteria (*Methanobrevibacter smithii*, *Methanobrevibacter arboriphilus*, *Methanobacterium* strain

G2R) were not affected by TAN concentrations up to 5,600 mg N/L (pH 7.0, UAN 61 mg N/L). Methane synthesis was completely inhibited under the same condition in *Methanospirillum hungatei*, and only 560 mg N/L TAN (pH 7.5, UAN 19 mg N/L) was severely toxic to *Methanothrix concilii*, the now-called *Methanosaeta concilii*. About 60% reduction in CH_4 yield was observed at about 2,800 mg N/L (pH 7.0, UAN 30 mg N/L) in case of another sensitive aceticlastic methanogen, *Methanosarcina barkeri*. This heterogeneity among different methanogens, with respect to sensitivity to ammonia, explains often-contradictory reports on maximum levels of ammonia at which methane formation is still feasible.

Certain methanogens have the ability to adapt to relatively high ammonia concentrations. A strain of *Methanosarcina mazei* (S6) was unaffected by ammonia levels 10 times above the optimal for growth but a 25-fold elevation resulted in lengthening of the lag phase and decreased rate of growth. The induction of a stress response was confirmed by observations of increased transcripts from a number of stress genes [Lange *et al.*, 1997].

The evidence of ammonia-induced injury of *Escherichia coli* (ATCC 25922) and *Enterobacter aerogenes* (ATCC 23355) was demonstrated after exposure to concentrations from 40 to 100 mg N/L TAN (pH 7.0, UAN negligible) [Naundorf and Aumen, 1989]. The injury was assessed by reduced ability to grow on m-Endo agar as compared with m-T7 agar. The ammonia-treated cells had an increased sensitivity to such m-Endo agar components as sodium deoxycholate.

2.2.1.10 Methods to mitigate ammonia inhibition

As described above, inhibition due to free ammonia increases with increasing temperature [Hansen *et al.*, 1998]. Several methods aiming at minimizing this effect during thermophilic (55°C) swine manure digestion were tested [Hansen *et al.*, 1999]. The original CH_4 yield was only 67 mL g VS^{-1} or 22.3% of the potential yield. Addition (w/w) of 1.5% activated carbon, 10% glauconite, or a combination of the two compounds resulted in an increase in CH_4 production to 126, 90, and 195 mL g VS^{-1}, respectively. The highest yield was comparable with the one observed during digestion at 37°C [Hansen *et al.*, 1998] and without any additives.

In cases of animal manure digestion, there is a potential for additional inhibition due to the presence of sulphide (as H_2S). Concentrations in excess of 50 mg S^{2-}/L inhibit methane generation [Parkin *et al.*, 1990; McCartney and Oleszkiewicz, 1993]. In addition, sulfate-reducing bacteria (SRB) compete with methanogens for substrates like acetate and H_2/CO_2, the competition being energetically in favour of the SRB. A combined inhibition of CH_4 production from ammonia and sulphide was addressed by Hansen *et al.* [1999]. Sulphide toxicity was found at H_2S level of 23 mg S^{2-}/L, a much lower level than the common one (see above). Additions of activated carbon and Fe^{2+} to the biomass digesting swine manure at 52°C mitigated toxic effect of sulphide and showed CH_4 production as high as in the unchallenged biomass.

Contrary to the findings by Hansen *et al.* [1999], Heinrichs *et al.* [1990] showed apparent improvement in CH_4 yield and soluble organic carbon removal in UAN-inhibited cultures in the presence of active sulfate-reducing bacteria and sulfate. This was

explained by the degradation of propionate, thermodynamically more favorable in the presence of sulfate, by the SRB to a level non-inhibitory for acetate-utilizing methanogens. Propionate accumulation is the result of increased partial pressure of H_2 caused in turn by UAN-induced inhibition of hydrogenotrophic methanogens [Wiegant and Zeeman, 1986].

Omil *et al.* [1995] reported on the acclimation of anaerobic sludge to saline wastewater as the primary substrate under high sulphide and ammonia stress. The values of up to 200 mg N/L UAN, less than 100 mg S^{2-}/L hydrogen sulphide, and 6-10 g Na/L salinity could be tolerated without process deterioration. Careful control of the influent protein content to avoid further free ammonia accumulation was necessary.

Other methods to address ammonia inhibition of methane production include sedimentation prior to wasting and feeding, addition of activated carbon and glauconite, and increase of HRT [Hansen *et al.*, 1999]. Addition of zeolite (2%w/v) to a thermophilic digester continuously fed cattle manure delayed onset of inhibition and helped to recover CH_4 formation after initial inhibition [Borja *et al.*, 1996 b]. Addition of 10% (w/v) powdered phosphorite ore enhanced CH_4 generation from previously inhibited poultry manure digester operating at ammonia levels up to 7,850 mg N/L TAN (pH not reported). This allowed for a partial recovery in the densities of proteolytic and methanogenic bacteria combined with the decrease in the numbers of sulfate-reducing bacteria [Krylova *et al.*, 1997]. In primary and waste activated sludge digestion, a 35% recycle of the digested sludge to the thickener lowered the concentration of ammonia in a digester from 2,700 mg N/L to 1,700 mg N/L [Melbinger and Donnellon, 1971]. This practice did not

affect the volatile solid reduction and CH_4 production at lower ammonia concentration, but made the process more stable.

2.2.2 Ammonia inhibition in high-solid digestion

The review presented above concerns dilute digestion systems. At high-solid level, an insufficient amount of free water can limit dissolution of substrates and intermediate products of bacterial metabolism, distribution of bacteria, mixing and mass transport. Because of that, efforts to mitigate ammonia toxicity are at greater risk of failure. Some common practices that can be used in slurry digestion may not be applicable in high-solid systems treating such residues as animal manures or organic fraction of municipal solid waste (OF-MSW).

There is little data available on ammonia toxicity at high-solid digestion. Lay *et al.* [1997] demonstrated solids content and pH dependence of methane generation from municipal sludge. With solids level varying from 4 to 10%, the relative specific methane production rate decreased by half over the range of pH of 6.6 to 7.8. The optimum pH for the highest solid treatment was 6.8 with the minimum lag of 9 days for CH_4 formation, while process failure was established anywhere outside of the pH 6.1 to 8.3 range. Since free ammonia concentration is linked to the pH, pH control is often indicated as that of outmost importance [Kayhanian, 1999].

The knowledge of the concentration or distribution of ammonia and organic forms of nitrogen in the organic fraction of municipal solid waste (OF-MSW) during biodegradation process is still incomplete [Burton and Watson-Craik, 1998]. This type of high-solid residue is low in organic nitrogen. On the other hand, animal excreta such as

chicken or pig excreta are high in organic nitrogen. During anaerobic digestion, nitrogenous organic compounds of the manure (excreta and bedding) are converted to ammonia. During poultry manure anaerobic digestion experiments conversion efficiency of 53% was obtained (Field *et* al., 1985; Webb and Hawkes, 1985]. However, the resulting ammonia will cause an early inhibition of the digestion performance. Chicken manure is one material that cannot be digested at its high original total solids because of ammonia inhibition but its presence could eventually contribute to pathogen inactivation.

2.2.3 Other abiotic parameters inhibitory to methane production

The pH alone can inhibit methane generation through decrease in enzymatic activity of methanogenic bacteria. This phenomenon is not exclusive to methanogens, however. Different enzymes have their optimum range of activity, which is pH-dependent. Obviously, the cells try to maintain a constant internal pH, but any deviation from the optimum pH may hamper their efforts.

Osmotic pressure is another killing mechanism. It removes water from the microorganisms (plasmolysis) when they are in solution high in salts or sugars. Loss of water interferes with the cell function and eventually leads to cell death [Black, 1996]. The susceptibility to osmotic pressure varies among different organisms [De Baere, 1984]. Gram-positive bacteria are more resistant than Gram-negative bacteria, as well resistance is dependent on the growth medium. Osmotic effect at the high NH_4^+ concentration can contribute to the already initiated UAN inhibition.

2.2.4 Applicability of anaerobic digestion to treat high-solid residues

In principle, anaerobic digestion is suitable over a wide range of solids concentrations. Traditionally, the process has been used for treating wastewater sludge and industrial wastewaters, but its application to treat even concentrated waste streams has been continuing to grow [Lusk *et al.*, 1996]. A better understanding of process biochemistry and development of improved tools to control the process has undoubtedly contributed to such outcome [Speece, 1996].

2.2.4.1 Animal residues (manure)

Poultry farming, including chicken, has been in the group of the most expanding and intense agricultural operations. Typical solids content of the manure is between 20 and 25% total solids (TS), largely depending on the degree of natural drying during storage [Webb and Hawkes, 1985]. Poultry manure has a higher fraction of biodegradable organic matter than other livestock wastes [Hill, 1983; Jewell & Loehr, 1977; Morris *et al.*, 1975]. Yet this substrate, rich in organic nitrogen, when anaerobically digested at its original solids content of 20-25%, can cause a reduction of process performance caused by ammonia accumulation. A common approach to this problem relies on dilution of the manure to 0.5-3.0% total solids (TS) thereby eliminating ammonia inhibition of the digestion. The resulting volume of the waste to be processed is larger. This, as well as an increased energy demand to heat the diluted manure to the operating temperature of a digester and maintain the temperature of the digester, make this method economically unattractive.

There have been some efforts made to treat the manure in its semi-solid state. Converse *et al.* [1981] operated a farm poultry digester fed with 11.4% TS manure, however high volatile acid content of the digestate and low volatile solids (VS) reductions obtained indicated the need for optimization of the digester's biogasification efficiency. Safley *et al.* [1985] reported better performance of their full-scale digester, though at a lower solids level (5.9% TS). Jantrania *et al.* [1985] attempted a laboratory scale digestion of poultry manure at 30-35% TS. Apparent build up of hydrogen sulphide to inhibitory levels in most of the reactors and overall reduced conversion efficiency with very long retention times employed pointed out the limitation of the application. Webb and Hawkes [1985] tested a broad range of solids from 1 to 10% TS and showed optimum substrate bio-conversion to methane at 4-6% influent TS. They suggested a two-phase operation with the first hydrolysis/acidogenesis phase and the second methanogenic phase, already pre-adapted to high levels of ammonia, as an alternative to a single stage design. The above experiments were carried out within the mesophilic temperature range (30-38°C). Huang and Shih [1981] conducted a thermophilic (50°C) digestion of diluted manure at different solids concentrations and retention times and concluded that maximum CH_4 production can be obtained at 6% VS and 4-day retention.

Problems with ammonia toxicity associated with increased load to a digester limit the strength of the manure that could be processed. Higher organic loading could have been possible had the ammonia content of the manure been lower.

2.2.4.2 Municipal residues (organic fraction of MSW)

Low nitrogen content of the organic fraction of municipal solid waste, coming mainly from the food waste component, makes this residue inadequate for biological processing. The common practice involves supplementation with wastewater sludge to co-digest under anaerobic condition

Co-digestion of organic residuals from municipal, agricultural and industrial sectors is practiced more widely than ever before [Braber, 1995, Lusk *et al.*, 1996]. In 1997, some 50 companies worldwide were reported to hold a license for developing and operating anaerobic digestion either in semi-solid or high-solid mode, with pilot or full-scale plant capacities of at least 100 tonnes per year [IEA, 1997].

The high-solid mode of operation is often preferred over the low-solid digestion that requires vast quantities of water to reduce the concentration of solids [Rivard *et al.*, 1993]. However, certain factors and operational conditions can limit the stability of high-solid digestion. Depending on the substrate (in parentheses), these include:

- concentration of macro- N, P, K, and micro- Fe, Cu, Ni, Zn, Co, Mo, Se, nutrients (organic fraction of the municipal solid waste, OF-MSW);
- buffering capacity of the system to reduce upsets due to rapid production and accumulation of organic acids relative to the methane generation stage (OF-MSW);
- organic or volatile solids loading to a digester that is normally higher than in low-solid systems, i.e. 5-14 g VS Kg^{-1} reactor mass d^{-1} versus 3.2-7.2 g VS L^{-1} d^{-1} (manures, OF-MSW, food processing), and the resulting inhibition due to the formation of ammonia (manures) or other by-products.

Co-digestion of OF-MSW with primary sludge is one example of synergistic application. There are a number of reasons for which wastewater sludge inclusion in the feed to a high-solid digester treating the OF-MSW is beneficial. Co-digestion of the OF-MSW and sludge offsets the need for nutrient supplementation of the anaerobic biomass [Rivard *et al.*, 1989a]. Sewage sludge inclusion in the feed was reported to enhance the startup of mesophilic digesters [Demirekler and Anderson, 1998] treating the OF-MSW. Usually, there is little alkalinity in the OF-MSW alone available to neutralize the organic acid buildup during anaerobic digestion. The acidogenic stage progresses faster as compared to methanogenic stage, which can lead to process upset. Addition of anaerobically digested sludge encourages digestion by inoculation of the OF-MSW digester and by addition of alkalinity to the digester. Primary sludge supplementation helps by means of addition of easily biodegradable organics, nutrients, etc. Supplementation of the OF-MSW with other wastewater sludges, e.g. waste activated sludge [Poggi-Varaldo and Oleszkiewicz, 1992], or with dairy manure [Rich *et* al., 1994], also enhanced biogas production rates and stability of digestion.

Co-digestion benefits municipalities also from the point of view of sludge processing cost. A portion of sludge can be set aside for co-digestion with OF-MSW, which translates to smaller volumes to be further dewatered and to less liquid from the dewatering to be further treated [Kayhanian *et al.*, 1991]. Also, enhanced co-digestion can actually be used to speed up hydrolysis and fermentation of organics to volatile fatty acids (VFA). The VFA, in turn, can be harvested and further used to provide easily biodegradable carbon source for nutrient removal from wastewater [Pavan *et al.*, 1998; Llabres *et al.*, 1999].

Hydrolysis is the rate-limiting step in the methane generation from solid waste degradation, with temperature having little effect once the process reached a steady-state condition [El-Fadel *et al.*, 1996]. Cellulose biodegradation in particular requires longer retention times as compared to the methanogenic stage [Noike *et al.*, 1985]. Municipal sludge is an additional supply of easily biodegradable organics that can actually help stimulate hydrolysis. Different reactor configurations, including phasing by separation of leachate coming out of the solids, have been proposed [Ramasamy and Abbasi, 2000]. Primary or biological sludge co-digested with the OF-MSW is thought to enhance leachate generation, in which case no extra water is needed to promote leachate generation.

Due to the benefits of OF-MSW and primary sludge co-digestion, it is important to know what is the effect of increasing the fraction of primary sludge solids on overall stability of the digestion. Particle size of the refuse is also thought to have an effect on the degree of feed compaction, allowing better distribution of the moisture and easier access of microorganisms to organic substrate. Interactions between primary sludge fraction and the particle size of the high-solid feed can be evaluated in terms of methane production as well as toxicity of un-ionized forms of ammonia or VFA.

2.2.5 Summary

Anaerobic digestion of wastes with high ammonia generation potential is possible. However, gradual acclimation is required to build up the resistance of methanogenic populations. The maximum achievable organic load and the resulting rate

of methane formation will be lower than the corresponding values at low ammonia concentrations.

The extent of resistance of methanogenic population to ammonia toxicity is reflected in the length of a lag phase in CH_4 production. This lag represents the time needed for the resistant strains in the sludge to grow.

Because approximately 70% of the methane formed in biogas digesters originates from acetate, the aceticlastic methanogens are of particular importance (Sprott *et al.*, 1986). Knowledge of the mechanism of ammonia inhibition and toxicity contributes to better control of anaerobic digestion process.

The gap that exists in the literature on high-solid anaerobic digestion needs to be filled in. The interactions between ammonia-rich or ammonia-deficient substrate and microbial population degrading it need better understanding. The thorough knowledge of process requirements and limitations would contribute to the overall applicability of the technology.

2.3 DISINFECTION OF LOW-SOLID AND HIGH-SOLID ORGANIC RESIDUES

Wastewater treatment plant effluent, wastewater sludges (primary, secondary, and chemical), and animal manures are a potential source of pathogenic microorganisms. They are usually associated with insoluble solids [Farrell *et* al., 1990] and once in environment, they pose a risk of infection to humans and to animals through contact with either contaminated receiving water or agricultural soil to which sludge or manure has been applied. Infection may take place during application through air borne route [Dowd

et al., 1997] or through direct contact with applied sludge following application [Ottolenghi and Hamparian, 1987]. Animals can become infected after grazing on contaminated grassland and, even after recovery, they may excrete pathogenic bacteria for months [Jones, 1980] thereby re-contaminating the environment. Contact of agriculture produces with animal manure or other pathogen sources during production and harvesting increases the risk of pathogen transmission to humans [Beuchat and Ryu, 1997].

There are a number of disinfection methods proven effective for dilute, wastewater effluents such as oxidation by chlorine or ozone, or UV radiation. Their use in sludge processing is of limited value due to higher solids content, which makes it economically unfavorable due to much higher dosing requirement or inability to fully disinfect the sludge. Efforts to minimize the risk of infection by enforcing appropriate sewage sludge treatment and disinfection practices prior to its use as agricultural fertilizer were undertaken [e.g. U.S. EPA, 1993; CEC, 1986].

Current trends in the area of sludge disinfection have been focusing on the establishment of environmentally sound technologies that would reduce initial costs, energy, and labor requirements.

2.3.1 Common pathogens associated with wastes, including high-solid residues

Concentrations of pathogenic bacteria and bacterial indicators of pathogen presence in treated material during different stages of treatment do not necessarily represent those densities found in the raw, untreated material. Unlike viruses and parasites, bacteria can multiply without a host. Therefore, their levels should be used only

as those representing the hygienic quality of the material at the particular stage of treatment tested.

2.3.1.1 Chicken manure

The intestinal tracts of chickens are normally inhabited by facultative and strict anaerobic bacteria, including pathogens [Salanitro *et* al., 1978; Barnes *et* al., 1972; Barnes and Impey, 1970]. Chicken excreta contain high counts of these bacteria. *Salmonella* are part of the normal micro-biota in the chicken's gut and feces [Black, 1996]. *C. perfringens* is another example of chicken intestinal micro-biota [Kondo and Tateyama, 1990]. In a study by Beery *et* al. [1985], it was shown that *E. coli* O157:H7 could colonize chicken intestine. The persistence of pathogenic species in the manures of other common farm animals makes the proper treatment of major importance prior to use as soil fertiliser. Kudva *et* al. [1998] demonstrated a long-term survival of *E. coli* O167:H7 in piled ovine and bovine manure, and manure slurry subjected to varying environmental conditions.

2.3.1.2 Municipal solid waste

The presence of pathogenic microorganisms in the OF-MSW is usually associated with the use of additives such as sewage sludge to biologically co-stabilize both residues

and supplement nutrients for optimal microbial activity. The literature data are scarce on this subject, with a couple of reports dealing with pathogen densities following a treatment process rather than prior to the treatment. In the pilot scale operation of OF-MSW and sewage sludge co-digestion at 55°C and 30-day retention, followed by drying at same temperature, total and fecal coliforms, *Streptococcus*, and *Enterococcus* were absent in the final humus-like product [Rich *et al.*, 1994]. Similar findings were reported by Deportes *et al.* [1998], where successful disinfection of aerobically composted MSW led to a decrease in fecal contamination indicators and disappearance of fecal pathogens, including *Salmonella* sp. *Shigella*, total and fecal coliforms, total and fecal streptococci, and *Ascaris* eggs. Apart from high temperature of a composting process, the action of indigenous organisms, such as fungi, can play role in inactivation too. Meekings *et al.* [1996] demonstrated progressing degeneration of *Ascaridia galli* eggs in compost environment, as compared with eggs in sterile compost or in control suspensions. Bacterial predation of protozoa is another suggested mechanism, by which inactivation occurs [Mallory *et al.*, 1983]. Based on the above evidence, the municipal solid waste has the potential to support pathogenic presence. The extent of treatment should be regarded on a case-by-case basis depending on the presence of fecal material in the waste.

2.3.1.3 Municipal sewage sludge

Presence of pathogens in municipal sludge is related to the knowledge of hygiene and sanitary conditions among the population in a given municipality. The type of industry in a region and co-treatment of its wastes with municipal waste can also influence the densities of pathogenic species. Because of the fecal origin, sewage sludge

contains pathogenic organisms mainly discharged by infected humans. Bacterial pathogens, relative to the total number of bacteria present, represent a rather minor part but remain highly infectious. Representative genera of bacterial pathogens include enteric *Salmonella*, *Escherichia*, *Campylobacter*, and *Yersinia*. High fever, diarrhoea, dehydration and other symptoms are often observed in the enteric pathogen related illnesses. In the review by Schwartzbrod *et al.* [1998], common *Salmonella* sp. concentrations in primary sludge are from none detected to 10^3 log/L. Bacterial indicators were quantified at 10^5-10^8/L for fecal coliforms, 10^7-10^9/L for fecal streptococci, and 10^6-10^9/L for the spores of *Clostridia*.

Viral pathogens such as adenovirus, enterovirus, reovirus, and rotavirus, cause gastro-enteritis symptoms of fever and vomiting. Enteric virus densities reported for sludge varied from 132 to 140,000 plaque forming units (PFU)/L [Schwartzbrod *et al.*, 1998].

Helminths (e.g. *Ascaris lumbricoides*, *Taenia saginata*) represent a wide spectrum of parasitic illnesses and their occurrence in sludge is especially common in areas with low level of sanitation. Common levels found in sludge from developing countries account for from 1 to 100 eggs/g TS, whereas in Europe these levels vary from none detected to 10 eggs/g TS [Gaspard *et al.*, 1995]. Hotez *et al.* [1997] estimated that some 63% of the population of China is infected with one or more of helminth parasites, *Ascaris lumbricoides* being one of them. Eggs of *Ascaris* can be produced by an adult worm at a rate of up to 200,000 per day [Little, 1986]. Gaspard *et al.* [1997] reported on a regular presence of human and animal parasites in urban sludges, lagoon sediments and composts intended for agricultural purposes in France. Nematode eggs (*Toxocara*,

Ascaris, *Capillaria*, *Trichuris*, *Ascaridia*, and *Enterobius*) represented 93.2% and cestode eggs (*Taenia*, *Hymenelopis*) accounted for 6.8% of the detected eggs. Similar findings were concluded by ThomazSoccol [1997] in Brazil where eggs of a human roundworm *Ascaris* and other egg species were identified in municipal sludge at significant concentrations of up to 20 eggs /L in liquid sludge or average of 4.85 eggs/g TS in dewatered sludge.

Eggs of *Ascaris* are the most commonly found in sewage sludge. Samples from 27 wastewater treatment plants in the United States identified *Ascaris* as the principal species, followed by *Toxocara* and *Trichuris* [Reimers et al., 1982]. The persistence of *Ascaris* eggs in soil is long, from 5 to 7 years, and possibly longer [Little, 1986]. These occurrences and high resistance to disinfection point to the need of enforcing effective treatment and quality assurance measures aiming at elimination of pathogens.

Wastewater chlorination has little effect on *Ascaris* eggs. Fortunately, the removal can be effectively achieved through sedimentation, as the eggs are heavy and settle well. Therefore, the primary concern is about sludge in which they accumulate.

The efficiency of removing nematode eggs from wastewater in stabilization ponds, low-cost and often used means of treatment in small communities, can be estimated by the following equation [Ayres *et al.*, 1992]:

$$R = 100\,[1 - 0.41\exp(-0.49\,\theta + 0.0085\,\theta^2)] \qquad (2\text{-}4)$$

where R is percentage nematode removal, and θ is retention time (days). The removal occurs through settling. This equation represents the lower 95% confidence limit

of the original equation, and recommended to meet the WHO [1989] guidelines of <1 human intestinal nematode egg/L wastewater used for irrigation purposes in agriculture.

The numbers of pathogenic microorganisms in secondary (biological) sludge and in mixed (primary plus secondary) sludge do not differ from those found in primary sludge as standard processes used in sewage treatment trains have limited effects on their inactivation. Unstabilized, dewatered sludge is also expected to have similar densities of pathogens.

2.3.2 Regulatory perspective on pathogens in residues

The probability of spreading pathogens into the environment with untreated residues of human and animal origin is high. Distribution of unstabilized sludge on land can also emit odours due to the high concentrations of sulphur compounds, nitrogen compounds, acids and organic compounds (aldehydes and ketones). Disinfection is mandatory to enhance public perception and build up their confidence regarding safe use of such residues. The following section is dedicated to demonstrating the efforts among legislators to establish the basis for safe use of municipal sewage sludge residues. There are no known pathogen or vector attraction reduction requirements regarding animal manures or other organic residues in any of the jurisdictions discussed below. Nevertheless, the technologies and methods used to disinfect sewage sludge as well as sludge regulations are applicable to both, animal manures and other organic residues.

2.3.2.1 U.S. Environmental Protection Agency

In the USA, advanced regulations (U.S. EPA, 1993) categorize sewage sludge into two classes based on the pathogen reduction requirements: Class A and Class B. There are two additional requirements, concerning vector attraction reduction (minimum 38% reduction in volatile solids) and pollutant (heavy metal) levels in sludge, but these are beyond the scope of this review.

The regulations designate biosolids as Class A with respect to pathogenic microorganisms such as viruses, bacteria, and helminth eggs. The U.S. EPA pathogen reduction requirements are technology-based, and list six alternatives for treating sludge to Class A level. All alternatives have to meet the following requirement:

either

the density of fecal coliform bacteria in the sludge is less than 1,000 most probable number (MPN) per gram total solids (TS, dry weight basis)

or

the density of *Salmonella* sp. bacteria in the sludge is less than 3 MPN per 4 g TS.

Under Class A - alternative 1, the sludge must be subjected to an adequate time-temperature regime, depending on the solids content. The temperature and time period can be determined using one of the following equations, depending on the sludge solids:

$$D = 131{,}700{,}000/(10^{0.14t}), \quad \text{when } TS \geq 7\% \qquad (2\text{-}5a)$$

$$D = 50{,}070{,}000/(10^{0.14t}), \quad \text{when } TS < 7\% \qquad (2\text{-}5b)$$

Where, D – time (days), t – temperature (^{O}C)

Different retention times are required to achieve the same degree of pathogen kill. Any time-temperature combination in Fig. 2-3 located on or to the right from the curves described by the above equations, would achieve necessary condition. The minimum temperature of sewage sludge should be at least 50°C. The minimum time is 20 min for the sludge at TS≥7%, and 30 min for sludge at TS<7%. From Fig. 2-3, the holding time for liquid sludge (TS<7%) required to achieve Class A product would be 5 days at 50°C, and 12 hours at 57°C.

Class A - alternative 2 requires that the pH of biosolids must reach above 12 for 72 hours. During this time, the temperature of the sludge should remain above 52°C for 12 hours or longer. In addition, at the end of the 72-hour period, the sewage sludge should be air dried to achieve a TS level in biosolids greater than 50%. Clearly, this alternative also includes temperature requirements and imposes additional condition with regard to the level of solids in treated biosolids.

Alternatives 3 through 6 require sewage sludge to be treated in: other pathogen treatment process (Alt. 3), an unknown pathogen treatment process (Alt. 4), a Process-to-Further-Reduce-Pathogens (PFRP) (Alt. 5), or a process equivalent to PFRP (Alt. 6). The PFRP's include: composting, heat drying, heat treatment, thermophilic aerobic digestion, beta ray irradiation, gamma ray irradiation, and pasteurization.

Alternatives 3 and 4 give room to new emerging technologies that can achieve pathogen destruction efficiency similar to that obtained by any of the processes under

Alternatives 1, 2, 5, or 6. However, to do so these are the only alternatives that have to meet two additional microbial reduction requirements:

the density of enteric viruses in the sludge is less than 1 plaque forming unit (PFU) per 4 g TS

and

the density of viable helminth ova in the sludge is less than 1 per 4 g TS.

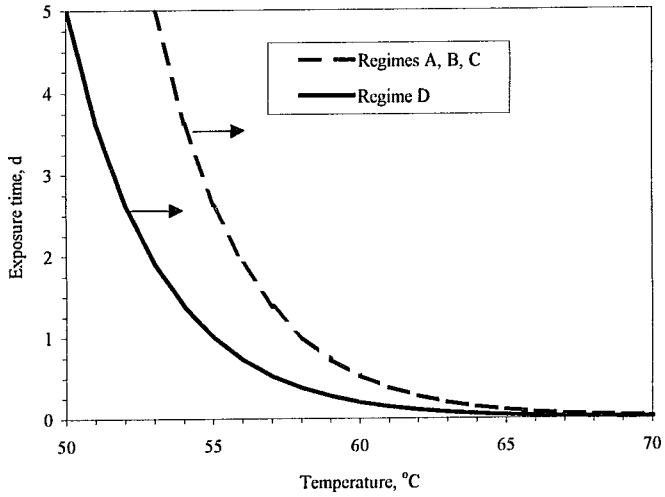

Fig. 2-3. Class A - Alternative 1 time-temperature requirement (thermal treatment) Biosolids must meet the time-temperature condition defined by any point to the right from corresponding curves [US EPA, 1993]
TS: total solids (dry weight)

REGIMES:
A-
temp. of sludge
at least 50°C
for at least 20 min
(all sludge at at least 7% TS)

B-
temp. of sludge
at least 50°C
for at least 15 sec
(sludge at least 7% TS, and
in the form of small particles
and heated by contact
with either warmed gases
or an immiscible liquid)

C-
sludge heated for
at least 15 sec
but less than 30 min
(sludge with less than 7% TS)

D-
temp. of sludge
at least 50°C
for at least 30 min
(sludge with less than 7% TS)

All Class A biosolids must meet the pathogen reduction criteria at the time it is used or disposed, prepared for sale or given away for application to the land.

Class B pathogen reduction requirements include three alternatives to confirm or obtain Class B status. Alternative 1 states that the density of fecal coliform bacteria shall be less than 2,000,000 MPN or colony forming units (CFU) per g TS. This should be confirmed by analysis of seven representative sludge samples during each monitoring episode. Under alternative 2, sludge should be treated in any Process-to-Significantly-Reduce-Pathogens (PSRP) such as aerobic digestion, air-drying, anaerobic digestion, composting, or lime stabilization, to achieve necessary pathogen reductions. Alternative 3 requires the sludge to be treated in a process that is equivalent to a PSRP. The equivalency is to be determined by the permitting authority. The last two alternatives do not require microbiological monitoring for regrowth of fecal coliforms or *Salmonella* sp.

In general, sludge with both Class A and B pathogen status are deemed protective of public health and the environment, even though Class B biosolids may still contain pathogens. The relation between the two classes can be pictured as follows [U.S. EPA, 1994]:

Class A = Class B + site restrictions + management practices

After recent revisions [1999], the U.S. EPA standards provide further incentive toward safe beneficial use of biosolids [Wu and Smith, 1999].

2.3.2.2 Council of European Communities

The EC directive concerning the use of sewage sludge in agriculture [CEC, 1986] requires its prior treatment, but authorizes the use of untreated sludge provided it is

injected or incorporated to the soil. The restrictions for use include prohibition from grazing animals on grasslands or forage land less than 3 weeks after sludge application. As well, the use of sludge is forbidden during growing and maturation of fruit and vegetable crops, and for 10 months preceding the harvest of the crops in contact with the soil and normally eaten raw. Although it stresses the risk of pathogenic contamination, the directive does not mention any specific allowable limits or densities of pathogenic or indicator microorganisms in sludge to be land-applied.

The other EC directive [1991], concerning discharges of urban and industrial wastewater on the environment, does not provide any limits on pathogens either. In one study [Barbier *et al.*, 1990], the "no grazing" limit of 3 weeks following sludge application was shown insufficient in ensuring safe use of the land after that period, as determined by the presence of eggs of parasitic Helminths.

There have been ongoing efforts to revise and enhance the sludge directive with scientific evidence and technological progress since its conception in 1986. The latest draft of the European Union sludge legislation is due sometime this year (2001).

2.3.2.3 Canadian legislative advances

Canada is in the process of adopting the general trend of change from the practice of restricted land disposal of sewage sludge to recycle of biosolids [Ho, 1997]. Soon provincial regulations in Manitoba and other provinces will become more strict and similar to those set by the U.S. EPA. At present, year round land disposal of Class B or unclassified biosolids (sludge) is still practiced (Winnipeg, Calgary), which is acceptable technically but with certain precautions. Incomplete information about the safety of

biosolids appears to undermine the public confidence in land application. Many municipalities are concerned that they will be faced with the need to upgrade to Class A, as per the U.S. EPA regulations, and that the Class B pathogen levels would be found indefensible in the public's eye.

The national standard, the Canadian Environmental Protection Act [MPWGS, 1999], defined the basis for establishing environmentally sound and sustainable technologies. Criteria for agricultural land application of sludge in Canada are set out by provincial and territorial governments.

In Manitoba, the Manitoba Environment Act requires anaerobic digestion at 30 days retention and temperature ≥20°C or an equivalent process such as storage of sludge solids for 1 year or composting [Van Den Bosch, 1999]. Land use is restricted for 3 years following sludge application (crop type is limited to cereals, forages, and oil seeds). In case of landfill disposal, sludge must be in a dewatered form (TS>20%).

In Alberta, the newly released sludge application guidelines [AE, 2001] recommend a 3-year period lapse before direct grazing of crops on applied land is resumed, with similar crop type limitations as in Manitoba. Ban on winter disposal of sludge to agricultural land is due to unsatisfactory conditions of incorporation to soil. Application to frozen, snow-covered or ice-covered soils is usually not permitted because of potential for surface run-off and surface water contamination. Specific numbers regarding the occurrence of pathogens in sludge are not mentioned in the guidelines.

The closest set of regulations that defines pathogen reduction requirements and can be applied to using municipal sludge as fertilizer is that of composts. The BNQ's Conformity Recognition for Composts requires that fecal coliform levels are less than

1000 MPN/g TS, and no *Salmonella* species are found in finished composts [BNQ, 1997]. There is an increasing trend to demand an official approval to sell organic waste-based products from compost producers in Canada [Allard, 2000].

The CCME [1996] guidelines for compost quality state that the compost shall be *Salmonella* free and that the density of fecal coliform bacteria shall be below 1,000 MPN/g TS (as per Class A U.S. EPA standard). Pathogenic organisms are sometimes present in the feedstocks used to make compost. As a result, the compost may also contain pathogens. To reduce any potential health concerns, treatment processes as well as biological specifications have been identified. If the compost does not originate from feedstock known to be high in human pathogens, the demonstration of pathogen-free product through microbiological testing may be sufficient to ensure good quality of the compost. Otherwise, one of the processes to ensure reduction in pathogens shall be used, such as the in-vessel or aerated static pile composting (at least 55°C for 3 d), or windrow composting (at least 55°C for a minimum of 15 d, windrow to be turned at least five times).

Under the Ontario's Draft Guideline for Aerobic Composting [MoE, 1998], if sewage sludge is used as feedstock, the testing for fecal coliforms or *Salmonella* is required, as per the CCME guidelines for compost quality [1996].

The recently instituted National Biosolids Partnership in U.S.A. is a model for similar Canadian action, on a national scale, to improve the existing standards for beneficial use of sewage sludge and sludge products, and develop a strategy for their implementation. First meeting took place in Toronto (24-26 IX, 2000) during the 1st

Canadian National Residuals and Biosolids Management Conference- Biosolids 2000, organized by the Water Environment Association of Ontario.

2.3.3 Disinfection processes

Pathogenic microorganisms are able to survive on crops and in sludge or manure-applied soil. Appropriate treatment must precede the use of such residues to ensure their pathogen-free status prior to use. The success of any disinfection process depends on the provision of a batch mode of operation. In cases where retention time is long enough to effect pathogen inactivation but the risk of microorganisms short-circuiting to the effluent exists, the success of disinfection remains questionable. Among processes commonly used to stabilize organic matter, some can serve the purpose of disinfection like in case of thermophilic anaerobic digestion, composting, or even long-term lagoon storage. Different degree of disinfection can be achieved with these processes, mainly depending on the temperature. Other processes, such as alkaline treatment, are done solely for disinfection. Depending on the dose and type of alkaline agents, the mechanism can be based on high temperature and pH, or high pH alone.

Variations in cell structure, physiology, and replication among different microorganisms are the main reason for differences in inactivation rates. The general order of microbial resistance to some forms of disinfection (e.g. except temperature treatment), starting from the least resistant, can be described as follows:

vegetative bacteria < enteric viruses < spore-forming bacteria < protozoan cysts < helminth eggs.

A wide variation between the resistances of various microbial pathogens within any of the above groups is also common [Bitton, 1999]. This variation is dependent on the target site(s) of inactivation. Multiple target sites at which disinfection progresses include the peptidoglycan layer of the cell wall, cytoplasmic membrane, outer membrane, structural proteins, enzymes, and nucleic acids [Russell *et al.*, 1997].

In general, there is very little that microorganisms can do to withstand physical disinfection such as thermal treatment. Evolutionary forms such as bacterial spores are one unique example of adaptation to adverse environmental conditions, including high temperature. In terms of the resistance of microorganisms to chemical treatment, such barriers like bacterial biofilms, viral aggregates, bacterial gram-negative outer membrane, spore coats, eggs of helminths, and cysts of protozoa can delay or prevent complete disinfection. Whereas high temperature can be a very effective means of inactivation, any form of chemical treatment has to be properly designed to ensure optimal dose for complete biocidal action.

Below are described a number of viable alternatives to affect disinfection and factors governing inactivation of pathogens.

2.3.3.1 Anaerobic digestion

Anaerobic digestion is one of the processes designed primarily to stabilize primary and waste activated sludges. Nevertheless, depending on temperature, it can also reduce pathogen densities of the treated sludges. It is known that at temperatures in excess of 50°C cell lysis occurs (mesophilic organisms), because of irreversible denaturation of the cell proteins. At mesophilic temperatures ranging from 35°C to 40°C

and at organic loading rates common to anaerobic digestion (standard: 0.5 – 1.6 Kg m^{-3} d^{-1}; high-rate: 1.6 – 4.8 Kg m^{-3} d^{-1}), very limited if any pathogenic reduction takes place. In the thermophilic range of 49 to 57°C the reduction is much higher and, depending on retention time and mode of operation, it can lead to complete inactivation of mesophilic pathogens. Much better results can be achieved with draw-and-fill mode of feeding a digester, and not *vice versa* [Farrell *et al.*, 1988]. The fill-and-draw mode would introduce pathogenic organisms to the already disinfected material, resulting in insufficient treatment. Different retention times are required to achieve the same degree of kill at different temperatures within this narrow range. The theoretical, statutory time [U.S. EPA, 1993] of 5 days at 50°C, and 13 hours at 57°C (Fig. 2-3) is required for achieving class A biosolids under alternative 1, the thermal treatment. Thermophilic anaerobic digestion is not classified under this alternative. However, it could be used while giving a large margin of safety at common to thermophilic temperature range retention of 10 days or more. For comparison, the thermophilic aerobic digestion (one of the PFRP, under alternative 5) at retention time of 10 days at 55°C to 60°C is required to meet the Class A requirement.

In a thermophilic anaerobic digestion at 53°C and sludge retention time as low as 10 d [Lee *et al.*, 1989], indicator bacteria (fecal coliforms, fecal streptococci), enterovirus, and viable *Ascaris* eggs were reduced to below detection limit. A two-phase (acid followed by methanogenic) digestion at 35°C reduced bacterial and viral indicators to a much lesser extent than in the thermophilic counterpart. Interestingly, greater bacterial reductions occurred in the acid phase of the system at either temperature.

Black *et al.* [1982] found that the number of viable eggs of *Ascaris*, endemic in biosolids in Central and Southern part of North America, was reduced by 23% in mesophilic anaerobic digestion and determined that their viability after anaerobic digestion was greater than after aerobic digestion.

Animal parasites were inactivated or found to be non-viable during batch, thermophilic digestion of cattle slurry at 53°C after 24 hours [Olsen and Nansen, 1987]. The mesophilic counterpart showed a different degree of reduction, depending on the species investigated, and was not effective in reduction of *Eimeria* oocysts at all. *Eimeria* oocysts, responsible for coccidiosis in poultry, were the subject of another investigation [Lee and Shih, 1988]. They were found to lose their infectivity during batch, thermophilic (50°C) anaerobic digestion of chicken manure at 7.5% TS. Longer detention of several days was needed to obtain similar reduction at 35°C.

In a study of animal viruses inactivation [Spillmann *et* al., 1987], it was found that a thermal treatment at 60°C is required to inactivate thermo-labile viruses, followed by an anaerobic mesophilic digestion to inactivate thermo-stable viruses. Burge *et* al. [1983] suggested that a rupture of the RNA chains in the lower temperature range (from 10 to 35°C) was the major, ammonia-induced virucidal mechanism, whereas at higher temperatures (from 35 to 60°C) denaturation by heat was the predominant mechanism.

2.3.3.2 Long-term storage

Storage alone is one practice of reducing pathogenic levels, but often it proves to be insufficient. After a hot and dry Australian summer and following rainfall at the beginning of the winter, repopulation of piled biosolids with fecal coliforms and

salmonellae previously undetected was observed [Gibbs *et* al., 1997]. The pathogens were able to survive undetected and, once at favourable conditions, they grew to levels higher than the original concentrations.

The decline in microbial pathogens appears to be temperature-dependent. Kearney *et al.* [1993] showed the increased rate of inactivation of *Salmonella typhimurium*, *Yersinia enterocolitica* and *Listeria monocytogenes* in beef cattle slurry during storage at 17°C as compared with 4°C-storage. Similar trend was observed by Munch *et al.* [1987], where cattle and pig slurries were subjected to storage at 18-20°C and at 6-9°C for up to 7 months. However, bacterial spores of *Clostridium perfringens* were found unaffected at both temperatures.

The two cases above concerned the storage of slurry. Ahmed and Sorensen [1995] demonstrated temperature-related inactivation of *Salmonella typhimurium*, *Yersinia enterocolitica*, *Campylobacter jejuni*, Bacteriophage f2, poliovirus, and *Ascaris suum* eggs during storage of dewatered biosolids. The storage at 5°C, 22, 38, and 49.5°C resulted in increased inactivation rates for all tested indicators. The eggs of *Ascaris suum* were least sensitive to temperature. In an extension of this work, Ahmed and Sorensen [1997] showed that minimal mixing (once or twice a month) of dewatered sludge could actually induce autoheating to 50°C and 57°C, thus showing the benefit of dewatered sludge storage over slurry storage. Combination of storage time and temperature led to the reduction of all pathogenic indicators, including eggs of *Ascaris suum*, to below detection levels.

In another storage case, the reported bacterial (fecal coliform, fecal streptococci, *Salmonella* sp.) reductions in lagoon-stored aerobically digested sludge were up to 2-logs

(or 99%) or more – to the levels low enough to classify the sludge as Class B, but too high for Class A biosolids designation [Ponugoti *et al.*, 1997].

During lagoon storage of anaerobically digested sludge in the southern U.S. climate (avg. temp. 25°C), the inactivation of pathogenic and indicator organisms was observed [Reimers *et al.*, 1990]. *Salmonella livingstone* and poliovirus Type 1 were inactivated within 6 months of storage, whereas total and fecal coliforms dropped 2 to 6 logs. The eggs of *Ascaris suum* lost their viability following 15-month storage. Kaneshiro and Stern [1986] reported on similar inactivation rates for *Toxocara* and *Ascaris* eggs in anaerobically digested sludge after 10- to 16-month storage at 25°C, although 4°C-storage retained viability of some of the eggs.

Storage provides some form of disinfection but the results largely depend on the specific storage conditions, including temperature. Indicators that are more conservative were best suited to assess the final quality of treated residues in this simple type of treatment. *Ascaris* eggs and bacterial spores of *C. perfringens* were shown to be among the toughest forms surviving for long times.

2.3.3.3 Freeze/thaw cycling

Freezing and thawing although mainly used for physical sludge conditioning, was found to contribute to pathogen reduction [Sanin *et al.*, 1994]. Artificial freeze/thaw systems are too expensive and costly to operate, but wherever natural freeze/thaw cycling occurs this can be a low-cost process to improve sludge dewatering and reduce its pathogen content. The rates of cooling and warming during freezing and thawing are among the most important factors influencing survival of microorganisms [Mackey,

1984]. At very rapid cooling of >100°C/min, the formation of intracellular ice crystals is retarded and survival increases. In natural freezing, however, the cooling rate is much slower leading to the formation of large extracellular crystals and increased concentration of solutes. The cells with high water permeability of the membrane and high cell surface/volume ratio are more prone to damage from the solutes. The primary site of freezing damage is often assumed to be the cell membrane. If cells are warmed slowly, as in the case of natural storage, intracellular ice crystals may grow and cause even more damage to the cells.

Cell damage in the context of the freeze/thaw cycling is more related to that of vegetative bacteria, as the spores are more resistant to mechanical damage caused by ice crystals. In vegetative bacteria, the repair times differ both within, and between, populations. The reported times for *E. coli* and *Salmonella* contaminated food samples can be as low as few hours [Mackey, 1984]. In case of sludge microorganisms, this time can be longer, but eventually the recovery of injured cells will occur unless additional treatment is provided.

2.3.3.4 Composting

In most composting operations, the primary effect on pathogen kill is high temperature. Due to microbial aerobic decomposition of organics in composting material, it can reach 60^{o}C and more. Frequent turning of compost pile is necessary to ensure that temperature distribution in the pile is uniform and to maintain aerobic conditions in the pile. From the operational standpoint, this condition is difficult to achieve in simpler, small-scale composting operations. Composting was compared with other management

alternatives such as alkaline stabilization, and thermal drying [Foess *et al.*, 1994]. On the basis of all factors involved (qualitative: public acceptance, reliability, product marketability; quantitative: costs both capital and operation and maintenance, land requirements), composting was ranked last overall. Alkaline stabilization and disinfection proved the best. Chemical treatment can offer a simpler solution to the complex issue of disinfection.

2.3.3.5 Alkaline disinfection of dewatered sludge

Alkaline disinfection is one of the processes that can effectively reduce the hardiest forms present in sludge that is destined for beneficial use [US EPA, 1993]. Toxicity of ammonia indigenous to wastewater sludges or animal manure, to microbial populations appears to provide alternative means of pathogen inactivation in this process. Its action depends on several factors including pH. Lime application is a commonly used practice that has two potential actions. Lime brings the pH of treated sludge to a level where most of the ammonia is converted to free ammonia form known from its disinfecting power. When dosed at very high concentrations, lime is hydrolyzed. This reaction is exothermic, which increases temperature of the treated sludge to the pasteurization level of 70°C and higher.

Mechanisms of alkaline disinfection include:

Heat (high-dose) -

$$CaO + H_2O \leftrightarrow Ca(OH)_2 + \underline{65.2\ kJ} \qquad (2\text{-}6)$$

High pH and concentration of ammonia gas (low-dose) -

$$Ca(OH)_2 \leftrightarrow Ca^{2+} + 2\,\underline{OH^-} \qquad (2\text{-}7a)$$

$$NH_4^+ + OH^- \leftrightarrow \underline{NH_3} + H_2O \qquad (2\text{-}7b)$$

The practice of lime treatment of wastewater sludges has been utilized for many years. Two forms of lime have been in use- hydrated or slaked lime, $Ca(OH)_2$, or quick lime, CaO. Major benefit of quick lime addition over the slaked lime is the release of heat during exothermic hydrolysis of CaO when in contact with water from sludge.

Depending on the dose of lime for sludge disinfection, the treatment can be classified as the process-to-significantly-reduce-pathogens, PSRP, or the process-to-further-reduce-pathogens, PFRP. In general, for the PFRP standard (pasteurization at temperature of 70°C or higher for at 30 minutes or longer) the lime requirement is much higher than for the PSRP time-pH standard (pH of 12 for two hours). In theory, 1 Kg of CaO produces 1140 kJ of heat and extracts nearly 1/3 of its weight in free water from the sludge. For the 28% solids sludge, theoretical temperature increase following 500 g CaO /Kg TS addition is almost 50°C [Roediger, 1987]. Another source [Burnham *et al.*, 1998] quotes similar requirements (sludge input temperature equals 20°C): theoretical quantity of lime needed per Kg TS would be approximately 510 g for sludge at 28% solids, and 1090 g for sludge at 15% solids. These additions would be high enough to reach the prescribed 70°C level. Such lime dose should be sufficient to ensure a significant degree of pathogen inactivation. Theoretical lime (CaO) requirements to estimate doses required

for Class A standard are depicted in Fig. 2-4. In practice the required CaO dose would have to be up to 50% higher to account for non-ideal conditions and to keep the required temperature (at least 70°C) for long enough (at least 30 min). For the PSRP, the amount of quick lime is much lower, anywhere from 150 to 250 g/Kg TS (Fig. 2-4).

Alkaline treatment to achieve Class A requirement with respect to pathogenic organisms is narrowed down to using high doses with the major killing mechanism being also the temperature. The action of elevated pH is one of the factors too, but time-temperature requirement, as per Class A - alternative 1, is the one stressed most. Temperature can be lower than 70°C (but not lower than 50°C), with the corresponding longer time needed to meet the requirement.

The drawbacks to the method are the high cost of lime, as well as serious concern because of the high residual pH of the treated material, which makes it less suitable for land application. High residual alkalinity increases the potential for odour development during, and following biosolids application on agricultural land. These concerns can be minimized by equivalent treatments such as addition of potassium ferrate (VI) rather than lime [De Luca *et al.*, 1996]. However, this strong oxidizing agent reduces ammonia to nitrate thereby excluding the ammonia as one of the factors governing pathogen inactivation.

It is expected that the resulting product would be virtually pathogen free, with fecal coliform indicator content of less than 1,000 MPN/g TS (Class A biosolids requirement). Heat inactivation alone is the most widely accepted and commonly used method but it is costly too.

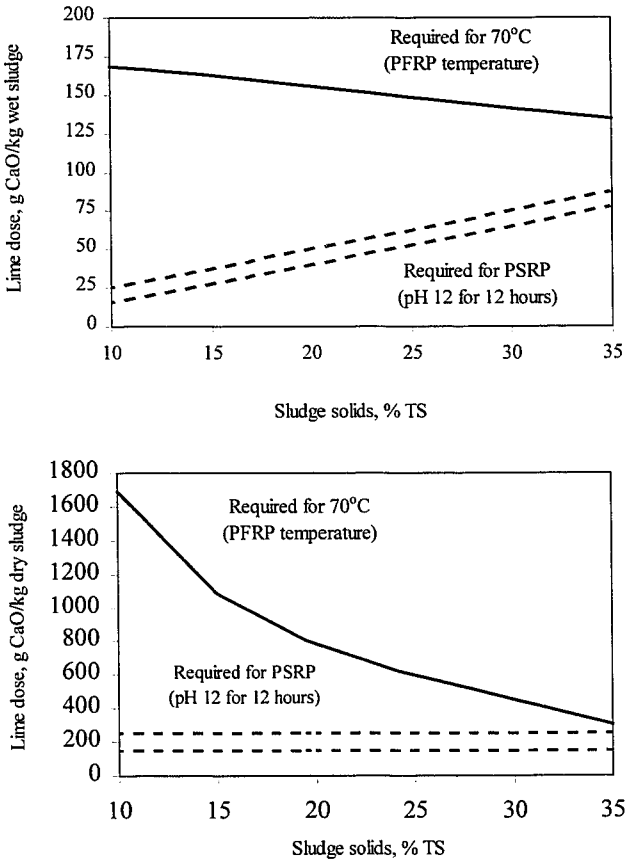

Figure 2-4. Theoretical lime requirements to effect PFRP or PSRP in sludge at different levels of solids

The upper graph relates solids level to lime dose expressed on wet weight basis, whereas the lower graph shows the relation based on dry solids in the sludge;
PSRP: process-to-significantly-reduce-pathogens;
PFRP: process-to-further-reduce-pathogens.

The N-Viro process is one of the few state-of-the-art alkaline disinfection processes relying on heat. As a result of high pH, elevated temperatures and aeration, the N-Viro process releases ammonia, odours and volatile organic compounds [Neil and Webster, 1994]. Release of these compounds occurs at each step of the process, with higher concentrations occurring when the material is disturbed or aerated.

Lime application can take place at different stages of sludge processing. Lime treatment of primary sludge was considered as an option to anaerobic digestion [Farrell, 1974], but the current contention is that sludge treated in such way is not fully stabilized.

Under current standards, liming of primary or combined sludge can only serve as a temporary solution before further processing takes place. The sludge still needs to be stabilized prior to its ultimate use or disposal to reduce its organic content and possible attraction of vectors (flies, rodents, etc.).

The U.K. Code of Practice [DoE, 1989] lists lime conditioning of liquid sludge as one of the treatment options. The pH should be not less than 12 for at least two hours. If the sludge was previously treated using e.g. anaerobic digestion, it can be used directly in agriculture. In case when untreated sludge is used and dewatered after lime conditioning it has to remain stored for at least 3 months prior to its ultimate use. Sludge disinfection experiments with 10 g $Ca(OH)_2$/L showed complete inactivation of *Salmonella typhimurium* in primary sewage sludge after one hour of exposure [Plachy *et al.*, 1996]. However, *Ascaris suum* eggs remained unaffected. Effective dewatering of high-pH liquid sludge following lime conditioning is harder to achieve [Paulsrud and Eikum, 1984]. In addition, processing or recycle of the high-pH return liquors to wastewater

treatment train may cause additional problems. Therefore, better results can be obtained with the use of quick lime.

Quinn and Hall [1984] reported on tests involving lime addition to undigested sludge. A quite low dose of 70 g/Kg TS allowed to reduce bacterial numbers by 98% after the pH reached the 9.5-10 levels, after which further reduction to 99.5% at pH 12 occurred. The remaining bacterial population was due to the presence of highly resistant bacterial spores that could survive the treatment.

Pathogen inactivation times, following lime addition, are often the subject of conflicting reports. Storage of sewage sludge following lime treatment reduced *Ascaris suum* eggs, provided the sludge was at pH of more than 12 and was stored for at least 3 months [Eriksen *et* al., 1996].

The data presented by Gaspard *et* al. [1997] indicated the presence of parasitic eggs in all urban sludges, lagoon sediments, and composts tested. The authors determined the effectiveness of lime treatment at doses of from 100 to 500 g CaO/Kg TS on 10 different sludges and found that only 7 out of 10 treatments achieved reductions to below detection limit. The failing treatments were: one at 150 g/Kg, one out of three at 250 g/Kg, and one out of two at 300 g/Kg. Surprisingly, the lowest dose of 100 g/Kg was effective, but there was no indication on the origin of the sludge and kind of treatment prior to lime addition. Viable egg concentrations of 10 eggs /100 g TS and higher were found in sludges treated by mesophilic anaerobic and aerobic digestion, composting, and liming. Anaerobic digestion was the only type of treatment that did not show complete reduction in viability, but only two samples were tested as oppose to 12 for prolonged aeration, 7 for composting and 8 for liming.

Montruccoli [1996] demonstrated decrease in coliform bacteria to below detection level after 10 days following lime treatment (78.5% CaO, 15.5% MgO) of poorly stabilized sludge (150 and 250 g CaO/Kg TS). After an initial increase, the pH markedly decreased which made application of the sludge more suitable in case of neutral or sub-alkaline soils.

Determination of the required lime doses has to be based on a common ground approach, for example reporting required doses based on the solid and water content of the treated sludge, specifying storage conditions, etc. Often, alkali-stabilized sludge using high doses of alkaline agent(s) require acidification prior to land application to make it suitable for plant growth [O'Brien and Barker, 1998]. If low-lime treatment would prove effective, the amount of alkalinity imparted by lime would be expected less significant. When sludge treated in such process would be applied to the soil, the soil buffering capacity shall ensure that the pH will remain in neutral range.

2.3.4 Role of ammonia in disinfection

Adverse effects of high ammonia concentrations can be used to inactivate pathogenic microorganisms in dewatered, digested sludge, and other high-solids residues. The effect of total ammonia, of un-ionized ammonia, and of high pH on pathogen kill remains insufficiently explored, as there is little data on that subject. For example, Seagren *et* al. [1991] concluded that ammonia (both forms) was not the primary toxic agent that caused inhibition of methanogenesis at pH above 8.1. High pH alone was claimed to be responsible for this impairment. Enzyme activity, membrane transport, and

proton-motive force were given as examples of functions likely being inhibited by the high pH.

Alkaline compounds raise the pH and denature proteins. Reactions that denature proteins include hydrolysis, oxidation, and the attachment of atoms or chemical groups. In denaturation, hydrogen and disulphide bonds are disrupted, and the functional shape of the protein molecule is destroyed [Black, 1996]. Pesaro *et* al. [1995] claimed that at pH values of more than 8.0, free ammonia was the major virucidal agent. Differentiation of pH-dependent free ammonia inhibition from the effects of high pH is very informative because of the different nature of inhibition [DePasquale and Montville, 1990; Cramer *et* al., 1983].

Anticipated presence of common pathogens in chicken manure makes it necessary to disinfect the product. Such pathogens as *E. coli*, *Salmonella* and *C. perfringens* are common inhabitants of chicken gut. Animal manure processed at mesophilic temperatures are not disinfected. Storage does not reduce pathogens significantly. The probability of success in alkaline treatment of manures is high as the ammonia generation potential during anaerobic digestion is high too.

A variety of alkaline stabilization procedures were used to treat chicken and hog manure with the objective of destroying manure pathogens, reducing odour, and improving physical properties [Logan *et al.*, 1999]. It was found that these objectives could be achieved by the use of moderately alkaline pHs (>9.5) and solids (>30%). Disinfection was achieved by the liberation of gaseous ammonia and the formation of air-filled porosity in which UAN can diffuse. Heat was not necessary to reduce levels of

Salmonella and *E. coli* to non-detectable levels. The actual pH and solids required is dependent on the levels of UAN in the manure.

The toxic effect of increasing levels of free ammonia on the eggs of *Ascaris suum* was seen following incubation in primary and aerobically digested sludge [Bowman *et al.*, 2000]. In case of digested sludge, ammonia was generated either by direct addition of ammonium sulfate to the sludge with an increased pH, or by direct addition of ammonia gas. In case of primary sludge, the addition of ammonium chloride was done prior to treatment with the Chemfix process. In the Chemfix process, Portland cement and sodium silicate are added to sludge, followed by chemical fixation, resulting in a soil-like product after 1 day of storage. Reduction of *Ascaris* egg viability in ammonium sulfate amended digested sludge varied depending on the dose, and was 62% at 50 mg salt/g TS and almost 100% at 500 mg salt/g TS, after 10 d from start. The concentration of sludge solids was not given, but if one assumes 5% TS, the 50-mg salt/g TS addition would translate to total ammonia concentration in the liquid phase of 265 mg N/L. For the 500-mg dose the ammonia concentration would be 2653 mg N/L, roughly 1 to 1.5 times the concentration of indigenous ammonia common in sewage sludge. The ammonia content in any of the sludges was not reported either, but its contribution to ammonia-induced egg inactivation was significant with no doubt. In ammonia gassing experiments, a dose of 1% was necessary to obtain complete inactivation after 10 d in sludge previously digested at 20-d and 30-d retention. In sludge with 10-d prior retention, the inactivation was 93% after 10 d. The relative concentration of the ammonia gas in sludge would be high regardless of solids level in sludge, accounting for 8,230 mg N/L and 24,700 mg N/L respectively. The complete reduction of egg viability in primary sludge subjected to

ammonia at 1% and 3% NH_4Cl/g TS concentration was achieved in 10 d and 7 d, respectively. If one assumes solids content in primary sludge of 5% TS, the resulting corresponding ammonia concentrations would be 131 mg and 393 mg N/L. In the above experiments, high pH of the digested sludge in case of ammonium sulphate salt addition, and high pH in the Chemfix treatment that followed upon adding the ammonium chloride salt to primary sludge, undoubtedly had an impact on the eggs, contributing to the overall outcome of the disinfection. In gaseous ammonia addition experiments, the doses were extremely high and represented a rigorous treatment regime. Overall, the use of a closed-system operation in all experiments was beneficial due to the increased vapor pressure of ammonia gas, allowing for an enhanced diffusion into the sludge matrix.

In another process called the N-Viro Soil, ammonia was one of the factors claimed responsible for pathogen inactivation, including eggs of *Ascaris* [Burnham *et al.*, 1990]. The process relies on the addition of cement kiln dust and lime to dewatered material (TS from 18 to 40%), which causes the increase of pH to at least 12.0 and rises the temperature to 52-63°C, followed by storage and drying of treated material. In 6 hours from start of the storage, the number of viable eggs decreased from 560 viable eggs/5 g TS to below detection level of 1 egg/5 g TS. It is difficult to determine the contribution of free ammonia in this rigorous treatment, as it involved both high temperature and high pH.

Apart from the obvious impact of free ammonia on pathogens during alkaline treatment, there are also a number of reported cases of its biocidal effect in other processes.

In a study of the effects of mesophilic and thermophilic anaerobic digestion on cysts of the protozoan *Eimeria tenella* [Lee and Shih, 1988], it was found that whereas temperature was the major killing factor at 50°C, other factors were involved at 35°C. The high total ammonia content of 4,500 mg N/L (pH 7.5-8.0, UAN 150-450 mg N/L) in the digester suggested that free ammonia was the possible toxic agent at this temperature.

A combination of high "ambient" pH in excess of 8.0 and corresponding high free ammonia concentration was indicated as the major cause of inactivation of five animal viruses and bacteriophage f2 during storage of aerated and non-aerated liquid and semi liquid animal wastes [Pesaro *et al.*, 1995]. In addition, viral inactivation progressed noticeably faster in liquid waste than in the manure pointing the shielding effect of the manure solids from direct contact with the disinfecting agent(s).

Another research on viral inactivation specifically proved that free ammonia is the virucidal driving force [Cramer *et al.*, 1983]. Separation of the effects of high pH (OH^- concentration) and ammonium ion (NH_4^+) from those of NH_3 on poliovirus Type 1 and bacteriophage f2 inactivation was conducted by preparing solutions with different levels of total ammonia nitrogen. The hydroxide ion had no significant effect on the viruses.

A more radical treatment, involving free ammonia at 1% of the manure wet weight, was conducted on chicken manure samples [Himathongkham and Riemann, 1999]. The manure was air-dried at 20°C to a solid content of about 90% TS, but the levels of target organisms (*Salmonella typhimurium*, *Escherichia coli* O157:H7 and *Listeria monocytogenes*) were only partially reduced. Additional treatment was undertaken by means of adding chemicals to increase pH {KOH} and generate ammonia gas {$(NH_4)_2SO_4$}. After 72 h of such treatment, an 8-log reduction of *Salmonella* and *E.*

coli and 4.5-log reduction of *Listeria* was observed. Clearly, such high inactivation was ammonia-induced, but concentration of the total and resulting free ammonia was higher than the one inherent to the manure.

2.3.5 Disinfection of high-solid residue from co-digestion of the OF-MSW and primary municipal sludge

Despite numerous advantages of high-solid anaerobic co-digestion of OF-MSW and municipal sludge, there are a number of concerns regarding the quality of the digested material. Such digestate is not completely stabilized. It often still exerts significant oxygen demand and is not suitable for use in such areas like gardening or horticulture. In the work done by Oleszkiewicz and Poggi-Varaldo, [1997], the high-solid digestate was found to have an oxygen uptake rate of 240 to 300 g O_2 Kg^{-1} d^{-1}. Post curing such as aerobic composting was suggested to lower this demand to below the Canadian guideline threshold value for cured compost of 150 g O_2 Kg^{-1} d^{-1} [CCME, 1996]. Such additional treatment minimizes possible attraction of vectors (rodents, flies, etc.), potential carriers of disease-causing microorganisms on to humans.

Land application of the material derived from co-digestion of the OF-MSW and wastewater sludges is often unsafe due to the pathogen content of the sludges. This risk is not significantly reduced following mesophilic anaerobic digestion or even post-curing, especially with regard to hardiest forms of pathogens, such as helminth eggs, protozoan cysts or bacterial spores. The survey done on urban sludges, lagoon sediments, and composts in France [Gaspard *et al.*, 1997] revealed parasitic contamination of all samples tested, despite different stages of standard treatment. While in most cases adequate post-

curing of digestate is simply achieved during aerobic composting, the potential for pathogen presence still poses a concern.

2.3.6 Summary

In the above review, the importance of pathogen control in residues of municipal and industrial origin prior to their use in agriculture was stressed out. Current legislation, available and continuously improved technologies, as well as increased public awareness, all make the engineering effort of providing safest possible product of outmost importance. A number of disinfection practices to treat these residues exist and their efficiency varies with anticipated degree of treatment. As the municipal budgets often prevent implementation of a more radical form of sludge disinfection, e.g. using the N-Viro® process or thermophilic stabilization, it is imperative to develop a low-cost technology of improving the hygienic quality of biosolids for the nearest future.

Abiotic factors such as temperature, pH, free ammonia, and storage or contact time were shown to influence pathogen levels in sludge and other residues. The effect of ammonia on pathogen survival was usually assessed as a result of multiple combination of the above factors. A single or double factor application (either high pH, or high pH and free ammonia concentration) has not been described in detail, however. Establishing a low-cost disinfection process designed to treat high-solids anaerobic digestion residues with the use of high pH and high free ammonia concentration seems feasible. Disinfecting potential of the ammonia indigenous to the residues investigated can hold promise.

The City of Winnipeg has been looking for ways to improve its sludge quality. The largest Winnipeg's wastewater treatment facility, the North End Water Pollution Control Centre (NEWPCC), utilizes anaerobic mesophilic digestion of combined primary and secondary biological sludges, and unstabilized mixed sewage sludges imported from two other plants in the city. The digested sludge is mechanically dewatered following polymer addition, and disposed of on agricultural land under the WinGro program. Usually, sufficient degree of sludge stabilization is achieved and the heavy metal content is low enough to meet the U.S. EPA Class A criteria. However, due to lack of disinfection, indicator and pathogenic presence in the dewatered biosolids is common. Soon upon an upgrade of the existing regulations by the provincial government, the sludge will no longer meet the requirements for agricultural land application. It is also expected that, prior to radically changing the regulations, winter land disposal will be banned and that the biosolids may have to be stored for at least 6 months prior to application. This storage time can be effectively utilized, based on the principle of long-term exposure, to maintain certain conditions that promote pathogen inactivation in the sludge.

Definition and optimization of the conditions leading to the improved pathogen inactivation in the Winnipeg biosolids became the practical aspect of the findings presented in the following parts of this document. The emphasis was on the need for a sustainable, low technology solution to the problem. Temperature and storage time was modelled to simulate respectively, weather conditions of the Canadian prairie, and technical storage capability of the City of Winnipeg facilities.

Chapter 3.

RESEARCH OBJECTIVES AND SCOPE OF WORK

The review of literature on ammonia and its effect on anaerobic digestion showed the experience mainly with low-solid or slurry systems. Knowledge of the impact of ammonia on anaerobic digestion and interactions at semi-solid or high-solid level is limited however. Optimization of high-solid systems is very important due to possible higher organic loading rates and resulting savings in reactor volume to treat undiluted substrate. The digestion at high-solid level can intensify problems of ammonia inhibition and/or toxicity. It is easier to properly address such problems in a system with low-solid substrate, however, the methods to mitigate ammonia toxicity may not be directly applicable to high-solid systems. In such case, practical limits to high-solid digestion have yet to be disclosed. Such obstacle can be expected when the substrate is of animal origin (manure), characterized by high nitrogen content.

On the other hand, nitrogen deficiency in high-solid digestion can lead to unbalanced operation. Apart from the requirement for common macronutrients, including nitrogen, the microbial biomass needs to be well balanced. Staged substrate biodegradation leads to the production of intermediate compounds that provide buffering of the system and serve as substrates for the bacteria involved in the next stage of anaerobic breakdown. For example, a potential imbalance by excess acid production is usually counteracted by alkalinity from organic nitrogen breakdown during hydrolysis and acidogenesis, and by utilization of the acetic acid by methanogens. Nitrogen deficiency can be expected in high-solid digestion of the organic fraction of municipal

solid waste (OF-MSW) having high Carbon-to-Nitrogen ratio. Nitrogen supplementation through sewage primary sludge addition is one solution to the problem. Balancing nutrients by means of combining different wastes appears to be quite attractive. Nevertheless, practical limits to this way of altering nutrient deficiency need to be addressed in more detail. Ammonia inhibition could occur in cases where resulting increased organic load to a digester adds to the ammonia balance, due to organic nitrogen breakdown.

Anaerobic digestion has been proven for years as effective means of waste stabilization. The common temperature range for the process (30-40°C) does not warrant disinfection however. Certain properties of anaerobic digestion residues, such as high content of ammonia, can actually help address the problem. Toxicity of ammonia, to be avoided in anaerobic digestion, can be promoted during subsequent disinfection. The need for often costly operations due to energy requirement (thermal inactivation) or high doses of chemicals such as lime (thermal inactivation, alteration of pH resulting in highly basic condition) can be minimized. Toxic effects of ammonia on different indicators of pathogen presence are to be determined. The toxic form of ammonia increases with increasing pH, but chemical doses to increase the pH and convert most of the ammonia to its un-ionized form are expected to be actually smaller than those to reach the extreme pH levels.

In order to define the contribution of free ammonia to pathogen inactivation, one needs to separate the effect of high pH alone from the combined effect of high pH and free ammonia concentration. The extent of UAN contribution is important due to expected higher rate of inactivation in residues with high indigenous ammonia. The

specific share of free ammonia in the inactivation process is not known. It may vary among different microorganisms.

This brings up the issue which microbial indicator of disinfection efficiency would be best fitted to assure that, once inactivated, it will loose its potential to recuperate and make the disinfection product safe to use. Among different indicators, especially when disinfection does not rely on temperature in excess of 55°C, the hardiest are bacterial spores and helminth eggs. Limits for the latter indicator in treated municipal sludge, or biosolids, have been already set [US EPA, 1993] and are often required to demonstrate product compliance with the regulation. A lengthy test for helminth eggs diminishes their value as indicators, however. A simpler procedure for bacterial spores could be used instead, but demonstration of the spore suitability to replace helminth eggs as indicator has not been done yet. It is also unclear so far if, apart from their taxonomic difference, there are enough similarities to use spores instead of eggs as indicators.

3.1 RESEARCH OBJECTIVES

In this research, the following objectives were addressed to contribute to the better understanding of the role of ammonia and other interrelated abiotic factors:

- Determination of the impact of ammonia on microbial activity during high-solid mesophilic anaerobic digestion of (1) chicken manure, and (2) organic fraction of municipal solid waste (OF-MSW) co-digested with municipal primary sludge (PS).
- Assessment of the feasibility of disinfection through effective use of free ammonia in a closed-system operation during (1) long-term storage alone, and

(2) low-dose alkaline inactivation (elevated pH), using such high-solid residues like dewatered municipal sewage sludge and digestate from co-digestion of the OF-MSW and PS.

- Determination of the contribution of free ammonia and high pH in pathogen inactivation.
- Selection of a convenient and reliable microbial indicator of the effectiveness of low-dose alkaline disinfection as a potential alternative to *Ascaris* eggs.

3.2 SCOPE OF WORK

The research comprises several parts with respective tasks as detailed below.

3.2.1 High-solid anaerobic digestion of chicken manure

Investigation of the performance of batch chicken manure digestion at 35-37°C under varying organic load and total solid level, and resulting varying ammonia nitrogen concentration, was approached as follows:

Batch reactors were set up at various solids levels of from 5% to 21.7% total solids (TS), with fresh chicken manure as primary substrate.

This resulted in varying initial organic load (from 30 to 134 g VS/Kg reactor mass) and ammonia nitrogen levels (from 1,500 to 6,600 mg N/L), with significant potential for further ammonia generation from organic nitrogen (N_{org}, 58 to 65% of TKN at start) breakdown.

Under such conditions, the course of manure degradation was evaluated based on methane production and extent of manure degradation, as assessed by volatile solids (VS) and chemical oxygen demand (COD) reductions by the end of incubations.

The effect of two different inocula, anaerobically digested sludge (ADS), and pit manure (PM), on the commencement of chicken manure degradation and extent to which it followed, was also evaluated.

3.2.2 Low and high-solid anaerobic digestion of the organic fraction of MSW and primary sewage sludge

The first stage was designed to start the digesters in a mesophilic mode (35-37°C) at a total solids of 2%, with seed biomass acclimated to digesting organic matter high in cellulose at a load of from 3.8 to 4.2 g VS L^{-1} d^{-1}. After commencement of the reactors, the TS level was gradually increased to about 30%. Initially (day 1 to 20), an increasing organic load was applied as a result of switching from one feed type (1.0 g VS Kg^{-1} d^{-1}, cellulose enriched PS, the usual feed for the seed biomass) to another (7.7 g biodegradable VS Kg^{-1} reactor mass d^{-1}, OF-MSW and PS). After that (day 22 until 80-90), the process of bringing TS up continued, but the load was held constant at 7.7 g biodegradable VS (BVS) Kg^{-1} reactor mass d^{-1}. The objectives approach was as follows:

Batch, intermittently fed reactors (every other day) were set up at 2% TS level. Initially (day 1 to 20), designated digesters were fed a decreasing fraction of the cellulose enriched PS feed (original feed for the seed biomass) and an increasing fraction of the high-solid OF-MSW and PS feed. The high-solid feed was prepared with the following contribution based on dry solids:

85% OF-MSW and 15% PS,

90% OF-MSW and 10% PS,

95% OF-MSW and 5% PS, and

100% OF-MSW and 0% PS.

This switch took 20 days to accomplish and showed the effect of varying organic load (as above) and total ammonia concentration (from 360 up to 830 mg N/L) on biomass acclimation in the solids range from 2% to about 8% TS.

Further on (day 22 to 80-90), the digesters were operated on the high-solid OF-MSW and PS feed with the constant load but increasing solids level, from about 8% to about 26-30% TS. The course of degradation was characterized based on the observed methane production rates, changing ammonia concentration in the digesters, and other performance parameters.

The second stage was designed to observe the effects of four different ammonia levels (due to four levels of PS solids in the high-solid feeds) among reactors operating at a constant organic load on the performance of high-solid anaerobic digestion. This objective was approached as described below:

After the solid level in reactors reached the maximum level of 26 to 30%, the operation was continued at a constant load of at 7.7 g BVS Kg^{-1} reactor mass d^{-1}. By then the ammonia concentration in respective reactors established at four different levels (from 530 to 1750 mg N/L). Its effect on methane production, extent of degradation, and process stability (pH, VFA levels) was determined.

3.2.3 Disinfection of high-solid residues from anaerobic digestion using long-term contained storage, with and without low-dose alkaline additives

3.2.3.1 Dewatered municipal sewage sludge

Several stages of experimental work were dedicated to investigation of inactivation of indicator and pathogenic microorganisms (fecal coliform bacteria, *Salmonella* sp. bacteria, bacterial spores of *C. perfringens*, and eggs of a helminth *Ascaris suum*) using two alkaline compounds (lime and fly ash) and conducting the process at two different temperature ranges (20-22°C, 4-6°C). Different doses of alkaline compounds resulted in different maximum pH levels, and the duration of treatment at these pH levels. When combined with different temperature conditions, the experiments allowed to observe the effect of pH and associated free ammonia concentration. The approach was as follows:

In stage I, six batches involving the sludge treated with various doses of lime (30, 60, 120, 150, 240, and 480 g CaO/Kg TS) were prepared, distributed among small containers, and stored at 20-22°C for six months. One batch involving untreated sludge was also included in the storage. The optimum dose for effective inactivation of each microbial indicator was selected.

In stage II, six batches involving the sludge treated with different doses of fly ash (300, 600, 900, 1,200, 1,500, and 1,800 g ash/Kg TS) were prepared, distributed, and stored at 20-22°C for ten days. The minimum dose for fecal coliform inactivation was selected.

In stage III, lime-treated biosolids (from stage I) were mixed with organic soil at three different ratios and stored open to the air at 20-22°C for about 8 weeks. The potential for regrowth was determined.

In stage IV, the sludge was treated with different doses of lime (20 and 40 g/Kg), fly ash (300, 600, and 900 g/Kg), and of lime and fly ash (20+300, 20+600. 20+900, 40+300, 40+600, and 40+900 g/Kg), distributed, and stored at 20-22°C and at 4-6°C for one month. The effects of temperature and combination of the alkalis on pH patterns were established.

In stage V, twelve batches involving the sludge treated with lime (20, 40, 80, 120, and 160 g/Kg), fly ash (300 and 600 g/Kg), and both lime and fly ash (combination treatment; 10+300, 20+300, 10+600, and 20+600 g/Kg) were prepared, distributed, and stored at 4-6°C for 9 months. One batch involving untreated sludge was also included in the storage. The effects of low temperature and of combination treatment on various indicators were determined.

3.2.3.2 High-solid residue from co-digestion of the OF-MSW and PS

This part of experimental work aimed at demonstrating the suitability of contained storage alone and in combination with low-dose lime treatment to disinfect high-solid material not entirely of sewage sludge origin. The objectives approach was as follows:

Four batches were prepared and involved the residues coming from the high-solid reactors digesting OF-MSW and PS at four different levels (see above), resulting in various ammonia concentrations.

Initially, the residues were stored without lime at 20-22°C for 17 d. After that, and in lieu of anticipated lack of influence on the spores of *C. perfringens*, lime was applied at 100 g/Kg TS and the residues stored at same temperature for another 9 d. The impact of the treatments, resulting different levels of total (and free) ammonia in the digestate, on the extent of pathogen inactivation was determined.

3.2.4 Synergism of high pH and high free ammonia concentration in inactivation of *C. perfringens* spores and *Ascaris suum* eggs

To study the effects of high pH and free ammonia and high pH alone, respectively, well-defined, solid-free, artificial media were prepared. The approach was as follows:

Two types of media were prepared, one with an ammonia nitrogen concentration common to digested sludge (~1,300 mg N/L), and the other ammonia-free.

Reductions in densities of *C. perfringens* spores and of *Ascaris* eggs were observed under different pH regimes (from 7.0 to 12.0) and incubation times (from 2 h to 48 h).

While one is aware of the complex characteristics of the sewage sludge influencing pathogen inactivation, such a shortcut (the use of well-defined artificial media) is the only feasible way of distinguishing between the effect of high pH alone and that of high pH in combination with high free ammonia concentration.

Chapter 4.

4 MATERIALS AND METHODS

The following section describes the experimental conditions and equipment used to address all aspects of work as described in the preceding chapter.

4.1 EXPERIMENTAL SETUP AND APPARATUS

4.1.1 Anaerobic digestion of high-solid organic residues

4.1.1.1 Chicken manure

This experiment was conducted in a batch mode to observe the kinetics of methane formation under different conditions of organic load and total solid content of each digester.

Apparatus

The laboratory apparatus consisted of 160 mL serum bottles (Wheaton) with the initial atmosphere of 50% carbon dioxide and 50% nitrogen gas. The bottles were sealed with rubber stoppers (Bellco) and crimp aluminum caps (Wheaton). Gas production was assessed indirectly using a calibrated pressure transducer connected to a multimeter (Micronta). Gas was vented to maintain equilibrium with the atmospheric pressure and the headspace samples were removed for qualitative analysis using a 1 mL gas tight syringe (Hamilton) equipped with a 22 gauge needle. Initial feeding, and sampling from the reactors at end of incubations was done using an anaerobic glove box. A transfer box

with a vacuum/purge cycle was used to move the materials into the glove box and to ensure an oxygen free environment. The headspace gas in the glove box was 20% carbon dioxide balanced with nitrogen. The serum bottles were incubated statically in a walk-in chamber at 35-37°C. A view of the experimental set up is shown in Fig. 4-1.

Experimental design

A batch, screening assay employed in this part of experimental work permits evaluation of a wide range of variables and thus, allows for sorting out of promising configurations. The assay has been designed in part using the principles of Biochemical Methane Potential (BMP) bioassay, as described by Owen *et al.* [1979]. It involved incubation of the fresh manure (FM) at total solids ranging from 5.0% to 21.7% with and without an inoculum of anaerobic biomass. The anaerobically digested sludge (ADS) served as one source of anaerobic biomass. It was decided to evaluate the use of the pit manure (PM) as inoculum, apart from the ADS. The solids content of this material was considerably higher (11.7% TS) than that of ADS (2.7% TS), potentially making it more suitable for inoculation of a high-solid digestion process.

The incubations were carried out for 17 weeks, a period much longer than 30 days being normally considered sufficient for assessing methane generation potential in slurry digestion. The aim was to provide enough time to determine the ultimate yields of biological conversion of the high-solid organic matter by the anaerobic microorganism consortia present in each reactor.

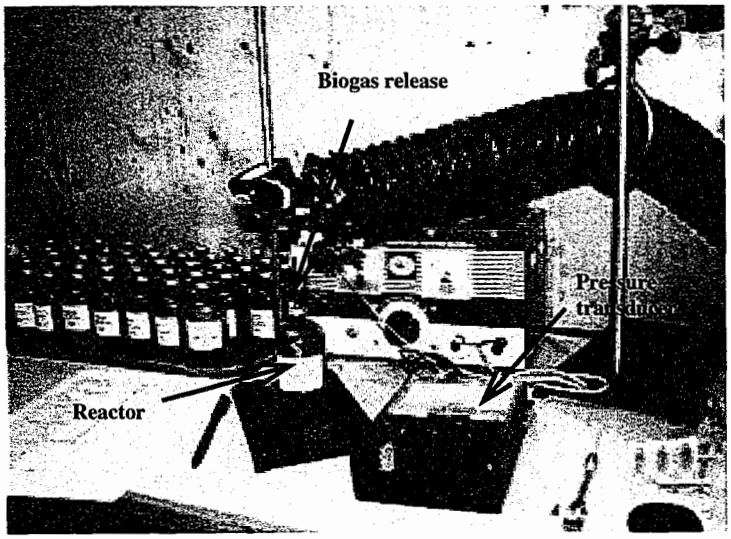

Figure 4-1. View of anaerobic digesters (serum bottles) degrading chicken manure with, or without, inoculum of anaerobic biomass
The reactors were incubated statically in a walk-in chamber at 35-37°C.

Eleven experiments were set, including 9 for the different combinations of substrate and inocula and 2 for the inocula alone. Out of the 9 experiments involving chicken manure, three involved digestion of the FM alone, next three for the FM and PM mixtures, and another three for the FM and ADS mixtures. The exact starting condition for these experiments, in terms of amounts of the fresh manure and inoculum, is reported in Table 4-1. Water for manure dilutions was deoxygenated prior to use applying several vacuum-purge systems using gas headspace replacement system, a manifold. The headspace gas was changed to 80% N_2 balanced with CO_2.

The reason for setting additional experiments with the inocula (PM and ADS) was to assess their contribution to the overall amount of methane generated in respective reactors involving these inocula. These two experiments are further referred to as *control* experiments.

Table 4-1. Experimental mixtures used in high-solid chicken digestion assay. There are three different groups of experiments distinguished which involve: fresh manure at different dilutions, fresh manure with pit manure at different ratios and dilution, and fresh manure with anaerobically digested sludge at different ratios and dilution.

FM alone	FM and PM mixtures	FM and ADS mixtures
100%* FM undil., 21.7% TS	90%* FM + 10%* PM	90%* FM + 10%* ADS
100%* FM dil. to 10% TS	40%* FM + 60%* PM	40%* FM + 60%* ADS
100%* FM dil. to 5% TS	90%* FM (dil. 1:2) + 10%* PM	90%* FM(dil. 1:2)+ 10%* ADS

Note- FM: fresh manure, PM: pit manure, ADS: anaerobically digested municipal sludge, % TS: percent total solids, dil: diluted, undil: undiluted, 1:2: one part fresh manure plus one part water, *: fraction of 50 g of the digester content (wet weight basis)

Each experiment consisted of eight replicate reactors to account for possible variability in digestion rates and methane generation. Prior to biogas release the serum

bottles were shaken for 10 s, in order to reach the equilibrium between gases present in both, the gas and the liquid phase. The effect of mixing on the overall performance of the system was observed by shaking the bottles manually for 1 minute daily, from the fortieth day on.

Start up of the reactors

All the feedstocks were prepared and placed into an anaerobic glove box (20% CO_2/ 80% N_2 atmosphere) together with 88 serum bottles (reactors). Each reactor was fed with 50 g of the feedstock and 1 mL of resazurin solution (0.1 g/L; reduction-oxidation indicator), and sealed. They were then removed from the glove box and the headspace of each bottle was changed to a 50% CO_2/ 50% N_2 atmosphere using a manifold connected to a tap water vacuum and a gas cylinder. The reason for using this particular gas mixture is that carbon dioxide acts as a buffer thereby reducing the impact of the expected elevated pH. The bottles were then placed in a walk-in chamber set up at 35-37°C. It took around 2 h for the content of the bottles to reach the incubation temperature. After that time, the headspace of each bottle was equilibrated with the atmospheric pressure.

4.1.1.2 Organic fraction of MSW and sewage primary sludge

This experiment was conducted in a semi-continuous mode to observe methane formation under the conditions of initially varying, and then similar, organic load and total solid content of each digester but different levels of nitrogen.

Apparatus

Each digester system consisted of a two-liter wide-mouth glass reactor sealed with rubber stopper and connected via tubing to a four-liter biogas collection bottle acting

as a additional headspace. The stopper was equipped with a Ø25-mm waste/feed pipe for sampling and feeding. Biogas production was measured directly using a liquid displacement system that was hooked up to the digester at time of biogas release. In addition, biogas production was also estimated indirectly by measuring the weight loss of each digester system upon biogas release from the system. Each time after release of biogas and equilibration of the headspace pressure with atmospheric pressure, a sample of the headspace was taken for analysis. Reactor contents were mixed before wasting and after feeding using a mechanical stirrer equipped with extendable blades, inserted through the waste/feed pipe. Sampling and feeding operations were performed under continuous purge of the reactor headspace with 80% N_2 balanced with CO_2. Following that, digester system headspace was deoxygenated by means of repeated vacuum/gas replacement system. Incubations (statically) of all reactors, as well as sampling and feeding, were done in a walk-in chamber at 35-37°C. A typical schematic of the set up is shown in Fig. 4-2.

Experimental design

Anaerobic digestion in a batch mode was chosen as it simplifies reactor design and eliminates the need for costly mechanical equipment. All high-solids digesters were started in a slurry mode with the aim to gradually bring the solids to a 30%-TS level. The seed biomass for the digesters was developed ahead of time in a slurry reactor (TS 3.0%) operating at an organic loading rate of 3.8-4.2 g VS L^{-1} d^{-1} and at 37°C. This "breeder" reactor, originally started using a mixture of anaerobically digested mesophilic sludge and dairy cow rumen fluid, was acclimated to, and maintained on, a cellulose-supplemented primary sludge feed. Inclusion of the fluid was designed to guarantee an

adequate supply of cellulose biodegrading bacteria to enhance anaerobic digestion of the cellulose component of the OF-MSW. During the first 20 days of reactors' operation the feed composition was changed by decreasing the primary sludge fraction in 10% increments and replacing it with the final formulation of the OF-MSW feed mixture, specific to the operation of each individual reactor. Organic load was determined based on the amount of biodegradable volatile solids (BVS) fed to reactors. The BVS were estimated by measurement of the acid insoluble lignin content of the OF-MSW components and subtracting it from the total volatile solid mass to exclude the unbiodegradable lignin. After the initial stage of switching from primary sludge to the high-solid OF-MSW feed, the BVS load to each digester was kept constant at 7.7 g BVS Kg^{-1} reactor mass d^{-1}.

The target TS level was designed to reach 30% in all reactors. Due to the expected significant removal of mass in the form of wet biogas the use of mass retention time (MRT) rather than volumetric input rate has been employed, and in this study reported based on the influent mass flow rate (MRT_{in}). The hydraulic or mass retention time was designed to be 30 days. Due to the small reactor size, the digesters were fed every other day.

The high-solid reactors with no primary sludge in the feed were treated as controls for methane production from biodegradation of the OF-MSW alone. Another control reactor operated in a slurry mode and fed only primary sludge, was included at each operating temperature to account for the portion of total methane gas that was produced from the sludge alone.

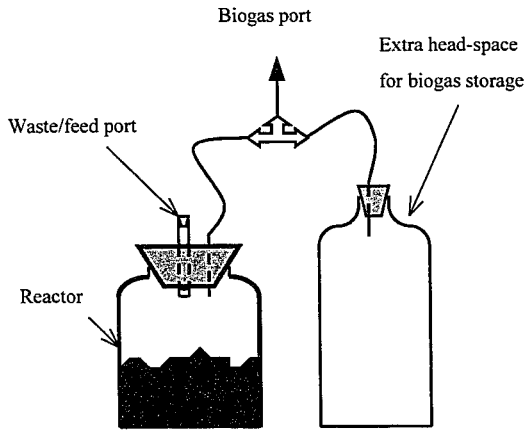

Figure 4-2. Typical schematic of the anaerobic digester system degrading the OF-MSW supplemented with primary sludge
OF-MSW: organic fraction of municipal solid waste

4.1.2 Disinfection of high-solid organic residues

There were five different stages of experimental work focused on disinfection of digested-dewatered sludge.

Confirmation of some of the findings from these stages was then conducted in the following part using another high-solid residue, the digestate from anaerobic co-digestion of OF-MSW and primary municipal sludge.

The last part of the work focused on the effects of high pH alone and in combination with free ammonia on pathogen inactivation in well-defined diluted artificial media.

4.1.2.1 Disinfection of digested-dewatered sewage sludge using closed-system, low-dose alkaline treatment at 20-22°C

All disinfection experiments were conducted in a batch mode to observe the effect of storage alone and in combination with alkaline treatment on pathogen reductions. Mixing of high-solid residues with alkaline additives was done using a Hobart mixer. A view of the Hobart mixer is shown in Fig. 4-3. The mixtures were ready for contained storage after 10 minutes of thorough mixing.

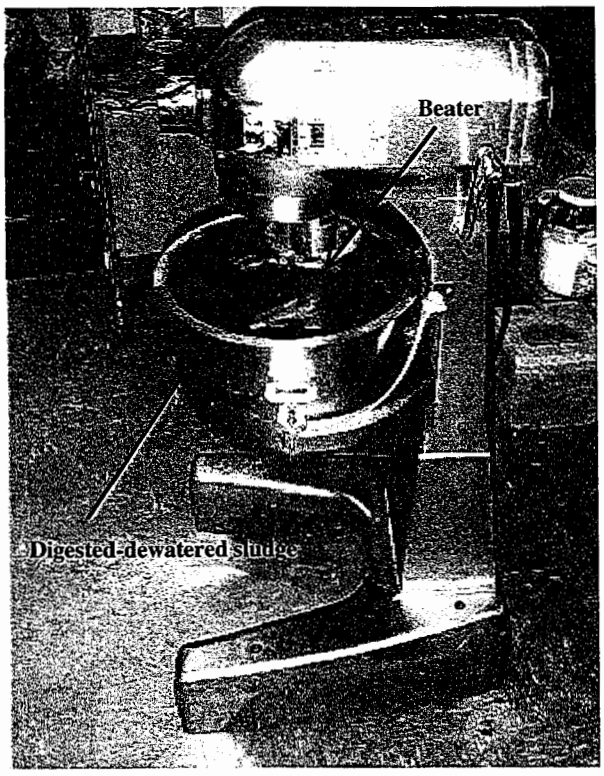

Figure 4-3. View of Hobart mixer used to mix sludge with alkaline agents and in pathogen spiking experiments

4.1.2.1.1 Disinfection using quick lime (stage I)

Lime was added to the digested-dewatered sludge (at 31.6% TS, VS=37.5% TS) at the doses of 30, 60, 120, 150, 240, and 480 g CaO/Kg TS (dry weight), followed by contained storage of the treated sludge at room temperature (20-22^{o}C). Sludge not treated with lime was also included in the storage. Fifteen plastic containers (per alkali dose) of 0.25 L capacity were fully packed with treated sludge and capped tightly for storage. The total storage time was 6 months. On each sampling occasion, one container was taken and its contents were used for analyses. A larger, 3.0 L container was also packed with the same sludge and served as a reference. The pH was measured in both small and large container for the purpose of verification of the same storage conditions. Sludge samples were monitored for pH, total solids, volatile solids, ammonia nitrogen, and density of fecal coliform bacteria, *Salmonella* sp. bacteria and *C. perfringens* spores.

4.1.2.1.2 Disinfection using fly ash (stage II)

Fly ash doses used were 300, 600, 900, 1200, 1500, and 1800 g/Kg TS (dry weight). Preparation and packing procedures were as in case with lime addition. Following contained storage at room temperature, treated samples were monitored for pH, total solids, volatile solids, and density of fecal coliform bacteria.

4.1.2.1.3 Pathogen regrowth experiment using lime-treated biosolids, and organic soil (stage III)

Following the 6-month anoxic storage, the biosolids previously treated with different doses of lime were used in topsoil formulations. The biosolids were mixed with soil high in organic content. The ratios used in topsoil formulations were 1 part biosolids to 1, 3, and 7 parts soil, by weight. The total weight of each mixture was 1 Kg. After preparation of all mixtures, they were placed in 3.0 L plastic containers with covers loosely placed on top. These containers were then stored at room temperature (20-22°C) for 57 days, and mixed occasionally (3 times/week). All biosolids-soil mixtures were monitored for pH, total solids, volatile solids, ammonia nitrogen, fecal coliform bacteria, *Salmonella* sp. bacteria and *C. perfringens* spores with the same analytical tests in place as in the lime and fly ash experiments.

4.1.2.1.4 Effect of various combinations of lime and fly ash (combination treatment) on pH patterns in treated sludge stored at 20-22°C and at 4-6°C (stage IV)

Lime doses were 20 and 40 g/Kg, fly ash doses were 300, 600, and 900 g/Kg, and lime and fly ash doses were 20+300, 20+600, 20+900, 40+300, 40+600, and 40+900 g/Kg. Three replicates (plastic containers of 0.15 L capacity) were used for each dose and temperature. The treated sludge was split into two batches and distributed among containers. Then, batch I was stored at 20-22°C and batch II at 4-6°C. Untreated sludge controls were also included at each temperature level. All containers were monitored for pH.

4.1.2.2 Disinfection of digested-dewatered sewage sludge using closed-system, low-dose alkaline treatment at 4-6°C

This part of experimental work was designated as stage V.

4.1.2.2.1 Disinfection using quick lime

Lime was added to the digested-dewatered sludge (avg. TS 28.3%) at the doses of 20, 40, 80, 120, and 160 g CaO/Kg TS (dry weight), followed by contained storage of the treated sludge at low temperature (4-6^{o}C). Sludge not treated with lime was also included in the storage. Twelve packets (per each dose) of treated sludge, previously spiked with *Salmonella* sp. bacteria and with eggs of *Ascaris suum*, were prepared. The packets were made out of a net commonly used for weed control in gardening. They were strong enough to contain the spiked material yet allowing free transfer of moisture in and out of them. Once filled with the sludge, the pockets were placed in a 16 L plastic bucket with cover, and packed around with sludge treated with same lime dose but non-spiked. The buckets were filled up to the top with treated sludge and tightly covered for storage. The total storage time was designed to be as long as expected inactivation would take place. The first three sampling occasions are covered in this document. Subsequent sampling has been on going and will have included a further storage but at room temperature.

On each sampling occasion, one packet was retrieved from the bucket and its contents were used for analyses. A 3.0 L container was also packed with the same sludge and served to replace the volume of each wasted packet. Sludge samples were monitored for pH, total solids, volatile solids, ammonia nitrogen, and densities of fecal coliform bacteria, *Salmonella* sp. bacteria, *C. perfringens* spores, and *Ascaris suum* eggs.

4.1.2.2.2 Disinfection using fly ash

Fly ash doses used were 300 and 600 g/Kg TS (dry weight). The average TS of the sludge were 28.6%. Preparation and packing procedures, as well as sampling schedules and analyses, were as in case with lime addition.

4.1.2.2.3 Disinfection using lime and fly ash

Lime and fly ash doses of 10+300, 20+300, 10+600, and 20+600 g alkali/Kg TS, respectively, were used. The average TS of the sludge were 27.9%. Preparation and packing procedures, as well as sampling schedules and analyses, were as in case with lime addition.

4.1.2.3 Disinfection of the digestate from anaerobic co-digestion of the organic fraction of municipal solid waste and primary municipal sewage sludge

This experiment was conducted in a batch mode to observe the effect of storage alone and in combination with alkaline treatment on pathogen reductions.

After the high-solid co-digestion experimental work was over, all mesophilic reactors stayed in the walk-in chamber at 37°C for the period of 24 months. Following this, the content of each mesophilic digester was analyzed for the levels of fecal coliform and *Salmonella* sp. bacteria, and bacterial spores of *C. perfringens*. The next step was then undertaken to obtain more digestate for this stage of the work. Four mixtures were prepared each by combining the digestate from reactors that were fed a feed with

different surface area of the paper components but same primary sludge (PS) content, i.e. 15%, 10%, 5%, and 0% (by weight). Prior to starting the high-solid digestion operation, it was found that the specific weight of each feed having same content of PS solids was similar regardless of the surface area of the paper. Therefore, it had no significant influence on the methane production in digesters receiving the same amount of primary sludge in the feed.

The mixtures, designated PS-15, PS-10, PS-5, and PS-0, were then supplemented with mesophilic digested-dewatered municipal sludge at a w/w (weight/weight) ratio of 3 to 1. The sludge, with naturally occurring fecal coliforms and spores of *C. perfringens*, was previously spiked with *Salmonella* sp. to the level similar to that of indigenous fecal coliforms to observe a meaningful, multi-log decline. Since during the 2-year storage the content of each reactor was continuously undergoing further biodegradation and inadvertent drying, the resulting mixtures had different moisture levels in the range from 21.5 to 25.9% TS. Therefore, upon dewatered sludge addition, the moisture was finally adjusted to a 22% TS level. The mixtures had four different levels of ammonia nitrogen, which allowed determining the influence of free ammonia concentration on microbial reductions. Each mixture was then split into 0.15 L capacity plastic containers with closures, and stored for 17 days at room temperature (20-22°C), during which time pH, total solids, volatile solids, ammonia nitrogen, and densities of fecal coliforms and *Salmonella* sp., and *C. perfringens* spores were determined.

At the end of this period, each mixture was treated with lime (calcium oxide) at the dose of 100 g CaO/Kg TS. This dose is high enough to bring the pH of treated material at least up to 12.0. . Mixing of the digester output with lime was done for 10

min, using the same mixer as in sludge disinfection experiments. Same containers as above were used to store treated material. The total storage time was 9 days at 20-22°C. The samples were monitored for same parameters as above.

4.1.2.4 Effect of high pH alone and in combination with free ammonia on pathogen inactivation in diluted media

Artificial medium with ammonia and without ammonia was prepared to study the effects of high pH and free ammonia and high pH alone, respectively, on reductions in densities of *C. perfringens* spores and of *Ascaris* eggs. This task was virtually impossible to accomplish using the sludge, mainly due to the lack of means to remove ammonia from the matrix without changing the properties of the sludge. Therefore, observation of the effect of high pH alone would not be possible to achieve.

In experiments to determine the effect of high pH and NH_3 concentration, the medium was prepared with ammonia content of 1,300 mg N/L, the level common to digested municipal sludge. In experiments to determine the effect of pH alone, the medium was without ammonia, but at equal salt concentration.

4.1.2.4.1 Experiment I

A two-day track study was performed. Both types of media were placed in 150 mL capacity Erlenmeyer flasks, and spiked with spores of *C. perfringens*. The initial spore density and ammonia nitrogen were determined, and pH adjusted to 12.0. Five experimental flasks were prepared for each medium. The level of liquid in all flasks

following pH adjustment was reaching the top of the neck. The flasks were covered with Para film and left at room temperature (20-22°C) until consecutive samplings began. The sampling for pH, ammonia, and spore density was done at the following intervals: 2 h, 6 h, 24 h, and 48 h. Control media spiked with spores and at neutral pH (~7.0) were also included in the test. At end of the incubations, the contents of each flask were analyzed for pH, ammonia, and *C. perfringens* spore density. The initial ammonia concentration and spore content were adjusted to reflect the final volume of each flask following pH adjustment.

4.1.2.4.2 Experiment II

In this experiment, the procedure used in experiment I was repeated, but the media were also spiked with *Ascaris suum* eggs. The collected data verified the spore inactivation observations obtained in experiment I.

4.1.2.4.3 Experiment III

A one-day study was performed using both types of media (with ammonia, and ammonia-free) and both indicator organisms: bacterial spores of *C. perfringens* and eggs of a helminth *Ascaris suum*. At start, the pH of each medium was adjusted to four different levels: neutral (7.0), 9.5, 10.0, and 12.0. The flasks used were as in the previous two experiments. The incubations lasted 24 h, after which final pH and ammonia nitrogen levels, and densities of indicator organisms were determined.

The pH levels were designed to reflect different levels of free ammonia in the ammonia-containing medium. The following free ammonia fractions (as % of total ammonia nitrogen, TAN~1,300 mg N/L) were expected at corresponding pH levels: ~0% at pH 7.0, 57% at pH 9.5, 80% at pH 10.0, and ~100% at pH 12.0.

4.2 ANALYTICAL PROCEDURES AND FREQUENCY OF SAMPLING

4.2.1 Anaerobic digestion of high-solid organic residues

4.2.1.1 Chicken manure

The methane and carbon dioxide contents in the headspace gas were measured on a GowMac gas chromatograph model 550, with a column temperature at 55^{o}C and thermal conductivity detector at 110^{o}C and helium as carrier gas. The column was packed with Porapak Q, 80/100 mesh. The biogas was also occasionally checked for the presence of O_2. Volatile fatty acids (VFA) were tested using a Antek gas chromatograph model 3000, with flame ionization detector at 200^{o}C and column set at 100^{o}C (initial temperature). Nitrogen served as carrier gas. A KjelTec Auto 1030 Analyzer was used to determine total Kjeldahl nitrogen (TKN); representing the organic and ammonia nitrogen components of the material, and ammonia nitrogen (AN). Uric acid was determined according to Marquardt [1983]. All other analyses were performed according to *Standard Methods* (*SM*) [APHA *et al.*, 1998]. The pH was measured using a Fisher Scientific Acumet pH meter model 50. Semi solid samples for VFA and soluble chemical oxygen demand (S-COD) analyses were prepared as follows: a known amount of sample was

diluted with de-ionized water to a desired level and vigorously shaken for 10 s or until a homogeneous liquid was obtained. The resulting slurry was then centrifuged for 10 minutes in a Damon IEC-HN-S centrifuge at 6000 rpm followed by filtration through a 0.45 μm nylon MicronSep membrane filter.

Gas volumes and composition were measured and gas vented according to the observed microbial activity. Sampling for pH, total solids (TS), volatile solids (VS), chemical oxygen demand (COD), S-COD, VFA, TKN, AN and uric acid determinations was done at the start and at the end of the assay.

4.2.1.2 Organic fraction of MSW and sewage primary sludge

The methane, carbon dioxide, nitrogen and oxygen contents in the headspace gas were measured on a GowMac gas chromatograph model 550, with a column temperature at 55°C and thermal conductivity detector at 110°C and helium as carrier gas. The column was packed with Porapak Q, 80/100 mesh. Volatile fatty acids (VFA) were tested using an Antek gas chromatograph model 3000, with flame ionization detector at 200°C and column set at 100°C (initial temperature). Nitrogen served as carrier gas. Acid insoluble lignin (AIL) content was measured using the method of Goering and Van Soest [1970].

All other analyses were performed according to *Standard Methods* (*SM*) [APHA *et al.*, 1998]. Total and volatile solids were measured using Method 2540 G of the *SM*. Total ammonia nitrogen (TAN) was measured using a KjelTec Auto 1030 Analyzer, and later replaced with the automated phenate method of the *SM* (Method 4500-NH_3 G). The pH was measured using a Fisher Scientific Acumet pH meter model 50, and the

principles of the Method 4500-H^+ B of the *SM*. During start-up and initial reactor operation the pH in slurry was measured using standard electrode, whereas during high-solid operation a dual probe system was used, with a high flow rate calomel electrode as the reference electrode and a universal glass pH electrode as the indicating electrode. The pH was taken directly in high-solid digestate and confirmed in slurred digestate. The slurry was prepared by weighing a 10-g sample of high-solid digestate and mixing it with 20 mL of 0.01 M $CaCl_2$ solution, followed by occasional slow mixing of the slurry and taking pH measurement after thirty minutes. Specific weight of feed mixtures was determined using uncompacted feed and 0.4 L glass container.

High-solid digestate samples for ammonia, VFA and soluble chemical oxygen demand (S-COD) analyses were prepared by weighing sample, diluting it with de-ionized water to a desired level and shaking until a homogeneous liquid was obtained. The resulting slurry was then settled and the supernatant filtered through a qualitative No. 1 filter (Whatman) followed by further filtration through a 0.45 µm nylon membrane filter.

Gas volumes and composition were measured and gas vented every day or every other day. Regular sampling for pH, alkalinity, total solids (TS), volatile solids (VS), total ammonia nitrogen (TAN), chemical oxygen demand (COD), S-COD, and VFA determinations was done every 7 to 10 days. In addition, a direct pH measurement in digestate was taken when there was an indication of reduced methanogenic activity, as determined from deteriorating biogas production and from increasing VFA concentration. Sodium bicarbonate, $NaHCO_3$, was used to supply alkalinity.

4.2.2 Disinfection of high-solid organic residues

4.2.2.1 Disinfection of dewatered sludge using closed-system, low-dose alkaline treatment at 20-22°C and at 4-6°C (stages I-V)

Most analytical determinations were performed according to the procedures in Standard Methods (*SM*) [APHA *et al.*, 1998]. Total and volatile solids were measured using Method 2540 G of the *SM*. Ammonia nitrogen was measured using the modified ammonia-selective electrode method and later replaced with the automated phenate method of the *SM* (Method 4500-NH_3 D and Method 4500-NH_3 G, respectively). The pH was measured using the principles of the Method 4500-H^+ B of the *SM*. A dual probe system with a high flow rate calomel electrode as the reference electrode and a universal glass pH electrode as the indicating electrode was used for this purpose. The pH was taken directly in sludge and confirmed by preparing slurry from the sludge. The slurry was prepared by weighing a 10-g sample of sludge and mixing it with 20 mL of 0.01 M $CaCl_2$ solution, followed by occasional slow mixing of the slurry and taking pH measurement after thirty minutes.

In the disinfection studies at 20-22°C (stage I and II), the frequency of sampling varied and decreased as the storage time progressed. In the lime addition experiments (stage I) during the first day, the sampling for microbiological tests (fecal coliforms, *Salmonella* sp. bacteria, *C. perfringens* spores) and pH was done at start, after 15 min, 30 min, 1 h, 2 h, 4 h, and after 24 h. Samples for total and volatile solids, and for ammonia nitrogen, were taken at start and after 24 hours only. On each following occasion, samples for determinations of all parameters were taken. In the fly ash addition

experiments (stage II), the sampling was done only for pH and for fecal coliform density determinations, and was performed at start, after 1 d, 2 d, 4 d, 7d, and 10 d from start.

In the pathogen regrowth studies (stage III), the sampling was performed at start, after 2 d, 5 d, 14 d, and after 57 d. All biosolids-soil mixtures were monitored using the same analytical tests in place as in the lime disinfection experiments.

In the lime-fly ash combination treatment studies (stage IV), the only parameter monitored was the pH. The pH was measured directly in treated sludge. Temperature compensation was applied to the measurements taken at 4-6°C. The frequency of monitoring was as follows: at start, after 1d, 8 d, and after 31 d.

In disinfection studies at 4-6°C (stage V), the frequency of sampling was: at start, after 1 day, and after about 9 months from start; and involved TS, TVS, pH, ammonia nitrogen, and microbiological tests (fecal coliforms, *Salmonella* sp. bacteria, *C. perfringens* spores, *Ascaris suum* eggs) for all alkali doses used. The extent of permanent *C. perfringens* spore injury due to high alkali/high pH condition after 9 months of storage was assessed by means of lysozyme inclusion in a standard agar used for spore enumeration (10 mg lysozyme/L agar). The lysozyme is an enzyme that can be used in lieu of naturally occurring spore-lytic enzymes in the spore coat, responsible for spore germination. These enzymes are removed from the coat following alkali treatment. Depending on the extent of injury due to disinfection, a fraction of the lytic enzyme-deficient spores may retain its ability to germinate once supplemented with the lysozyme.

4.2.2.2 Disinfection of the digestate from anaerobic co-digestion of the organic fraction of municipal solid waste (OF-MSW) and primary municipal sewage sludge (PS)

Most analytical determinations were performed as in the studies with digested-dewatered sludge [APHA *et al.*, 1998]. Ammonia nitrogen was measured using the automated phenate method of the *SM* (Method 4500-NH_3 G). The extent of permanent *C. perfringens* spore injury due to high alkali/high pH condition was assessed by means of external lysozyme source, as described in the previous section.

During initial 17-d long contained storage of the high-solid residues (without lime added), sampling was done at start, after 7 d, and at the end. Following that, lime was added to the residues, and the storage continued for another 9 d. The sampling during this period was done on day 1, and on day 9 of lime treatment. On each sampling occasion, the parameters monitored included TS, TVS, ammonia nitrogen, and microbial indicators (fecal coliforms, *Salmonella* sp., spores of *C. perfringens*).

4.2.2.3 Effect of high pH alone and in combination with free ammonia on pathogen kill in diluted media

The diluted medium was buffered dilution water, but without magnesium salt ($MgCl_2$) to avoid precipitation of ammonia as magnesium-ammonium-phosphate, or struvite ($MgNH_3PO_4$ * 6 H_2O), at pH above 7. The medium was prepared with ammonium chloride (NH_4Cl) for use in studies to determine the effect of pH and NH_3, and with sodium chloride (NaCl) for use in studies to determine the effect of pH alone.

The chloride concentration was adjusted to yield equal final concentrations in both media.

In experiment I, spores of *C. perfringens* were the only indicators studied. Spore density was measured at the beginning and after 24 h from start in all media.

In experiments II and III, spores of *C. perfringens* and eggs of *Ascaris suum* were used as indicators. Spore and egg densities were measured at the beginning and after 24 h from start in experiment II, and at start, after 2 h, 6h, 24h, and after 48 h from start in experiment III.

Ammonia nitrogen was measured using the modified ammonia-selective electrode method of the *SM* (Method 4500-NH_3 D). The pH was measured using the principles of the Method 4500-H^+ B of the *SM*. A combination electrode (model) was used for this purpose. The pH was taken directly in liquid media. Ammonia nitrogen and pH were measured each time samples were taken for microbiological tests.

4.2.3 Microbial indicator and pathogenic organisms used in disinfection studies

The microbial indicator and pathogenic microorganisms used in this research were: fecal coliform bacteria, *Salmonella* sp. bacteria, bacterial spores of *C. perfringens*, and eggs of parasitic helminth *Ascaris suum.*

Sample preparation for fecal coliform and *Salmonella* tests was done according to the U.S. EPA [1992]. This preparation was also used for *C. perfringens* analysis. Neutralization step was required in case of alkali treated high-solid residues. During initial stage of sample preparation [U.S. EPA, 1992] the pH of the lowest dilution (10^{-1}) was gradually adjusted with sulfuric acid (6*N* and 1*N* H_2SO_4) to about 7.0 and the volume

was adjusted to obtain the desired dilution. The neutralization step would usually take up to 10 minutes to complete.

Sample preparation for *Ascaris suum* egg recovery from treated biosolids and quantification was done by weighing the appropriate quantity of sludge, diluting the sample, and neutralizing with sulfuric acid immediately thereafter.

A quality assurance program was used throughout the study. Quality control consisted of monitoring incubation temperatures. The sterility of the media and other materials used was always verified.

In all bacteriological tests involving direct plating methods, at least ten distinct colonies (10 Colony Forming Units) had to be found on each plate to meaningfully quantify the bacteria of interest. Counts lower than 10 CFU and at lowest dilution used were utilized to confirm the presence or absence of an organism. In this case, just one colony at a given dilution would determine the detection limit of the test.

The five-tube and three-tube most probable number (MPN) test for enumeration of different bacteria was also used. In the lime addition experiments at 20-22°C, a five-tube MPN test was used in determinations made within first 24 hours following addition, and was then replaced by the three-tube MPN test. The three-tube MPN test was utilized throughout all other experiments that followed.

Microbiological methods used for respective microorganisms and some modifications to the methods are listed below.

4.2.3.1 Fecal coliform bacteria

Fecal coliforms in sludge were analyzed in accordance with the *SM* fecal coliform MPN Procedure (Method 9221 E) and fecal coliform Membrane Filter Procedure (Method 9222 D). The latter procedure was modified and filters were excluded from the procedure. Direct plating from prepared dilutions yielded satisfactory performance.

The Standard Operating Procedure can be found in Appendix E.

4.2.3.2 *Salmonella* sp. bacteria

Salmonellae were analyzed in accordance with the method of Kenner and Clark [1974] with one exception: heat killed *Salmonella paratyphi* A was not added to the Dulcitol Selenite Broth in the initial stage of isolation. Also, the optional test with Lysine Iron Agar slants was omitted in the procedure.

The Standard Operating Procedure can be found in Appendix E.

4.2.3.3 Bacterial spores of *C. perfringens*

C. perfringens spores were determined using Tryptose Sulphite Cycloserine (TSC) agar [Harmon *et al.*, 1971]. Confirmation of *C. perfringens* spores was performed using buffered motility nitrate medium [Harmon and Kautter, 1978] and lactose gelatin medium [Hauschild and Hilsheimer, 1974]. A portion of sporulating culture in sludge dilution, after heating at 75°C for 20 minutes to kill vegetative cells and activate heat-resistant spores, was used to prepare higher dilutions. These dilutions were used in the TSC agar plating procedure. The plates were allowed to solidify before placing into anaerobic jars for incubation at 37°C.

The Standard Operating Procedure can be found in Appendix E.

4.2.3.4 Eggs of parasitic helminth *Ascaris suum*

The *Ascaris suum* eggs were obtained from Excelsior Sentinel, Inc., Ithaca, U.S.A. Originally, the eggs were collected from the feces of naturally infected pigs to assure their maturity. This method of acquiring eggs is preferred over the other one where eggs are retrieved from the lower part of the uteri of an adult worm, in which case the eggs may not be fully developed and more vulnerable to environmental stress.

The stock egg suspension was preserved in 0.5% formalin and at a density of 500,000 to 600,000 eggs per vial (3-4 mL). Prior to using them for spiking purposes, the stock suspension was diluted to the desired level. Spiking of sludge was performed using this working suspension and administered using 5-mL capacity automatic pipette. The mixing of sludge during spiking was done in a Hobart mixer. It took around 10 min to complete the spiking.

The method used to enumerate eggs in treated sludge was a modification of the original method of Little [1999], Tulane University School of Public Health and Tropical Medicine, New Orleans, LA, U.S.A. The modifications included discarding all supernatant from settling eggs over a nylon sieve (the nylon sieve with smaller diameter to fit in a 400 mL beaker was used instead of a metal, standard sieve for soil particle analysis), and repeated floatation to possibly recover more eggs from the sludge.

The Standard Operating Procedure can be found in Appendix E.

4.3 CALCULATIONS

Calculations of the free (un-ionized, gaseous) forms of ammonia and VFA are presented below. All other calculations have been described in detail in Appendix A.

Ammonium ion (NH_4^+) and free ammonia (NH_3) are the two principal forms of ammonia nitrogen in aqueous solution. The free or un-ionized ammonia nitrogen (UAN) was calculated based on the total ammonia nitrogen (TAN) concentration and experimental temperature (35-37°C, 20-22°C, 4-6°C) and pH using the following equilibrium equation:

$$UAN = TAN\ (1 + 10^{(pKw-pKb-pH)})^{-1} \qquad (4\text{-}1)$$

where the values of the ionization constant for water (K_w; 2.09×10^{-14}, 6.809×10^{-15}, and 1.846×10^{-15}, at 35°C, 20°C, and 5°C, respectively) and the dissociation constant of ammonia (K_b; 1.849×10^{-5}, 1.710×10^{-5}, and 1.479×10^{-5}, at 35°C, 20°C, and 5°C, respectively) were obtained from Weast [1987].

Similarly to ammonia, volatile fatty acids (VFA) can exist in solution as un-ionized molecules (UVFA). The un-ionized volatile fatty acids were calculated based on the total VFA concentration by the following equilibrium expression:

$$U\text{-}VFA = VFA\ (10^{(pKa-pH)}) / (1 + 10^{(pKa-pH)}) \qquad (4\text{-}2)$$

where the dissociation constant values of acids in water (K_a; 1.728×10^{-5} for acetic acid, 1.310×10^{-5} for propionic acid, 1.439×10^{-5} for butyric acid) at 35°C were taken from Weast [1987].

4.4 STATISTICAL EVALUATION

The statistical appraisal of the collected data was done using common statistical functions featured in Microsoft Excel 2000 for Windows software.

Chapter 5.

5 RESULTS AND DISCUSSION

5.1 HIGH-SOLID MATERIALS- CHARACTERISTICS

5.1.1 Substrates for the anaerobic digestion experiments

5.1.1.1 Chicken manure

Chicken manure was the primary substrate for high-solid digestion. It was obtained from a commercial farm housing about 18,000 laying hens. The fresh manure (FM), with an estimated age of up to 3 h, was collected from the area just below the cages, in a semi-solid form (21.7% TS). It was stored in closed plastic buckets filled up to the cover, at 6^{o}C and, within 6 h, used for analysis and preparation of the feed. Chicken manure characteristics are shown in Table 5-1.

Table 5-1. Characteristics of the fresh manure, pit manure, and anaerobically digested sludge used in high-solid anaerobic digestion assay.

Material	TS	TVS	pH	COD	TKN	TAN	VFA
	%	% TS		g O_2/L	g N/L	% TKN	g HAc/L
FM	21.7	61.8	8.0	263.7	19.0	34.7	50.3
PM	11.7	68.9	6.6	150.6	10.2	51.3	32.0
ADS	2.7	42.2	7.5	2.0	1.8	56.9	0.1

Note- FM: fresh manure, PM: pit manure, ADS: anaerobically digested sludge, TS: total solids, TVS: total volatile solids, COD: chemical oxygen demand, TKN: total Kjeldahl nitrogen, TAN: total ammonia nitrogen, VFA: volatile fatty acids, HAc: acetic acid or acetate.

5.1.1.2 Organic fraction of municipal solid waste

The organic fraction of municipal solid waste (OF-MSW) feed consisted of office paper (54% dry weight), newspaper (18%), food waste (11%), and grass clippings or yard waste (17%). This particular composition was chosen to reflect the typical MSW composition in North America, characterized by a high fraction of paper components [Rich *et al.*, 1994]. During preparation of the feed, office paper and newspaper components were added at three different particle sizes (circles of 4.6, 7.0, and 12.0 mm in diameter) having surface areas of 0.17, 0.39, and 1.13 cm^2, thereby producing three different batches of the OF-MSW feed. Each of the three mixtures was then split into four batches, three of which were amended with different levels of primary sludge and the fourth not amended. This resulted in twelve different feed mixtures, each having an unmatched combination of the surface area of the paper components of the feed and of the amount of primary sludge (PS) solids. All feed mixtures were prepared based on dry solids content of each component. The total dry solids content of each mixture was designed to be 30% TS. The actual average solids content of all mixtures was 29.82 (Std. dev. 0.77). In the sludge-amended feeds, out of the 30% dry solids the OF-MSW solids supplied a fraction of 0.85, 0.90, or 0.95 and the remaining was balanced with the PS solids. These various levels of primary sludge produced feeds with varying specific weight of each mixture (Table 5-2).

Table 5-2. Specific weight[a] (SW) and C/N ratio of feed fed to high-solid anaerobic digesters.

Surface area of paper components		Primary sludge (PS) solids in feed, % dry weight			
		15	10	5	0
0.17 cm^2	SW, Kg m^{-3}	480 (10)[b]	436 (5)	400 (6)	370 (4)
	C/N	37.7	40.4	49.1	62.5
0.39 cm^2	SW, Kg m^{-3}	471 (9)	443 (7)	397 (4)	360 (4)
	C/N	39.6	42.4	49.6	61.1
1.13 cm^2	SW, Kg m^{-3}	476 (5)	437 (10)	398 (10)	350 (4)
	C/N	42.5	44.4	54.7	65.3
Mean value for all three sizes	SW, Kg m^{-3}	475 (4.5)	439 (3.8)	398 (1.3)	360 (9.9)
	C/N	39.9 (2.4)	42.4 (2.0)	51.1 (3.1)	63.0 (2.2)

[a] SW of uncompacted feed; [b] values in parentheses represent standard deviation

Carbon-to-Nitrogen ratio for each feed was determined based on the results of elemental analysis using an atomic absorption spectrophotometer. The values have been adjusted to exclude the non-biodegradable carbon by subtracting the carbon content of the lignin. The contribution of inorganic carbon was assumed negligible and was omitted from the carbon balance. Table 5-2 shows the range of C/N ratios in the prepared feeds.

5.1.1.3 Primary sewage sludge

The sludge used in feed preparation had an average solid content of 3.5% TS, with the volatile solid fraction of 78.1% TS.

5.1.2 Substrates for the disinfection experiments

5.1.2.1 Anaerobically co-digested organic fraction of municipal solid waste and primary sludge

The high-solid residues (digestate) from reactors that were fed a feed with different surface area of the paper components but same primary sludge (PS) content, i.e. 15%, 10%, 5%, and 0% (by weight) were combined together, resulting in four different mixtures. The mixtures were designated PS-15, PS-10, PS-5, and PS-0, reflecting the fraction of primary sludge solids in the original feed to the digesters. The mixtures were then supplemented with digested (35-37°C) and dewatered municipal sludge from Winnipeg's NEWPCC, at a w/w (weight/weight) ratio of 3 to 1. The sludge, with naturally occurring fecal coliforms and spores of *C. perfringens*, was previously spiked with *Salmonella* sp. to the level similar to that of indigenous fecal coliforms to observe a meaningful, multi-log decline. The resulting mixtures had different moisture levels in the range from 21.5 to 25.9% TS. Therefore, upon dewatered sludge addition, the moisture was finally adjusted to a 22.0% TS level. The initial ammonia nitrogen concentrations and pH in the mixtures are reported in Table 5-3.

Table 5-3. Characteristics of the high-solid residue from anaerobic co-digestion of the OF-MSW and PS, used in disinfection studies (20-22°C).

Parameter	Mixture type			
	PS-15	PS-10	PS-5	PS-0
TAN, mg N/L	2,561	2,197	1,849	1,148
pH	8.33	8.25	8.25	7.28
UAN, mg N/L	195	156	113	9

PS-15, PS-10, PS-5, PS-0 - numeric values give fractions (% by weight) of primary sludge (PS) solids in feed fed to high-solid digesters; TAN- total ammonia nitrogen; UAN - un-ionized ammonia nitrogen or free ammonia; values in parentheses represent standard deviation.

5.1.2.2 Anaerobically digested-dewatered sewage sludge

Throughout the experimental work, samples of anaerobically digested dewatered sludge were collected from the North End Water Pollution Control Centre, Winnipeg, Manitoba, Canada. The sludge had a pH range of 7.1 to 7.8, temperature of 35-36 ^{o}C at time of collection, and total solids (TS) of about 25% to 36%. Levels of fecal coliforms, the pathogenic indicator bacteria, in the collected sludge were usually high. Measurements over the period of six months ranged from 6.6 $\times 10^{6}$ to 2.1$\times$ 10^{7} colony forming units (CFU) or most probable number (MPN) /g total solids (TS, dry weight). The presence of other, pathogenic microorganisms was verified each time by determinations of *Salmonella* species (none detected to 4.73 x 10^{2} MPN /g TS) and spores of *Clostridium perfringens* bacteria (1.9 x 10^{6} to 4.0 x 10^{6} CFU /g TS).

5.1.2.3 Alkaline additives

Calcium oxide (lime), dry in a fine, pulverized form, was obtained from a local distributor - manufactured by the Mississippi Lime Company - sealed in 50 pound brown paper bags.

Fly ash, as a dry, fine powder, was obtained from the Manitoba Hydro power generation station in Brandon, Manitoba. The average fly ash composition was (mg/Kg): Calcium (17.3), Silica (16.6), Aluminium (8.77), Iron (6.64), Magnesium (1.63), Sodium (1.10), Sulfur (0.69), Potassium (0.30), Phosphorus (0.103), Copper (0.005), and Nickel (0.002).

5.1.2.4 Organic soil for pathogen regrowth experiments

The soil used in topsoil formulations was at TS 28.1%. The average volatile solids content was 83.4% TS, and the pH was 5.7. The soil was free of fecal contamination (no fecal coliform and *Salmonella* bacteria detected). Native heterotrophic population of the soil was above 1.0×10^4 MPN/g TS. The average total spore anaerobic heterotrophic population was 8.9×10^3 CFU/g TS. Spores of *C. perfringens* were naturally occurring in the soil at the average spore density of 3.9×10^3 CFU/g TS. Despite different methods used to determine total spore counts and heterotrophic bacterial counts, it can be seen that majority of the soil bacteria were the spore-forming bacteria.

5.2 MICROORGANISMS- CHARACTERISTICS

5.2.1 Seed biomass used in the high-solid anaerobic digestion experiments

5.2.1.1 Inocula for the chicken manure digestion

The first inoculum used was anaerobically digested municipal sludge (ADS). This type of inoculum for digester start-up is very easy to acquire, usually from an existing

digester treating municipal sludge. It is also often most appropriate to use for seeding purposes due to already established methanogenic population. The ADS was received from the mesophilic (35-37°C) digester at the North-End Water Pollution Control Centre, Winnipeg, Manitoba, Canada. Upon delivery to the laboratory, the sludge was stored in a walk-in chamber at 37°C until start of seeding. The average oxidation-reduction potential (ORP), indicative of anaerobic condition of the sludge, was –376 mV. The characteristics of ADS used to seed the high-solid digesters are shown in Table 5-1.

Since the chicken manure has a high-solid content, the use of another inoculum with solids content higher than that of ADS was considered. Faster start-up would normally be expected using an inoculum already pre-adapted to digesting high-solid material. Due to the difficulty in obtaining a high-solid inoculum from an already existing digester, it was decided to use pit manure. In the normal chicken barn operation, the droppings are flushed from the floor and transported to a pit using a scraper system twice a day. The resulting slurry, referred to as pit manure (PM), is normally stored in the pit for half a year and then spread onto a nearby field as fertilizer. Due to the prolonged storage under anaerobic condition in the pit the prevalent active bacterial population of the manure was expected to comprise of mostly anaerobes, including methanogenic bacteria. An attempt to validate this hypothesis was done by sampling the headspace in the pit chamber and analyzing for the presence of methane gas. Possible inflow of the air through unsealed cover of the pit made it hard to detect any methane, however. No other tests to determine methanogen presence were initiated. The only evidence of anaerobic condition in the pit was obtained by measuring the ORP of the manure. The average ORP was –255 mV, indicative of a moderately reductive environment. This ORP however was

higher than the optimum level of around –360 mV for methanogenic growth [Smith and Hungate, 1958]. The pit was sampled at a depth of 1.5-1.8 m to ensure anaerobic condition of the manure drawn for the seeding of high-solid digesters. The manure was then stored at room temperature in closed plastic buckets filled up to the cover and, within 6 h, used for analysis and preparation of the mixtures. Pit manure characteristics are summarized in Table 5-1.

Farm practices involve co-disposal of some bedding (*i.e.* straw) and broken eggs from the packaging plant and the barns with scraped FM into the pit, resulting in volatile fraction of PM being higher than that of FM.

5.2.1.2 Inoculum for the co-digestion of the OF-MSW and PS

The seed biomass for the digesters was developed ahead of time in a slurry reactor (TS 3.0%, volatile solids were 59.1% of the TS) operating at an organic loading rate of 3.8-4.2 g VS L^{-1} d^{-1} and at 37°C. This "breeder" reactor was originally started using a mixture of anaerobically digested mesophilic sludge and dairy cow rumen fluid. It was acclimated to, and maintained on, a cellulose-supplemented primary sludge feed. Cellulose supplementation was accomplished by addition of toilet paper to degritted wastewater (100 g per 5 L). The paper was soaked and incorporated in the wastewater by means of high-speed mixing. The wastewater was then used to dilute the primary sludge from an original total suspended solids (TSS) level of from 2.9 to 3.9%, to a final concentration of 1.0-1.2% TSS.

5.2.2 Indicator and pathogenic microorganisms used in disinfection experiments

The microorganisms analyzed for in the disinfection studies were fecal coliform bacteria, *Salmonella* sp. bacteria, spores of *Clostridium perfringens* bacteria, and eggs of a helminth *Ascaris suum*.

5.2.2.1 Justification for the choice of microbial indicators of disinfection

The selection of fecal coliform and *Salmonella* sp. bacteria as indicator organisms in assessing biosolids quality is based on their common presence in wastewater sludge. Fecal coliforms belong to the family *Enterobacteriaceae* and usually include *Escherichia coli* as well as various members of the genera *Enterobacter*, *Klebsiella*, and *Citrobacter*. In general, they are non-pathogens (some can be opportunistic pathogens), but are regarded as indicators of pathogen presence in sludge. *Salmonella* sp. bacteria all are pathogenic and have cell structure similar to that of fecal coliform bacteria. Both groups of these bacteria are similar in terms of survival upon exposure to common disinfecting agents.

Enteric, pathogenic bacteria (e.g. *E. coli* O157:H7 - a member of fecal coliform group, *Salmonella* sp.) are the most susceptible to environmental changes, as they had not developed any protection mechanisms that would otherwise minimize the impact of external factors. Yet, *Salmonellae* and fecal coliforms are regulated [U.S. EPA, 1993] and their use in this work was justified.

Clostridia are another group of bacteria, anaerobic, that are mainly used to indicate the presence or absence of remote faecal pollution. Similar to fecal coliform and

Salmonella bacteria, the presence of these organisms in the faeces of all warm-blooded animals is the basis for this practice. The ability to form spores that resist dryness, heat, and aerobic conditions make the *Clostridia* ubiquitous in the environment [Hippe *et al.*, 1992]. Apart from intestinal tract of man and animals, they are considered ubiquitous in soil, water, air (dust), mud, marine sediments, and have frequent and universal geographic distribution.

Spore production is an evolutionary process whose purpose is the protection against unfavorable environmental conditions. During spore production, the protoplasm is condensed, partially dehydrated, and enclosed by exine, an impermeable wall. The fully formed spore structure is consisted of an inner core enclosed by the inner membrane, a cortex surrounded by the outer membrane, and the exterior coat. The major components of the spore coat are structural proteins [Aronson, 1968]. Germination of the spore occurs when a number of spore cortex-lytic enzymes get activated. The activation is triggered by a functional environmental sensory mechanism, once conditions are favorable. A dormant spore is becoming converted into a vegetative cell. Due to the action of the enzymes, the exine becomes more permeable and the spore starts losing its resistance in a sequential fashion, during which dipicolinic acid and Ca^{2+} are released. This occurs only in a medium that is capable of supporting vegetative cell forms. Under conditions normally not permissive for the germination, the activation can still occur, either by sublethal heat or by alcohols [Craven and Blankenship, 1985].

Some of the spore-forming bacteria are pathogenic but remain not regulated because of the longevity of the spores and the lack of direct link to the source of contamination. Because of the resistance to heat, the spores constitute an excellent

indicator of disinfection effected by other abiotic mechanisms. Similar to parasite eggs, these forms are dormant and capable of withstanding higher temperatures, and are rather resistant to conventional, chemical disinfection practices. The much lower water content of the spore coat or eggshell limits the exchange between its interior content and outside environment, thus also contributing to limited free ammonia (NH_3) influx.

Thermotolerant clostridia were found to be reliable indicator of pathogenic contamination in aerosols generated during land application of the biosolids [Dowd *et* al., 1997]. They were present in the highest proportion among other bacterial indicators studied. The authors suggest this is due to condition of higher temperature during anaerobic digestion that favours thermotolerant clostridia. Significantly higher fraction of sporogenous population exists in the thermophilic temperature range, as compared with the mesophilic counterpart mainly comprised of asporogenous bacteria [Chen, 1987], which points the resistance to temperatures in thermophilic range (45-65°C). *C. perfringens* spores were not affected by prolonged, 7-month storage in non-aerated pig slurry (6% TS) at 7°C and 20°C [Munch *et* al., 1987]. This and the previous cases demonstrate their longevity in common stabilization processes.

Yanko [1988] reported on the regular occurrence of helminth ova in one composting operation and in four commercially marketed, compost based soil amendment products. He proposed that *Ascaris* eggs be used as an indicator of other parasites due to their abundance and resistance. The persistence of *Ascaris* eggs in soil is long, from 5 to 7 years, and possibly longer [Little, 1986]. The structure of a shell allows for protecting the embryos from adverse conditions. The outer and subsequent layers of the egg shell consist mostly of proteins and chitin and are surrounding the inner lipoid or

vitelline membrane. The outer irregular coat, characteristic to *Ascaris* species, forms a secondary envelope consisting mostly of uterine secretions [Fairbairn, 1957]. During development to a larval stage or embryonation, eggs do not require any external nutrients as they contain considerable amounts of glycogen and lipids. The only waste product excreted is carbon dioxide. In addition, a wide spectrum of chemicals including 9% sulfuric acid and 12% formalin does not prevent eggs from embryonation. Even when sufficient concentrations of acid or alkali are present to dissolve the external coat and the hard shell as well, the vitelline membrane remains a sufficient protection for the embryo. Only gaseous compounds, including ammonia [Passey and Fairbairn, 1955], are capable of permeating this inner membrane. This can prevent embryonation and lead to ultimate loss of viability. Such durability of the eggs of *Ascaris* species and other parasitic eggs with the resistance similar to that of *Ascaris* decided on establishing a limit of less than 1 viable egg/4 g TS in biosolids destined for unrestricted land application [U.S. EPA, 1993].

The only indicator of pathogen presence used in this work that is not regulated by the U.S. EPA's regulation is *C. perfringens* spore. However, one of the aims of this work was to determine if the resistance of the spores to disinfection is similar to that of *Ascaris suum* eggs.

5.2.2.2 Fecal coliforms

The density of fecal coliforms inherent to sewage sludge was high enough (10^6-10^7 MPN or CFU/g TS) to observe a meaningful decline. Therefore, no spiking was necessary to obtain a desired level of this indicator in the sludge.

5.2.2.3 *Salmonella* sp.

Since the density of autochthonous *Salmonella* sp. cells is usually low in Winnipeg's digested-dewatered sludge, spiking in studies involving this pathogen was necessary. Spiking was performed on all sludge samples, with the exception of sludge used in stage I (lime treatment at 20-22°C).

Salmonella sp. cultures used for spiking were isolated from samples of wastewater and sludge taken at different stages during treatment, at the NEWPCC in Winnipeg. The procedure for culture isolation is included in the SOP for *Salmonella* sp. (see Appendix E). The isolated strains were purified from primary plates by means of repeated transfers.

The selection of particular strain(s) for spiking was based (1) on sample origin in treatment train (the further in the train, the higher resistance of an isolate from that sample) and (2) on growth rate under optimum condition.

The growth rates of isolated strains were determined by transferring a small volume of a log phase *Salmonella* sp. culture to tryptic soy broth, incubating the culture for up to 3 h and measuring the percent absorbance using a spectrophotometer in half hour intervals. In parallel, plating of the culture was performed to determine the density of *Salmonella* sp. strain.

Two isolates were selected for spiking sludge, one from raw sewage and the other from digested-dewatered sludge. The cultures were combined to maintain a sufficiently high genetic pool of the working culture used for spiking. The results of selective *Salmonella* sp. isolation and enumeration are included in Appendix D.

5.2.2.4 Spores of *Clostridium perfringens*

The density of *C. perfringens* spores inherent to sewage sludge was high enough (in the 10^6 CFU/g TS range) to observe a meaningful decline. Therefore, no spiking was necessary to obtain a desired level of this indicator in the sludge.

However, for the inactivation studies using well-defined, artificial media, the spores had to be added in spike from an external source. The spores were produced according to the procedure in SOP for *C. perfringens* enumeration (see Appendix E). The results of spore production and enumeration for the use in spiking are included in Appendix D.

5.2.2.5 Eggs of a helminth, *Ascaris suum*

Since there were no *Ascaris suum* eggs or any other parasitic eggs detected in Winnipeg's digested-dewatered sludge, spiking in studies involving this pathogen was necessary. Spiking was performed on sludge samples used in later stages of experimental work (stage V of disinfection work involving alkali treatment at 4-6°C, and in experiments using well-defined, artificial media) due to delay in acquiring the eggs.

The levels of *Ascaris* ova in spiked material can be found later in the sections describing their inactivation in the corresponding treatments.

5.3 ANAEROBIC DIGESTION AT HIGH-SOLID LEVELS - CHICKEN MANURE

Chicken manure digestion was conducted in a solids range from 5.0% TS to 21.7% TS- the original solids content of the manure. The Biochemical Methane Potential (BMP) bioassay was used to determine the extent of manure degradation. The performance was assessed in terms of methane production, efficiency of organic substrate conversion to methane, nitrogen transformations and the resulting effects on degradation. Particular attention was given to free ammonia (UAN) and free VFA (U-VFA) inhibition.

This section summarizes the data collected during operation of the digesters. The duration of the assay was 17 weeks, or 119 days. The decision to terminate experiments was based on the observed decreased activity in methanogenic reactors and on the lack of biodegradation progress in highest solids reactors. Besides, it was expected that after that time the possible further extent of degradation would have been marginal. Svendsen and Blackburn [1986] have shown that complete degradation of high-solid swine manure (TS 15.5%) in a batch process required 100 days at 35°C. Due to similarity in characteristics of chicken manure and of pig manure, including high ammonia load, it was decided that 17 weeks is enough to effect almost complete degradation of the organic matter in the manure.

5.3.1 Biogas production

The cumulative methane production in corresponding digesters is presented in Fig. 5-1. The yields have been reported with respect to the initial volatile solid fraction in

the contents of the digesters, thus providing a common basis for comparison among different experiments. Methane passing above 60% (volumetric basis) in the biogas was considered a sign of balanced digestion. The minimum time it took to reach this level in methanogenic reactors coincided with onset of the maximum rates of methanogenesis (i.e. methane formation). Plots of average methane content in biogas from different digesters are depicted in Fig. 5-2.

As the incubation proceeded, different phases of the batch anaerobic digestion process were distinguished. Beginning of each phase has been marked with an arrow in Fig. 5-1. The phases were established using the method of least squares. A range of data points was selected to find the maximum slope (equal to maximum rate of methanogenesis) on each cumulative methane production curve. The criterion used to determine the phases was that the coefficient of determination (r^2) of the slope be higher than or equal to 0.95.

During batch anaerobic digestion, the following phases can be distinguished:

phase I- acclimation of microbial population to organic substrate, often characterized by a lag in methane production; if inhibitory/toxic substances are present this phase will be longer; depending on the robustness of seed biomass, phase I can be very short, which results in almost instantaneous and high production of methane;

phase II- period of exponential growth, which corresponds to highest activity where substrate is not limited; this results in highest rates of methane production;

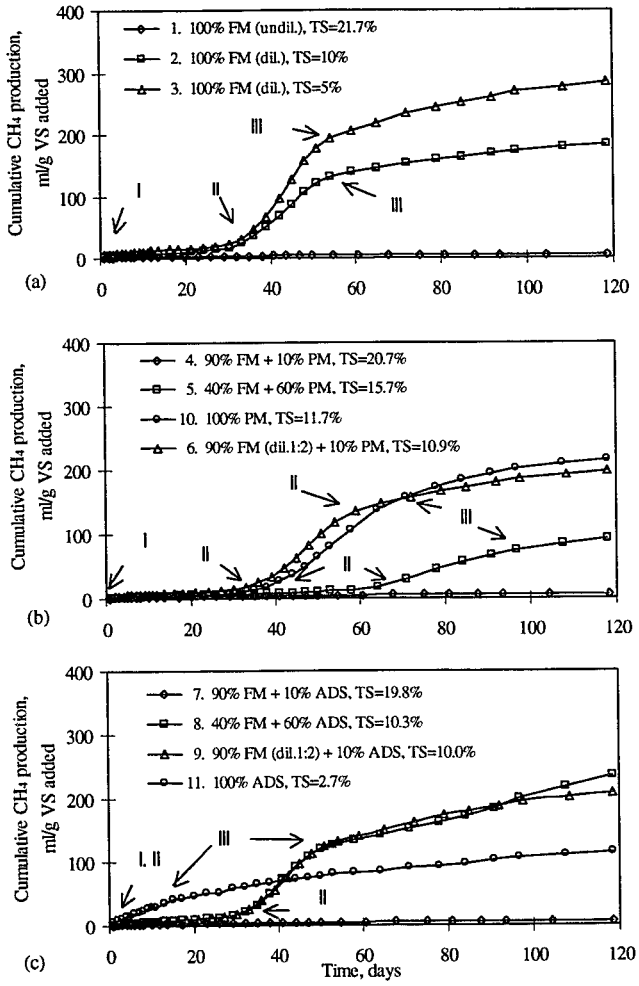

Figure 5-1. Cumulative methane production for experiments, with fresh manure (FM), pit manure (PM) and anaerobically digested sludge (ADS), involving: (a) FM alone, (b) FM + PM and (c) FM + ADS, at different total solids levels

Arrows indicate the beginning of each phase; dil.: diluted; undil.: undiluted; TS: total solids; VS: volatile solids

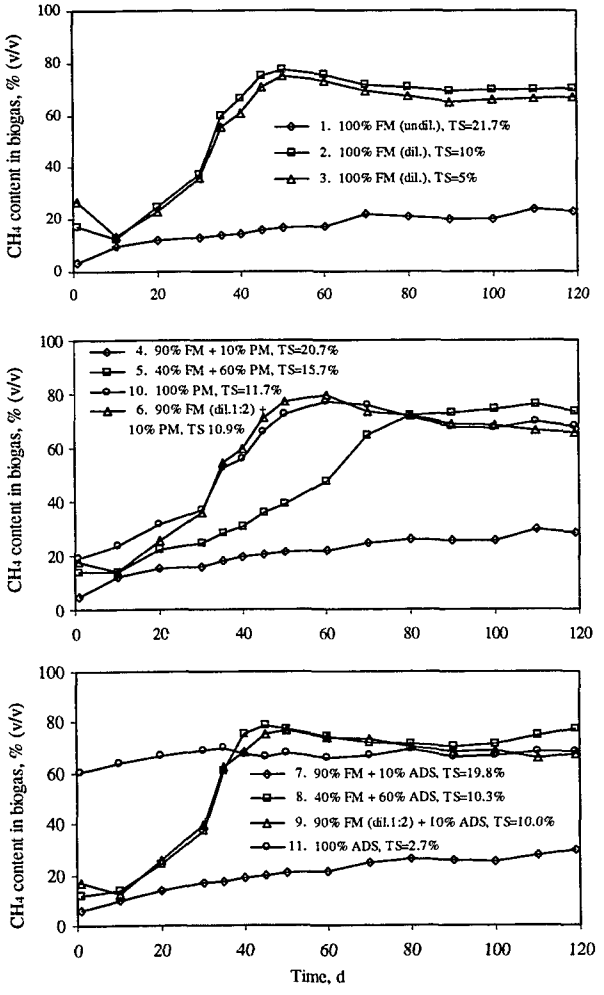

Figure 5-2. Methane content of biogas for experiments, with fresh manure (FM), pit manure (PM) and anaerobically digested sludge (ADS), involving: (a) FM alone, (b) FM+PM, and (c) FM+ADS, at different solids levels
dil.: diluted; undil.: undiluted; TS: total solids (initial)

phase III- substrate concentration decreases causing shift in equilibrium between cell growth and lysis; activity decreases as marked by decreased methane yields; this phase can also result from inhibition or toxicity to one or more groups in microbial consortium (e.g. methanogens utilizing acetate; acetogenic bacteria utilizing propionate) often leading to accumulation of intermediate products of microbial metabolism and further inhibition, (substrate-mediated inhibition).

Data presented in Table 5-4 characterize each experiment from the point of biodegradability and methane generation potential from chicken manure. The experiments were arranged in groups, depending on the presence and type of inoculum used, and numbered according to the decreasing initial total solid content in each digester. In digesters operating at an initial total solids level ranging from 5.0 to 11.7% (experiment no. 2, 3, 6, 8, 9, and 10), all three phases are distinguished. The {40% FM + 60% PM} experiment with a TS of 15.7% had a longer lag such that it had hardly gone to completion of phase II at the end of the incubation. These experiments involving undiluted manure, with resulting higher solids concentrations (experiment no. 1, 4, and 7), exerted a limited digestion course and typical phases during anaerobic breakdown could not be seen.

Phase I

All experiments involving the fresh manure, during the first day of incubation, produced a significant amount of biogas. The average volumes recorded were from 45ml up to 90 mL (at STP- standard temperature and pressure) and correlated with increasing concentration of the FM in the digester mixture. The biogas consisted of 73.0 to 96.7% carbon dioxide (by volume). After that, the biogas production rates decreased. The

control experiment involving pit manure had a low biogas yield (13 mL) with the corresponding CH_4 content of only 19%. The other control, anaerobically digested sludge, produced 9.5 mL biogas with high CH_4 content of 60%.

Methane production rate and its content in the biogas decreased during the first 10 days but then recovered to their initial levels after 20 to 30 days of the incubation following exponential rise in the subsequent phase, except for the three high TS experiments (experiment no. 1, 4 and 7). The shortest lag periods of 30 days and the resulting accelerated maximum CH_4 yields were recorded for experiments involving the diluted FM alone as well as the mixtures of FM and ADS at initial TS ranging from 10.0 to 10.3%. Phase I for the {90% FM (dil. 1:2) + 10% PM} experiment (no. 6) continued until day 40. For the fourth highest TS experiment (no. 5, 15.7% TS), the lag period was extended to over 60 days.

It was anticipated that the pit manure due to its long storage condition would have an already established anaerobic population of the bacteria, including methanogens. In spite of that, the PM control experiment required 45 days to adjust. The only biomass that did not go through phase I, that is a lag phase, was the one of ADS control experiment (no. 11). This biomass was incubated without addition of an organic substrate, and methane yield was due to the residual biodegradable matter still present in sludge. It still gave an expected high overall methane yield of 534 mL CH_4/g VS removed (Table 5-2) and satisfactory biogas quality of 60.6-69.4% CH_4 (Fig. 5-2). This observation confirmed ADS suitability as the seeding material.

Table 5-4. Anaerobic biodegradability of chicken manure at varying solids levels with and without inoculum of anaerobic biomass.

No., Experiment	Initial TS	Duration of phase I and II	$R(CH_4)_{Max}$	CH_4 yield in phase: I, II, III[(B)]	BMP[(C)] (per C-COD: fed, removed)	Conversion efficiency[(D)] total, net	CH_4 yield (per VS: fed, removed)
	%	D	mL/g VS_{fed} d^{-1}	% total	mL/g COD	%	mL/g VS
FM alone							
1. 100%[(A)] FM (undil.)	21.7	-	-	-	5, 28	1	5, 13
2. 100% FM (dil.)	10.0	36, 15	5.9	19, 47, 34	192, 354	55	184, 283
3. 100% FM (dil.)	5.0	36, 15	9.2	16, 46, 38	308, 654	88	286, 548
FM and PM mixture							
4. 90% FM + 10% PM	20.7	-	-	-	6, 33	2, -	7, 23
5. 40% FM + 60% PM	15.7	64, 33	1.8	19, 62, 19	87, 160	25, -	93, 256
6. 90% FM (dil. 1:2) + 10% PM	10.9	39, 20	5.0	17, 51, 32	201, 423	57, -	198, 378
FM and ADS mixture							
7. 90% FM + 10% ADS	19.8	-	-	-	7, 40	2, -1	7, 22
8. 40% FM + 60% ADS	10.3	35, 12	6.7	14, 33, 53	241, 362	69, 49	234, 473
9. 90% FM (dil. 1:2) + 10% ADS	10.0	33, 15	5.9	13, 42, 46	216, 403	62, 58	207, 335
Controls							
10. 100% PM	11.7	44, 20	5.0	17, 46, 37	190, 384	54	215, 436
11. 100% ADS	2.7	1, 14	2.4	6, 30, 64	118, 362	34	115, 534

Note- FM: fresh manure, PM: pit manure, ADS: anaerobically digested municipal sludge, TS: total solids, VS: volatile solids, COD: chemical oxygen demand, C-COD: carbonaceous COD, dil: diluted, undil: undiluted, 1:2: one part fresh manure + one part water, $R(CH_4)_{Max}$: max. rate of methane prod. (during phase II),

[(A)]: fraction (percent) of 50 g of the digester content (wet weight basis);

[(B)]: fraction (percent) of total CH_4 generated in phases I, II, III, (equal to fraction of carbonaceous COD, C-COD, converted to methane in respective phases);

[(C)]: Biochemical Methane Potential, at STP; measures sample biodegradability (background value from seed-control is subtracted from total CH_4 produced);

[(D)]: conversion efficiency of organic matter to methane; total: in sample, net: fresh manure; based on theoretical BMP of 350 mL CH_4/g COD converted at STP.

Phase II

This phase was characterized by the highest rates of biogas production that correlated with higher than 60% methane content of the biogas (Fig. 5-2). The maximum rates of CH_4 generation, $R(CH_4)_{Max}$ (Table 5-4), varied among experiments and correlated with the initial total solid content in digesters. The highest average $R(CH_4)_{Max}$ of 9.2 mL/g VS fed d^{-1} was observed in the digesters with most diluted manure (5% TS) and the lowest (1.8 mL/g VS fed d^{-1}) in the highest initial TS (15.7%) methanogenic reactors.

In most methanogenic experiments, phase II was significantly shorter than the other two phases, the lag and the III-rd phase (Table 5-4). Among the experiments with the shortest lag periods (Figure 5-1), the ones involving FM and ADS yielded methane during this phase that accounted for 33 to 42% of the total amount of CH_4. The diluted FM experiments demonstrated 46% (at 5% TS) and 47% (at 10% TS) CH_4 yield. The remaining methanogenic experiments, involving FM and PM, showed a CH_4 yield of 51% (no. 6) and 62% of the total (no. 5).

Phase III

Due to a batch nature of the process and to possible effects of high ammonia and VFA, for the well performing experiments the biogas production decreased markedly, thereby manifesting the beginning of the last phase, phase III. Methane content in the biogas, however, was maintained at levels of 65% and more. For the experiments with the highest TS contents (no. 1, 4, and 7) after the maximum biogas yields observed on day 1, no substantial increase in the rate of biogas production followed. The CH_4 component was limited and increased gradually over the length of the incubation period

approaching only about the 30% content at the end of the experiment. The biochemical methane potential (BMP), Table 5-4, was virtually zero for all highest-solid experiments.

The methanogenic reactors continued to generate methane, but at rates much lower than in phase II. When comparing CH_4 yields in digesters with starting TS around 10% (no. 2, 6, 8, 9) during this phase with those in phase II, higher contributions were observed from digesters seeded with ADS (53%, 46% of total CH_4) than from PM-seeded and non-seeded digesters (32% and 34%, respectively). Slightly higher BMP values from the ADS-seeded reactors were noted too.

The BMP was found highest from digesters degrading most diluted manure (5% TS, no. 3), 308 mL CH_4/g COD fed or 88% of the theoretical BMP of 350 mL CH_4/g COD (Table 5-4). This conversion value is commonly used in the engineering field, and is based on the theoretical amount of oxygen (here as COD) required to oxidize methane to carbon dioxide and water (COD equivalency of methane: 350 mL CH_4 = 1 g COD; see Appendix A). The majority of CH_4 from ADS control experiment (no. 11) was generated during phase III. The remaining organic carbon present in the already digested sludge was mostly refractory, thus requiring more time for further degradation. The conversion efficiency of 34% (Table 5-4) was low as well. In experiments started at about 10% TS (no. 2, 6, 8, 9) was in the range from 55% for the fresh manure alone, up to 69% for the fresh manure supplemented with 60% ADS, the anaerobic biomass seed. When expressed in terms of net conversion efficiency, this highest percent of 69% in the 10%-TS group was mainly due to the CH_4 from the anaerobically digested sludge. When this contribution was accounted for, the organics conversion in the fresh manure alone was calculated as only 49%. The net conversion in pit manure-amended experiments was not

established, as it would be erroneous to treat the pit manure as a seed only. It contained significant amount of organics contributing to the overall load and required initial adaptation (phase I), unlike the ADS.

Similar three-phase patterns of batch anaerobic degradation were described by Svendsen and Blackburn [1986], who conducted un-inoculated experiments with swine manure at TS from 16 to 23% as primary substrate.

Final reductions in VS, and COD were the lowest for the high-TS samples, which coincided with low biogas and low methane production rates. It should be noted here that though ADS had the lowest VS and COD removal rates, its S-COD reduction performance of 88.0% was the highest observed (already digested sludge with a low VS content of 42.2% TS). Methane production in successful methanogenic experiments at an initial TS of about 10% varied from 184 to 234 mL CH_4/g VS fed, and was 286 mL/g VS in the 5%-TS experiment.

The specific methane productivity (SMP), expressed as mL CH_4/g VS removed, observed among experiments coincided with the patterns and the ultimate CH_4 yields shown in Fig. 5-1. This productivity (Table 5-4) varied from 256 to 548 mL/g VS and from 13 to 23 mL/g VS for high methane yield experiments (no. 2, 3, 5, 6, 8, 9, 10, 11) and low methane yield experiments (no. 1, 4, 7), respectively. The diluted manure experiment at an initial TS of 10% (no. 2) produced 283 mL CH_4/g VS removed which was lower than for the other two diluted manure treatments supplemented with 10% PM (no. 6), and with 10% ADS (9). The highest rates were achieved in the experiment involving most diluted manure (5% TS, no. 3), followed by the ADS control and the {40% FM + 60% ADS} experiments.

The SMP from anaerobic digestion of organic substrate was proven to be approximately constant for volatile solids regardless of substrate type [McCarty, 1964b; *Metcalf and Eddy, 1991*], with about 500 mL CH_4 generated per g VS removed. This direct correlation has been attributed to the fact that the only mechanism for removing organic carbon is the conversion of organic matter (VS) to methane and carbon dioxide. This figure closely corresponds with the already described theoretical methane yield of 350 mL/g COD (carbonaceous). The second highest SMP of 534 mL/g in the control ADS experiment coincided with the anticipated high CH_4 yield per COD removed of 362 mL CH_4/g. The first highest SMP of 548 mL/g VS removed recorded in case of the most diluted experiment (no. 3) corresponded to 308 mL/g COD fed, but was 654 mL/g COD removed, almost double the theoretical CH_4 yield. Higher than theoretical methane yields reported elsewhere were as high as 750 mL/g VS removed, from poultry manure [Safley et al., 1985], and 540 mL/g VS and 430 mL/g COD from dairy manure [Fisher et al., 1979]. Yields in this bioassay from other methanogenic reactors, in terms of mL CH_4/g COD removed, were also higher (up to 423 mL/g). The cause of such significant difference between theoretical and experimental values is unknown. The measured methane yield expressed as mL/g VS removed was in agreement with the theoretical value of 500 mL/g. The yield per amount of C-COD removed was in a few cases higher than the theoretical value of 350 mL/g. Hill [1983] pointed out that the usual 500/350=1.42 conversion factor of cell mass COD to cell mass biodegradable VS may not hold for the conversion of raw substrate COD to biodegradable VS.

Mixing of the reactors started on day 40 and did not have any effect on the performance of the digesters, as the methane productions remained unchanged (Fig. 5-1).

There was no oxygen detected in any of the reactor headspaces during the entire duration of experiments, documenting the provision of an oxygen-free environment.

5.3.2 Nitrogen transformations

The higher nitrogen content of chicken manure, as compared with manures of other farm animals, is due to the common route of urine and feces excretion in chickens. Such excretory course is characteristic of all monogastric animals (as oppose to ruminant animals), including poultry. Nitrogen (N) is excreted mainly in the form of uric acid ($C_5H_4O_3N_4$), a non-protein nitrogenous compound and principal product of purine metabolism. Urinary sources contribute about 70% of the total N and the remaining 30% comes from faecal matter. Uric acid represents 88% of urinary N (Krogdahl and Dalsgard, 1981), which translates to almost 62% of the total N. The uric acid in the FM accounted for as much as 6% of dry solids, which demonstrates that it was the major organic nitrogenous compound found in the manure. There was no uric acid detected in the PM and in the ADS. There was also no uric acid present in any of the digesters' contents after terminating the incubation.

Total Kjeldahl nitrogen (TKN) in all experiments remained almost unchanged by the end of the assay. This observation confirmed that nitrogen is conserved during anaerobic breakdown, unlike in nitrification-denitrification during aerobic-anoxic mode of degradation commonly used to remove nitrogen from wastewater. An ammonia nitrogen (TAN) portion of the TKN increased significantly as the nitrogenous organic compounds of the manure, including uric acid, were converted to ammonia (Table 5-5).

Table 5-5. Summary of organic and ammonia nitrogen, VFA, and pH data, and the corresponding un-ionized ammonia and VFA.

No., Experiment	N_{org} mineraliz. total, net	pH -		Ammonia TAN (UAN)* as N, mg/L		Volatile fatty acids VFA (U-VFA)* As acetic acid, mg/L	
	%	Init.	Final	Initial	Final	Initial	Final
FM alone							
1. 100%[(A)] FM (undil.)	71.6	8.0	7.39	6,598 (666)	16,310 (437)	50,307 (31)	60,296 (149)
2. 100% FM (dil., TS 10%)	69.0	7.9	8.07	3,040 (249)	6,578 (766)	23,183 (18)	8,401 (4)
3. 100% FM (dil., TS 5%)	77.6	7.9	7.69	1,520 (124)	4,049 (211)	11,592 (9)	5,714 (8)
FM and PM mixture							
4. 90% FM + 10% PM	70.7, 71.3	7.8	7.27	6,462 (427)	15,796 (323)	48,478 (47)	61,940 (200)
5. 40% FM + 60% PM	61.8, 64.5	7.2	8.19	5,784 (101)	11,405 (1,689)	39,331 (151)	27,746 (11)
6. 90% FM (dil. 1:2) + 10% PM	79.4, 78.9	7.6	8.04	3,493 (149)	7,403 (811)	25,840 (40)	9,767 (5)
FM and ADS mixture							
7. 90% FM + 10% ADS	73.1, 73.0	7.9	7.30	6,040 (494)	14,803 (324)	45,291 (35)	55,290 (166)
8. 40% FM + 60% ADS	66.2, 66.6	7.7	8.00	3,250 (173)	6,159 (621)	20,209 (25)	2,860 (1)
9. 90% FM (dil. 1:2) + 10% ADS	70.0, 66.4	7.8	8.00	3,071 (203)	6,321 (638)	22,653 (22)	10,146 (6)
Controls							
10. 100% PM	55.3, 0	6.6	8.11	5,241 (23)	8,061 (1,018)	32,013 (483)	11,834 (5)
11. 100% ADS	22.8, 0	7.5	7.31	1,018 (35)	1,074 (24)	144 (0)	41 (0)

Note- FM: fresh manure, PM: pit manure, ADS: anaerobically digested municipal sludge, dil: diluted, undil: undiluted, 1:2: one part fresh manure plus one part water, TKN: total Kjeldahl nitrogen, TAN: total ammonia nitrogen ($NH_4^+ + NH_3$), UAN: un-ionized or free ammonia (NH_3), VFA: total volatile fatty acids, U-VFA: un-ionized or free VFA,

* values in parentheses represent concentrations of un-ionized forms of ammonia and of VFA

(A): fraction (percent) of 50 g of the digester content (wet weight basis),

(B): mineralization of organic Nitrogen (N_{org}); total: in sample, net: in fresh manure; calculated as a ratio of (TKN-TAN) at start to (TKN-TAN) at end of experiments.

Organic nitrogen (N_{org}) mineralization in digesters treating fresh manure (FM) was ranging from 61.8% to as high as 79.4%. Less efficient conversion of 55.3% took place in the pit manure (PM) control experiment. The storage of PM in the pit, prior to use in the experimental work, allowed an earlier uric acid conversion to ammonia. The lowest N_{org} mineralization of 23.2% was observed in the anaerobically digested sludge (ADS) control experiment (Table 5-5).

Conversion of N_{org} to ammonia at least doubled TAN levels in most experimental digesters, as confirmed by the end of incubations. A 35%-TAN increase was measured in the PM control experiment whereas stable ammonia concentration of around 1 g N/L was maintained in ADS control digesters.

Depending on the pH of a solution, ammonia (TAN) exists as ammonium ion (NH_4^+) and/or as free gaseous species (NH_3). The final initial un-ionized free ammonia (UAN) levels in the three highest solids reactors were in the range from 427 to 666 mg N/L, which is considered inhibitory to methanogens. By the end of incubation, the free ammonia content decreased because of drop in pH. For the methanogenic experiments, the final free ammonia concentrations exhibited a significant increase as compared to initial levels (Table 5-5).

5.3.3 Volatile fatty acids utilization

In all experiments at the beginning of incubation, the acetic acid (HAc, 61-73% VFA total) dominated over the propionic acid (HPr, 19-20% VFA total, expressed as HAc), with the average HPr/HAc ratio of 0.3. Butyric-n acid was the third predominant VFA accounting for 6-15% VFA total and the remaining fatty acids were at negligible

concentrations of 1-2%. After the termination of the experiment, the characterization of VFA into different acids in the three experiments with highest solid content and proportion of the FM did not show any significant changes in fraction of propionic acid. The decrease in acetic acid and increase in butyric-n acid was noted, however, resulting in net increase in the total VFA concentration of 20 to 28%. In the fourth highest TS (15.7%) experiment, a ratio of HPr/HAc by end of incubation was 1.1, with butyric-n acid approaching the level similar to the level of HAc and HPr (30% acetic acid, 33% propionic acid, 27% butyric-n acid; as fraction of VFA total).

The VFA distribution for all experiments that were started at around 10% TS and involved diluted FM alone or in combination with 10% ADS or 10% PM (no., 2, 6 and 9), allowed propionic acid accumulation over acetic acid to be identified, but the relative propionic acid concentration increased only slightly. Butyric-n acid concentrations remained constant but valeric-iso acid increased. The PM control experiment showed similar characteristics. The {40% FM + 60% ADS} experiment (no. 8), classified in the same, close to 10% TS, series though with much higher inoculum content of the mixture, depicted the most stable course of digestion in the ~10%-TS group, with a ratio of HPr/HAc of 0.13, however; butyric-iso acid and valeric-iso acid have also accumulated. The highest ratio of HPr/HAc of 15.6 was noted in the experiment with most diluted FM (no. 3, TS 5%). In addition to butyric-iso acid and valeric-iso acid build up, propionic acid concentration doubled. The VFA concentrations in the ADS control experiment were significantly lower as compared to the remaining experiments, with acetic acid being the primary VFA present.

Similarly to free ammonia the un-ionized form of VFA (U-VFA) or free VFA can also be toxic if at higher levels, and its concentration increases with decreasing pH of solution. Inhibitory levels can be as low as 10 mg/L as HAc; however, acclimations in the range of 30-60 mg/L as HAc were reported [Kroeker *et al.*, 1979]. The very high initial and final concentrations of total VFA and U-VFA (Table 5-5) as well as high initial total and free ammonia levels recognized as those revealing a severe toxicity, were recorded in the high total-solid (19.8-21.7%) digestions.

5.3.4 Effects of seed inoculum and acclimation on commencement of the reactors

The beginning of methane generation (phase II) in experimental methanogenic reactors ended the initial period of acclimation (Table 5-4). The adaptation time was similar in experiments where anaerobically digested sludge was used as a seed (33 to 35 days), and in experiments digesting fresh manure alone at initial TS of 5% and 10% (36 days). A few days more were necessary for such observation in pit manure-amended reactors. Even longer period of 45 days was necessary for biomass adaptation in the pit manure control experiment (TS 11.7%). Highest total solid methanogenic reactors (15.7%) showed longest acclimation of 64 days.

The sources of anaerobic biomass inocula used, *i.e.* the anaerobically digested sludge and the pit manure, were not adapted to digesting the fresh manure, and it was expected to observe an initial lag period in each of the experiments. Regardless of the dilution and/or fraction of the inoculum used, an adequate adaptation period was necessary for onset of CH_4 generation.

The uric acid, as the principal nitrogenous constituent in chicken manure when anaerobically decomposed, significantly contributes to the formation of ammonia. Two principal, obligate anaerobes capable of anaerobic decomposition to CO_2, acetic acid and ammonia are *Clostridium acidiurici* and *C. cylindosporum* [Vogels and Van Der Drift, 1976], the latter organism producing insignificant amounts of glycine and formic acid as well. Complete anaerobic decomposition of one part uric acid eventually yields just ¼ parts methane, 4¼ parts carbon dioxide, and 4 parts ammonia (theoretical decomposition based on stoichiometry, see Appendix A). Acetic acid is an intermediate product during uric acid breakdown, and its ultimate conversion to methane and carbon dioxide depends on whether the significant increase in ammonia can be tolerated by methanogenic populations. In the study by Lacey *et al.* [1981], complete utilization of the uric acid was confirmed after first 24 hours of diluted (5% TS) poultry manure digestion. Based on initial and final uric acid and TAN portion of TKN, it was concluded that uric acid was completely converted to ammonia. In another study of batch poultry digestion [Webb and Hawkes, 1985], the initial biogas production was high but the biogas was mainly consisting of carbon dioxide. Rapid accumulation of VFA followed.

In this bioassay, the biogas production within first day of incubation was significant in all experiments involving fresh manure, regardless of its portion in the digester mixture. The small up to 30% fraction of CH_4 in biogas, recorded at that time and during initial stage of incubation, is likely the result of two possible actions: (a) an ongoing adaptation of anaerobic biomass, and (b) uric acid breakdown. Low methane yield expected from uric acid degradation together with the immediate acetate surge and lack of microorganisms' adaptation can, therefore, affect successful start-up of the

digestion process. Although the latter action was not validated, it is indicated as the second most probable cause for the lag. Third explanation is that there was a small population of acetate-utilizing methanogens, thus limiting the rate of CH_4 production. This conclusion was reached in a study of batch fermentation of un-inoculated swine manure at semi-solid levels (16-23% TS) [Svendsen and Blackburn, 1986]. If this was the case however, the experiment inoculated with 60% ADS (no. 8) would have shown much shorter lag phase due to the likely abundant presence of acetoclastic methanogens from the seed sludge.

The only indirect evidence of uric acid breakdown in the experiments involving fresh manure during first 24 hours of operation was the lack of excessive biogas production in the pit manure control experiment. This observation was concluded to be due to the absence of uric acid in the PM, suggesting that uric acid breakdown in the fresh manure fed experiments contributed to such high gas production after day 1.

The above observations allowed making three important conclusions. First, the native anaerobic population of the fresh manure was capable of initiating degradation and generating methane within a time span and at rates similar to the anaerobic seed from a municipal sludge digester. Secondly, under similar condition (TS 10.9%, i.e. close to 10%), the pit manure delayed onset of phase II. When digested alone at TS 11.7% it underwent even longer transition. Considerably higher volatile solid content of the pit manure as compared to the VS of the fresh manure (Table 5-1) lowers the organic loading threshold that can be applied to a digester. Methane production eventually followed even at highest methanogenic TS of 15.7%, in the {*40% FM + 60% PM*} mixture, but the process was visibly retarded. Therefore, the pit manure should rather be

regarded as a substrate and not a source of inoculum for fresh manure digestion. Third, the indirect evidence of uric acid breakdown by day 1 in all experimental reactors fed fresh manure suggests that the final ammonia levels recorded at the end of incubation could exist in early stages of the digesters' operation, even prior to establishment of the methanogenic population. Therefore, higher than original levels of un-ionized ammonia could be present in these reactors actually shortly after they were commenced.

5.3.5 Impact of organic load (VS) and resulting ammonia on methane production

The methanogenic activity during phase II was plotted against initial concentration of the solids in the reactors, in Fig. 5-3. The strong correlation between the two parameters (r^2=0.96) points to the impact of TS on the performance of chicken manure degradation. As the total solids concentration increased, the resulting increased organic load caused drop in overall maximum rate of CH_4 generation. The increased organic loading, associated with total solids, decreased the potentially high CH_4 productivity clearly indicating that methanogenic population was inhibited most. The BMP of 308 mL CH_4/g COD obtained in experiments digesting most diluted manure (5% TS) corresponded to 88% conversion efficiency, whereas in experiments with higher starting total solids concentration the conversion was lower (Fig. 5-4). The ultimate methane production in the most diluted experiment of 287 mL/g VS fed compares well with the yield of 270 mL/g VS in a chicken manure experiment at 50°C and at similar VS loading [Huang and Shih, 1981].

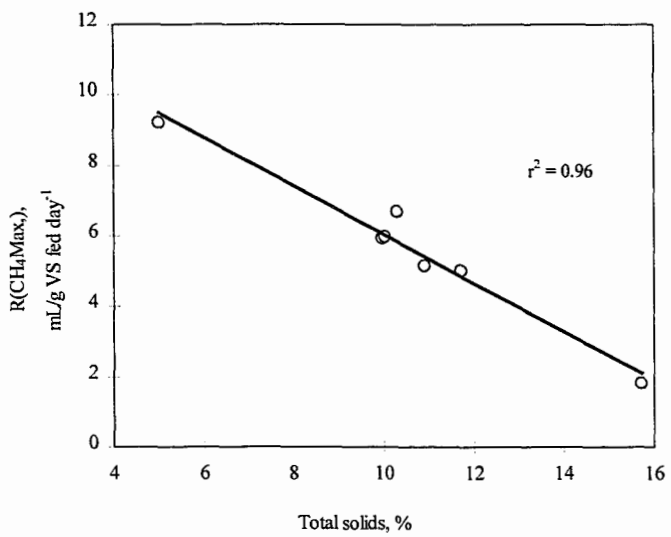

Figure 5-3. Maximum rate of methane production in methanogenic reactors, recorded during Phase II

RCH_4Max: maximum rate of methanogenesis

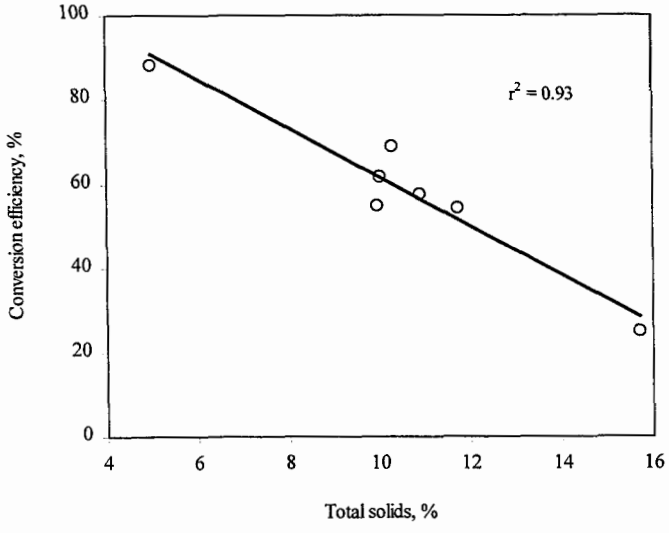

Figure 5-4. Overall conversion efficiency of organic matter to methane in methanogenic reactors

Based on theoretical BMP of 350 mL CH_4/g COD converted at STP; BMP: biochemical methane potential; COD: chemical oxygen demand; STP: standard temperature and pressure

High organic nitrogen conversions to ammonia of up to around 80% proved that the native hydrolyzing bacterial population of the manure was very active. Lower organic N conversion efficiency of 53% was obtained by Field *et al.* [1985], and by Webb and Hawkes [1985], but this could be attributed to the older age of manure used in these studies. In this research, the lack of uric acid in the pit manure at start of digestions coupled with a higher VS fraction as compared to the fresh manure indicated that N_{org} was of another origin. The inclusion of wastes from the egg packing plant in the pit contributes the extra load. The 55.3% N_{org} mineralization in the pit manure is therefore the result of prior storage of the manure in the pit and the presence of nitrogen containing organics other than uric acid.

High degree of organic nitrogen mineralization was coupled with efficient conversion of the carbonaceous COD (C-COD, the portion of total COD after subtraction of the nitrogenous COD) to VFA. This was observed in all experimental reactors, including the most inhibited non-methanogenic reactors (experiment no. 1, 4, 7) where a high degree of conversion of the C-COD to VFA of from 70 to 74% was determined by the end of incubation. Even higher conversion of 94% was noted in the highest TS (15.7%) methanogenic experiment. This significant degree of particulate matter solubilization to VFA indicates the lack of impact due to high-solid operation on fermentative bacteria as well.

The trend shown in Fig. 5-5 substantiates the previous correlation. Organic loads ranging from 30 to 80 g VS/Kg digester content were feasible, however, the methane generation decreased linearly as the TS increased.

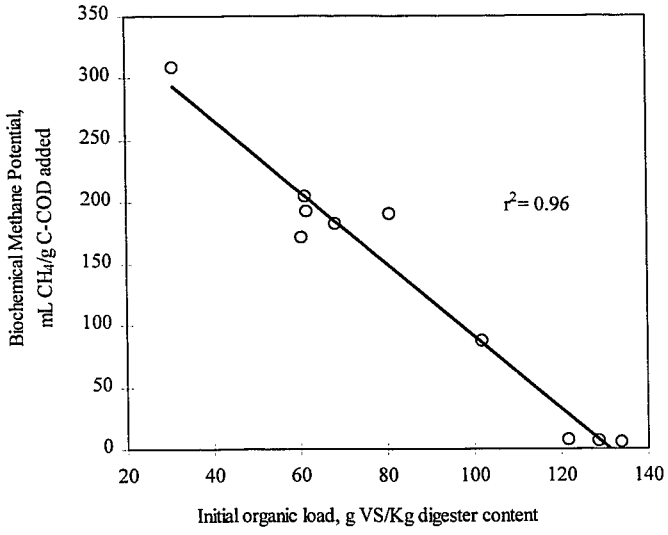

Figure 5-5. Impact of organic load on methane production
C-COD: carbonaceous chemical oxygen demand; VS: volatile solids

The loads of more than 80 g VS/Kg digester content would not be desirable. Such limitation points at two factors associated with high TS: (a) ammonia and (b) volatile fatty acids concentrations.

The extra ammonia likely generated in the very early stage of incubations could cause an early inhibition of the digestion performance. In methanogenic experiments, despite varying organic matter conversions to CH_4, the presence of phase II and significant extent of degradation indicate that a gradual acclimation of the methanogenic population to ammonia was feasible. Even though the biogas production decreased by the end of phase II, the CH_4 content in biogas remained high until the end of experiments (Fig. 5-2).

Though biogas composition with regard to methane varied after that, it was at a level higher than 65% at all times. This range of values agrees well with the methane composition from poultry digestion reported by others. The average level quoted by Aubart and Fauchille [1983] and Hobson *et al.* [1980] was 69%.

As discussed in Chapter 2, at higher VFA salt concentrations (>9g NH_4Ac/L, as HAc) any toxicity to acetate utilizing methanogens is indirectly associated with salt toxicity mainly dependent on the cations of the salts [McCarty and McKinney [1961]. The increasing levels of ammonium (NH_4^+) upon contact with the bicarbonate ion result in significant increase in buffering capacity of a digester, thus leading to an increased pH and free ammonia. Very high initial concentrations of ammonium and the presumable rapid mineralization of the N_{org} to ammonia did not allow acclimation of the highest initial solids reactors (no. 1, 4 and 7) and caused acute inhibition of methane production prevailing throughout the duration of the experiment. The remaining experimental trials

performed significantly better; only the 15.7% TS experiment was affected to a greater extent, based on methane production evidence.

As a result of increased ammonia concentration and pH, a higher portion of CO_2 would be expected to exist in the bicarbonate form (HCO_3^-). From Fig. 5-2, one can observe a consistent increase in biogas methane composition in all methanogenic reactors up to a maximum point which, in all but one experiment (no. 5, TS 15.7%), corresponded with the end of phase II. Until that point, an improved transfer of CO_2 from biogas to the semi-liquid phase in the digesters would cause even further pH increase. Such an increase could be high enough to convert even more ammonia to the free form resulting in ultimate reduction in rates of methane generation. The end of phase II in those reactors was followed by the change in biogas composition. During the next three to four weeks methane content dropped by at least 5 to 10% from the maximum value. Inhibition by UAN of hydrogenotrophic methanogens could cause an overall drop in CH_4 content, even though increased bicarbonate and resulting higher dissolved CO_2 would rather stimulate these bacteria. The fresh manure experiment started with 60% ADS as seed (no. 8) was the only one that showed gradual increase in methane composition of biogas until the end of incubation. Lowest concentration of VFA in the reactors' output by end of incubation confirmed a gradual increase in methanogenic activity. Biogas composition can indirectly indicate possible shifts in the digester operation but in this case the change in CH_4 content was already the result of such changes.

As stated before, acclimations to high total ammonia and to free ammonia concentrations are possible. Successful digestion operations quoted earlier were conducted with UAN up to 345 mg/L [Ripley *et al.*, 1985] or even twice as much [Lapp

et al., 1975] when a very long acclimation period was used. The effect of free ammonia on methane production, depicted in Fig. 5-6, clearly indicates that there is a trend of a moderately strong negative exponential form (r^2=0.78). However at UAN concentrations of up to 250 mg N/L, the relationship remained independent.

No conclusive interpretation can be made at initial free ammonia concentrations ranging from 250 mg N/L to 400 mg N/L. At some point within this range with a small increase in UAN, methanogenic activity sharply decreased. The findings of this bioassay showed that there is not an absolute threshold for methanogens at UAN of more than 150 mg N/L [McCarty and McKinney, 1961].

Both ammonium and free ammonia are inhibitory to the production of methane from hydrogen (H_2). In the research by Wiegant and Zeeman [1986], TAN concentrations above 3500 mg N/L (pH 6.7-6.9) caused failure of methanogenesis from H_2 and CO_2 resulting in hydrogen concentration increase and propionic acid accumulation. An increase in H_2 up to 100 ppm makes the conversion of propionate to acetate energetically unfavorable [Speece, 1996], which leads to propionate accumulation. Acetic acid that is considered the least toxic of all volatile fatty acids when accumulated over 3000 mg/L can reveal an acetic acid product inhibition of butyric acid [Ahring and Westermann, 1988]. Initial UAN concentrations found in those experimental digesters with the starting TS of about 10% (no. 2, 6, 8, and 9), were close to the inhibitory level of 250 mg N/L and were significantly above that level at termination of the bioassay.

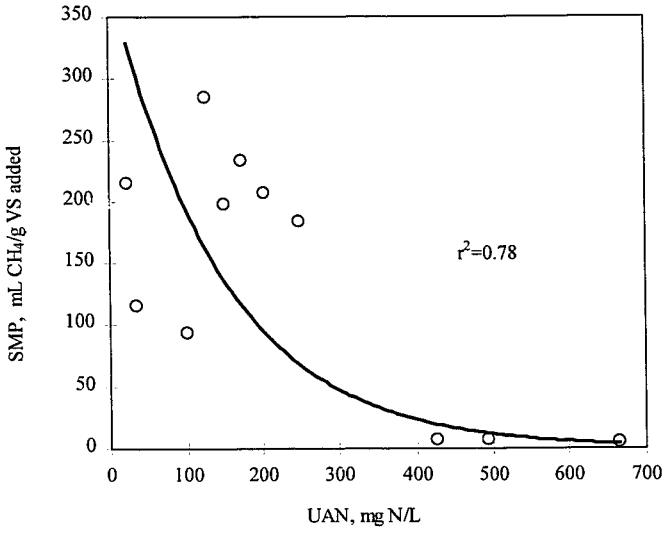

Figure 5-6. Effect of initial levels of free ammonia on specific CH_4 production
SMP: specific methane production; UAN: un-ionized (free) ammonia nitrogen; VS: volatile solids

Therefore, it is believed that although the VFA reduction occurred at such high UAN levels, a possible accumulation of H_2 over 100-ppm threshold could have caused propionic acid accumulation. Regardless of the rate of substrate inhibition in acetic acid degraders, this would ultimately lead to significant reduction of their activity in the high-solid digesters as well.

During the anaerobic breakdown of organic matter, acetoclastic methanogens produce approximately 70% of methane [Kugelman and McCarty, 1965; Jeris & McCarty, 1965; Smith & Mah, 1966]. The remainder (30%) is produced from reduction of CO_2 by H_2, and to some extent by other intermediate electron donors [Hashimoto *et al.*, 1980]. According to Kaspar and Wuhrmann [1978], 10-15% of the 70% comes from propionic acid breakdown to acetic acid and to H_2. Mah *et al.* [1990] showed that as much as 35% of the overall methane produced comes from propionic acid breakdown, with 20% accounting for direct conversion to acetic acid and the remaining 15% for oxidation to H_2. Whether H_2 build up was the reason that syntrophic bacteria could not degrade propionic acid cannot be stated here. Since the relative propionic acid concentrations in the experiments started at about 10% TS did not change that significantly but overall VFA degradation proceeded, this could indicate that the native populations of propionic acid degraders (acetogens) in the reactors were low. Koster [1986] recorded no decrease in the maximum specific methanogenic activity in the adapted sludge at U-VFA values for acetic acid of 9.7 mg/L and for propionic acid of 24.6 mg/L, but degradation of propionic acid was retarded. Inability to recover from possible initial U-VFA inhibition can be the reason for low activity of the acetogenic bacteria [Ozturk, 1991].

McCarty and Brosseau [1963] showed that propionic acid slowed the breakdown of complex organic matter to propionic and acetic acids but did not affect acetoclastic methanogens; up to 6000 mg/L propionic acid at a neutral pH was tolerated. On the other hand, Gourdon and Vermande [1987] found that propionic acid up to 6000 mg/L and at pH $\geq$ 7.4 had little or no effect on the acetogenic step but delayed substrate degradation. This could be the case of the most diluted FM experiment (no. 3, at 5% TS) where very low acetic acid but high propionic acid presence was identified, giving a ratio of HPr/HAc of 15.6. This ratio was much higher than the ratio of 1.4, suggested as the maximum allowable prior to inevitable failure of methane generation [Hill *et al.*, 1987].

The ammonia nitrogen to volatile fatty acid ratio, proposed by Georgacakis [1982], is another process stability indicator. This ratio for successful manure digester operation should remain above 0.5. In all experiments involving fresh manure, and including the pit manure control this ratio at start of incubations was very low, from 0.13 to 0.16. By the end of the experiment, this ratio was found higher than 0.5 for all but three highest solids experiments (no. 1,4, 7) and the most inhibited (with the longest lag) methanogenic experiment (no. 5). In the latter case, the increasing methane content and the final TAN/VFA ratio of 0.41 were the signs of progressing recovery from the initial organic load and ammonia inhibition.

The high initial U-VFA concentrations in highest solid reactors (no. 1, 4, 7) and in experiments with the large portion of pit manure (no. 5, 10) were at or above the toxic level of 30-60 mg/L, reported by Kroeker *et al.* [1979]. Nevertheless, by the end of incubations the only reactors with extreme U-VFA levels (from 149 to 200 mg/L) were those operating at the TS range common to fresh, undiluted manure.

At the elevated pH, the concentration of U-VFA remains extremely low, even at excessive total VFA concentration. Because of the large buffering capacity, such system is able to withstand environmental stresses that normally cause a rapid increase in the total VFA concentration. This was the case for all methanogenic experiments where final total VFA concentrations of up to 11,834 mg HAc/L were tolerated. The inhibited methanogenic reactor (no. 5, initial TS 15.7%) could still operate at 27,746 mg HAc/L (U-VFA 11 mg/L), as confirmed by satisfactory CH_4 content in the biogas. An almost doubled increase in butyric-n acid from 4,087 mg HAc/L could result from the product inhibition of degradation. The rate of butyrate degradation to acetate and hydrogen is dependent on the concentrations of the products [Kaspar and Wuhrmann, 1978]. The relative concentration of acetic acid decreased from 26,373 mg/L at start to the final value of 8,246 mg/L, but possible inhibition of hydrogen-utilizing methanogens might have led to hydrogen accumulation leading to butyric acid accumulation.

5.3.6 Conclusion

It can be concluded from this study that the digestion of the most concentrated substrates was visibly inhibited. Inhibition of the methanogenesis was directly proportional to the organic load and the resulting concentration of free ammonia. The efficiency of organic matter conversion to methane was found to decrease with increasing organic loads to the digesters. The high efficiency of organic nitrogen conversion to ammonia (TAN), ranging from 61.8% to as high as 78.9%, was achieved in most experimental digestions. This points at relative independence of this phenomenon, and

corresponding hydrolytic/acidogenic activity, from high initial ammonia or VFA concentrations.

An expected increase in methane yield with increasing influent volatile solid concentrations did not take place because of the concomitant increase in reactor ammonia and pH, leading to increased levels of UAN. Highest solid reactors suffered from extremely high VFA (>45,000 mg HAc/L) and TAN (>14,800 mg N/L) levels and digestion was limited to fermentation. High-solid anaerobic digestion of chicken manure was shown feasible at initial solids concentration up to 11% TS, despite high initial concentrations of total ammonia (up to 3,500 mg N/L), free ammonia (up to 250 mg N/L) and of VFA (up to 25,800 mg HAc/L), (Table 5-3). The accumulation of VFA was partially neutralized by alkalinity generated from ammonia yielding reactions. Chicken manure digestion at an initial TS of 15.7% was possible, although not practical, since the longest lag phase of 62 days and overall poor organic conversion efficiency to methane of 25%was noted.

Suitability of anaerobically digested municipal sludge and of pit manure to seed digesters treating chicken manure was found to be of limited value. Initial acclimation was necessary in all investigated experiments. The experiment using a mixture of 40% fresh manure and 60% anaerobically digested sludge showed the most complete digestion of all experiments in the close to 10% total solids range in terms of methane production (69% conversion efficiency) and final volatile fatty acid content. However, net conversion of the organics from fresh manure alone in this experiment was only 49%, as compared to 54% in the experiment with fresh manure digestion at 10%-TS level but without seeding.

The transformation of organic nitrogen into ammonia affected the process; methanogenesis took place at free ammonia concentrations of up to 250 mg N/L above which methane production ceased. The highest biochemical methane potential of 308 mL CH_4/g COD was found with fresh manure diluted to 5% total solids, confirming that the dilution factor has a critical effect on digester performance. Chicken manure digestibility was confirmed high but the extent of degradation largely depended on process conditions. High solids anaerobic digestion of high organic nitrogen substrates required a prolonged acclimation period. One strategy for successful initiation of chicken manure digester operation would be to gradually increase the organic load and total solid level to allow proper acclimation. Inoculation of a digester with already digested manure or other-already acclimated to high ammonia- type of seed, is another option. Svendsen and Blackburn [1986] showed reduction in degradation time from 100 to 60 days after inoculation with digested manure.

5.4 ANAEROBIC DIGESTION AT HIGH-SOLID LEVELS - ORGANIC FRACTION OF MUNICIPAL SOLID WASTE AND PRIMARY MUNICIPAL SEWAGE SLUDGE

In this phase of experimental work, anaerobic digestion of another high-solid organic substrate, the organic fraction of municipal solid waste (OF-MSW), co-digested with primary sludge (PS) was investigated. The principal difference between chicken manure and the OF-MSW is a much lower organic nitrogen and ammonia content of the MSW. Co-digestion with PS was designed to offset possible nitrogen deficiency and promote digestion that is more balanced. The effect of similar organic loading, at a level common to high-solid OF-MSW anaerobic biodegradation, but different levels of nitrogen on the methane generation was studied.

5.4.1 Reactor start up and acclimation to high-solid operation

At the start up the digesters were seeded with biomass from a slurry reactor, so called "breeder". Such mode of start-up was proven best in cases where high-solid anaerobic biomass inoculum is not available [Rivard *et al.*, 1989b]. During the first 20 days the composition of feed fed to reactors was changed by gradually (in 10% increments) decreasing the primary sludge fraction and replacing it with the final formulation of the high-solid OF-MSW & PS feed mixture, specific to the operation of each individual reactor. The target total solids (TS) level was designed to reach 30%, the optimal level for non-mixed, batch digestion [Wujcik and Jewell, 1980]. After completely switching to the high-solid feed a common routine involved feeding a feed previously

oven-dried overnight at 70°C. This operation aimed at a faster TS increase in reactors, as compared to using the fresh feed (at 30% TS) instead. Efficient hydrolysis and fermentation of complex organic substrates was achieved by ensuring that the MRT_{in} was at least 30 days. High retention times, in excess of 20 days, ensure that substantial, up to 80% at 37°C, cellulose biodegradation takes place [Vinzant *et al.*, 1990]. The weight of digestate to be wasted each time for biomass retention control was actually less than the required amount (one-fifteenth of the reactor content every two days to keep MRT_{in}=30 d) due to the increasing, significant production of biogas. In high-solid anaerobic digestion the biogas production is much higher than in slurry digestion and the assumption of equal inflow and outflow rates is incorrect. The approach of using mass units to define retention time [Kayhanian *et al.*, 1996] was justified as the method that better approximates the actual retention. At times, the weight loss due to biogas was more than the weight of the oven-dried feed but the designed load of 7.7 g BVS Kg^{-1} reactor mass d^{-1} was still maintained using the fresh feed. Feeding using the dried feed could be then resumed. Even though primary sludge quantities in the OF-MSW feed varied, the BVS concentration was similar for all feed mixtures (Table 5-6). The organic loading rate to all digesters was, therefore, also same.

Table 5-6. Estimation of feed biodegradability based on acid insoluble lignin content.

Feed type/component	TS	VS	AIL	BVS	BVS
	%	% TS	% VS[b)]	% VS[c)]	% TS[c)]
Office paper (OP)	95.4[b)]	84.5[b)]	0.6	99.4	84.0
News paper (NP)	93.2[b)]	97.3[b)]	21.0	79.0	76.8
Yard waste (YW)	92.4[b)]	87.0[b)]	4.8	95.2	82.8
Food waste (FW)	33.8[b)]	94.8[b)]	1.3	98.7	93.6
100%[a)] OF-MSW	29.8[b)]	89.3[b)]	13.1	86.9	77.6
95%[a)] OF-MSW + 5%[a)] PS	30.0[c)]	89.0[c)]		87.6[d)]	77.6
90%[a)] OF-MSW + 10%[a)] PS	30.0[c)]	89.0[c)]		88.2[d)]	77.6
85%[a)] OF-MSW + 15%[a)] PS	30.0[c)]	89.0[c)]		88.9[d)]	77.7

TS: total solids; VS: volatile solids; AIL: acid insoluble lignin; BVS: biodegradable fraction of the volatile solids; OF-MSW: organic fraction of municipal solid waste consisting of 54% NP, 18% OP, 17% YW, and 11% FW, on dry weight basis; PS: primary sludge; [a)] – fraction of dry solids; [b)] – measured value; [c)] – calculated value; [d)] – PS total solids were 3.8% and volatile solids, assumed fully biodegradable, were 78.1% TS

The specific methane production (SMP) rates recorded during this phase, and later in high-solid digester operation, are plotted in Fig. 5-7. The reported values are averages of SMP for each of the four sets of reactors, based on the amount of PS solids in the high-solid OF-MSW feed, from 0% to 15% dry solids. There was no significant difference in SMP rates among the reactors fed feed with same level of PS solids, even though three different particle sizes were used to prepare the feed. The reason for this correlation lies in the fact that the specific weights of these feed mixtures were essentially the same (Table 5-2), resulting in equal water activity and provision of organic substrate to microbial populations. This apparent similarity among SMP in reactors in each set justified the decision to treat these reactors as triplicates for data processing, with the following character representation: PS-15 (15% primary sludge solids in feed to reactor), PS-10, PS-5 and PS-0. The inherent heterogeneity of the feed material was relatively low, allowing for similar methane production observed under similar operating condition.

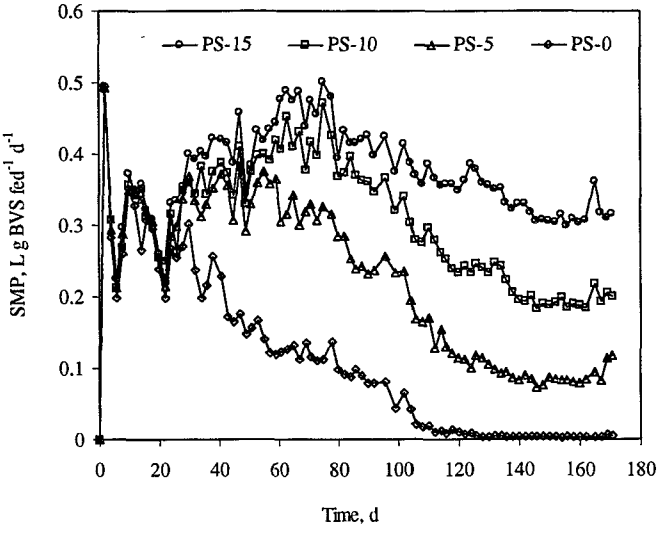

Figure 5-7. Profile of specific methane production rates in digesters at four different levels of primary sludge solids in feed

Each point represents the mean of 3 observations from 3 reactors fed same amount of primary sludge; the maximum standard deviation was 0.015;

STP: standard temperature and pressure; SMP: specific methane production; BVS: biodegradable volatile solids

Gradual build-up of the solids in reactors resulted in more variation among the parameters of interest. The average SMP in both PS-10 and PS-15 sets of reactors increased from 0.3 up to 0.5 L CH_4 g BVS^{-1} d^{-1} in the first 80 days. In the same period, the PS-5 reactors had a stable SMP of around 0.3 L CH_4 g BVS^{-1} d^{-1}. In contrast, a gradual decrease from 0.3 on day 30 down to 0.1 L CH_4 g BVS^{-1} d^{-1} was seen in all PS-0 reactors (fed OF-MSW alone). Such patterns were observed throughout the period of bringing reactors to high-solid operation. Methane production varied during acclimation time due to changes in quality of the feed VS, i.e. from composition typical of primary sludge to the high-carbohydrate OF-MSW. The biomass used for seeding reactors was acclimated to an organic load of 3.8 to 4.2 g VS L^{-1} d^{-1}. However, to accommodate change in substrate characteristics due to moving from cellulose-supplemented primary sludge feed to the OF-MSW feed, this load was decreased to 1.0 g BVS Kg^{-1} d^{-1} at start, and then gradually increased up to 7.7 g BVS Kg^{-1} d^{-1} by day 20.

The effort of minimizing the impact of substrate change and increasing organic load to digesters was not successful in all PS-0 reactors. The approximate time it took to reach the ultimate TS was 82-92 d for the PS-0 set (30% TS), 92-99 d for PS-5 (27.8% TS), and 99 d for PS-10 (27.4% TS) and PS-15 (26.0% TS) sets.

During the time of bringing solids level to its final level, alkalinity and pH were closely monitored to avoid any excessive buildup of acidity, which could result in excessive alkalinity consumption for buffering organic acids and consequent decrease in pH to below 6.5, the minimum value in the pH range considered optimal for CH_4 production [Speece, 1996]. The PS-0 reactors, fed the feed with no primary sludge solids, were more prone to pH upsets and alkalinity ($NaHCO_3$) administration started early in the

operation. Addition of $NaHCO_3$ was on intermittent basis, but controlled to not exceed sodium levels considered toxic to methanogens if applied without prior acclimation, i.e. 7 g Na/L [Anderson *et al.*, 1982]. Despite pH neutralization and addition of alkalinity the PS-0 reactors failed to maintain the high initial methane productivity. In addition, completeness of the sodium bicarbonate powder dissolution decreased with increasing solid level in digester, which practically limits its capacity to manage alkalinity.

The initial lag in biogas production of six days from start was followed by an increased CH_4 yield in all reactors. Since the organic load to all digesters was almost doubled in three weeks after start, such substrate boost increased rates of methane generation in all but PS-0 reactors, and continued further into high-solid operation. The patterns of cumulative net methane production in Fig. 5-8 show that highest CH_4 yields from respective reactors continued until day 36 (PS-0), 66 (PS-5), 86 (PS-10), and until day 99 for the PS-15 reactors. These methane yields were solely from the decomposition of the OF-MSW, corrected for background methane produced from primary sludge degradation, as measured in the control reactor that had no added OF-MSW.

One can consider a complete turnover of the biomass in each reactor after one MRT (30 d) and the fact that the period of switching from the primary sludge to the high-solid feed ended by day 20. In such case, the declining CH_4 production in PS-0 reactors early in operation, i.e. after day 36, was the result of inhibition due to both organic overload and depleting nitrogen content through feeding the high C/N feed (Table 5-2). In all other cases, the eventual decline in CH_4 productivity (days 66 through 99) was due to reasons other than organic overloading.

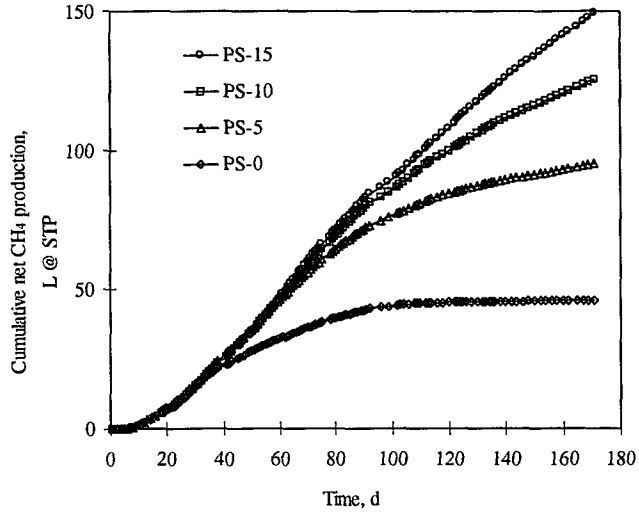

Figure 5-8. Effect of the level of primary sludge in the feed on cumulative net (from the OF-MSW alone) methane production

Each point represents the mean of 3 observations from 3 reactors fed same amount of primary sludge; The final standard deviation was 3.3, 2.1, 2.9, and 2.8 for PS-15, PS-10, PS-5, and PS-0 data, respectively;

STP: standard temperature and pressure; OF-MSW: organic fraction of municipal solid waste

The lack of vitamins and minerals, as a possible cause of limited digestion in PS-0 reactors, was verified by the addition of these media to the reactors on day 60 from start. Nutrient limitations have an impact on cellulose degradation [Rivard *et al.*, 1989]. All typical symptoms of nutrient deficiency, such as lower CH_4 production, increased carbon dioxide content of biogas, high VFA, and deteriorating pH [Kayhanian *et al.*, 1991], were apparent in these reactors.

Mineral and vitamin solutions, prepared according to Sparling [1988], were added directly to the reactors, and not to the feed, to ensure their long-term effect. This cause of poor digester performance was finally excluded, as it did not result in any significant increase in biogas production or improved stability.

Once all the reactors reached the maximum achievable TS of around 26 to 30%, the addition of fresh feed was permanently resumed. The period of bringing all reactors to a high-solid operation lasted about 3 months. The actual solids levels in reactors with high methane yields never reached 30% TS because of the rapid volatile solids destruction, similar to the observation by Rivard *et al.* [1990].

5.4.2 High-solid digester operation

The designed solids loading rate of 7.7 g BVS Kg^{-1} reactor mass d^{-1} was quite high, but there was no attempt to determine whether this was the maximum attainable loading. The maximum rates for mixed high-solid OF-MSW systems reported elsewhere were 7.2 g BVS Kg^{-1} reactor mass d^{-1} [Kayhanian *et al.*, 1991], 9.5 g VS Kg^{-1} reactor mass d^{-1} [Rivard *et al.*, 1990], and up to 14.0 g VS L^{-1} reactor volume d^{-1} [Rivard *et al.*, 1993]. Similar organic loads of up to 9.36 g VS Kg^{-1} reactor mass d^{-1} were reported for

both mixed and non-mixed high-solid OF-MSW and wastewater sludge co-digestion [Stroot *et al.*, 2001]. In addition, the non-mixed condition proved to be more stable of the two regimes. The above findings suggest that the organic loading used here was close to the upper limit for successful digester operation, and that non-mixing operation could actually enhance stability of the process. Feed composition is known to affect the maximum loading rates [Rivard, 1993]. Although the maximum loading for the system was not established, different levels of primary sludge in the feed and under similar loading condition had distinct effect on the digestion performance.

Summary of the digester characteristics during high-solid operation is given in Table 5-7. Most significant BVS reductions were obtained for reactors fed organic substrate containing highest fraction of primary sludge solids, whereas decreasing destructions correlated with smaller fractions of PS solids. Lignin makes up the highest portion of refractory volatile solids that are recalcitrant to biodegradation. It actually artificially increases the biodegradable portion of the waste and has to be subtracted from the total volatile solid concentration. The BVS for the feed mixtures were in the range of 86.9 to 88.9% VS, and around 77.6% TS (Table 5-6). The lignin method as the way of predicting BVS fraction of VS gives only rough estimate. Kayhanian [1995] showed that the BVS fraction of OF-MSW was actually smaller by the factor of 0.82 as determined by pilot digester operation than that estimated by the lignin method or long-term anaerobic biodegradation batch tests. Therefore, the volatile solids destruction rates obtained in this experiment might have been actually higher than those estimated from the lignin content.

Table 5-7. Reactor characteristics during high-solid operation (days 106-172, 2 months)

Feed type/ parameter	Status*	Range	Mean (Std. dev.)
85%[a] OF-MSW + 15%[a] PS			
TS, %	S	24.81-27.36	25.83 (0.71)
BVS removal efficiency, %	↓(18)S	51.1-66.3	56.3[c] (4.5)
pH	S	7.61-8.04	7.86 (0.11)
TAN, mg N/L	S	1495-1983	1752 (126)
UAN, mg N/L	S	91-232	149 (34)
VFA, mg HAc/L	S	677-1301	977 (174)
U-VFA, mg HAc/L	S	0.6-1.2	0.8 (0.2)
90%[a] OF-MSW + 10%[a] PS			
TS, %	S	26.88-29.40	27.80 (0.54)
BVS reduction, %	↓(18)S	31.3-52.1	35.5[c] (3.9)
pH	S	7.22-7.94	7.56 (0.21)
TAN, mg N/L	S	1231-1549	1378 (88)
UAN, mg N/L	S	29-151	67 (34)
VFA, mg HAc/L	↑(18)S	1121-2901	2439[c] (259)
U-VFA, mg HAc/L	↑(18)S	1.1-7.3	4.4[c] (1.4)
95%[a] OF-MSW + 5%[a] PS			
TS, %	S	26.37-29.75	28.74 (0.72)
BVS reduction, %	↓	12.3-33.2	-
pH[b]	S[b]	6.54-7.59	6.88 (0.23)
TAN, mg N/L	S	849-1131	976 (72)
UAN, mg N/L	S	4.4-40	10.6 (7.4)
VFA, mg HAc/L	↑	2,099-10,823	-
U-VFA, mg HAc/L	↑	9.4-122	-
100%[a] OF-MSW			
TS, %	S	29.37-31.38	30.25 (0.48)
BVS reduction, %	↓	0.3-7.4	-
pH[b]	S[b]	6.11-6.96	6.53 (0.21)
TAN, mg N/L	S	383-655	532 (76)
UAN, mg N/L	S	0.8-5.8	2.5 (1.3)
VFA, mg HAc/L	↑	11,131-27,100	-
U-VFA, mg HAc/L	↑	78-783	-

OF-MSW – organic fraction of municipal solid waste; PS – primary sludge; [a] – fraction of dry solids; HAc – acetic acid; [b] pH was controlled at >5.8 by $NaHCO_3$ addition; [c] mean for the steady-state period only; * indicates trend for a given parameter: ↑ - increasing trend; ↓ - decreasing trend; S – steady-state value; ↓(18)S - decreasing for 18 days and then steady-state; data for TS, pH, TAN, UAN, VFA, U-VFA are the mean of 8 observations; BVS reduction=100 (Kg BVS removed Kg BVS fed^{-1}), %, where BVS removed is based on the mass of dry CH_4 from anaerobic degradation (corrected for water uptake).

The rate of BVS reduction and corresponding methane production are also generally slower under non-mixing condition. A near complete digestion at mesophilic temperatures would require months whereas under thermophilic regime may be as short as weeks [Rivard *et al.*, 1989b].

A closer observation of both Fig. 5-7 and Fig. 5-8 points to a certain degree of reduction in biological conversion rates on days 80 through 100. This was a critical time during digester operation in that most digesters reached their maximum solid levels. Decreasing slope of the cumulative net CH_4 production lines indicates this transition period. At this point from start any change in digestion performance would be largely attributed to the type of feed, rather than influence of the original biomass due to a 30-day turnover. Progressing instability further into high-solid operation was observed in PS-5 and in PS-0 reactors. Second attempt to correct for possible vitamin and/or mineral deficiency in PS-0 reactors on day 99 did not bring about any improvement. Addition of vitamin and mineral solutions to all other reactors at same time was also non-stimulatory, with no significant impact on either CH_4 yield or VFA concentration in the effluent.

Particle size reduction of the feed is believed to improve the organic substrate conversion in any biodegradation process [Barlaz *et al.*, 1990]. The density or compaction of the feed is a function of particle size and increases with the decreasing particle size. In this experiment, the feed density was meant to be adjusted by using different sizes of its two components, newspaper and office paper. The amount of primary sludge rather than surface area of the paper components was found to influence the specific weight (SW) of the mixtures (Table 5-2). Because of a similar specific weight of feed mixtures containing equal amount of PS solids, regardless of the surface

area of paper components of the feed, it was expected, and later confirmed, that biogas production among respective reactors would not differ significantly.

Effect of particle size on gas production was researched by Kayhanian *et al.* [1991] and Hills and Nakano [1984]. In the first study, biogas production increased by 25% when the office paper particles had their diameter reduced from 215 mm to 41 mm. Similar results were obtained in the second quoted work, where anaerobic digestion was performed using tomato solid wastes as substrate. Particle size influences degradation rates in that it increases or decreases the compaction of the feed material. Feed specific weight, or density, has an impact on the density of biomass in biological reactor, which leads to different ratio of surface area to mass of the reactor contents. The surface areas of the paper component of 0.17, 0.38, 1.13 cm^2 used in this experiment corresponded to the particle size of 4.6, 7.0, and 12.0 mm in diameter, respectively. They were well below the lowest particle size investigated in the quoted works and no direct comparison can be made. Nevertheless, it was expected to see the continuation of their trend in the narrow Ø 4.5-12.0-mm range. In another work by Hamzawi *et al.* [1999], the range of particle sizes under investigation was from 0.8 to 8.0 mm, and the digestions were conducted at a broad total solids concentration range from 7.9 to 22.1%. Both experimental an empirical data were indicative of enhanced performance through particle size reduction. Our results show very little if any dependence of the digestion rates on particle size of the paper components. It was the primary sludge that influenced the actual density of the feeds and consequent biodegradation profiles.

Fermentation results in production of volatile fatty acids (VFA) and consequent pH decrease. When uncontrolled, this phenomenon may hinder methane formation in the

terminal stage of anaerobic digestion. The VFA concentration data (Table 5-7) were indicative of the degree of acidogenesis and acetic acid production, as well as its utilization by methanogenic bacteria. Extremely high VFA in PS-0 reactors were responsible for termination of CH_4 production. Biogas quality started to deteriorate early in operation and by the time high-solid level was reached the CH_4 content in biogas dropped to 25%. Fig. 5-9 shows the decreasing trend of VFA, expressed as % soluble chemical oxygen demand (S-COD), with increasing fraction of primary sludge solids in the feed. The suitable range for degradation to CH_4 and CO_2 is below 25% VFA as COD. Small proportion of VFA in S-COD of less than 25% demonstrated feasibility of high solids digestion with 10% and 15% primary sludge solids in the feed. A similar study [Borzacconi *et al.*, 1997] of batch digestion of the OF-MSW yielded values of 25 to 35% VFA in the output COD as those causing decline of CH_4 production.

Inhibition of acetogenesis (acetic acid formation from higher organic acids) was visible in high VFA/COD reactors (PS-0, PS-5) and was due to the accumulation of un-ionized volatile fatty acids (U-VFA), as the result of decreasing pH. The U-VFA values observed in these reactors were in the range from 9.4 to 783 mg/L as acetic acid (HAc). Kroeker *et al.* [1979] reported acclimations of bacterial populations to un-ionized VFA in the range of 30-60 mg HAc/L, above which retardation of higher VFA degradation to acetic acid was noted. Acetic acid, or acetate, is the major substrate for methane production accounting for as much as 70% of methane [Kugelman and McCarty, 1965; Jeris and McCarty, 1965; Smith and Mah, 1966]. The remainder, 30%, comes from reduction of CO_2 by H_2, and to some extent by other intermediate electron donors [Hashimoto *et al.*, 1980]. Acetate was the major VFA produced from highest primary

sludge amended feeds and it was easily converted to CH_4, as indicated by low levels of VFA/S-COD (Fig. 5-9). Greater quantities of propionic acid and higher volatile fatty acids (butyric, valeric) were recorded in the PS-10 reactors that had equal, and at times higher, proportion of propionic acid over acetic acid. According to Kaspar and Wuhrmann [1978] 10-15% of the 70% comes from propionate breakdown to acetate and to H_2. Mah *et* al. [1990] have shown that up to 35% of the overall methane produced comes from propionate breakdown, with 20% accounting for direct conversion to acetate and the remaining 15% for oxidation to H_2. In the PS-5 reactors, the major acids present were propionic and butyric acids. Accumulation of propionic acid in these reactors points inhibition of acidogenic bacteria responsible for conversion of propionate to acetate. This in turn leads to poor CH_4 production or cessation of methanogenesis due to less favorable thermodynamic conditions of increased partial pressure of H_2 and consequent inhibition of methanogens. In the PS-0 reactors, the addition of fresh feed was providing a continuous supply of organics that, once broken down by hydrolyzing bacteria, were easily converted to VFA.

Gradual VFA build-up in the reactors fed no primary sludge resulted in pH values of 6.2 and lower. Alkalinity addition to the reactors to offset acid accumulation provided only temporary buffering and did not allow recovery of the reactors to reach methanogenic stage.

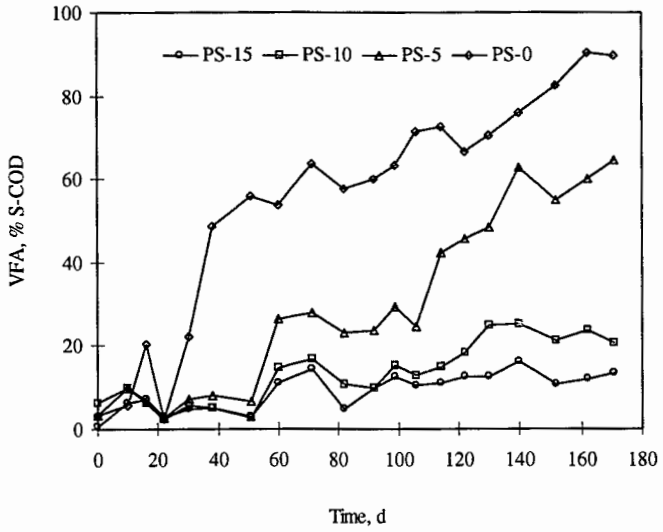

Figure 5-9. Relative volatile fatty acid share in the overall, readily biodegradable, carbon balance as indication of digestion efficiency

Each point represents the mean of 3 observations from 3 reactors fed same amount of primary sludge; the final standard deviation was 1.1, 2.4, 2.9, and 2.3 for PS-15, PS-10, PS-5, and PS-0 data, respectively;

VFA: volatile fatty acids; S-COD: soluble chemical oxygen demand

Nitrogen in the organic matter gets easily converted to ammonia and is biochemically available whereas not all of the carbon is biodegradable. The original C and N concentrations to determine the C/N had to be adjusted to account for the non-available carbon found primarily in the lignin. The C/N ratios (Table 5-2) clearly show that the level of N was a critical parameter. With increased N value, the biochemical conversion of the feed was higher.

In the most productive reactors with the highest proportion of primary sludge solids in the feed, ammonia concentrations were significantly higher as compared to other reactors (Table 5-7). The low PS reactors, in turn, had much lower ammonia content, which was linked to insufficient concentration of nitrogen to stimulate methane production.

Conversion of organic nitrogen to ammonia during hydrolysis of organic matter under anaerobic condition proceeds readily and ammonia is one of the major products in this process. This nutrient, essential to anaerobic bacteria, was present in digester output in wide range from almost 2,000 mg N/L for PS-15 reactors to 532 mg N/L in PS-0 reactors (Table 5-7). Kayhanian *et* al. [1991] demonstrated optimum performance of their high-solid reactor at ammonia concentration in the range of 650 to 800 mg N/L, with only 50% of the optimum performance observed at both tails of the curve marking 300 mg/L and 1500 mg/L levels. The results of this study indicate that insufficient ammonia content of the OF-MSW feed mixture with no primary sludge was one of the major reasons for the failure of PS-0 reactors. On the other hand, the highest portion of primary sludge solids gave much higher ammonia levels and one would expect only limited, around 50% performance, which was not the case in this study however. These (PS-15) reactors gave

highest methane yields (Fig. 5-7). It appears from this study that, as long as ammonia levels stay above 1230 mg N/L, the digestion course will yield sufficient CH_4 quantity indicative of healthy performance. Ammonia levels in anaerobic reactors closely correlated with Carbon-to-Nitrogen ratios (Table 5-2) of the corresponding feeds and were higher with decreasing C/N values.

Increasing the fraction of primary sludge solids in the feed from 15% to a higher level would likely result in TAN concentrations in excess of 2,000 mg N/L. This, in turn, could lead to free ammonia toxicity of the methanogenic stage. Free ammonia, when compared with ammonium ion, is recognized as more toxic of the two species to bacterial metabolism [Kroeker *et al.*, 1979]. Free ammonia concentration increases with the increasing temperature and pH. A one-tenth variation of pH unit within the exponential part of the curve described by equation (4-1) causes about 5% change in NH_3 concentration. The inhibiting effect of NH_3 is exerted because of its capability to diffuse into the bacterial cells. The range of values reported to inhibit anaerobes, including methanogens, starts from 150 mg N/L [McCarty and McKinney, 1961]. Another threshold of 500 mg N/L for unacclimated methanogens was given by Lay *et al.* [1998]. Digestion operations conducted at extremely high NH_3 concentration were reported; one with up to 345 mg N/L [Ripley *et al.*, 1985] or even twice as much but with a very long acclimation period [Lapp *et al.*, 1975].

Free ammonia levels in PS-15 reactors were already at or above the inhibitory level of 150 mg N/L. It is possible that the reduced CH_4 production in PS-15 digesters during high-solid operation, as compared with methane yields at lower solid levels was a direct consequence of the high NH_3 concentration. Since 15% primary sludge inclusion in

the feed was the highest used in this study, it is indeed difficult to fully validate this conclusion.

With every increment in PS fraction in the feed to high-solid digesters, the increasing trend in net ultimate CH_4 yield (Fig. 5-7) and in BVS reduction (Table 5-7) is quite apparent. This trend is not linear however, as seen through comparison of the methane yield data (Fig. 5-10). Similar trend can be seen when plotting average concentrations of un-ionized ammonia at steady-state digester operation against the CH_4 values (Fig. 5-11). The probable NH_3 inhibition in PS-15 reactors can, therefore, be further elucidated. This decreasing trend in CH_4 production is unlikely to be caused by complete consumption of the BVS, since this condition was not achieved in any of the digesters.

Adjustment of the feed C/N ratio and dilution of reactor contents with water were given as remedial actions to suppress possible inhibition due to high NH_3 concentration in high-solid digestion [Kayhanian, 1999]. These remedial actions were not undertaken in this experiment. Process optimization through dilution of digester solids and/or addition of carbon-rich material is still to be pursued.

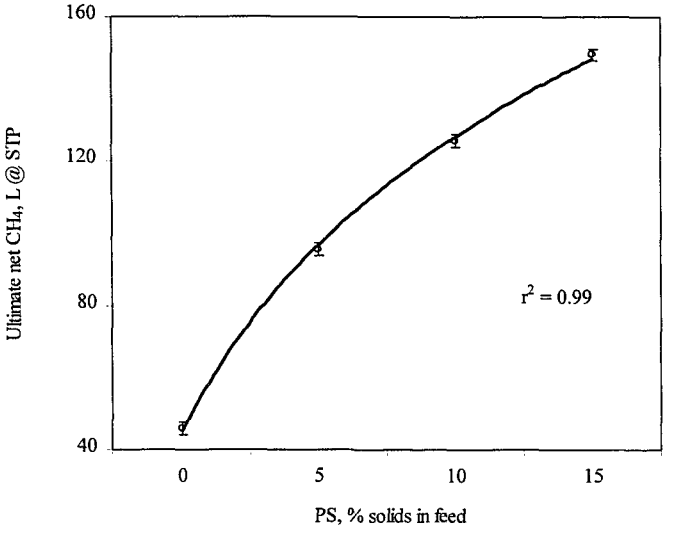

Figure 5-10. Effect of primary sludge solids in the feed to high-solid digesters on the ultimate CH_4 yield (from conversion of the OF-MSW alone)

Each point represents the mean of 3 final CH_4 yield observations from 3 reactors fed same amount of primary sludge; STP: standard temperature and pressure; OF-MSW: organic fraction of municipal solid waste; PS: primary sludge

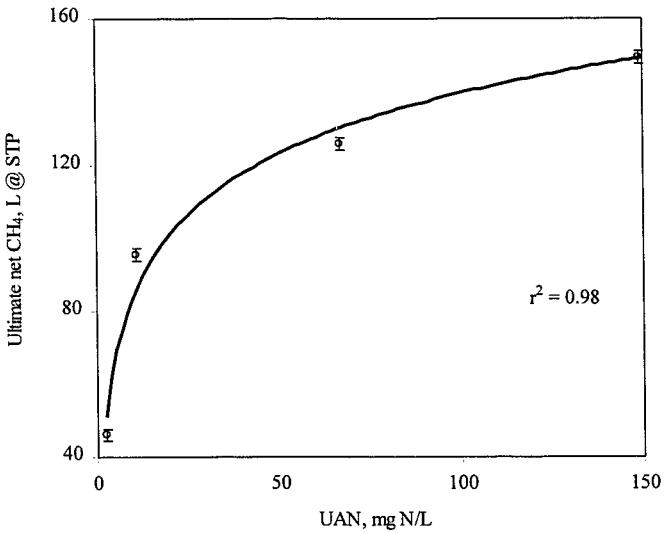

Figure 5-11. Impact of the un-ionized ammonia concentration on the net ultimate CH_4 production during steady state, high-solid digester operation

Each point represents the mean of 3 final CH_4 yield observations from 3 reactors fed same amount of primary sludge;

UAN: un-ionized (free) ammonia nitrogen; STP: standard temperature and pressure

5.4.3 Conclusion

The findings reported here point to the importance of primary sludge inclusion in the OF-MSW feed to high-solid digesters. Since primary sludge is a source of pathogenic organisms that are not significantly reduced in mesophilic digestion, an additional treatment for the high-solid anaerobic residue is required to meet final disposal regulations. The following observations summarize this research:

The highest net (from degradation of the OF-MSW alone) methane yield was achieved in reactors fed a feed with highest portion of primary sludge.

Specific methane production rates of 0.3 to 0.5 L CH_4 g BVS^{-1} d^{-1} corresponded with BVS conversion efficiency of 50 to 66%.

Surface area of the paper component in the high-solid feed of 17 mm^2, 39 mm^2, and 1.13 mm^2, had no effect on the specific weight of the feed but the amount of primary sludge solids had. As a result, reactors fed the feed with different particle size but equal amount of primary sludge had similar methane yields. If a feed to high-solid digesters has a varying particle size but in a similar range, the likely observation would be to see no significant impact on the process performance.

The most productive digesters could tolerate high concentrations of un-dissociated ammonia (NH_3). However, the evidence of decreasing ultimate CH_4 production with increasing fraction of primary sludge in the feed and NH_3 concentration shows the impact of the free ammonia on the extent of BVS reduction.

VFA accumulation in digesters fed the OF-MSW alone points limitation of this experiment. The C/N ratio of the feed was too high to promote stable digestion.

5.5 DISINFECTION OF DEWATERED SLUDGE USING CLOSED-SYSTEM, LOW-DOSE ALKALINE TREATMENT AT 20-22°C

5.5.1 Effect of lime on reduction of indicator and pathogenic microorganisms (stage I)

Lime (CaO) was added to dewatered sludge (TS 31.4-31.7%) at doses of: 20, 30, 60, 120, 150, 240, and 480 g/Kg TS (dry weight of sludge solids). These doses, when expressed on wet weight basis, were: 6.3, 9.5, 19, 38, 47, 76, and 152 g/Kg sludge. Contained storage followed for a period of 6 months, during which indicator bacteria (fecal coliforms, *Salmonella* sp., and spores of *C. perfringens* sp.) were tested to assess the degree of disinfection in treated sludge.

5.5.1.1 pH patterns in treated sludge

Addition of lime to the dewatered sludge resulted in a very rapid increase of pH (Fig. 5-12). The highest initial pH values were attained within 15 minutes of lime addition. The rate of pH increase was lower for the two smallest lime doses of 20 and 30 g/Kg TS. At doses of 60 g/Kg and higher the rate of pH increase was independent of the amount of CaO added. In these treatments, the average pH value reached after first 15 minutes was 12.4. Temperature increase in all treatments was insignificant. At the highest dose of 480 g/Kg any heat formed was dissipated due to a 10-min long mixing.

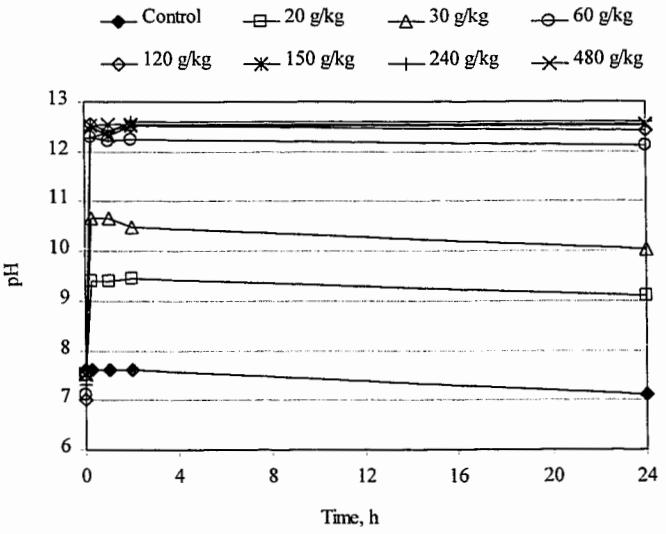

Figure 5-12. pH increase in lime-treated, dewatered sludge during first 24 h of contained storage at 20-22°C

At 120 g/Kg TS and above a pH of at least 12 persisted for the duration of the storage, i.e. 6 months. During this storage, the average pH values noted for the respective lime treatments were 12.4 (120 g/Kg TS), 12.5 (150 g/Kg TS), 12.5 (240 g/Kg TS), and 12.5 (480 g/Kg TS).

Christy [1990] and Burnham [1992] reported the maximum pH attainable in lime treated biosolids to be 12.5, which is in agreement with the results of this study. Clearly, there is a limit to the pH level that can be reached following lime addition. Extra lime addition would not increase the pH significantly and therefore would have little or no effect on pathogen inactivation. This limit is determined by the slight solubility of final product, $Ca(OH)_2$, following lime (CaO) reaction with water. The solubility product constant of the calcium hydroxide, Ksp, is 1.4×10^{-6} at 25°C. Therefore, the final pH obtained would be equal to about 12.15. The reactions occurring in dewatered biosolids are much more complicated than those taking place in water alone. Other lime reactions, both with inorganic constituents such as calcium, phosphorus, and carbon dioxide, and with organic constituents, including fatty acids and fat, occur also [WEF and ASCE, 1998]. Lime stabilization of digested sludge would mainly involve the former constituents, as organic compounds would have already been mineralized. Additional lime consumption to accommodate the above reactions would obviously affect the required lime dose.

Anoxic storage regime used in this research allowed prolonged periods of high pH maintained from the beginning, as shown in Figure 5-13. Gradual decrease in the pH was apparent in case of the 20, 30 and 60 g/Kg and was inversely proportional to the amount of lime added. The 20-g/Kg treatment showed very rapid pH decline taking anywhere

between 6 to 44 days to reach the level similar to the untreated solids, or the "Control" treatment. The 20-g/Kg treatment was later excluded from further testing. During the first 24 days in the 60-g/Kg treatment a gradual decrease from pH 12.4 to 11.1 took place. The average pH of 11.1 was maintained until the end of experiment. The pH in 30 g/Kg TS biosolids showed a decrease from the initial 10.7 to 9.6 during the first 15 days, after which it further declined to the final value of 8.5. The overall balance of pH in depicted in Fig. 5-14.

Several reasons can be given for the decline observed. Once the pH drops below a certain level, the bacterial action would start again thereby leading to the production of organic acids and CO_2 and subsequent further pH decrease. For example, during routine monitoring of *C. perfringens* spore density in the biosolids where gradual pH decline was observed (30 and 60 g/Kg TS) it was found that other, spore forming bacteria more resistant than *C. perfringens* were present. Spore forming non-sulphite reducing bacteria (white colonies on TSC agar) were noted. Slow reactions between lime and sludge solids containing the aforementioned constituents are given as a second reason, besides the bacterial action, for gradual pH decline in these treatments. The consumption of residual alkalinity by atmospheric carbon dioxide is excluded from this typical balance as the treated sludge was packed and there was no free headspace left in storage containers.

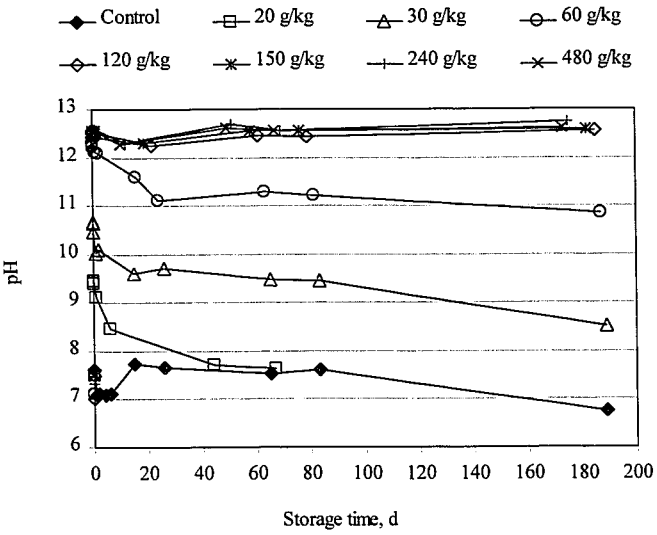

Figure 5-13. pH patterns in lime-treated, dewatered sludge during the entire course of contained storage at 20-22°C

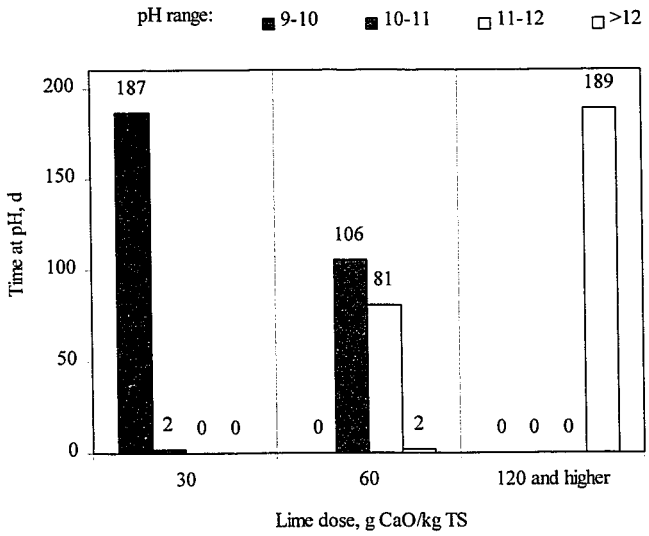

Figure 5-14. Time-dependent distribution of pH in lime-treated, dewatered sludge stored at 20-22°C

5.5.1.2 Reductions in levels of indicator and pathogenic bacteria

Fecal coliform monitoring over the duration of the anoxic storage demonstrated effective reductions of these bacteria in all treatments. The effects of lime addition, expressed as the $\log_{10}$ of the survival ratio (N_T/N_0) or (($MPN/g)_T$ / $(MPN/g)_0$), of the fecal coliform bacteria are presented in Figure 5-15. The average initial fecal coliform density in biosolids prior to lime addition was 4.02×10^7 MPN/g TS (1.87×10^7 CFU/g TS). In the first 15 min, the rates of fecal coliform reduction to obtain at least 5-log inactivation varied and, for the lowest CaO doses (30 to 120 g/Kg), were proportional to the amount of lime added. Further reductions occurred at lower rates, but eventually reached an 8-log level. For the lowest lime dose of 20 g/Kg TS, the fecal coliform decline was tested using the direct plating method. A lower limit for quantitative determination allowed for only 3-log reduction to be observed anywhere between 9 and 24 h from start of the storage.

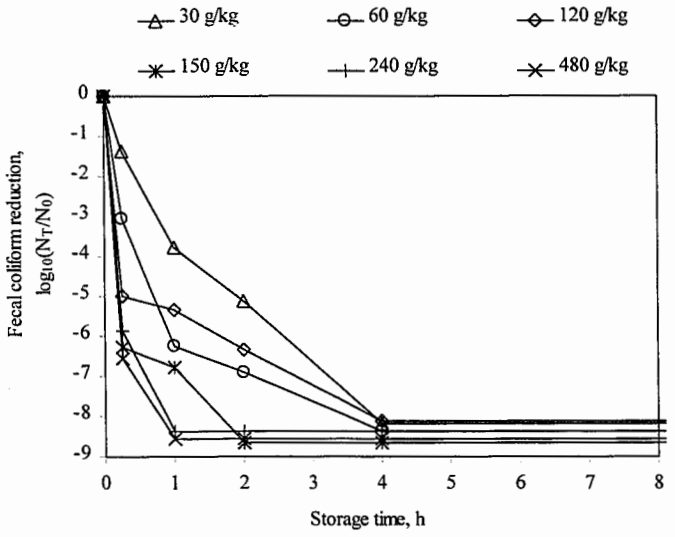

Figure 5-15. Reduction in the density of fecal coliforms in lime-treated, dewatered sludge during first hours of contained storage at 20-22°C

These significant inactivations, well below the Class A biosolids requirement of less than 1000 MPN/g TS (or, for these particular densities in sludge, a minimum of 6 logs), have been achieved within first 4 hours of storage and confirmed after 1 day of storage. Fig. 5-16 illustrates storage time predicted for the required reduction below Class A limit. Points at which trendlines (corresponding with respective times) intersect the line described as "Class A" indicate the minimum time for the corresponding lime dose needed to meet the Class A requirement. Graphical representation shown in Fig. 5-17 is an alternative way of predicting time and dose required to effect disinfection. A common engineering practice of using product of disinfectant concentration and contact time (C×T) offers a quick and practical way of estimating these parameters to obtain anticipated reductions. The C×T values necessary to obtain Class A biosolids product under the specified conditions of storage would be 100 g h/Kg TS and higher, with the minimum effective lime dose of 30 g/Kg TS.

Testing during subsequent storage, with the inclusion of the presence-absence (P-A) test, showed no fecal coliform presence in any of the lime treated biosolid samples. In the last two testing events the neutralized biosolids slurry (10^{-1} dilution) was left at room temperature for up to 4 days and the P-A test preformed once again to see if there was regrowth. In all cases there was no fecal coliform reoccurrence.

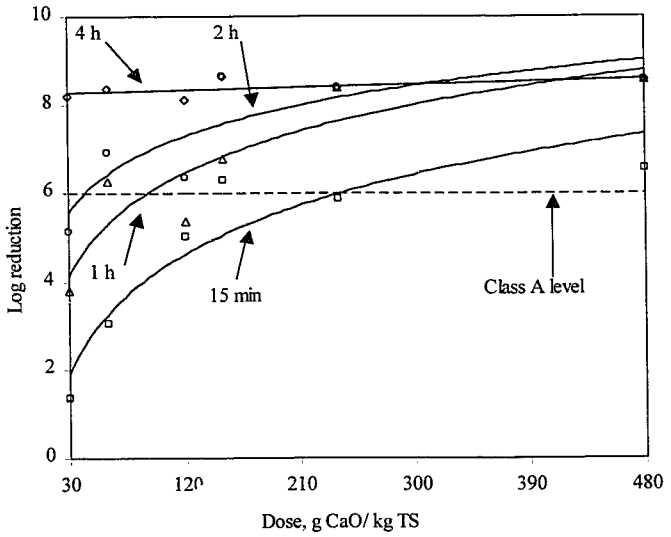

Figure 5-16. Predicted times to inactivation of fecal coliform bacteria in sludge to Class A level during contained storage at 20-22°C following treatment with lime

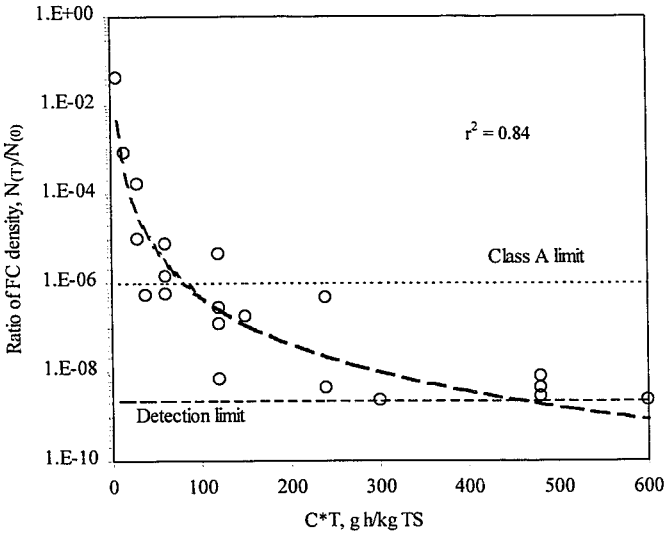

Figure 5-17. Kinetics of inactivation of fecal coliform bacteria with lime in dewatered sludge (TS 31.4-31.7%) during contained storage at 20-22°C

A 6-log inactivation occurs at C×T of up to 100 g h/kg TS; an 8-log inactivation occurs at C×T >300 g h/kg TS;

C×T: product of disinfectant concentration (g CaO) and contact time (d);

TS: total solid content of dewatered sludge;

FC: fecal coliform.

The average density of the *Salmonella* sp. bacteria determined in the dewatered, digested sludge prior to lime addition, was 3.08×10^2 MPN/g TS. This particular density proved to be within the common range for the Winnipeg sludge of from none detected to 4.73×10^2 MPN /g TS. The reported range of *Salmonella* sp. bacteria in activated sludge and in digested sludge is anywhere from none detected to 4.0×10^2 MPN /g TS [Yanko *et al.*, 1995]. As expected similarly to the fecal coliform inactivation, *Salmonella* reduction was equally rapid. After 1 h from start there was no *Salmonella* sp. detected in 30-g/Kg TS treatment. Attempts to isolate *Salmonella* from sludge samples treated with higher lime doses failed after first 15 min from start. Within one day of lime addition, all attempts to isolate *Salmonella* species from the treated biosolids at each lime dose failed. P-A tests performed on each sampling occasion yielded negative result.

Since the initial density of this pathogenic bacterium was relatively small, the observed reductions following lime application are only indicative of qualitative response to treatment. A meaningful, 3-log reduction or higher would have been much more useful in terms of assessing the effectiveness of liming. Current extension of the experimental work presented in this paper has incorporated *Salmonella*-spiked sludge. *Salmonella* sp. bacteria were added to the sludge at levels similar to these of indigenous fecal coliform bacteria, and similar survival patterns have been observed. There are no known characteristics that would significantly improve survival of one over another group of the bacteria. It is thus postulated that during quick lime treatment, when at its commonly low numbers in sludge, *Salmonella* can be considered inactivated as long as fecal coliform inactivation is achieved. In general, estimates on potential *Salmonella* reduction can be

based on analogous efficient fecal coliform reductions (3-log and higher) as seen in this research.

Bacterial spores of the pathogenic *Clostridium perfringens* decreased below the minimum level for quantitative determination (2.6 x 10^3 CFU /g TS, equivalent to 3-log or 99.9% reduction) after day 1 at lime dose of 480 g/Kg TS (Fig. 5-18). The 240-g/Kg treatment was effective in reducing the spores after 12 days from start. It took 22 days for a similar pattern to be observed in 120 g/Kg biosolids. On day 81 of storage, no spores were quantified in the 60-g/Kg biosolids sample. However, a semi-qualitative determination of the spores (at least one colony present using the lowest, 10^{-2}, biosolid dilution; detection limit one log lower than for the quantitative method, 2.6 x 10^2 CFU/g TS), revealed the presence of *C. perfringens* spores in the 60-g/Kg biosolids until the end of storage. Spore presence in all biosolid samples with the lime dose of 120 g/Kg and higher was qualitatively confirmed positive (with the P-A test) until day 69 through day 79 (depending on treatment). There was no testing scheduled for these higher lime treatments in between and spore inactivation could have taken place well before then. This earlier (prior to day 69-79) inactivation would have to be confirmed in a more narrow storage time range. The overall reductions in *C. perfringens* spore densities, dependent of the lime dose, used are shown in Fig. 5-19.

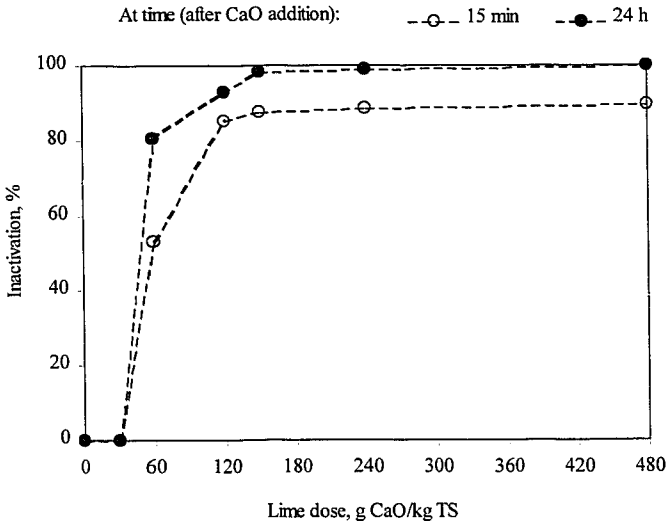

Figure 5-18. Course of *Clostridium perfringens* spore inactivation dependent on the lime dose used to treat dewatered sludge during contained storage at 20-22°C

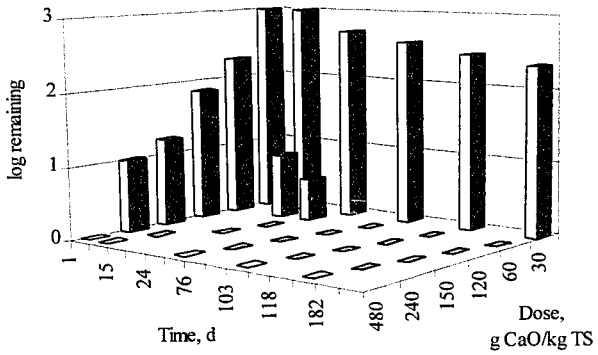

Figure 5-19. Inactivation of the spores of *Clostridium perfringens* in dewatered sludge treated with lime (30 g to 480 g CaO/kg TS) and stored for 6 months at 20-22°C

Determination of the *C. perfringens* spore density can be used as a confirmation of the disinfection efficiency. During mesophilic and thermophilic anaerobic digestion of animal slurry [Olsen and Larsen, 1987], all vegetative bacteria, including *Salmonella*, were significantly reduced whereas spores of *C. perfringens* remained unaffected. Munch *et al.* [1987] have shown that long-term storage (7 months) of non-aerated pig slurry at 6% TS and at 20°C and 7°C did not reduce *C. perfringens* spore density. Similarly, we found that the spore levels were unaffected in biosolids where with lime (30 g/Kg TS) or without lime addition. As expected, *C. perfringens* spore elimination varied from treatment to treatment. Lime doses of at least 120 g/Kg TS were required to effectively reduce this pathogenic bacterium when sufficient time for anoxic storage was provided.

The results of the experimental work presented here suggest that in order to reduce fecal coliform density to below the U.S. EPA Class A (U.S. EPA, 1993) limit of 1000 MPN/g TS a dose as low as 30 g/Kg TS and anoxic storage time of 1 day is effective. Storage of sludge alone reduced fecal coliform density to below that level anywhere between day 64 and day 83. The Class A limit for the *Salmonella* bacteria of less than 3 MPN/4g TS was also met. The low indigenous density of this pathogen in Winnipeg sludge made it easier to achieve the required reduction.

The storage of untreated sludge can itself be effective too. As seen in our experiments, densities of fecal coliform and *Salmonella* bacteria were significantly reduced. The results of die-off during storage alone will be discussed in a separate section.

5.5.1.3 Ammonia levels in treated sludge

Average total ammonia nitrogen (TAN) concentration in the dewatered sludge was 2,250 ± 40 mg/L (as N). In theory, some 80% of the TAN in water is converted to free or un-ionized ammonia form (UAN) at a pH of 10.0 and at 20°C. As a result of the high initial pH following lime addition, free ammonia concentrations of 1780 to 2175 mg/L were observed (Fig. 5-20). The free ammonia release potential was utilized in all lime-applied sludges at pH ranges observed; anywhere from over 50% to 100% of the TAN was in the UAN form. Significant decline in fecal coliform and *Salmonella* numbers, as depicted above, correlates with the high initial pH and free ammonia pointing out the synergistic effect of the two parameters as the major mechanism responsible for this reduction. *C. perfringens* spore inactivation would have been affected to a lesser extent. Apparently, the alteration of the spores by high pH inactivates a lytic system that normally is responsible for degradation of the cortex of the spore. Diffusion of the free ammonia through the spore wall is rather limited as the water content of the spore itself is very low.

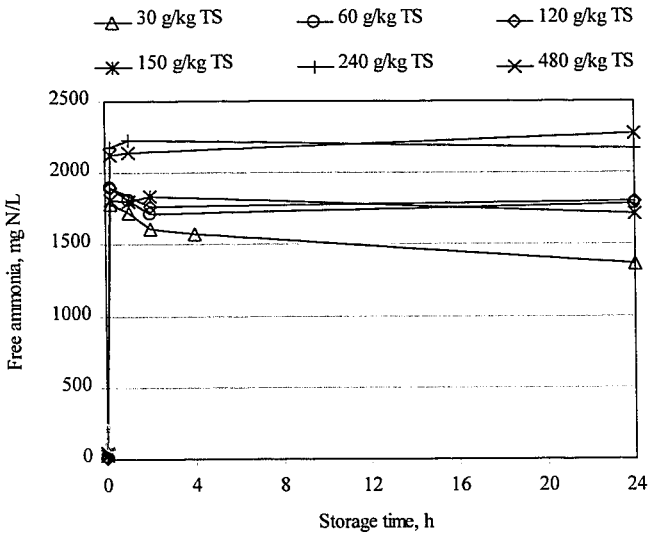

Figure 5-20. Free ammonia release in lime-applied, dewatered sludge during first 24 h of contained storage at 20-22°C

Common quick lime doses to obtain Class B biosolids are in the range of 15% to 25% of the dry weight of sludge, or 150 to 250 g CaO/Kg TS [Burnham *et al.*, 1998]. Montruccoli [1996] reported successful disinfection of poorly stabilized wastewater sludge by means of lime addition at 150 g/Kg TS and 250 g/Kg TS in ten days after application, with fecal coliform reductions to below detection. Inactivation of other sludge microorganisms was not reported. The experimental work presented here demonstrates that at doses usually considered sufficient to achieve fecal coliform reductions below 2,000,000 MPN/g TS, or the Class B biosolids, the actual disinfection effect is far more efficient.

5.5.1.4 Summary

For the Process-to-Significantly-Reduce-Pathogens (PSRP) and obtain Class B biosolids, sufficient quantity of lime is added to raise the pH of the biosolids to 12 after two hours of contact [U.S. EPA, 1993]. However, results similar to those when using PFRP lime treatment can be achieved with smaller CaO dosages that would also produce high pH.

Disinfection of dewatered sludge (TS 31.4-31.7%) with lime (CaO) at doses much smaller than those recommended for Class B biosolids was effective in inactivation of fecal coliform and *Salmonella* bacteria. The treatment involved lime addition to the sludge and mixing for 10 min, followed by contained storage at room temperature (20-22°C). A dose of 30 g CaO/Kg TS of sludge reduced fecal coliform by more than 8 logs in four hours from start. This degree of reduction far exceeded the requirement for Class A biosolids quality (<1,000 MPN/g TS), with regard to this particular bacterial indicator.

The *Salmonella* sp. bacteria were reduced to Class A level (<3 MPN/4 g TS) after 1 h at this dose, and after 15 min at any higher dose. The absence of culturable *Salmonella* species was confirmed after 1 d from start of the storage. The threshold for the pH to eliminate fecal coliform and *Salmonella* bacteria appears to be 9.5, i.e. the initial pH reached in the 20-g/Kg treatment, corresponding to the free ammonia concentration of about 1,200 mg N/L.

Third bacterial indicator, the spores of *C. perfringens*, was much harder to disinfect when compared with the non-spore forming fecal coliform and *Salmonella* species. A marked resistance to high pH and free ammonia concentration resulted in a prolonged storage required to observe a 3-log reduction (99.9% inactivation). Such reduction was achieved in sludge treated with lime doses of 60 g/Kg and higher, after 10 to 11 weeks of storage. Nevertheless, spore presence (P-A test) was confirmed in the 60-g/Kg dose even at the end of the storage, making the next higher dose the minimum effective dose. The pH required for this inactivation had to be higher than 12.0, with the corresponding free ammonia concentration of 2,250 mg N/L, or ~100% of the TAN.

5.5.2 Effect of fly ash on reduction of indicator organisms (stage II)

This part of the 20-22°C study was performed in a collaborative form [Liu, 2000]. Fly ash doses utilized were 300, 600, 900, 1,200, 1,500, and 1,800 g/Kg TS dry weight, or 80, 160, 240, 320, 400, and 480 g/Kg wet sludge. Upon mixing with fly ash for 10 min, the dewatered sludge (TS 26.8%) was stored under contained condition for up to 10 d. Fecal coliform bacteria were the indicators tested.

When compared with lime, much larger doses of fly ash were required to achieve the same reduction in fecal coliform. The fly ash addition of 900 g/Kg TS produced Class A biosolids, with respect to this indicator after 4 days of anoxic storage whereas at 600 g/Kg TS, fecal coliform reductions below 1000 MPN (CFU)/g TS were obtained after 10 days (Fig. 5-21).

Fecal coliform density was not significantly affected by the lowest fly ash dose of 300 g/Kg. The fecal coliform decline in Fig. 5-21, and earlier in lime disinfection in Fig. 5-15, was dependent on the pH with the minimum effective pH of 9.5. At this pH, in theory, the fraction of free ammonia would reach around 55% of the total ammonia in the liquid phase. This pH threshold was proven to be adequate in achieving adequate fecal coliform reductions in the case of lime treated sludge, as shown in Fig. 5-16.

At the same fly ash dose, sludge with higher initial TS (prior to fly ash administration) could obtain significantly higher pH values than that with lower TS (Table 5-8). Similar fecal coliform reductions would be expected as long as same pH levels are reached in the sludge.

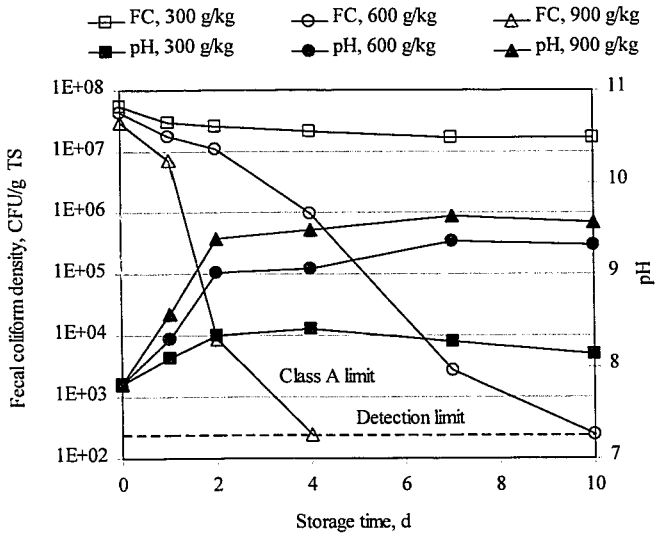

Figure 5-21. Changes in fecal coliform density and pH of treated sludge following fly ash application and contained storage at 20-22°C
FC: fecal coliform

Table 5-8. *The influence of total solids of the sludge on the maximum attainable pH after fly ash addition*

	pH levels in two different sludge samples treated with fly ash						
	Fly ash dose, g/Kg TS						
TS, %	0	300	600	900	1200	1500	1800
26.8	7.7	8.4	9.6	9.8	9.8	10.4	10.5
36.0	7.6	9.2	9.7	10.8	11.2	11.4	11.5

The pH values reported are the maximum values that can be achieved at a corresponding fly ash dose

Although popular concerns are often expressed about land application of materials containing harmful substances (heavy metals, etc.), the levels found in Brandon fly ash are much below the Canadian Council of Ministers of the Environment [CCME] Soil Quality Guidelines [1997] for agricultural use, and land application of fly ash treated sludge would be permitted (Table 5-9). At current sludge application rates of 55 tonnes dry solids/ha (one time only) and assuming fly ash dose of 600 g/Kg TS, the heavy metal contents of the soil following the application would still stay below the recommended values. At this dose, the soil buffering capacity will more likely ensure that the pH will drop to neutral range, or will not rise substantially at all.

Table 5-9. Comparison of selected characteristics of the sludge (from Winnipeg's NEWPCC), fly ash (from Brandon power plant), and soil* applied with fly ash-treated sludge, with the values recommended by the CCME [1997] for soil

Constituent	Average values, mg/Kg (dry weight)			
	Sludge	Fly ash	Soil mixture	CCME
Barium	239	0.33	4.5	12
Copper	1482	0.005	27.6	63
Nickel	53	0.002	1.1	150
Vanadium	14	0.0058	0.36	130
Boron	11	<0.0001	0.30	2

*Levels of selected pollutants in the soil were assumed negligible

Usually processes to meet Class A designation are cost-prohibitive for two reasons: cost of lime and added cost of trucking the product. A low-cost process such as alkaline stabilization would provide an attractive biosolids management alternative for many municipalities [Oerke, 1999; RT&W, 1998]. Direct access to such alkaline compounds as lime or fly ash makes the process very attractive. Lime improves overall quality of the biosolids for agricultural use. In areas where acidic soils prevail many farmers routinely apply lime alone to raise the soil pH. Therefore, such combination of nutrient-rich biosolids and lime makes it very attractive [Outwater, 1994]. Fly ash, in turn, is a byproduct of the combustion of solid fuels such as coal. Depending on the quality of the coal used, its residue can contain a variety of potentially hazardous substances and improper disposal or management could cause considerable environmental impacts [Bilski *et al.*, 1995]. The disposal of coal fly ash is often a problem as ash has little value, other than some aggregate additive. Fly ash is also generated as cement kiln dust (CKD) or lime kiln dust (LKD) in the cement and lime manufacturing, respectively. Such proprietary alkaline sludge stabilization processes like N-Viro Soil and BIOFIX (Bio Gro Systems) use fly ash as primary reagent and the ratio

of fly ash to sludge solids is from 1:1 to 2:1. In spite of the effectiveness of this combination, such ratio increases the mass of the treated biosolids thereby reducing their nutrient value (Burnham *et al.*, 1992). Also, in case of naturally alkaline soils, agricultural utilization of alkali-treated biosolids at commonly recommended doses for stabilization is not appropriate (Haug, 1998). The effective use of low-dose fly ash treatment of sludge, as demonstrated with fecal coliform bacteria as indicator of disinfection efficiency, is possible. A closer investigation into its use in sludge disinfection, possibly with small doses of lime to optimize the increase in pH, will be elucidated in part IV and part V of this research.

5.5.2.1 Summary

Fly ash doses and contained storage times at 20-22°C to inactivate fecal coliform bacteria were 600 g/Kg TS in 10 d, and 900 g/Kg TS in 4 d. The 300-g/Kg TS dose was ineffective in reducing fecal coliform levels to below Class A threshold. This implies that the disinfecting potential of the fly ash used was about 20 times lower than that of quicklime (CaO). The time required to obtain necessary reduction (6 logs) was also longer.

At lowest effective dose of 600 g fly ash/Kg TS, if the treated sludge is land-applied at common application rates, there is no risk of passing over the heavy metal limits for soil. In fact, the addition of fly ash would dilute heavy metal concentrations found in Winnipeg sludge making it possible to apply sludge treated with higher fly ash doses as well.

Dewatered sludge with a higher solids level (36.0% as oppose to 26.8%) may be easier to disinfect, as indicated by significantly higher attainable pH with same amount of fly ash. Improvement of disinfection with increased TS level would have to be confirmed by measuring levels of bacterial indicators, as their survival may improve due to increased difficulty of ensuring a homogenous mixture of the sludge and the disinfectant.

5.5.3 Pathogen regrowth study using lime-treated sludge and organic soil (stage III)

The sludge previously treated with various doses of lime (stage I) was used in topsoil formulations. The soil used was high in organic content, thereby providing good condition for possible regrowth of sludge microorganisms, including pathogens.

The acidic character of the soil did not have any major effect on the pH values of the (1+1) biosolid-soil mixtures containing sludge previously treated with 120 g/Kg and higher (Table 5-10). Only after 5 days from start the pH begun to decline considerably. With the increased ratio of soil, the pH of respective mixtures was much lower than that of the sludge right from start. Initial insignificant pH increase in mixtures with lowest doses of lime to the sludge was only temporary.

Prior to preparation of the sludge-soil mixtures, fecal coliform absence was confirmed by the P-A test in all previously limed sludge samples and in the Control treatment (raw sludge). Despite that, after 5 days of storage, regrowth occurred in formulations containing sludge previously treated with up to 60g CaO/Kg TS (Table 5-11). Formulations containing raw sludge stimulated fecal coliform regrowth too. Fecal coliform numbers were at low but stable levels. No fecal coliform regrowth occurred in

mixtures involving sludge at higher lime doses in mixtures containing sludge previously treated with up to 60 g CaO/Kg TS; P-A tests performed on these samples were always negative. None of the mixtures regained fecal coliform densities that would be even close to the Class A pathogen limit of less than 1000 MPN/g TS.

Similar to fecal coliform determination and due to a potential for regrowth, *Salmonella* sp. bacteria were also monitored. The P-A test consistently gave negative results in all mixtures prepared, even after 57 days of storage. There is some evidence that some Gram-negative bacteria, such as *Salmonella* sp. bacteria, can remain viable but non-culturable in soil [Turpin *et al.*, 1993]. The cells can enter a dormant state in which they are not cultivable using standard techniques. An enzyme-linked immunosorbent assay (ELISA) and a microwell fluorescent antibody (FA) test in the quoted study were both consistent in demonstrating the presence of viable *Salmonella*. However, the high minimum detection level of these two methods (10^6 *Salmonella* cells per gram of soil) makes it impossible to apply these tests for the presence of *Salmonella* in alkali-treated sludge.

Table 5-10 Changes in pH and free ammonia in sludge-soil (topsoil) mixtures during 57 days of storage

Lime dose* sludge+soil (wet weight)	pH and free ammonia concentration (mg N/L)									
	Storage time, d									
	0		2		5		14		57	
Control										
1+1	7.1	(10)	7.0	(8)	6.9	(4)	6.7	(1)	7.0	(3)
1+3	6.3	(0)	6.7	(1)	6.8	(1)	6.6	(0)	6.9	(1)
1+7	6.1	(0)	6.4	(0)	6.6	(1)	6.3	(0)	6.7	(1)
30 g/Kg TS										
1+1	7.6	(63)	7.9	(124)	8.2	(220)	8.2	(147)	7.7	(81)
1+3	7.2	(11)	7.7	(35)	7.6	(30)	7.4	(13)	7.1	(15)
1+7	6.8	(2)	7.1	(5)	7.1	(5)	7.0	(3)	6.8	(9)
60 g/Kg TS										
1+1	9.1	(727)	8.9	(442)	8.3	(224)	8.1	(109)	8.2	(238)
1+3	8.2	(63)	8.0	(55)	8.1	(67)	8.0	(50)	7.6	(36)
1+7	7.5	(9)	7.5	(10)	7.5	(12)	7.3	(6)	7.0	(11)
120 g/Kg TS										
1+1	11.1	(1612)	10.7	(1004)	10.2	(871)	9.0	(159)	8.3	(204)
1+3	9.3	(437)	8.4	(105)	8.3	(72)	8.3	(79)	8.1	(199)
1+7	8.0	(24)	8.2	(45)	8.2	(79)	7.7	(15)	7.5	(38)
150 g/Kg TS										
1+1	12.0	(1318)	1.5	(834)	11.1	(869)	9.7	(186)	8.3	(219)
1+3	9.9	(610)	9.5	(412)	8.5	(121)	8.3	(78)	7.9	(101)
1+7	8.6	(85)	8.1	(35)	8.5	(79)	7.7	(12)	7.6	(33)
240 g/Kg TS										
1+1	12.8	(1128)	12.9	(915)	12.6	(843)	11.7	(180)	8.2	(126)
1+3	10.6	(659)	10.7	(470)	10.5	(500)	8.7	(49)	8.4	(79)
1+7	9.1	(190)	8.6	(91)	8.3	(48)	8.2	(43)	7.9	(29)
480 g/Kg TS										
1+1	12.6	(904)	12.8	(705)	12.5	(646)	12.5	(138)	12.3	(484)
1+3	12.0	(563)	12.5	(505)	12.1	(463)	11.8	(225)	8.3	(49)
1+7	10.5	(321)	10.4	(303)	10.4	(307)	8.7	(39)	8.1	(34)

*Quick lime dose used to treat dewatered sludge (g CaO/ Kg TS, dry weight); the sludge was treated with different doses of quick lime and stored under anoxic condition at room temperature for a period of 6 months prior to their use in topsoil formulations

Stable population of the spores of *C. perfringens* was maintained in formulations containing raw sludge and sludge previously treated with 30 g CaO/Kg TS. In the latter case, *C. perfringens* remained unaffected despite of the previous, long-term exposure to increased pH and free ammonia concentration. From this observation, it can be concluded

that sufficient increase in pH is mandatory for achieving any marked disinfection effect on the spore density. *C. perfringens* spores in (1+3) and (1+7) sludge-soil formulations containing 60g CaO /Kg TS sludge, were reduced to below the limit for quantitative determination but were not eliminated completely (semi-qualitative test was positive). This decrease is not believed to be due to the activity of the soil bacteria since the soil native *C. perfringens* spores constituted a major fraction (almost 100%) of the *C. perfringens* spores present in the mixtures.

The spore density in the (1+1) mixture decreased to below 7.9 $\times 10^2$ CFU/g TS during the first five days of storage. After that, however, the spore count increase by over one log from day 5 level until last day (day 57) of storage seems to coincide with the pH drop by day five. This pH was maintained until end of storage.

Table 5-11 Fecal coliform regrowth in sludge-soil (topsoil) mixtures

Lime dose* Sludge+soil (wet weight)	Fecal coliform Presence-Absence test, + or - (fecal coliform density, log MPN/g TS)									
	Storage time, d									
	0		2		5		14		57	
Control										
1+1	-	+	(1.2)	+	(1.5)	+	(-0.1)	+	(<-0.3)	
1+3	-	+	(0.3)	+	(0.9)	+	(-0.3)	+	(<-0.3)	
1+7	-		-	+	(-0.1)	+	(-0.5)	+	(<-0.3)	
30 g/Kg										
1+1	-		-	+	(-0.1)	+	(0.5)	+	(1.4)	
1+3	-	+	(<-0.3)	+	(1.0)	+	(0.9)	+	(0.4)	
1+7	-	+	(<-0.3)	+	(1.7)	+	(-0.2)	+	(-0.5)	
60 g/Kg										
1+1	-		-	+	(<-0.3)	+	(<-0.3)	+	(-0.2)	
1+3	-		-	+	(<-0.3)	+	(<-0.3)	+	(<-0.3)	
1+7	-		-	+	(<-0.3)	+	(<-0.3)	+	(<-0.3)	

*Quick lime dose used to treat dewatered sludge (g CaO/ Kg TS, dry weight); the sludge was treated with different doses of quick lime and stored under anoxic condition at room temperature for a period of 6 months prior to their use in topsoil formulations

Spores of *C. perfringens* originating from the soil did not survive in any of the topsoil mixtures containing sludge that was previously treated with 120 g CaO/Kg TS and higher. Bacterial non-pathogenic growth was observed in all topsoil formulations from 30-g/Kg and 60-g/Kg treatments, and in (1+3) and (1+7) topsoil formulations from 120-g/Kg treatment. This growth demonstrated the ability of soil bacteria to acclimate to the conditions after mixing with low-dose lime-treated sludge. Due to higher lime doses in the remaining sludge treatments, high pH levels found in the topsoil mixtures did not allow for any bacterial growth except for the growth of few spore formers (qualitative determination).

As a result of open storage, the moisture loss from topsoil formulations proceeded to a different extent and depended on how high the fraction of soil in the mixture actually was. With the higher quantity of soil the natural drying process occurred faster. The highest TS levels of up to 70% by the end of the storage were achieved in the (1+7) topsoil combinations.

Levels of un-ionized ammonia in formulations containing sludge with 60 g CaO/Kg TS decreased markedly as a result of decline in pH of the mixtures (Table 5-10). Increase in TS levels corresponded to the increase in total ammonia, as expressed per volume of the remaining moisture. The rate of ammonia volatilization from mixtures during the storage was limited and was directly linked to the pH of the mixture material.

Even though there are no known commonly regarded methods for assessing stability of treated biosolids, biological stability of the product must meet toughest criteria especially in case of land application [Switzenbaum *et al.*, 1997]. The U.S. EPA vector attraction reduction requirement for sludge volatile solids is at least 38% [1993].

In well-stabilized biosolids, microbial biota successfully competes with pathogens and their regrowth is minimized or eliminated [Haug, 1993; Ho, 1997]. However, certain enteric bacteria can regrow in digested sludge if the degree of stabilization is unsatisfactory.

Human pathogens tend to convert into the non-culturable state more or less rapidly in the environment. The question arises, whether these cells are still viable. Different factors such as population size and interactions with other groups of microorganisms, available food and nutrients, environmental conditions affect survival of bacteria and other microorganisms in sludge. Discriminate nutrient requirements of some pathogens make the effort of enumerating them even more complicated.

The problem of pathogen regrowth in finished products from sludge processing is of great concern. Gibbs *et al.* [1997] reported on fecal coliform and *Salmonella* bacteria repopulation in soil amended with biosolids and biosolids alone after 50 weeks of storage. Tight handling procedures of biosolids products to reduce risk of repeated regrowth must be followed at all times [U.S. EPA, 1993]. Russ and Yanko [1981] pointed to a number of parameters contributing to the survival of *Salmonella* bacteria in finished compost from sludge, such as moisture (≥20%), temperature (20-40^{O}C), and nutrients (C/N>15). There are reported cases of the resistance of microbial populations to disinfection processes. Scully *et al.* [1999] described increased resistance to routine chlorine disinfection of fecal coliform bacteria originating from oxidation towers as oppose to fecal coliforms from other sources at treatment plant under investigation.

The issue of viability, as assessed by traditional microbiological methods, often leads to inconclusive results. The use of resuscitation techniques such as non-selective

enrichment media for the isolation of the bacteria of interest, before they are transferred to selective media for identification, is still a method of choice. However, the ability to recover these bacteria may be limited by the fact that other non-target microorganisms are being recovered too. These organisms may interfere with the target bacteria by means of competition or outnumbering due to faster recovery rates. Selective enrichment media are one way of overcoming this problem but may prove detrimental to the injured cells of a target microorganism.

Currently there are a number of new methods that can be used for enumeration of bacteria also in complex environmental samples such as wastewater or wastewater sludge. They include molecular techniques such as enzyme-linked immunosorbent assay (ELISA), fluorescent antibody (FA), and polymerase chain reaction (PCR). The major advantage over the use of traditional microbiological media is the ability to detect the target organism or group of organisms in a fast and precise way (Cooper and Danielson, 1997). There are a number of disadvantages to these techniques that limit their application, however. The ELISA and FA methods suffer from the lack of sensitivity. Often, the condition for having certain minimum number of organisms present in a sample cannot be met.

The ELISA and FA techniques are antibody based. Cross-reactivity of antibodies among some of diverse bacterial populations found in wastewater is possible and false positive reaction can result. For example, enteric bacteria have certain antigens (lipopolysaccharide) that are sufficiently similar among species to cause cross-reactions [Martinko, 2000]. The tests are also limited in their inability to measure viability of the detected organism [Cooper and Danielson, 1997].

The only technique that appears to be promising is the PCR technique that relies on the recovery of relatively intact DNA, but non-disruptive extraction techniques serving that purpose may not be that efficient. Frequently amplification (augmentation) of the target DNA sequence is required. Direct probing is only satisfactory if the microorganisms of interest are in sufficient quantity in the investigated sample.

Application of any of the above techniques to confirm the absence of an organism of interest after performing standard microbiological procedure would certainly be of value. In the pathogen regrowth experiments, the focus was on the non-selective enrichment and recovery of the injured microorganisms. The issue of competitive recovery of other microorganisms and problems with their interference would certainly be minimized. From the bacteriological quality standpoint and based on standard resuscitation techniques, the resulting biosolid-soil product can be considered stable and beneficially used.

5.5.3.1 Summary

The fecal coliform regrowth in topsoil formulations progressed to a limited extent and in mixtures involving sludge previously treated at up to 60 g CaO/Kg TS. The regrowth was to densities well below those required for Class A product classification. There was no *Salmonella* sp. regrowth observed in any of the topsoil formulations. The spores of *C. perfringens* did not regrow in topsoil mixtures containing sludge previously regarded as (*C. perfringens*) spore-free.

5.5.4 Conclusion

The low-dose alkaline treatment using either lime or fly ash benefits from the combination of two factors; high pH and resulting high free ammonia concentration. High pH is the stimulating factor whereas combined action of high pH and free ammonia is responsible for pathogen kill. Anoxic storage slows down the natural decline in pH of treated sludge thereby ensuring necessary disinfection within the prescribed time frame.

In terms of compliance with the U.S. EPA Class A pathogen standard [1993], the treatment was effective in reducing fecal coliform and *Salmonella* sp. bacteria at lime dose as low as 30 g/Kg TS (~10 g/Kg wet weight) after 4 h, and at fly ash dose of 600 g/Kg (160 g/Kg wet weight) after 10 d. Regardless of the type of alkaline chemical and the level of solids in sludge (in the range of 26.8 to 36.0%), effective inactivation took place when the pH level reached 9.5, or higher. This pH level corresponded to free ammonia concentration of at least 1,200 mg N/L.

Under Alternative 3 and 4, any new treatment deemed to produce Class A biosolids has to be proven to reduce enteric viruses (<1 PFU/4 g TS) and viable helminth ova (<1 ovum/4 g TS). Even though reductions in bacterial densities were in agreement with Class A requirement, likewise, the low-dose alkaline treatment would need to be effective against viruses and parasite eggs. These parameters were not established however. As well, the effect of alkali at storage temperatures other than 20-22°C needs to be established.

Although not regulated by the U.S. EPA, the spores of *C. perfringens* were also under investigation and were found much harder to disinfect. Lime doses of 120 g/Kg TS (38 g/Kg wet weight) and higher resulting in pH≥12.0 during entire storage, and storage

times of 10 to 11 weeks were required for complete elimination of spore viability (P-A test). Although not established with the use of fly ash, the spore reduction appears unlikely to be observed at any fly ash dose, even one considered high (more than 1,000 g/Kg TS or 270 g/Kg wet weight of sludge at 26.8% TS). This would be due to limited alkalinity generation potential of the fly ash used in this research. The highest fly ash dose of 1,800 g/Kg TS (480 g/Kg wet weight) resulted in a maximum pH value of 10.5 only.

The marginal regrowth of fecal coliforms in sludge-soil mixtures incorporating sludge previously treated with up to 60 g CaO/Kg TS was much lower than the Class A limit of <1,000 MPN/g TS. The suppression by other, native to the soil bacteria is believed responsible for such behavior. There were no cases of *Salmonella* sp. regrowth in all mixtures under investigation. Although standard resuscitation techniques were the only methods used to recover potentially present but injured microorganisms, the use of molecular PCR technique, is recommended for future studies.

5.6 EFFECT OF VARIOUS COMBINATIONS OF LIME AND FLY ASH (COMBINATION TREATMENT) ON PH PATTERNS IN TREATED SLUDGE STORED AT 20-22°C AND AT 4-6°C (STAGE IV)

A preliminary collaborative study to observe a possible benefit of combining lime and fly ash, and assessing their contribution to the pH level in the sludge was initiated [Liu, 2000]. When lime was combined with fly ash at the respective doses of 25 g/Kg and 500 g/Kg, this mixture was much more effective in rapid and sufficient pH increase than any of the alkaline additives used separately (Fig. 5-22). Such marginal lime supplementation of just 25 g/Kg could stimulate an additional alkalinity release from fly ash, which would obviously lead to a much higher pathogen destruction potential.

The relative increase in [OH^-] (hydroxide ion concentration) in combination treatment (lime + fly ash) was 3.7, 6.8, and 8.3 times higher than the sum of increases in [OH^-] in sludge treated with either alkali alone (at 30 min, 4h, and 20 h from start, respectively). This points the applicability of such combination treatment in cases where chemical disinfection and not physical disinfection, e.g. by heat, would be employed. Lower lime consumption to achieve the prescribed pH and higher final solids content of the treated sludge due to fly ash addition are the benefits of this combination.

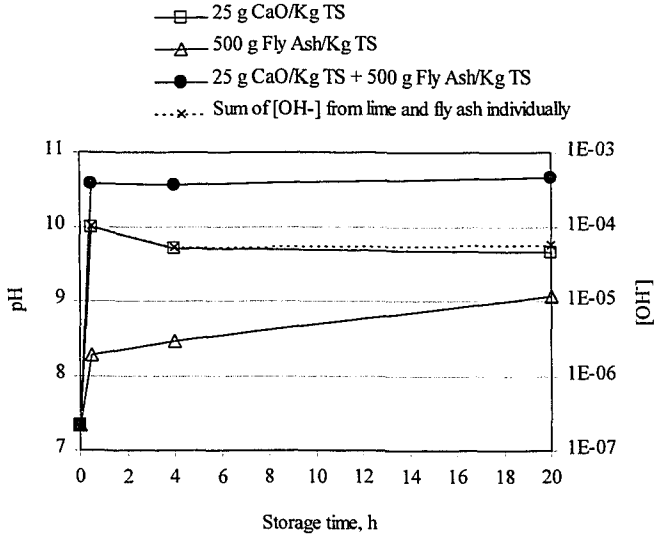

Figure 5-22. Comparison of pH and [OH^-] in stored sludge (20-22°C) following treatment with lime, fly ash, and combination of lime and fly ash

Following the promising results of the preliminary experiment, a more detailed protocol was followed to expand the knowledge of observed interactions between lime and fly ash. In this part of the work, various treatments involving lime and fly ash were set up at 20-22°C (batch I) and at 4-6°C (batch II). The lime and fly ash doses tested were 20+300, 20+600, 20+900, 40+300, 40+600, and 40+900 g/Kg, respectively. In addition, control treatments were set up and included 20 and 40 g CaO/Kg, and 300, 600, and 900 g fly ash/Kg TS. These treatments were designed to compare the contribution of each chemical when used separately, and in combination. Untreated sludge was also set up for storage at each temperature range.

The average pH measurements taken over 31-d period at respective temperatures are shown in Fig. 5-23 for 20-22°C, and in Fig. 5-24 for 4-6°C. The reported pH values are relative numbers as they were measured at distinctly different temperature regimes. Therefore, interpretation of the results is based on relative increase or decrease and not differences between corresponding pH values at different temperatures.

Incremental increase in lime dose while keeping the fly ash dose constant, resulted in an average pH increase of about 0.5 unit at both temperatures. Every increase in the quantity of fly ash at same lime dose led to a mean pH increase of around 0.3 units at 20-22°C and 0.2 units at 4-6°C. Such close correlation of pH in the range of doses under observation makes it possible to increase pH to the level necessary for disinfection.

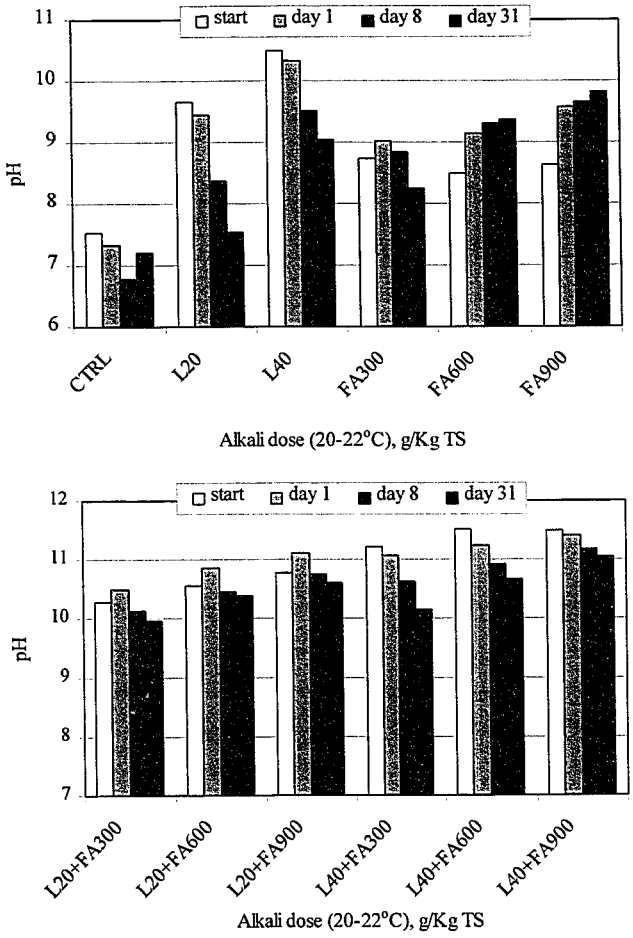

Figure 5-23. Impact of lime and fly ash combination on pH levels in dewatered sludge during contained storage at 20-22°C (batch I)

TS: total solids, CTRL: sludge without alkaline additives, other abbreviations: e.g. L20 means lime at dose 20 g CaO/Kg TS, FA: fly ash

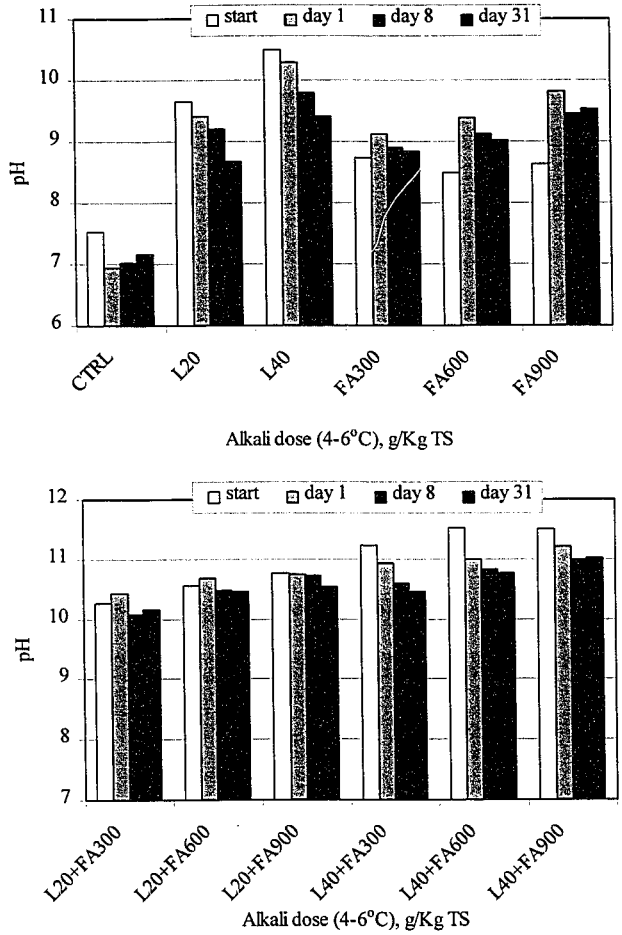

Figure 5-24. Impact of lime and fly ash combination on pH levels in dewatered sludge during contained storage at 4-6°C (batch II)

TS: total solids, CTRL: sludge without alkaline additives, other abbreviations: e.g. L20 means lime at dose 20 g CaO/Kg TS, FA: fly ash

At both temperature regimes by the end of treatment, the drop in pH in lime-treated sludge was higher (at least one pH unit) than in any other treatment. In sludges treated with lowest doses of lime (20 g/Kg TS) and of fly ash (300 g/Kg TS) at 20-22°C, the higher decline in pH from the maximum level was due to increased rates of recovery of biological activity. The 4-6°C temperature of storage retarded this activity and allowed for smaller fluctuation of pH levels until the end of incubations. With the increased contribution of lime (from 20 to 40 g/Kg TS), the maximum pH that was attained was obviously higher. Also, the benefit of increased contribution of fly ash (from 300, to 600, and to 900 g/Kg TS) resulted in a more stable treatment, preventing the pH from faster decline. The use of low dose of lime to support alkalinity release from fly ash certainly reduces fly ash requirement, and could be utilized to reduce otherwise high fly ash doses, as in commercial alkaline disinfection processes.

The overall gain of alkalinity observed in any combination treatment investigated was significantly higher than the sum of alkalinities generated in those treatments where either of the alkalis was used alone. Table 5-12 shows the relative increases of hydroxide ion concentration, $[OH^-]$, in combination treatment over the sum of those $[OH^-]$-s measured in corresponding lime and fly ash treatments alone. At least 30% higher initial increases (on day 1) in hydroxide ion concentration (i.e. ratios of 1.3 and higher) led to even more improved alkalinity generation and maintenance of high pH further, during the entire storage.

Table 5-12. Relative increase in [OH^-] in combination treatment (lime + fly ash) as compared with the sum of increases in [OH^-] in sludge treated with either alkali alone

Combination treatment	Ratio ([OH^-]L+FA) / ([OH^-]L + [OH^-]FA)						
	20-22°C (batch I)				4-6°C (batch II)		
	Time during storage, d						
	Start*	1	8	31	1	8	31
L20+FA300	1.2	2.5	4.5	13.7	2.2	1.5	4.0
L20+FA600	2.4	5.3	3.9	3.2	3.0	3.1	5.8
L20+FA900	3.8	6.2	3.8	1.9	1.9	3.8	2.8
L40+FA300	1.7	1.6	3.4	3.6	1.3	1.8	2.7
L40+FA600	3.4	2.3	5.1	4.2	1.4	2.8	4.9
L40+FA900	3.2	3.2	6.2	4.4	2.0	3.4	5.4

*Following a 10-min mixing of alkalis with sludge in Hobart mixer prior to storage; underlined values represent maximum ratio in corresponding treatment

The ability to optimize disinfection using fly ash and supplementation with small quantities of lime would allow for more control over the anticipated increase in pH. Since the higher the alkalinity in treated sludge the less fitted it becomes for land application.

5.6.1 Conclusion

Combination treatment using fly ash as the primary alkaline agent and lime as supplementary chemical benefits from lime' ability to induce much higher alkalinity generation (2.7 to 13.7 times higher) than anticipated, based on alkalinity generation from fly ash and from lime when used separately. The decline in pH of treated sludge is less steep than in case of lime or fly ash when used alone. This allows for an improved control over the process where duration of treatment at specified pH is the key for complete disinfection. Since with every increase in fly ash or in lime there is a significant correlation in pH increase (0.5 units for lime, 0.2 to 0.3 units for fly ash), the proper

administration of both alkalis makes it easier to obtain anticipated pH. The temperature of storage had little impact on pH level observed in all mixtures.

5.7 DISINFECTION OF DEWATERED SLUDGE USING CLOSED-SYSTEM, LOW-DOSE ALKALINE TREATMENT AT 4-6°C (STAGE V)

This was the last stage of work on sludge disinfection- stage V.

The effectiveness of low-dose disinfection was investigated at temperature range a few degrees above freezing. This so called "cold" temperature study, with storage at 4-6°C following alkali addition, was conducted to see if temperature - a physical factor - has an effect on pathogen inactivation due to chemical factor(s) - pH and concentration of free ammonia. The duration of storage was 280 d, or 9 months, to mimic low-tech contained sludge storage (e.g. in a shed or other enclosed structure) at temperatures just above freezing, without accounting for the effect of freezing.

In the work presented so far, the assessment of *Salmonella* sp. inactivation was incomplete because of low indigenous densities of this pathogen in Winnipeg sludge. This extension of the work presented so far has incorporated *Salmonella*-spiked sludge. *Salmonella* bacteria were added to the sludge at levels similar to these of indigenous fecal coliform bacteria. As well, the sludge was spiked with *Ascaris suum* eggs (absent in Winnipeg sludge) to determine if, indeed, the low-dose alkaline treatment and long-term contained storage are effective in their inactivation. Another improvement was made as to determination of the extent of permanent injury to *C. perfringens* spores. Spore germination can be induced by the addition of cortex-lytic enzymes, such as lysozyme

[Ando, 1979]. Its inclusion in the medium enhances or permits germination when the indigenous to the spore coat germination lytic enzyme is absent or inactivated, such as in alkaline treatment [Duncan *et al.*, 1972].

The dewatered sludge had an average TS range of 28.1-28.7%. The doses used were, for lime: 20, 40, 80, 120, and 160 g/Kg TS, for fly ash: 300 and 600 g/Kg TS, and for the combination treatment (lime and fly ash): 10+300, 10+600, 20+300, and 20+600 g/Kg TS. The treated sludge was stored contained for about 280 d.

5.7.1 Effect of lime, fly ash, and both lime and fly ash on reduction of indicator and pathogenic microorganisms

This study (4-6°C) gave similar results as to the extent of fecal coliform inactivation. As long as the pH remained at 9.5 or higher, the inactivation proceeded to the extent that made the treatment highly efficient. In lime-treated sludge, similar reductions were observed (Table 5-13), as both groups of microorganisms are incapable of producing forms that are more resistant.

By day 1, in sludge treated with lime doses of 40 g/Kg TS and higher, fecal coliform and *Salmonella* sp. densities were below Class A limits for the respective organisms. This confirms that fecal coliforms and *Salmonella* bacteria possess similar survival characteristics, and justifies the selection of either indicator to demonstrate Class A biosolids quality. In fly ash-treated sludge, and in sludge disinfected by combination treatment, after 1 d from start, the reductions were lowest- 0.4-log for *Salmonella*, and 0.2-log for fecal coliform- in the 300-g fly ash/Kg TS treatment. Highest reductions after 1 d of 4.6-log for *Salmonella* and 4.4-log for fecal coliform, were observed in the 20-g

lime plus 600-g fly ash/Kg TS treatment, insufficient to classify the sludge as Class A. Interestingly, after one day of storage in 10-g CaO plus 600-g fly ash/Kg TS treated sludge and in 20-g CaO plus 300-g fly ash/Kg TS applied sludge, the reductions in fecal coliform and *Salmonella* sp. densities were similar. The 3.3-log fecal coliform and 5.5-log *Salmonella* inactivations were observed. The pH of both mixtures was at about 10.

By the end of storage (day 280), there were no fecal coliform and *Salmonella* sp. bacteria detected in any of the treatments under investigation (P-A test negative). The inactivation of non-spore forming bacteria, using fecal coliform and *Salmonella* sp. as model microorganisms, was very effective.

Table 5-13. Log inactivation of fecal coliform and *Salmonella* bacteria during contained storage of limed sludge at 4-6°C (≥5-log inactivation is needed to obtain Class A biosolids).

Organism	Storage time, d	Lime dose, g CaO/Kg TS					
		Control	20	40	80	120	160
Fecal coliforms	1	0.5	1.9	8.2	8.2	8.2	8.2
	280	2.3	8.2	8.2	8.2	8.2	8.2
Salmonella sp.	1	0.2	1.4	7.7	7.7	7.7	7.7
	280	2.8	7.7	7.7	7.7	7.7	7.7

Reductions in *C. perfringens* spore densities varied among different treatments. The issue of bacterial spore injury was looked at by assessing the extent of damage to the spores of *C. perfringens* in alkali-treated sludge. The damage was assessed upon neutralization of the sludge in lysozyme-supplemented TSC agar.

In lime-treated sludge at doses of 80 g/Kg TS and higher, spore recovery using standard medium (TSC agar) failed. Lysozyme addition allowed only a small population

to recuperate and re-gain its viability (Fig. 5-25). The extent of irreversible or permanent injury, assessed for the first time after 280 days of storage, was found proportional to the amount of lime added (based on the ratio of log-transformed spore density in media with lysozyme to the density in media without the enzyme). Only 0.27, 0.26, and 0.18% of the initial spore population was recovered from sludge treated with lime doses of 80, 120, and 160 g/Kg TS. Because of the detection level set at a level corresponding to 99.9% or 3-log inactivation, it was impossible to quantify un-injured spores (if such ones still existed).

A potential for repair of the recoverable damage to the spores under environmental conditions after land application of spore-containing sludge is yet to be determined. Although the presence of enzymes such as lysozyme under natural condition in soil is possible, a more detailed research would have to be conducted as to their concentrations found in soils, and the ability to repair alkali-injured spores. At lime doses of 20 and 40 g/Kg TS, the extent of permanent inactivation was limited to 0.1 to 0.3-log reductions. There were no visible differences between the recovery using standard and lysozyme-supplemented agar.

The complete inhibition of germination as a consequence of treatment could have resulted from a combination of spore death, direct physical inhibition of germination, or permanent damage to some unknown part of the lysozyme-inducible germination pathway. Demonstration of permanent damage to the spore germination mechanism is crucial in assessment of disinfection efficiency. In addition, evaluation of disinfection efficiency using indigenous spores, as in this research, offers inclusion of the natural variability in their resistance at different times.

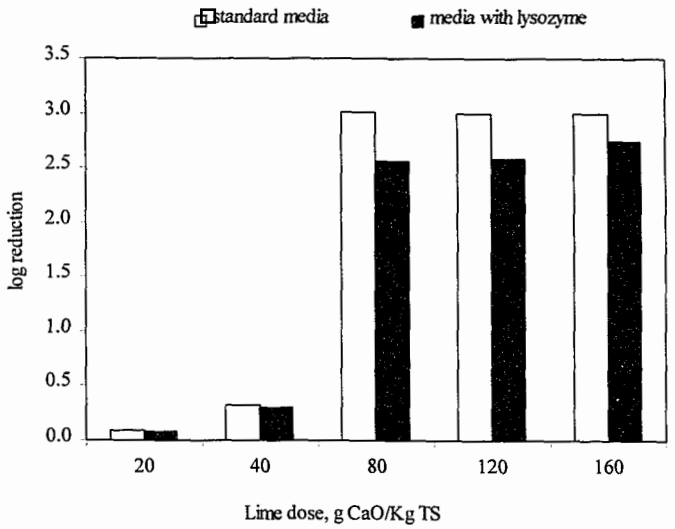

Figure 5-25. Effect of lysozyme addition on recovery of injured *Clostridium perfringens* spores in lime-treated sludge on day 280 of contained storage at 4-6°C

3-log inactivation corresponded with the threshold for quantitative determination of spore density

Fly ash and combination treatments were ineffective in reducing the spores even by the end of storage, on day 280. Lowest reduction was noted in 300-g fly ash/Kg TS sludge treatment, and increased up to 0.5 logs in treatments with highest doses of lime and fly ash. The comparison of counts from standard media and those from lysozyme-supplemented media showed no differences in any of the fly ash or combination treatments. As in the case with lowest lime doses, it appears that this part of spore population is weaker than the rest, and attempts to reverse their injury are unsuccessful. Some 20% (0.1-log reduction in 20 g CaO/Kg TS treatment) to 70% (0.5-log reduction in 20+600 g/Kg TS combination treatment) of the total spore population was inactivated at pH levels 8.5 to 11.0. Inactivation of the hardiest fraction required pH levels in excess of 12.0.

Using the data from both storage conditions of 20-22°C and 4-6°C, one can estimate what is the required lime dose and time to effect inactivation of the spores (below the level for quantitative determination). The concept of using the product of disinfectant concentration and contact time (C×T) for *C. perfringens* spores is depicted in Fig. 5-26. The C×T values required to obtain spore reduction to below the limit for enumeration (equivalent to 3-log or 99.9% reduction; NT/Ninit. of 0.001) are much higher than those shown earlier for fecal coliform inactivation (Fig. 5-17). The trendline describes the spore reduction dependence of C×T, valid only for lime doses of 60 g CaO/Kg TS and higher. A 2-log or 99% inactivation occurs at C×T of up to 240 g d/Kg TS, whereas 3-log inactivation (99.9%) occurs at C×T>2600 g d/Kg TS. Such degree of spore reduction does not mean that the product of disinfection is spore-free.

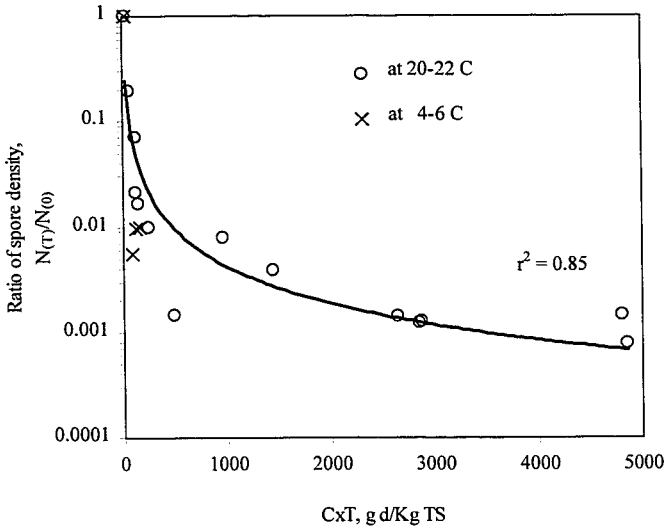

Figure 5-26. Kinetics of inactivation of *Clostridium perfringens* spores with lime in dewatered sludge stored at 20-22°C and at 4-6°C

C×T: product of disinfectant concentration (g CaO) and contact time (d);
TS: total solid content of dewatered sludge

The remaining spore population, like the eggs of *Ascaris*, is still capable of posing risk to human health. Therefore, it is suggested to set a limit for this pathogen at a level for detection (by means of P-A test), similar to using the U.S. EPA approach stating the threshold density, e.g. for *Salmonella* and helminth ova.

The inactivation of *Ascaris suum* eggs proved most difficult to obtain even with highest doses of alkaline agents used. Inactivation is based on the assessment of egg viability. Four major stages of egg development are: 1) one cell starts splitting to a two-cell organism, 2) cytokinesis begins, 3) formation of higher number of cells proceeds, 4) larva becomes visible from outside the egg shell. Egg viability following concentration of eggs from sludge is assessed after a minimum 28-day incubation at 25°C and higher. The sample is examined for total egg numbers and viable (embryonated) egg numbers. The criterion for distinguishing embryonated eggs from dead eggs is the presence of a visible worm within the egg shell. The morphological criteria used for identification of the dead eggs are: 1) cytolysis of egg cell (after cytokinesis has begun), 2) formation of large refractile granules within the cell, 3) vacuolation or hyalinization in the cytoplasm, 4) shrinkage of the egg, 5) disintegration of the membrane surrounding the egg cell, and 6) collapse of the egg shell.

Since sludge treated with alkaline compounds was stored at 4-6°C and in oxygen-depleted environment, no unwanted embryonation was observed. Depending on the extent of damage made to the eggs, they will not embryonate in the same way as they would have, had they been stored in water or other medium providing safe environment. During the incubation period (30 d), the eggs are kept in 0.5% buffered formalin solution and, therefore, the impact due to microbial activity affecting the eggs (bacteria and fungi

attacking the egg shell and preying on its contents) can be omitted. Different stages of development verify the effectiveness of inactivation. With a portion of eggs undeveloped or just partially developed (not all the way to the larval stage) one can confirm at least partial injury to an egg and retardation of its usual developmental pattern.

The results of *Ascaris suum* egg inactivation are depicted in Fig. 5-27. In all cases, a rather limited reduction, if any, took place. The initial density of this pathogen following spiking ranged from ~700 to ~1,000 eggs/g TS, the level high enough to observe a 3-log reduction. The initial density of this pathogen in untreated sludge was designed to be ~1,000 eggs/g TS. Due to "dilution" effect caused by addition of alkaline compounds to sludge, or additional solids, this density decreased with the increased alkali dose.

The highest reduction of 0.7-log, equivalent to 80% loss of viability, was obtained using lime at a dose of 160 g/Kg TS. The reduction of viability appeared proportional to the lime dose, but effectiveness of treatment was visibly impaired at this range of doses. Similar degree of inactivation was seen in lime-treated sludge at 80 g CaO/Kg TS, and in combination treatment using 20 g CaO and 600 g fly ash/Kg TS. This phenomenon was initially thought to be related to the pH in both sludge samples. However, the pH of the lime-treated sludge at the end of storage was significantly higher (12.05) than the pH in the corresponding combination treatment (9.95). Although not confirmed by any solid evidence, some property of the fly ash could hold responsible for this increased inactivation at much lower pH level. Low-dose combination treatment using low doses of fly ash and lime could contribute additional mechanism by which disinfection occurs.

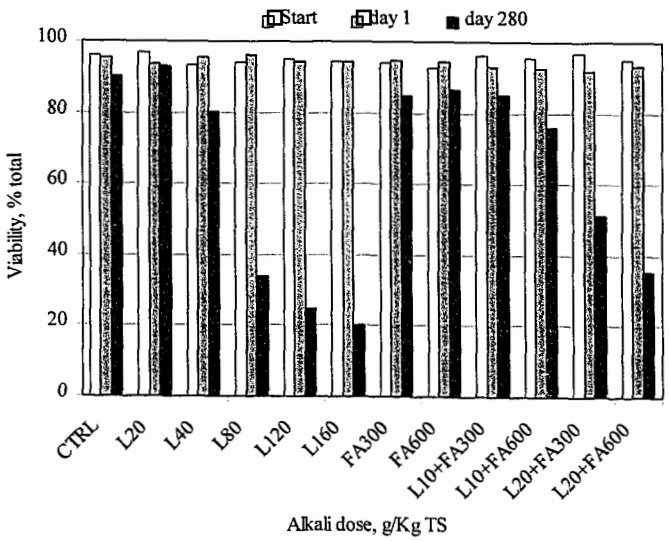

Figure 5-27. Loss of *Ascaris suum* egg viability in dewatered sludge following lime and fly ash treatment and contained storage at 4-6°C

CTRL: stored sludge without alkaline additive, L20: lime dose of 20 g CaO/Kg TS; FA: fly ash

If such mechanism exists and additionally weakens the egg shell structure, the effect of free ammonia would be far more destructive to the ova. At this pH and temperature the free ammonia would account for about 60% of the total ammonia, or 1,400 mg N/L.

The slightly lower viability of *Ascaris* eggs in the control treatment, as compared with that in the sludge treated with lowest lime dose of 20 g/Kg TS, was noted. This could be due to the fact that, although greatly reduced, the activity of other microorganisms praying on the eggs in the control treatment decreased their viability. In the case with low lime supplementation, this amount of lime acted as a preservative, similar to the action of formalin in the viability test, where the potential for loss of egg viability due to preying is avoided.

The quality of the *Ascaris suum* ova in the batch used for spiking was confirmed by measuring their viability at the end of 280-d storage. The eggs were stored in 0.5% formalin at 4-6°C at an average density of about 1,000 eggs/mL, throughout the duration of storage. The initial viability was 97%, whereas the final viability was 94.7%.

During any storage, helminth eggs are subject to environmental stress. They will embryonate when conditions for their development are met. Depending on temperature of storage they may or may not embryonate. Eggs may embryonate no matter what is the pH and other characteristics of the surrounding material as long as the temperature stays at 20°C and higher, and an adequate oxygen tension is present. In case of *Ascaris* species, the eggs are adapted to the very low partial pressures of oxygen that are characteristic of the gut lumen of their hosts [Lee and Atkinson, 1976]. Freshly collected feces of humans or of pigs do not contain embryonated eggs. It is also unlikely that egg development

would occur in a lagoon-stored sludge due to anaerobic or anoxic condition, even in a warm climate. However, in soil to which untreated sludge was applied, such embryonation is possible and some of the eggs may be well into embryonation and at different stages of development, thereby increasing the risk of infection.

High survivability of the eggs in non-treated sludge is due to the temperature of storage. A few studies related the persistence of *Ascaris* eggs during storage to low temperature. While complete inactivation of *Ascaris suum* eggs occurred in both aerobically and anaerobically digested biosolids stored at 25°C for 16 months, a large portion of the ova stored in both types of sludge at 4°C for 2 years was still viable [O'Donnell *et al.*, 1984]. No significant decrease in *Ascaris suum* egg viability was observed following 5°C storage of dewatered biosolids for 60 d [Ahmed and Sorensen, 1995]. Similar to the above observations, the reduction in egg viability in the control sludge was insignificant, and the reductions observed in the remaining sludge treatments were the result of chemical treatment with lime and with fly ash.

5.7.2 Conclusion

In all parts of the experimental work discussed so far, the combined effect of high pH, high free ammonia, and other factors showed to have a tremendous impact on bacterial activity. High pH alone is responsible for denaturation of bacterial proteins in non-spore formers and for lysozyme removal in spore formers, which also leads to

inactivation. Unlike ammonium ion (NH_4^+), ammonia gas (NH_3) - as a small molecule - can diffuse into bacterial cells and alter the chemical equilibrium between the cells and the solution. Certain enzymes are no longer capable of functioning properly and the cell dies. Therefore, free ammonia is recognized as the more toxic of the two forms to bacterial metabolism [Kroeker *et al.*, 1974; McCarty and McKinney, 1961]. Osmotic pressure is another killing mechanism. It removes water from the microorganisms (plasmolysis) when they are in solution high in salts or sugars. Loss of water interferes with the cell function and eventually leads to cell death [Black, 1996].

The major factor that would allow microorganisms to survive is the shielding effect of the bacterial cell and organic matter (solids) clusters that are formed in sludge. Digested-dewatered sludge provides limitations in influx of the external species to such clusters. This makes certain disinfection methods unsuitable for the use in sludge treatment. Possible formation of bacterial aggregates can lead to higher resistance to disinfection. Such knowledge already exists in case of viral aggregates where all virions within such cluster must be inactivated before the whole cluster is considered non-viable [Grant, 1995].

This research demonstrated that addition of alkaline agents at low-dose to promote levels of pH high enough to stimulate free ammonia evolution from sludge were efficient in bacterial pathogen kill. Nevertheless, lime doses to inactivate bacterial spores of *C. perfringens* were up to four times higher than those to effectively reduce non-spore forming fecal coliform and *Salmonella* sp. bacteria (120 g/Kg TS versus 30 g/Kg TS at 20-22°C, 80 g/Kg TS versus 20 g/Kg TS at 4-6°C). These higher doses were capable of increasing the pH of treated sludge to >12.0, as compared to about 10.5 to 9.5 only (30

g/Kg at 20-22°C and 20 g/Kg at 4-6°C, respectively). The potential for regrowth in treated sludge-organic soil mixtures during subsequent storage allowed for the fast decline in pH, which stimulated beneficial bacterial populations native to the soil.

Even though highest pH in combination treatment using 20-g CaO and 600-g fly ash/Kg TS reached only 10.2, the extent of inactivation of the *Ascaris* ova of about 80% (0.74-log) was similar to that achieved in sludge at pH>12.0, treated with 80 g CaO/Kg TS. This gives a niche for application of low-dose combination treatment, but would have to be confirmed as to the ability to further inactivate bacterial spores of *C. perfringens* that resisted pH<12.0 at both temperature ranges investigated, following 6 months (20-22°C) to 9 months (4-6°C) of contained storage. This relationship would have to be confirmed based on the C×T approach, by combining the effect of disinfectant concentration and storage time.

5.8 DISINFECTION OF DEWATERED SLUDGE USING LONG-TERM CLOSED-SYSTEM STORAGE AT 20-22°C AND AT 4-6°C

5.8.1 Effect of storage alone on inactivation of microbial indicators

The reference samples (without alkali addition) used during disinfection experiments, were also observed for fecal coliform, *Salmonella* and *C. perfringens* spore inactivation during anoxic storage at the two temperature ranges. At 20-22°C, initially there was no significant change in fecal coliform population for 15 days. A one-log decline of fecal coliform density took place between day 15 and 26. By day 64, fecal coliform density dropped to 1.58×10^4 CFU/g TS. On day 83 and day 120, the fecal

coliform level was less than 0.6 MPN/g TS, however, the P-A test on both occasions was positive. Only at the end of the 6-month storage period (day 189) the P-A test gave a negative result. This reduction in the control treatment can be attributed to the natural die-off of fecal coliform bacteria. At 4-6°C, the fecal coliform density remained at above the U.S. EPA Class B fecal coliform level of less than 2,000,000 MPN/g TS.

Reduction of *Salmonella* sp. under the condition of contained storage without alkaline additives followed similar to fecal coliform inactivation pattern (data from cold storage study with spiked sludge, Table 5-13). A 2.3-log reduction for fecal coliform bacteria corresponded with a 2.8-log reduction for *Salmonella* sp. bacteria. The competition with other active microorganisms in sludge can affect pathogenic species too. Those in low numbers, such as indigenous *Salmonella*, are at obvious disadvantage. The higher storage temperature of 20-22°C, and thereby increased bacterial activity, promotes this behavior. The kill mechanism is not known although predation by other organisms or a toxic effect of their metabolites (bacteriocins) may have an effect [Ward *et al.*, 1999]. As expected, spores of *C. perfringens* were not affected by the storage alone.

A higher temperature of storage could also promote natural die-off of the bacteria. Kearney *et al.* [1993] reported on a more rapid decline of *Salmonella typhimurium*, *Yersinia enterocolitica*, and *Listeria monocytogenes* in beef cattle slurry stored at 17°C, as compared with the storage at 4°C. Similar to farm slurry storage, the long-term contained storage of dewatered sludge, despite much higher solids level, proved to be effective.

These experiments confirmed our suspicion that winter storage alone would do little to inactivate pathogens. The effect of freezing and subsequent thawing, as it proceeds in nature, was not investigated in this part of experimental work. However, a parallel study where the author collaborated with an M.Sc. student performed for the City of Winnipeg [Liu, 2000], showed that the freeze/thaw treatment did not have any effect on fecal coliform bacteria.

Three different storage temperatures of 20-22°C, 4-6°C and –22°C were used to determine the long-term influence on fecal coliform inactivation in digested-dewatered sludge not treated with alkaline agent(s). The effect of storage under such condition is shown in Fig. 5-28. The inactivation was ineffective over the duration of the cold storage at 4-6°C and –22°C. The initial fecal coliform density of 2.1×10^7 CFU/g TS remained within the same order of magnitude in the next 90 d. The cold storage did not effect even one log reduction that would have allowed to reach Class B level of less than 2×10^6 CFU/g TS. The conditions in sludge stored at 20-22°C initiated a gradual decrease in fecal coliform density to below Class B requirement of 2,000,000 CFU/g TS in 22 to 34 days, and further to below Class A requirement of less than 1000 MPN/g TS around day 70 of the storage. On day 90 the fecal coliform density fell to below the detection level of 300 CFU/g TS.

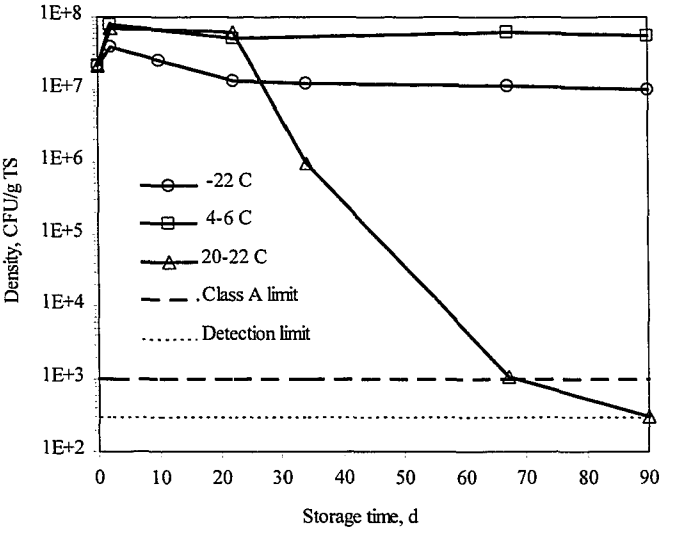

Figure 5-28. Effect of temperature on fecal coliform inactivation in dewatered sludge during contained storage without alkaline additives

Detection limit: detection limit for quantitative determination; CFU: colony forming units; TS: total solids

In the context of winter storage, it can be seen that the anticipated fecal coliform reduction can only proceed to a limited extent, if any. Temperatures in excess of 4°C are expected to promote bacterial growth. The ambient temperatures of 20-22°C allow successful competition of other microorganisms with fecal coliform bacteria whereas at lower temperatures this competition is less apparent. Bacterial activity, as measured by the growth, doubles with every 10°C increase.

Freezing has some effect on pathogen survival. The fecal coliforms proved however very resistant to cold– their die-off was in fact very limited in conditions imitating cold climates of –22°C. The freezing of sludge samples, although in a matter of hours, was relatively slow. Moving average interpretations of the average temperatures during the course of freezing and thawing are shown in Fig. 5-29. It is known that apparently slow freezing is more detrimental to pathogens that rapid freeze. During slow freeze, larger ice crystals are formed outside the cells leading to the exposure to concentrated solutes. The fast freeze (20°C/min and higher) leads to the formation of intracellular ice. In both cases, damage to the cell can occur [Mazur, 1966]. From Fig. 5-16, the rates of freezing and thawing were rather slow around the freezing point of 0°C to –4°C, whereas faster increase or decrease in temperature followed beyond this range. Such slow freezing could have a role in a visible decrease in fecal coliform counts in the frozen sludge (-18°C), as compared to an unchanged fecal coliform population in the sludge following 4-6°C storage (Fig. 5-28).

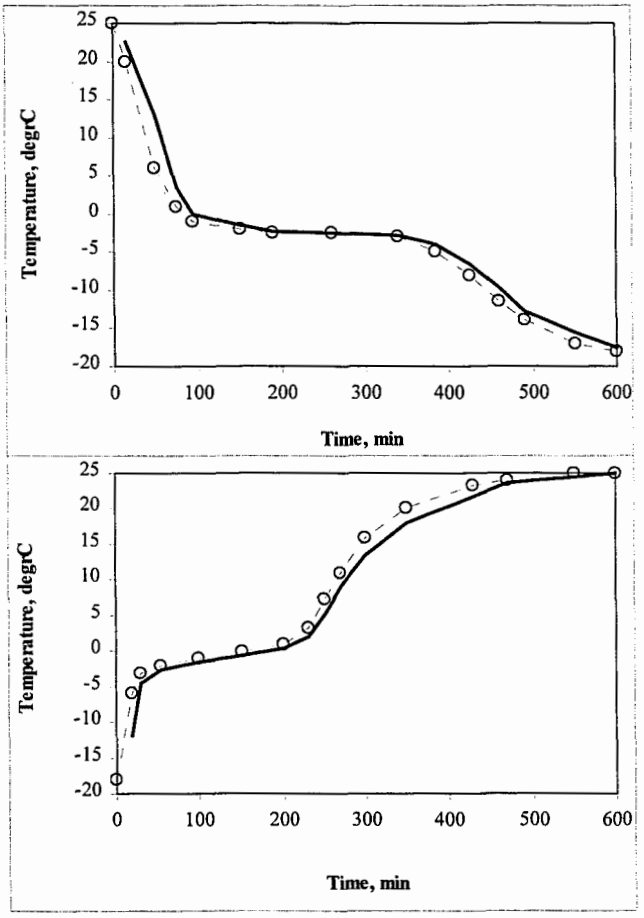

Figure 5-29. The rates of freezing and thawing at start and at termination of storage cycle, respectively, for dewatered sludge stored at –18°C

The solid lines represent moving averages of the data points; top figure shows the rate of freezing; bottom figure shows the rate of thawing

One would expect that the freezing and subsequent thawing would destroy the structure of solids leading to further water release and the increase in TS content of sludge in systems where leachate can be collected. In this particular case, just one freeze-thaw cycle neither produced any additional leachate due to water release nor contributed to a noticeable reduction in fecal coliform numbers.

Usually, following storage at 4-6°C or at –22°C, the microbial activity in the sludge, determined based on the higher fecal coliform counts, slightly increased- within same order of magnitude- and then returned to its previously established level from prior to the storage.

5.8.2 Conclusion

Natural elimination of non-spore forming pathogenic and indicator bacteria during sludge treatment is often site-specific and limited. Higher temperature leads to faster inactivation, like in the 20-22°C-sludge storage study. It took anywhere from 64 d to 83 d to inactivate fecal coliform bacteria to a level corresponding with Class A requirement at this temperature. With decreasing temperature, the rate of reduction is expected to decrease, and longer detention is required to observe similar reductions in pathogenic indicator levels. The cold storage study (4-6°C) was effective in over 2-log reductions in both fecal coliform and *Salmonella* sp. densities at the end of 280-d period. There was no attempt to observe the progress of inactivation on any earlier occasion, that is, in between day 1 and day 280 of the storage.

Freezing of sludge, and subsequent storage at –22°C for up to 90 d, did not effect inactivation to a satisfactory level, but the impact of slow freeze and thaw on fecal

coliforms was visible when compared with the storage a few degrees above freezing (4-6°C). There was no effect on these bacteria during the 90-d period.

5.9 DISINFECTION OF THE DIGESTATE FROM ANAEROBIC CO-DIGESTION OF THE ORGANIC FRACTION OF MUNICIPAL SOLID WASTE AND PRIMARY MUNICIPAL SEWAGE SLUDGE

Application and benefits of anaerobic co-digestion of the OF-MSW and sewage sludge has been demonstrated elsewhere [Hamzawi *et* al., 1998; Rintala and Jarvinen, 1996; Poggi-Varaldo and Oleszkiewicz, 1992; Cecchi *et* al., 1988]. However, implication of the pathogenic potential of the digestion residue was not addressed. Rich *et* al. [1994] demonstrated the absence of pathogenic indicators (total and fecal coliform bacteria, *Streptococcus* and *Enterococcus*) in the humus produced by the thermophilic high-solids anaerobic-composting/aerobic-biodrying process. Identification of the kill factor was not attempted, but digestion at 53°C pointed the temperature.

5.9.1 Effect of storage alone on pathogen reduction

It is known that mesophilic digestion (temperature range of 30-40°C) at common retention times of 15 to 30 d does not significantly contribute to the reduction of pathogenic microorganisms. At human body temperature, most pathogenic species thrive regardless of the presence of other competing microorganisms such as the natural biota found in human feces, including fecal coliforms. However, a long-term storage does

contribute to pathogen die-off, especially in case of non-spore forming bacteria and enteric viruses.

In this work, primary sludge, although found to stimulate the digestion process while co-digested with the OF-MSW, contained pathogenic microorganisms. In a collaborative study done by Frosk [1998], pathogenic potential of the digested material was assessed. The reactors with highest primary sludge proportion and highest compaction were selected for microbiological analyses including such pathogenic indicators as total and fecal coliforms, fecal streptococci and enterococci, coliphage and *Bacteroides*. The digestion in the broad range of solids studied (2 to ~26% TS) in mesophilic conditions was not effective in reducing levels of these indicators of pathogen presence. Another part of the high-solid co-digestion studies involved thermophilic digestion at 55°C (the data are not reported in this document). Some of the indicator organisms were affected due to the effect of heat. During the initial part of the thermophilic experiment (at 2 to 6% TS) all pathogenic indicators (fecal streptococci and enterococci, total and fecal coliforms) except coliphage and *Bacteroides* remained under detection limit. However, despite high temperature, once TS reached higher levels (from 8% up to 16%; analyses not continued at higher solids) all organisms except total and fecal coliforms revived and showed significant resistance. The reason for such outcome was thought to be due to possible shielding effect of the digester solids.

A long-term (2-year) storage of the digestate from all high-solid mesophilic reactors, upon completion of digesters operation, was effective in reducing fecal coliform bacteria to below detection. Although the presence of *Salmonella* in the feed mixtures was not determined at the time digesters were in operation, there were no *Salmonella* sp.

detected after the storage ended. Spores of *C. perfringens* were found at levels in the range from 10^4 to 10^5 CFU/g TS and considered significant. The spores were not assayed for in the digestate before, but such density is not indicative of significant reduction. As already observed in storage studies with dewatered sludge, the storage alone was capable of effectively reducing non-spore forming bacteria such as fecal coliforms or *Salmonella* sp. However, more resistant forms such as bacterial spores remained intact. Similar resistance would be expected in case of protozoan cysts or helminth eggs.

The impact of storage alone in this study was verified by mixing digested-dewatered municipal sludge previously seeded with *Salmonella* sp. and containing indigenous fecal coliforms and *C. perfringens* spores, with the high-solid mesophilic digestate. This step was designed to bring the densities of pathogenic and indicator bacteria in each mixture to a level high enough to observe a meaningful decline during subsequent disinfection. The mixtures were prepared as described in the Materials and Methods section, and were designated PS-15, PS-10, PS-5, and PS-0, depending on the fraction of primary sludge (PS) solids in the feed fed to the high-solid digesters. During the first 17 d, the mixtures were stored in containment and monitored periodically for the bacterial indicators. The average total ammonia content, pH, and corresponding free ammonia concentration in each mixture found during this storage period is shown in Table 5-14. Since the fraction of primary sludge solids in high-solid feed varied, this contributed to the concentration of ammonia in the high-solid digestate.

Table 5-14. Ammonia and pH in high-solid digestate during contained storage (20-22°C).

Parameter	Mixture type			
	PS-15	PS-10	PS-5	PS-0
TAN, mg N/L	2,473 (86)	2,267 (154)	1,703 (136)	1,313 (25)
pH	8.26 (0.06)	8.21 (0.06)	8.17 (0.08)	7.06 (0.39)
UAN, mg N/L	164 (26)	133 (21)	92 (18)	7 (4)

PS-15, PS-10, PS-5, PS-0 - numeric values give fractions (% by weight) of primary sludge (PS) solids in feed fed to high-solid digesters; TAN- total ammonia nitrogen; U-AN - un-ionized ammonia nitrogen or free ammonia; values in parentheses represent standard deviation

Data in Table 5-15 show the extent of fecal coliform and *Salmonella* inactivation. These bacteria were originally supplied to the digestate in numbers sufficiently high to ensure successful competition with other, indigenous, microorganisms found in it prior to dewatered sludge addition. A rapid decline in fecal coliform and *Salmonella* sp. numbers to below detection was seen in the PS-15, PS-10 and PS-5 mixtures and by day 17 was further confirmed by lack of their presence in the first two mixtures. The PS-5 mixture was found positive for the presence of fecal coliforms and *Salmonella* sp., while only up to 2-log reduction was seen in the PS-0 mixture.

Table 5-15. Influence of contained storage (20-22°C) on non-spore forming bacteria in high-solid digestate.

Organism	Parameter	Mixture type			
		PS-15	PS-10	PS-5	PS-0
Fecal coliforms	Initial density, MPN/g TS	1.96×10^{7}	1.09×10^{7}	1.08×10^{7}	8.70×10^{6}
	Log reduction after:				
	7d	3.78	3.22	3.10	1.43
	17d	$8.18^{(-)}$	$7.92^{(-)}$	$7.92^{(+)}$	1.65
Salmonella sp.	Initial density, MPN/g TS	1.96×10^{5}	4.13×10^{5}	4.12×10^{5}	1.09×10^{6}
	Log reduction after:				
	7d	3.26	3.32	3.32	1.52
	17d	$6.18^{(-)}$	$6.50^{(-)}$	$6.50^{(+)}$	2.22

PS-15, PS-10, PS-5, PS-0 - numeric values give fractions (% by weight) of primary sludge (PS) solids in feed fed to high-solid digesters; MPN/g TS- Most Probable Number in one g total solids; $^{(-),\ (+)}$ Qualitative (presence-absence) test results: negative, positive.

These different inactivation patterns correspond with the level of pH and free ammonia (UAN) concentration, with the inactivation progressing at increasing pH. The contribution of UAN at this pH is quite evident, with only 6 to 7% of total ammonia nitrogen as UAN at a pH 8.3. Free ammonia concentrations in excess of 130 mg N/L led to fast inactivation of fecal coliforms and *Salmonella* sp., virtually within a couple of weeks. Lower pH and TAN of the mixture with no primary sludge showed limited inactivation, which makes this method unsuitable to disinfect the high-solid residue upon confirmation of its pathogenic content.

5.9.2 Effect of lime on pathogen reduction

The contained storage had no impact on the spores of *C. perfringens* in all four mixtures. Disinfection of such material is therefore necessary to ensure a pathogen-free product and compliance with regulations concerning its final use/disposal, e.g. with the U.S. EPA Final Rule [1993]. A lime dose of 100 g CaO/Kg TS, much smaller than the doses of 500 to 1,000 g/Kg TS commonly used in conventional disinfection, was capable of raising the pH to above 12 in a matter of minutes and converting all of the ammonia (Table 5-14) to its un-ionized form. This dose was selected based on the previous work with digested-dewatered sludge. The prolonged storage at room temperature followed, which resulted in a minimum 3-log reduction in the spore density in all samples (Fig. 5-30).

Assessment of the treatment efficiency and potential for spore repair and reacquisition of infectivity was done by including lysozyme in the TSC agar medium used to enumerate *C. perfringens* spores. Lysozyme, an enzyme responsible for *C. perfringens* spore germination and likely removed during alkaline treatment, was used to allow for higher recovery of injured spores from the mixtures by the end of storage. After one day from lime addition, the reduction in recoverable spore population was 1-log lower than in total population. After 9 days of contained storage, there were no detectable spores in treated material.

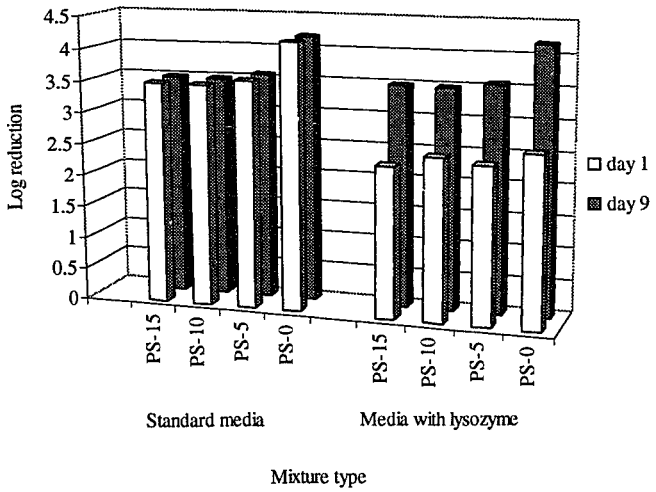

Figure 5-30. The extent of *Clostridium perfringens* spore injury and permanent inactivation in low-dose alkaline disinfection of high-solid digestate*

PS-15, PS-10, PS-5, PS-0: numeric values give fractions (% by weight) of primary sludge (PS) solids in feed fed to high-solid digesters;

*higher reduction was observed in the PS-0 mixture due to higher initial spore density

5.9.3 Conclusion

Simple disinfection practices, such as long-term storage and low-dose lime treatment followed by contained storage, have been demonstrated. The storage alone reduced fecal coliform and *Salmonella* sp. bacteria to below detection in mixtures with minimum total ammonia concentration of 1,700 mg N/L. The densities of these indicators stayed above Class A pathogen limit, even though the total ammonia level was still high, around 1,300 mg N/L. Higher pH in the mixtures with efficient reductions (min. pH 8.17) pointed to the disinfecting effect of free ammonia (min. 92 mg N/L).

The alkaline treatment in particular is a reliable and easy way of reducing hardy forms such as bacterial spores, a property unlikely to be attained during long-term digestate storage alone. Lime dose of 100 g CaO/Kg TS was effective in achieving over 3-log reductions in *C. perfringens* spore densities. The extent of recoverable damage was minimal, as less than 1% of the initial population could be recovered (reduction > 2-log) after day 1 from start. After 9 d of storage, the lysozyme-supplemented medium was incapable of inducing any growth, with both quantitative (direct plating) and qualitative (P-A test) methods in place.

5.10 EFFECT OF HIGH PH ALONE AND IN COMBINATION WITH HIGH FREE AMMONIA CONCENTRATION ON PATHOGEN KILL IN DILUTED MEDIA

Determination of the primary disinfection mechanism during alkaline treatment is not an easy task as many interrelated mechanisms, including disinfecting action of free ammonia and high pH, can contribute to microorganism inactivation. A better explanation of the inactivation mechanisms is believed to yield information useful in understanding of how this process actually works and how to optimize it. Determination of the mechanism by which pathogen inactivation occurs would have far-reaching implications.

There are conflicting reports on the nature of bacterial inactivation, including inactivation by chemical treatment. In general, it is very difficult to separate the effects of NH_3 from those of high pH (or high OH^-) on bacteria in alkali treated biosolids. Differentiation of pH-dependent free ammonia inhibition from the effects of high pH can be very informative because of the different nature of inhibition [DePasquale and Montville, 1990; Cramer *et al.*, 1983]. Pesaro *et al.* [1995] claimed that, at pH values of more than 8.0, free ammonia was the major virucidal agent. On the other hand, Seagren *et al.* [1991] showed that ammonia (both forms) was not the toxic agent that caused inhibition of methanogenesis at pH above 8.1. Enzyme activity, membrane transport, and proton-motive force were the factors cited by the authors as those suffering from high pH alone. Although the majority of pathogenic bacteria differs in their characteristics from methanogenic populations, they all experience the lack of a protection mechanism that

would let them survive under adverse conditions of high pH and free ammonia. In addition, hostile conditions within sludge have an impact on some bacterial pathogens such as *Salmonella* sp. [Yanko *et al.*, 1995]. Spore forming pathogenic microorganisms such as *C. perfringens* are much more difficult to inactivate.

In this part of the research program, an attempt to determine primary causes of inactivation and assess their contribution was undertaken. As indicated before, it would not be possible to distinguish between the effects of high pH and of high free ammonia concentration in such complex material like sewage sludge. This is because removal of ammonia from the sludge to observe the effect of high pH alone cannot be achieved without altering physical and chemical characteristics of the sludge. In this study, well-defined artificial media, with and without ammonia nitrogen, were used. Such approach was successfully used in a study of virucidal agent determination [Ward and Ashley, 1977]. One major characteristic making the media different from the sludge was that they were solids-free, which did not let accounting for the effect of solids. Another important difference was the reduction-oxidation potential. Raw dewatered sludge had an ORP value of –250 mV, whereas diluted media were clearly aerobic with an ORP of +100 mV. Although not directly transferable, the anticipated results were thought sufficient to describe the relative impact of high pH and of free ammonia concentration on pathogen inactivation.

5.10.1 Impact on the spores of *C. perfringens*

In experiments I and II (two sets of data), the spores of *C. perfringens* were added to both media with pH adjusted to 12.0, and spore inactivation was measured at intervals

(sampling at 2h, 6h, 24h and 48h) over the period of 2 days. Fig. 5-31 (experiments, I and II) shows the impact of the combination treatment (high pH & UAN concentration) and high pH alone on the survival of *C. perfringens* spores. Medium with no free ammonia but subjected to the same pH condition is called "Control", while the ammonia-containing medium is called "UAN". In the case of spore recovery from both media using standard TSC agar without lysozyme supplementation, there were no spores detected on first sampling occasion, i.e. after 2 h from start of incubations. Obviously, it was impossible to draw any conclusion as to the difference, if any, in inactivation patterns in either medium. However, when lysozyme was added to the standard agar, the obtained progressing inactivation curves in both media were evident. The major observation is that the inactivation proceeded at similar rates in both ammonia-free medium, and in ammonia-containing medium. This finding implies that the major sporocidal agent was high pH alone. The presence of free ammonia upon conversion from ionic form and at a concentration common to dewatered sludge, that is ~1,300 mg N/L, did not play any significant role in this inactivation. Higher ammonia concentrations were not tested. The assessment of possible increase of inactivation following ammonia supplementation was beyond the scope of experimental work conducted.

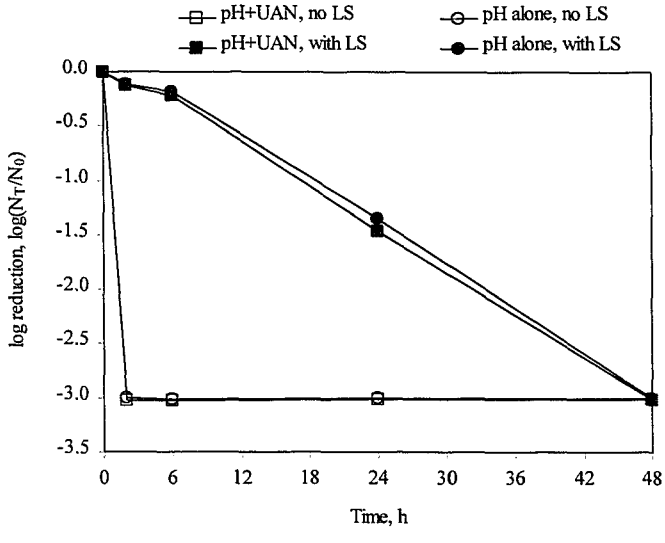

Figure 5-31. Impact of high pH alone (12.0) and in combination with high free ammonia concentration (1,325-1,223 mg N/L, ~100% TAN) on inactivation of *Clostridium perfringens* spores during 2-day storage at 20-22°C

LS: lysozyme, TAN: total ammonia nitrogen, UAN: un-ionized ammonia nitrogen

It is known that alkaline environment is responsible for the inactivation of spore lytic enzymes, including lysozyme. The inclusion of lysozyme allowed for a quantitative recovery of the fraction of original spore population that was reversibly injured. Lysozyme is found in the spore cortex, one of the layers of the spore coat [Duncan *et al.*, 1972]. Over 90% (equivalent to 1-log) reduction in the number of spores following high pH treatments in both lysozyme-supplemented media after 24 h suggests that the site of inactivation is in the upper layer of the spore coat. It is not known if any other damage to the spore occurred and what effect it might have had on the overall reduction in spore viability. The difference in inactivation rates observed between lysozyme-supplemented spore cultures and those without the enzyme also indicates that the high pH played the primary role. There are no documented cases of free ammonia altering any of the spore lytic enzymes, and so the high pH (12.0) is deemed to be the major cause of the observed inactivation.

In experiment III, the spores were subjected to four different pH levels in both, ammonia-containing and ammonia-free, media for 24 h. The pH values were 7.0, 9.5, 10.0, and 12.0. The spore levels in both media at pH 7.0 were treated as a reference pertaining to the initial condition found in the media at higher pH levels, and was taken as the basis for calculation of inactivation. As shown in Fig. 5-32 (experiment III), there were no significant reductions observed at pH 9.5 and 10.0. Hence, the pH alone and in combination with the corresponding free ammonia concentration of 750 mg N/L (pH 9.5) and 1,050 mg N/L (pH 10.0), in ammonia-free and ammonia-containing media at pH 9.5 and 10.0 respectively, had no impact on the spores. Since the next higher pH was 12.0, it was impossible to determine what would be the effect of pH in between these two

levels. At this highest pH investigated, the extent of inactivation in both media was similar to what was observed in experiments I and II after 24 h from start. Spore reduction to below detection level equivalent to a 3-log reduction, was seen in standard agar with no lysozyme added, whereas lysozyme supplementation allowed for over 1.5-log lower reduction.

Since the survival of the spores was based on the ability to form colonies on agar, it was impossible to determine whether injury involved one of components of the germination or post-germination system. The major observation concerning the nature of spore inactivation led to the conclusion that high pH alone is the killing factor for spores of *C. perfringens* and that free ammonia has limited, if any, contribution.

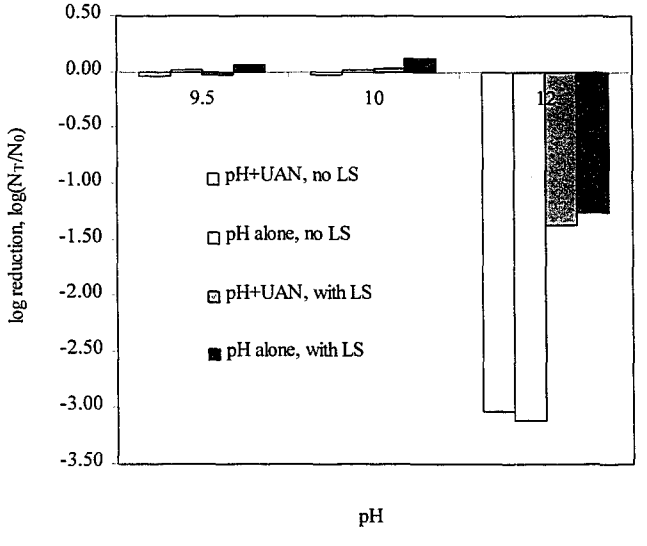

Figure 5-32. Impact of high pH alone and in combination with high free ammonia on inactivation of *Clostridium perfringens* spores during 24-hour storage (20-22°C) at pH of 9.5, 10.0, and 12.0 (corresponding to the pH calculated free ammonia fractions of TAN are 57% or 663 mg N/L, 80% or 973 mg N/L, and ~100% or 1,237 mg N/L)

Control: no ammonia nitrogen present; LS: lysozyme; TAN: total ammonia nitrogen; UAN: un-ionized (free) ammonia nitrogen

5.10.2 Impact on the eggs of *Ascaris suum*

In experiments II and III, eggs of *Ascaris suum* were also used as indicators in addition to the spores of *C. perfringens*. In experiment II, egg densities (similar to spore densities), were measured at start, after 2 h, 6 h, 24 h, and after 48 h from start. In experiment III, the measurements were done at the beginning, and after 24 h from start.

The results of experiment II show that both, high pH and high free ammonia concentration were the factors contributing to egg inactivation. As seen in Fig. 5-33, the progress of inactivation was higher in ammonia-containing medium. The initial viability was 93.9% in the control medium (ammonia-free medium), and 94.6% in the UAN medium (with ammonia).

The relative decrease of viability at corresponding times due to un-ionized ammonia alone (UAN), obtained by subtracting the % decrease in the Control medium (high pH) from that observed in the ammonia medium (high pH and UAN), has been estimated as 56.2% at 2 h, 66.9% at 6 h, 62.9% at 24 h, and 54.3% at 48 h. With storage time up to 6 h, the relative share of un-ionized ammonia in the overall inactivation increased, but then it decreased. The lack of lag in inactivation suggests that the egg shell became permeable from the beginning of the treatment allowing the ammonia molecules to enter inside the eggs. This, in turn, led to the progressing loss of viability. Similar inactivation was observed after 2 h (and 6 h) in ammonia-containing medium, and after 24 h (and 48 h) in ammonia-free medium.

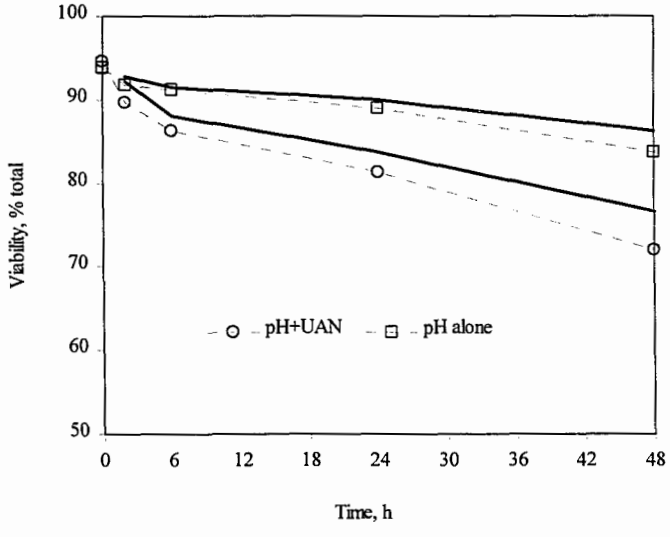

Figure 5-33. Impact of high pH alone (12.0) and in combination with high free ammonia concentration (1,325-1,223 mg N/L, ~100% TAN) on viability of *Ascaris suum* eggs during 2-day storage at 20-22°C

The solid lines represent moving averages of the measured values; TAN: total ammonia nitrogen; UAN: un-ionized ammonia nitrogen

In experiment III, the toxic effect of ammonia after 1 d of storage was further confirmed (Fig. 5-34). When the fraction of viable eggs was determined in each medium, it was evident that larger impact was in the media with ammonia. Another interesting observation was made about the extent of inactivation at lower pH levels of 10.0 and 9.5. In ammonia-containing media, the corresponding free ammonia concentrations were 663 mg N/L (57% TAN), 973 mg N/L (80% TAN), and 1,237 mg N/L (~100% TAN). At these pH levels, decrease in egg viability can be directly related to the increased fraction of free ammonia in the medium. In the Control medium, the effect of high pH alone was only visible at the highest tested pH of 12.0. Egg inactivation by high pH alone requires the hydroxide ion concentration of two orders of magnitude higher than in the case with ammonia containing medium.

There is a correlation between the concentration of free ammonia and the extent of inactivation, as depicted in Fig. 5-35. Viability of the eggs in Control (high pH alone) media in the pH range from 9.5 to 12.0 varied from 95.1% to 89.6%, whereas a linear trend of decreasing viability was observed in the ammonia-containing media. Similar level of 9.5 was previously defined as the minimum value for effecting inactivation of fecal coliform and *Salmonella* sp. bacteria in digested-dewatered sludge.

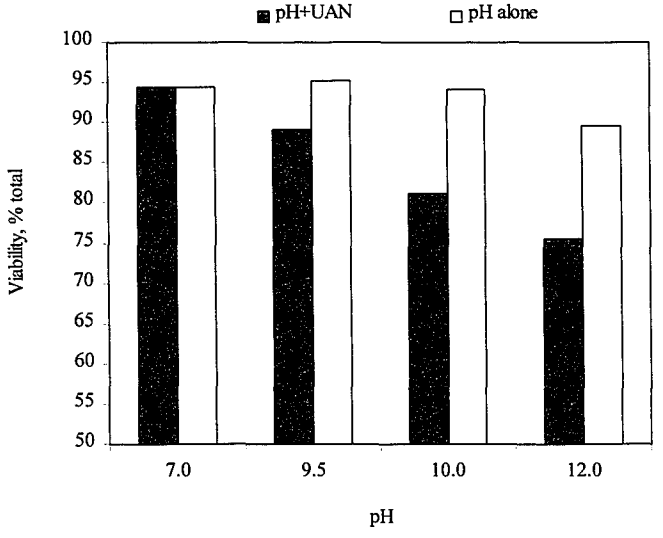

Figure 5-34. Impact of high pH alone and in combination with high free ammonia concentration on inactivation of eggs of *Ascaris suum* during 24-hour storage (20-22°C) at pH of 9.5, 10.0, and 12.0 (corresponding to the pH calculated free ammonia fractions of TAN are 57% or 663 mg N/L, 80% or 973 mg N/L, and ~100% or 1,237 mg N/L)
TAN: total ammonia nitrogen, UAN: un-ionized (free) ammonia nitrogen

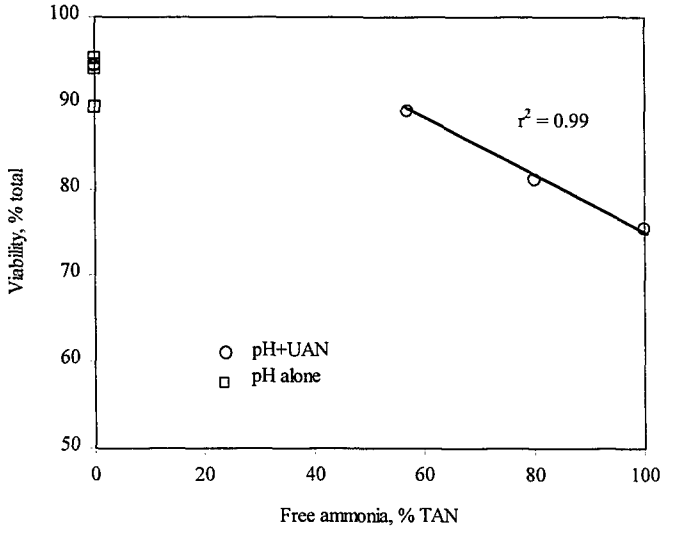

Figure 5-35. Impact of free ammonia on inactivation of *Ascaris suum* eggs during 24-hour storage (20-22°C) at pH of 9.5, 10.0, and 12.0 (corresponding to the pH calculated free ammonia fractions of TAN are 57% or 663 mg N/L, 80% or 973 mg N/L, and ~100% or 1,237 mg N/L)

TAN: total ammonia nitrogen, UAN: un-ionized (free) ammonia nitrogen

There is a niche for application of molecular technique(s) such as polymerase chain reaction (PCR), potentially useful in assessing *Ascaris* egg viability indirectly. The recovery of RNA from, or selection of a marker that would be turned on by growth in, *Ascaris* would allow proper identification and even quantification of *Ascaris* RNA. The pioneering work in this area was done on expression of both DNA and RNA during several stages of *Ascaris* embryogenesis [Davis *et al.*, 1999]. The embryos of *Ascaris* were used as a model to demonstrate the application of biolistic transfection, or particle bombardment, a technique that can complement other biochemical tools available (e.g. *in vitro* whole-cell embryo extracts for transcription, RNA processing, translation). So far, there are no other similar reports. Such technique would surely overcome the current shortcomings of available methods, such as the need for a month-long incubation to allow eggs develop from unicellular to larval stage. This lengthy test means longer retention of a treated biosolid sample prior to land application to confirm the absence of viable ova.

5.10.3 Conclusion

In this part of work, the independence of *C. perfringens* spore inactivation from the presence of free ammonia at a level of up to ~1,300 mg N/L was demonstrated. *Ascaris* egg inactivation was, in turn, dependent on free ammonia concentration. The extent of permanent spore inactivation was high. The surviving fraction, following recovery in lysozyme supplemented medium, accounted for only 3.4% of the initial population after 1 d of storage. Spore inactivation was complete after 2 d of storage. The impact of free ammonia on *Ascaris* eggs was significant accounting for 54 to 67% inactivation from free ammonia alone. Although affected by, both, high pH and

corresponding high free ammonia concentration, the eggs were much more resistant than the spores of *C. perfringens*. The highest reduction of about 0.1 log was noted in medium at pH 12.0 after 2 d of storage.

5.11 CORRELATION OF *C. PERFRINGENS* SPORE REDUCTION WITH THE REDUCTION IN *ASCARIS SUUM* EGG DENSITY

A comparison, using the collected data, was attempted to find out if *C. perfringens* spores can be used as a surrogate human pathogen (bacterial) in lieu of helminth ova, such as *Ascaris* species. Obviously, spore presence does not necessarily indicate the presence of parasitic eggs, but demonstration of correlation between their removal rates would offer a number of advantages. The *C. perfringens* spore enumeration method is much simpler, easier, and less time-consuming as compared with that of *Ascaris* eggs. Analytical time to quantify spores is much shorter (2 d) than that needed for *Ascaris* eggs recovery from sludge and viability test (2 d + 28-30 d). The ability of bacterial pathogens to regrow once conditions improve is another important factor, whereas the number of parasitic eggs will always be equal to, or lower than, the initial number. On the other hand, this correlation would have to be strong enough to prevent from not accounting for even a few viable eggs in the sample, as the potential infectivity level for parasite eggs is very low (1 viable egg).

The preliminary work on the surrogacy of *C. perfringens* spores with regard to *Ascaris* eggs was done by Tulane University (New Orleans, LA, U.S.A.) researchers. In their report [Blanker *et al.*, 1992], the evaluation was performed using sludges treated

chemically in three classified processes. These were the Synox process (an acid/ozonation/nitrous acid treatment), thermophilic alkaline processes (Chemfix, RDP, N-Viro, BFI; various alkalis and solidifying agents are added and the pH and temperature are raised), and a heat treatment/alkaline process (the Pori Biosolids Treatment Technology). It was found that although the log reduction relationship between *C. perfringens* spores and *Ascaris* eggs was not significantly linear, the reduction of spores to less than 100 CFU/g TS of sludge allowed to estimate *Ascaris* egg reduction to less than 1 egg/25 g TS. This indirect relation was valid for both types of processes using either heat or biocidal chemical agent or both mechanisms.

One comment has to be made as to the method these researchers used for *C. perfringens* determination. The method utilized nutrient agar supplemented with sodium sulphite as an antioxidant, and with ferrous ammonium sulfate to detect bacterial reduction to sulphide and black colony growth as a result of reaction with Fe^{+3} to form FeS. However, there were no means to differentiate the *C. perfringens* from other *Clostridia* in the sludge. It is, therefore, proper to treat the results of Blanker *et al.* [1992] as those regarding spores of sulfate-reducing *Clostridia* and not *C. perfringens* alone. Nevertheless, in the context of disinfection, it does not change the extent, by which bacterial spores were reduced and the relation between the densities of these two pathogens would still hold true.

In the work reported here, the comparison of *C. perfringens* spore reductions with those of *Ascaris* was made using two different materials: digested-dewatered sludge, and diluted media. In the sludge disinfection experiments, a spore reduction of 2.56 log was equivalent to a 0.73-log reduction in viable eggs at 80 g CaO/Kg, after 280 d of contained

storage (Table 5-16). When the lime dose was doubled (160 g/Kg), the spore reduction increased to 2.74 logs and egg reduction to 0.98 logs. In both treatments, the pH stayed above 12.0 for the entire duration of storage. Although limited, but similar, reduction of 0.30 logs for the spores and 0.30 logs for the eggs was noted in 40-g CaO/Kg TS treatment. The spore densities quoted above are these of lysozyme-supplemented cultures. It is believed that limited reduction in *Ascaris* egg viability was due to low temperature of storage of 4-6°C. Unfortunately, higher reductions in both indicators e.g. to below limit for quantitative determination, were not observed. Thus, it was impossible to observe the inactivation that would take place in the entire range of pathogen densities.

The extent of inactivation for both pathogenic forms was different, but when spores of *C. perfringens* were reduced by 99.8%, a corresponding reduction of about 80% in viable *Ascaris suum* egg population was seen.

In the experiments using diluted media, at pH of 12.0 the reductions were 1.38-1.47 logs for the spores and 0.048-0.079 logs for the eggs after 1 d of storage at 20-22°C. Additional storage for one more day effected further inactivation to below detection for quantitative determination (>3.0 log) for the spores and almost 0.1 logs for the eggs. The rates of inactivation in treated dewatered sludge were lower than the rates observed in diluted media. This can be contributed to the following factors different for each type of material: (1) the temperature of storage was higher in the studies with diluted media, which could trigger higher susceptibility of both pathogenic forms to high pH alone and in combination with high free ammonia concentration; (2) the difference in reduction-oxidation potential in both materials could have an impact too, although the mechanism by which inactivation progressed would seem similar; (3) in the diluted media, there was

also no interference from any other chemical interactions like these taking place in treated sludge and decreasing the disinfecting potential of lime and/or fly ash mixtures.

Table 5-16. Comparison of inactivation of the *C. perfringens* spores with eggs of *Ascaris suum* during alkaline treatment in different media.

Medium type, condition of storage	pH, storage time		C. perfringens spores	Ascaris suum eggs	
			Log Reduction	Log Reduction	Viability loss*, $(V_{final}/V_{initial})10^2$
Dewatered sludge, storage ("anoxic") at 4-6°C, high pH and UAN	Lime added				
	pH>12.0, g/Kg TS +,	80-160 280 d	2.56 - 2.74	0.73-0.98	63.9-78.7
	11.0>pH>12.0, g/Kg TS,	40 280 d	0.30	0.34	14.0
Diluted medium, stor. (aerobic) at 20-22°C,	NaOH added				
High pH and UAN	pH ~ 12.0,	1 d	1.38-1.47	0.048-0.079	14.1-20.0
		2 d	>3.0	0.097	23.9
	pH ~ 10.0,	1 d	0	0.057	14.1
	pH ~ 9.5,	1 d	0	0.014	5.7
high pH alone	pH ~ 12.0,	1 d	1.26-1.35	0.023-0.033	5.2
		2 d	>3.0	0.043	10.9
	pH ~ 10.0,	1 d	0	0.004	0.4
	pH ~ 9.5,	1 d	0	0.003	0

* A 90% loss of viability corresponds to 1-log reduction

Limited inactivation in experiments using diluted media does not allow for drawing any conclusions as to the correlation between inactivation of eggs and spores. As well, a limited number of measurements were taken to claim the correlation between spore and egg survival (36 in sludge disinfection studies, and 18 in studies with diluted media). More work would need to be conduced at higher storage temperatures to confirm

the suspicion of increased rates of inactivation. Nevertheless, from the sludge disinfection experiments at 4-6°C, it appears that even though total ammonia was completely converted to the free ammonia (at pH of 12.0 and higher) the inactivation was only limited. Increased rate of inactivation is believed linked to higher doses of alkaline compounds. Thus, lime doses in excess of 160 g/Kg TS would have to be used to observe a broader egg inactivation within the 9-month period. As well, longer detention at 160-g/Kg TS dose could effect further inactivation. The storage of treated sludge at 4-6°C has been continued beyond day 280 (data not available at the time of this writing), and an effective C×T product under this condition is yet to be established.

Egg viability is defined as the ability of the eggs to develop to the third larval stage upon cultivation, whereas spore viability is defined as the ability to grow to a visible size in agar medium as a colony. The direct plating method allowed for estimation of up to 3-log, i.e. meaningful, reduction in spore density. The use of an MPN technique rather than the direct plating for the calculation of spore numbers would allow lowering the detection level for quantitative determination, and shall be pursued in future research on the subject.

Another factor to be considered while making comparison between the two indicators is the difference in handling and recovery techniques. The effect of alkaline pH and ammonia on permeability of an egg shell is evident. Once recovered from sludge or from diluted media, the eggs were free of alkaline residue and were ready to undergo incubation for around 40 to 50 days at 28°C in 0.5% formalin (incubation fluid) to determine their viability. This extra time over the standard time of 28 days was usually given to allow for a complete development of possibly damaged eggs. However, if the

eggs were damaged, they would be incapable of further maturation anyway. Once the injury occurs there is little possibility that the eggs would develop.

Certain part of unembryonated egg population had internal structure of multicellular appearance, even after prolonged incubation. The lack of full development points at possible inhibition by factors other than alkaline treatment itself. The morphology of eggs recovered from sludge was never observed on samples prior to start of incubation, and so we were unable to determine the stage of development. However, sludge storage at 4-6°C, in a virtually oxygen-free environment, automatically limits the possible development to none. In studies with the use of diluted media, the egg morphology was occasionally checked prior to viability test. There were no aforementioned anomalies observed though the treatment time of up to 2 d was obviously much shorter than in the sludge treatments. With this in mind, a couple of other causes are elucidated.

First, increased egg permeability can lead to the unwanted loss of some of the nutrients from within the egg, otherwise needed for full development to larval stage. Resulting deficiency might have allowed for only partial maturation.

As the result of weakening the shell structure the formalin can, with time, enter the interior of an egg and prevent from developing to the larval stage being considered the evidence of viability. The formalin is used to prevent growth of fungi and other microorganisms capable of preying on eggs, but it can become toxic to the eggs having weakened wall structure as well. No other media were used to incubate eggs in the viability test, but the ability of some of the eggs to develop to a multicellular stage and then stop the process suggests that there is an impact due to culture fluid.

5.11.1 Conclusion

Applicability of using *C. perfringens* spores instead of *Ascaris* eggs as indicator of alkaline disinfection efficiency was addressed. Confirmation of egg viability often requires the expertise of a parasitologist. Determination of *C. perfringens* spore density requires less troublesome approach to determine. The extent of inactivation for both pathogenic forms varied. At pH 12.0 and higher, when spores of *C. perfringens* were reduced by 99.8%, a corresponding reduction of about 80% in viable *Ascaris suum* egg population was seen. The observations come from a limited sample population and further studies would have to be pursued to expand the database for making the comparison and to validate the conclusions. Nevertheless, the results indicate the independence of *C. perfringens* spore inactivation from free ammonia concentration, at least in the range investigated (UAN from 660 mg N/L up to 1,270 mg N/L). The eggs of *Ascaris suum* were inactivated at increased rate under the influence of free ammonia.

Chapter 6.

6 CONCLUSION

6.1 LOW- AND HIGH-SOLID ANAEROBIC DIGESTION

The research on the stimulating function of ammonia, as well as inhibition by the free ammonia in high-solid anaerobic digestion led to the following conclusions:

6.1.1 Anaerobic digestion of chicken manure

(1) Chicken manure digestibility was confirmed to be high but the extent of degradation largely depended on process conditions. The most concentrated substrates severely inhibited methane generation. It was directly proportional to the organic load and the resulting concentration of free or un-ionized ammonia (UAN). The efficiency of organic matter conversion to methane was found to decrease with increasing organic loads to the digesters because of the concomitant increase in reactor ammonia and pH, which led to increased levels of UAN. Methane production took place at free ammonia concentrations of up to 250 mg N/L, above which methane production ceased.

(2) Highest solid reactors (initial TS ~20-22%) suffered from extremely high VFA (>45,000 mg HAc/L) and TAN (>14,800 mg N/L) levels. Digestion was limited to fermentation only.

(3) Chicken manure digestion at initial TS of 15.7% was possible, although not practical, as it exhibited the longest lag phase (62 d) and the overall organic conversion efficiency to methane was only 25%.

(4) High-solid anaerobic digestion of chicken manure was shown feasible at initial solids concentration up to 11% TS, despite high initial concentrations of total ammonia (up to 3,500 mg N/L), free ammonia (up to 250 mg N/L) and of VFA (up to 25,800 mg HAc/L). Alkalinity generated from ammonia yielding reactions allowed for partial neutralization of accumulating VFA.

(5) The highest biochemical methane potential of 308 mL CH_4/g COD was found with fresh manure diluted to 5% total solids, confirming that the dilution factor has a critical effect on digester performance.

(6) The organic nitrogen conversions to ammonia (TAN), ranging from 61.8% to as high as 78.9%, were achieved in most experimental digestions. Such high conversions point at relative independence of hydrolytic/acidogenic activity from high initial (total and free) ammonia or VFA concentrations.

(7) Suitability of anaerobically digested municipal sludge and of pit manure to seed digesters treating chicken manure was of limited value. Initial acclimation was necessary in all investigated experiments.

6.1.2 Anaerobic co-digestion of the OF-MSW and primary sludge

(1) The importance of appropriate ammonia nitrogen level in anaerobic digestion of organic matter high in carbon was demonstrated on the example of the organic fraction of the municipal solid waste (OF-MSW). This substrate with high C/N ratio of 63, when digested alone regardless of proper digestion start-up, resulted in un-balanced operation evidenced by high volatile fatty acid (VFA) accumulation and inhibited methane production. The possible influence of other factors, such as insufficient minerals or

vitamins to effect biodegradation, was excluded by lack of improvement in additional degradation or methane production following addition of these compounds to the digesters.

(2) Supplementation of the OF-MSW with primary municipal sludge (to adjust the C/N to values of from 51.1 to 39.9) resulted in improved methane production and biodegradable volatile solids (BVS) destruction at all three levels of addition (5%, 10%, 15% of dry weight as primary sludge solids). The highest net (from degradation of the OF-MSW alone) specific methane yield of 0.3 to 0.5 L CH_4 g BVS^{-1} d^{-1}.was achieved in reactors fed a OF-MSW feed supplemented with highest portion of primary sludge solids of 15% (dry weight). This yield corresponded with BVS conversion efficiency of 50 to 66%.

(3) The most productive digesters could tolerate high concentrations of un-ionized ammonia (NH_3) of up to 230 mg N/L. However, the evidence of decreasing ultimate CH_4 production with increasing fraction of primary sludge in the feed and NH_3 concentration shows the impact of the free ammonia on the extent of BVS reduction.

(4) Surface area of the paper component in the high-solid feed of 17 mm^2, 39 mm^2, and 1.13 mm^2, had no effect on the specific weight of the feed but the amount of primary sludge solids had. As a result, reactors fed the feed with different particle size but equal amount of primary sludge had similar methane yields. If a feed to high-solid digesters has a varying particle size but in a similar range, the likely observation would be to see no significant impact on the process performance.

6.2 LOW-DOSE ALKALINE DISINFECTION

The research on low-dose alkaline disinfection in the aspect of the effective use of free ammonia and other abiotic factors, led to the following developments:

(1) Inactivation of non-spore forming bacterial indicators, including fecal coliforms and *Salmonella* species, using low-dose alkaline disinfection was found feasible. When using fecal coliforms as a model indicator of pathogen presence and quick lime, the minimum value for the product of disinfectant concentration and contact time (C×T) for Class A biosolids designation, was 100 g h/Kg TS, indicating fast inactivation. The C×T value is valid at lime dose of 30 g/Kg TS (e.g. 9.5 g/Kg wet weight for the sludge at 31.5%TS) and higher. The minimum pH for non-spore formers' inactivation was found to be 9.5, with the corresponding free ammonia concentration of about 1,200 mg N/L. The inactivation of fecal coliform and *Salmonella* sp. bacteria was virtually complete in the entire range of temperatures from 4°C to 22°C in 1 day from start.

(2) The spores of pathogenic *C. perfringens* were much harder to disinfect. The C×T>2600 g d/Kg TS was needed to obtain 3-log inactivation (99.9%). Complete inactivation required a dose of 80 g CaO/Kg, pH≥12.0 and 10 to 11 week-long contained storage (C×T>5,600 g d/Kg TS). The corresponding free ammonia concentration in high-solid residues would be more than 2,000 mg N/L, or ~100% of the total ammonia. The use of lysozyme supplementation to assess the extent of recoverable spore injury was found useful. It demonstrated the ability of hardiest spores to regain their viability and, potentially, infectivity. Lysozyme addition in standard media is suggested for routine use in spore recovery of any spore-forming pathogen.

(3) Fly ash doses had to be up to 20 times higher to achieve similar degree of fecal coliform reduction as when using lime. Time to Class A equivalent inactivation was also longer, 4 d for 900 g/Kg TS and 10 d for 600 g/Kg.

(4) Combination treatment using fly ash and small quantities of lime was found to increase the alkalinity generation from fly ash by the factor of 2.7 to 13.7. The decline in pH of treated sludge was less steep than in case of lime or fly ash when used alone at lowest doses used. An improved control over the process was related to the ability to predict the average pH increase following alkali additions; 0.5 units for lime for every 20-g increment, and 0.2 to 0.3 units for fly ash for every 300-g increment. The proper administration of both alkalis makes it easier to obtain anticipated pH, regardless of the storage temperature.

(5) When lime was used at the highest dose of 160 g/Kg TS, the 280-d storage at 4-6°C resulted in a limited inactivation of *Ascaris* eggs of ~1.0 log. Combination treatment using 20 g CaO and 600 g fly ash/Kg TS resulted in similar egg inactivation as seen in 80-g CaO/Kg TS treatment (80% or 0.74-log), even though the maximum pH attained was significantly lower than in the limed sludge (10.2 versus >12.0).

(6) The disinfecting potential of contained storage of digested solids alone (i.e. without alkaline additives) is related to the temperature of storage. Storage of digested-dewatered sludge for 9 months at 4-6°C resulted in limited 2- to 3-log inactivation of non-spore formers, fecal coliforms and *Salmonella* species. Storage for up to 83 d at 20-22°C was required to reduce fecal coliform levels to Class A requirement of <1000 MPN/g TS. Free ammonia had little if any effect in the above cases with sludge, as pH stayed in the neutral range for the duration of storage. However, when high-solid

digestate from co-digestion of the organic fraction of municipal solid waste and primary sludge was stored at 20-22°C, similar inactivation took only 17 d. Such fast inactivation was linked to the disinfecting effect of free ammonia. The minimum recorded pH was ~8.2, and corresponding free ammonia concentration >90 mg N/L.

(7) In the research on contribution of free ammonia concentration to the overall inactivation, the independence of *C. perfringens* spore inactivation from the presence of free ammonia at a level of up to ~1,300 mg N/L (pH 12.0) was demonstrated. *Ascaris* egg inactivation was, in turn, dependent on free ammonia concentration, and progressed linearly with UAN ranging from 57% to 100% of the total ammonia (from 663 mg N/L at pH 9.5 to 1,237 mg N/L at pH 12.0). The share of the free ammonia in overall inactivation was 54 to 67%. The extent of permanent spore inactivation over time was much higher as compared with the eggs of *Ascaris suum*. Irreversible spore inactivation was complete after 2 d of storage, whereas the resulting decrease in viability of *Ascaris* ova was only 0.1 log. The extent of inactivation for both pathogenic forms was different, but when spores of *C. perfringens* were reduced by 99.8%, a corresponding reduction of about 80% in viable *Ascaris suum* egg population was noted. The ova, although affected by high pH and corresponding high free ammonia concentration, were much more resistant than the spores of *C. perfringens*.

In general, the synergistic effect of a combination of factors with particular emphasis on the role of ammonia in effecting inactivation was demonstrated.

Chapter 7.

7 ENGINEERING MILESTONES AND FUTURE WORK

7.1 HIGH-SOLID ANAEROBIC DIGESTION OF CHICKEN MANURE

7.1.1 *Milestones*

The research defined the maximum total solid level, at which methane generation from the manure is still feasible. This level was found to be around 10-12% TS. Significant savings in digester volume of up to 4 times lower than in case of diluted manure (3% TS) processing can result.

It also determined the maximum initial un-ionized ammonia concentration, at which biological stabilization using un-acclimated biomass was possible.

7.1.2 Future work

The experiments were conducted without prior acclimation to high ammonia levels. More work is required on the successful initiation of chicken manure digester operation by means of acclimation to high-solid operation. Inoculation of a digester with already digested manure or other- already acclimated to high ammonia- type of seed, is another suggested option.

7.2 ANAEROBIC CO-DIGESTION OF THE ORGANIC FRACTION OF MSW AND PRIMARY SLUDGE

7.2.1 Milestones

The research demonstrated the effects of ammonia concentration on anaerobic digestion of carbon-rich substrate, such as OF-MSW. A technique for the proper start-up of high-solid operation from low-solid level, including seeding with acclimated biomass and gradual increase in organic load to a digester was shown.

Thresholds of vulnerability of the OF-MSW co-digestion with sewage sludge were established. Although already practiced in full-scale applications, OF-MSW co-digestion can become vulnerable to increased ammonia concentrations from sludge inclusion, as seen in this work.

7.2.2 Future work

Further research on the feasibility of adjusting the C/N ratio of the OF-MSW feed with a material rich in carbon easy to assimilate, in cases of the confirmed progressing ammonia inhibition, needs to be done. In addition, the possibility of pH control to reduce the free ammonia fraction of the total ammonia in digester contents should be explored.

7.3 LOW-DOSE ALKALINE DISINFECTION

7.3.1 *Milestones*

It was demonstrated for the first time that alkali doses below those regarded as necessary for Class B biosolids quality were effective in fast non-spore forming bacterial inactivation corresponding with Class A level, and in spore kill following prolonged contained storage to preserve alkalinity.

This research paved the way for less expensive method of upgrading the product of mesophilic anaerobic digestion - the mainstay of sludge stabilization in Canada and elsewhere. The U.S. EPA municipal sludge disposal regulations are becoming a model for Canada. However, they tend to rely on heat as the means of disinfection without taking into account other factors, such as ammonia content and storage time.

The low-dose disinfection can serve as a form of post-treatment for use by any municipality concerned with land application of unclassified sludge, bringing it to the range of at least Class B biosolids depending on the dose applied.

Bacterial spores of *C. perfringens* were used as indicator of disinfection efficiency with success. The spores are proposed as a model pathogen for further disinfection studies, including their use as surrogate forms in lieu of helminth ova.

The insight into mechanisms and relative share of free ammonia in pathogen inactivation allowed for better understanding of the disinfection. High dependence of helminth ova inactivation on free ammonia concentration, and lack of such in case of bacterial spores of *C. perfringens* contributed to this knowledge.

7.3.2 Future work

Further extension of the work in terms of pathogen inactivation should involve: (1) testing for enteric viruses, the only group that has been regulated (under Class A requirement), but was not included in the protocol; (2) determination of C×T product for complete inactivation of *Ascaris* eggs at doses investigated, and at temperature ranges other than 4-6°C. In this kind of treatment, depending on the local climatic conditions, the minimum time length and auxiliary conditions to effect a treatment should be appropriately adjusted.

The use of alkali doses effective in pathogen inactivation during low-dose disinfection in bench scale studies shall be investigated at a pilot or full scale to account for the effect of natural variability in environmental factors like temperature. Another major factor, and obstacle, to account for the effect of scale up is believed to be the provision for efficient mixing. In alkaline stabilization, it is very important to provide efficient mixing of alkaline chemicals and high-solid residue. Incomplete penetration into sludge aggregates may result in pockets of lower pH, leading to limited inactivation of pathogens. This problem has been usually avoided by using high lime doses and relying on the heat effect rather than on high pH.

Depending on the length of contained storage in low-dose alkaline disinfection, the cost can vary substantially. Cost analysis of the treatment as function of storage time, as well as alkali doses and marketability of the product once Class A-compliant, is required.

The studies on the effect of high pH alone and in combination with high free ammonia concentration were conducted using diluted media containing dissolved

oxygen. In order to arrive at a reduction-oxidation condition closer to that in anaerobically digested residues it is recommended to conduct further work using pre-reduced media. Total ammonia concentrations higher than ~1,300 mg N/L shall be tested to find out if independence of spore inactivation from free ammonia concentration is valid at all ammonia levels found in anaerobic digestion residues. As well, even though the *C. perfringens* spore population comprised of various isolates to maintain high genetic pool, the use of all *Clostridia* instead (using Shahidi-Ferguson-Perfringens agar - the TSC agar antibiotic-free base, or other popular media) would be useful to account for a wider variation in resistance to disinfection among the species. This condition is not necessary for parasitic eggs of Helminths other than *Ascaris*, as the *Ascaris* ova are commonly regarded the hardiest.

8 REFERENCES

Ahmed A. U. and Sorensen D. L. [1995]. Kinetics of pathogen destruction during storage of dewatered biosolids. *Wat. Environ. Res.*, **67** (2), 143-150.

Ahmed A. U. and Sorensen D. L. [1997]. Autoheating and pathogen destruction during storage of dewatered biosolids with minimal mixing. *Wat. Environ. Res.*, **69** (1), 81-94.

Ahring B. K. and Westermann P. [1988]. Product inhibition of butyrate metabolism by acetate and hydrogen in a thermophilic coculture. *Appl. Environ. Microbiol.*, **54**, 2393-2398.

Alberta Environment [2001]. *Guidelines for the Application of Municipal Wastewater Sludges to Agricultural Lands.* Municipal Program Development Branch, Environmental Sciences Division, Environmental Service, Alberta, Canada, 32 p.

Albertson O. E. [1961]. Ammonia nitrogen and the anaerobic environment. *J. Wat. Pollut. Control Fed.*, **33** (9), 978-995.

Allard S. [2000]. National standard compost criteria CAN/BNQ 0413-200/97-01-27, organic soil conditioners- composts. In: *CD-ROM Proceedings of the 1st Canadian National Residuals and Biosolids Management Conference – Biosolids 2000*, Water Environment Association of Ontario, 24-26 September, Toronto, Canada.

American Public Health Association; American Water Works Association; Water Environment Federation [1992]. Standard Methods for the Examination of Water and Wastewater (Greenberg A. E., Clesceri L. S., Eaton A. D., eds), 18th Edition, Washington, DC, USA.

Anderson G. K., Donnelly T. and McKeown K. J. [1982]. Identification and control of inhibition in the anaerobic treatment of industrial wastewaters. *Process Biochem.*, **17**, 28-32, and 41.

Ando Y. [1979]. Spore lytic enzyme released from *Clostridium perfringens* spores during germination. *J. Bacteriol.*, **140**, 59-64.

Angelidaki I. and Ahring B. K. [1994]. Anaerobic digestion of manure at different ammonia loads: effect of temperature. *Wat. Res.*, **28**, 727-731.

Angelidaki I. and Ahring B. K. [1993]. Thermophilic anaerobic digestion of livestock waste: effect of ammonia. *Appl. Microbiol. Biotechnol.*, **38**, 560-564.

Aronson A. I. and Fitz-James P. C. [1968]. Biosynthesis of bacterial spore coats. *J. Molecular Biol.*, **33**, 199-212.

Aubart C. L. and Fauchille S. [1983]. Anaerobic digestion of poultry wastes- part 1, biogas production and pollution decrease in terms of retention time and total solids content. *Proc. Biochem.*, **18** (2), 31-34 and 37.

Ayres R. M., Alabaster G. P., Mara D. D. and Lee D. L. [1992]. A design equation for human intestinal nematode egg removal in waste stabilization ponds. *Wat. Res.*, **26**, 863-865.

Bailey W. R. and Scott E. G. [1966]. *Diagnostic microbiology. A textbook for the isolation and identification of pathogenic microorganisms.* C. V. Mosby Company, Saint Louis, MI, U.S.A.

Barbier D., Perrine D., Duhamel Ch., Doublet R. and Georges P. [1990]. Parasitic hazard with sewage sludge applied to land. *Appl. Environ. Microbiol.* **56** (6), 1420-1422.

Barker H. A. [1981]. Amino acid degradation by anaerobic bacteria. *Ann. Rev. Biochem.*, **50**, 23-40.

Barlaz M. A., Ham R. K. and Schaefer D. M. [1990]. Methane production from municipal refuse: a review of enhancement techniques and microbial dynamics. *Critical Reviews in Environment Control*, **19** (6), 557-584.

Barnes E. M. and Impey C. S. [1970]. The isolation and properties of the predominant anaerobic bacteria in the caeca of chickens and turkeys. *British Poultry Sci.*, **11** (4), 467-481.

Barnes E. M., Mead G. C., Barnum D. A. and Harry E. G. [1972]. The intestinal flora of the chicken in the period 2 to 6 weeks of age, with particular reference to the anaerobic bacteria. *British Poultry Sci.*, **13**, 311-326.

Beery J. T., Doyle M. P. and Schoeni J. L. [1985]. Colonization of chicken cecae by *Escherichia coli* associated with hemorrhagic colitis. *Appl. Environ. Microbiol.*, **49** (2), 310-315.

Beuchat L. R. and Ryu J. H. [1997]. Produce handling and processing practices. *Emerg. Infect. Diseases*, **3** (4), 459-465.

Bilski J. J., Alva A. K., Sajwan K. S. [1995]. *Fly ash*. In: Soil amendments and environmental quality. (Agriculture and environment series, Rechcigl J. E., ed.). Chapter 9, CRC Press, Inc., Boca Raton, FA, U.S.A., 327-363.

Bitton [1999]. *Wastewater microbiology*. 2nd ed., John Wiley & Sons, Inc., New York, NY, USA.

Black J. G. [1996]. *Microbiology: principles and applications*. 3rd Edition, Prentice-Hall, Inc., Upper Saddle River, NJ, USA.

Black M. I., Scarpino P. V., O'Donnell C. J., Meyer K. B., Jones J. V. and Kaneshiro E. S. [1982]. Survival rates of parasite eggs in sludge during aerobic and anaerobic digestion. *Appl. Environ. Microbiol.*, **44** (5), 1138-1143.

Blanker E. M., Little M. D., Reimers R. S. and Akers T. G. [1992]. Evaluating the use of clostridium perfringens spores as an indicator of the presence of viable *Ascaris* eggs in chemically treated municipal sludges. Unpublished report, Departments of Environmental Health Sciences and Tropical Medicine, School of Public Health and Tropical Medicine, Tulane University, New Orleans, LO, U.S.A., 14 p.

Borgmann U. and Borgmann A. I. [1997]. Control of ammonia toxicity to *Hyalella azteca* by sodium, potassium and pH. *Environ. Pollut.*, **95** (3), 325-331.

Borja R., Sanchez E. and Weiland P. [1996a]. Influence of ammonia concentration on thermophilic anaerobic digestion of cattle manure in upflow anaerobic sludge blanket (UASB) reactors. *Process Biochem.*, **31** (5), 477-483.

Borja R., Sanchez E. and Duran M. M. [1996b]. Effect of the clay mineral zeolite on ammonia inhibition of anaerobic thermophilic reactors treating cattle manure. *J. Environ. Sci. Health Part-A: Environ. Sci. Engng. Toxic Hazard. Subst. Control*, **31** (2), 479-500.

Borzacconi L., Lopez I. and Anido C. [1997]. Hydrolysis constant and VFA inhibition in acidogenic phase OF-MSW anaerobic digestion. *Water Sci. Technol.*, **36** (6-7), 479-484.

Braber K. [1995]. Anaerobic digestion of municipal solid waste: a modern waste disposal option on the verge of breakthrough, *Biomass and Bioenergy*, 9(1-5), 365-376.

Braun R., Huber P. and Meyrath J. [1981]. Ammonia toxicity in liquid piggery manure digestion. *Biotechnol. Letters*, **3**, 159-164.

Bowman D. D., Reimers R. S., Little M. D., Jenkins M. B., Bankston W. S. and Atique M. M. [2000]. Assessment and comparison of *Ascaris* egg and *Cryptosporidium* oocysts inactivation with respect to biosolids processing. In: CD ROM Proceedings of the 14th Annual Residuals and Biosolids Management Conference, Boston, MA, U.S.A., p. 1-12.

Bureau de Normalisation du Québec (BNQ) [1997]. *Organic soil conditioners – Composts*, CAN/BNQ 0413-200/97-01-27, Montreal, Canada.

Burge W. D., Cramer W. N. and Kawata K. [1983]. Effect of heat on virus inactivation by ammonia. *Appl. Environ. Microbiol.*, **46** (2), 446-451.

Burnham J. C., Donovan J. F., Forste J., Gschwind J., Zenz D. and Logan T. J. [1998]. *Production and distribution of municipal sewage sludge products.* In: Municipal sewage sludge management: a reference text on processing, utilization and disposal. WQML-Vol. 4, 2nd ed., (Lue-Hing C., Zenz D. R., Tata P., Kuchenrither R., Malina J., Sawyer B., eds), Technomic Publishing Co., Inc., Lancaster, PA, U.S.A., 591-641.

Burnham, J. C., Hatfield, N., Bennett, G.F., and Logan, T. J. [1992]. Use of Kiln Dust with Quicklime for Effective Municipal Sludge Pasteurization and stabilization with the N-Viro Soil Process. In: *Innovations and Uses for Lime*, ASTM STP 1135, D. D.

Burton S. A. Q. and Watson-Craik I. A. [1998]. Ammonia and nitrogen fluxes in landfill sites: applicability to sustainable landfilling. *Waste Manage. Res.*, **16** (1), 41-53.

Canadian Centre for Occupational Health and Safety [1988]. *Chemical hazard summary-ammonia.* CAS-7664-41-7, CCOHS, Hamilton, ON.

Canadian Council of Ministers of the Environment [1996]. *Guidelines for Compost Quality*. CCME-106E, Ottawa, Canada.

Canadian Council of Ministers of the Environment (CCME) [1997]. *Recommended Canadian Soil Quality Guidelines*. ISBN 1-895-925-92-4, Ottawa, Canada.

Cecchi F., Pavan P., Mata Alvarez J., Bassetti A., Cozzolino C. [1991]. Anaerobic digestion of municipal solid waste: thermophilic vs. mesophilic performance at high solids. *Waste Manage. Res.*, **9**, 305-315.

Cecchi F., Traverso P. G., Perin G. and Vallini G. [1988]. Comparison of co-digestion performance of two differently collected organic fractions of municipal solid waste with sewage sludges. *Environ. Technol. Letters*, **9**, 391-400.

Chen M. [1987]. Difference in sporogenous bacterial populations in thermophilic (55°C) and mesophilic (35°C) anaerobic sewage digestion. *Appl. Environ. Microbiol.*, **53** (10), 2414-2419.

Christy R. W. [1990]. Sludge disposal using lime. *Wat. Environ. Technol.*, **2** (4), 56-61.

Cooper R. C. and Danielson R. E. [1997]. *Detection of bacterial pathogens in wastewater and sludge*. In: Manual of environmental microbiology. (Hurst C. J., Knudsen G. R., McInerney M. J., Stetzenbach L. D., Walter M. V., eds.). Chapter 23, ASM Press, Washington, DC, U.S.A., 222-230.

Converse J C; Evans G W; Robinson K L; Gibbons W [1981]. Methane production from a large-size on-farm digester for poultry manure. In*: Livestock Waste: A Renewable Resource*. Proceedings of the Fourth International Symposium on Livestock Wastes-1980, ASAE Publication, Amarillo, Texas, USA, 122-125.

Council of the European Communities [1986]. Council directive of 12 June 1986 on the protection of the environment, and in particular the soil, when sewage sludge is used in agriculture (86/278/EEC). *Official J. European Communit.*, **L 181**, 6–12.

Cramer W. N., Burge W. D. and Kawata K. [1983]. Kinetics of virus inactivation by ammonia. *Appl. Environ. Microbiol.*, **45** (3), 760-765.

Craven S. E. and Blankenship L. C. [1985]. Activation and injury of *Clostridium perfringens* spores by alcohols. *Appl. Environ. Microbiol.*, **50** (2), 249-256.

Crittenden G. [2001]. Toronto's new bylaw and disposal strategy for biosolids impacts industry and sets a national precedent. *Hazard. Materials Manage.*, Dec/Jan, 6-16.

Davis R. E., Parra A., LoVerde P. T., Ribeiro E., Glorioso G. and Hodgson S. [1999]. Transient expression of DNA and RNA in parasitic Helminths by using particle bombardment. *Proc. Natl. Acad. Sci.*, **96**, 8687-8692.

De Baere L. A., Devocht M., Van Assche P. and Verstraete W. [1984]. Influence of high NaCl and NH_4Cl salt levels on methanogenic associations. *Wat. Res.*, **18** (5), 543-548.

De Luca S. J., Idle C. N. and Chao A. C. [1996]. Quality improvement of biosolids by ferrate (VI) oxidation of offensive odour compounds. *Wat. Sci. Technol.*, **33** (3), 119-130.

Demirekler E., and Anderson G. K. [1998]. Effect of sewage sludge addition on the start-up of the anaerobic digestion of OF-MSW. *Environ. Technol.*, **19**, 837-843.

Department of the Environment [1989]. *Code of Practice for Agricultural Use of Sewage Sludge*, HMSO, London, England.

Deportes I., BenoitGuyod J. L., Zmirou D. and Bouvier M. C. [1998]. Microbial disinfection capacity of municipal solid waste (MSW) composting. *J. Appl. Microbiol.*, **85** (2), 238-246.

Dowd S. E., Widmer K. W. and Pillai S. D. [1997]. Thermotolerant clostridia as an airborne pathogen indicator during land application of biosolids. *J. Environ. Quality*, **26** (1), 194-199.

Duncan C. L., Labbe R. G. and Reich R. R. [1972]. Germination of heat- and alkali-altered spores of *Clostridium perfringens* type A by lysozyme and an initiation protein, *J. Bacteriol.*, **109** (2), 550-559.

El-Fadel M., Findikakis A. N., and Leckie J. O. [1996]. Temperature effects in modeling solid waste degradation. *Environ. Technol.*, **17**, 915-935.

Emerson K., Russo R. C., Lund R. E. and Thurston R. V. [1975]. Aqueous ammonia equilibrium calculations: effect of pH and temperature. *J. Fish. Res. Board Can.*, **32**, 2379-2383.

Eriksen L., Andreasen P. and Ilsoe B. [1996]. Inactivation of Ascaris suum eggs during storage in lime treated sewage sludge. *Wat. Res.*, **30** (4), 1026-1029.

European Union [1991]. Council directive of 21 May 1991 concerning urban waste-water treatment (91/271/EEC). *Official J. European Communit.*, **L 135**, 40–52.

Evison L. M. [1988]. Comparative studies on the survival of indicator organisms and pathogens in fresh and sea water. *Wat. Sci. Technol.*, **20** (11/12), 309-315.

Fairbairn D. [1957]. The biochemistry of *Ascaris*. *Experimental Parasitology*, **6**, 491-554.

Farrell J. B., Erlap A. E., Rickabaugh J., Freedman D. and Hayes S. [1988]. Influence of feeding procedure on microbial reductions and performance of anaerobic digestion. *J. Wat. Pollut. Control Fed.*, **60**, 635-644.

Farrell J. B., Salotto B. V. and Venosa A. D. [1990]. Reductions in bacterial densities of wastewater solids by three secondary treatment processes. *J. Wat. Pollut. Control Fed.*, **62**, 177-184.

Farrell J. B., Smith J. E. Jr., Hathaway S. W. and Dean R. B. [1974]. Lime stabilization of primary sludges. *J. Wat. Pollut. Control Fed.*, **46** (1), 113-122.

Field J. A., Reneau R. B. Jr., Kroontje W. and Caldwell J. S. [1985]. Nutrient recoveries from plug-flow anaerobic digestion of poultry manure. *Agric. Wastes*, **13**, 207-216.

Foess G.. Fredericks D. and Coulter F. [1994]. Evaluating biosolids stabilization technologies. *Wat. Environ. Technol.*, April issue, 55-59.

Frosk P. [1998]. *Pathogenic potential of the organic fraction of municipal solid waste during dry anaerobic co-digestion with primary sludge*, Final report submitted to the Department of Microbiology, University of Manitoba, Winnipeg, MB, Canada, 7 p.

Gaspard P., Wiart J. and Schwartzbrod J. [1995]. Urban sludge reuse in agriculture: waste treatment and parasitological risk. *Bioresource Technol.*, **52**, 37-40.

Gaspard P., Wiart J. and Schwartzbrod J. [1997]. Parasitological contamination of urban sludge used for agricultural purposes. *Waste Manage. Res.*, **15** (4), 429-436.

Georgacakis D., Sievers D. M. and Ianotti E. L. [1982]. Buffer stability in manure digesters. *Agric. Wastes*, **4**, 427-441.

Gibbs R. A., Hu C. J., Ho G. E., and Unkovich I. [1997]. Regrowth of fecal coliforms and salmonellae in stored biosolids and soil amended with biosolids. *Wat. Sci. Technol.*, **35** (11-12), 269-275.

Goering H. K. and Van Soest P. J. [1970]. *Forage Fiber Analyses*, U. S. Department of Agriculture, Agricultural Research Service, Washington, DC, Agriculture Handbook No. 379, Jacket No. 387:598.

Gourdon R. and Vermande P. [1987]. Effects of propionic acid concentration on anaerobic digestion of pig manure. *Biomass*, **13**, 1-12.

Gottschalk G. [1979]. *Bacterial metabolism*. Springer-Verlag, New York, NY, USA, 213-221.

Grant S. B. [1995]. Inactivation kinetics of viral aggregates. *J. Environ Engng.*, **121** (4), 311-319.

Guerrero L., Omil F., Mendez R. and Lema J. M. [1999]. Anaerobic hydrolysis and acidogenesis of wastewaters from food industries with high content of organic solids and protein. *Wat. Res.*, **33** (15), 3281-3290.

Hamzawi N., Kennedy K. J. and McLean D. D. [1998]. Technical feasibility of anaerobic co-digestion of sewage sludge and municipal solid waste. *Environ. Technol.*, **19**, 993-1003.

Hansen K. H., Angelidaki I. and Ahring B. K. [1998]. Anaerobic digestion of swine manure: inhibition by ammonia. *Wat. Res.*, **32** (1), 5-12.

Hansen K. H., Angelidaki I. and Ahring B. K. [1999]. Improving thermophilic anaerobic digestion of swine manure. *Wat. Res.*, **33** (8), 1805-1810.

Harmon S. M. and Kautter D. A. [1978]. Media for confirming *Clostridium perfringens* from food and feces. *J. Food Protect.*, **41** (8), 626-630.

Harmon S. M., Kautter D. A. and Peeler J. [1971]. Improved medium for enumeration of *Clostridium perfringens*. *Appl. Microbiol.*, **22** (4), 688-692.

Hashimoto G. [1986]. Ammonia inhibition of methanogenesis from cattle wastes. *Agric. Wastes*, **17**, 241-261.

Hashimoto A. G., Chen Y. R. and Varel V. H. [1981]. Theoretical aspects of methane - production: state-of-the-art. In: *Livestock Waste: A Renewable Resource*. Proceedings of the Fourth International Symposium on Livestock Wastes- 1980, ASAE Publication, Amarillo, Texas, USA, 86-91 and 95.

Haug R., Kuchenrither R., Malina J. F. Jr., Oerke D., Tata P., Sawyer B., Soszynski S., Zenz D. [1998]. *Sludge processing technology*. In Municipal sewage sludge management: a reference text on processing, utilization and disposal. WQML-Vol. 4, 2nd ed., (Lue-

Hauschild A. H. W. and Hilsheimer R. [1974]. Enumeration of food-borne *C. perfringens* in egg yolk-free tryptose-sulfite-cycloserine agar. *Appl. Microbiol.*, **27** (3), 521-526.

Heinrichs D. M., Poggi-Varaldo H. M. and Oleszkiewicz J. A. [1990]. Effects of ammonia on anaerobic digestion of simple organic compounds. *J. Environ. Engng.*, ASCE, **116** (4), 698-710.

Hill D. T. [1983]. Simplified Monod kinetics of methane fermentation of animal wastes. *Agric. Wastes*, **5**, 1-16.

Hill D. T., Cobb S. A. and Bolte J. P. [1986]. Using volatile fatty acid relationships to predict anaerobic digester failure. *Trans. ASAE*, **30** (2), 496-501.

Hills D. J., and Nakano K. [1984]. Effects of particle size on anaerobic digestion of tomato solids wastes. *Agric. Wastes*, **10**, 285-295.

Himathongkham S. and Riemann H. [1999]. Destruction of *Salmonella typhimurium*, *Escherichia coli* O157:H7 and *Listeria monocytogenes* in chicken manure by drying and/or gassing with ammonia. *FEMS Microbiol. Lett.*, **171**, 179-182.

Hing C., Zenz D. R., Tata P., Kuchenrither R., Malina J., Sawyer B., eds). Chapter 6, Technomic, Inc., Lancaster, PA, U.S.A., 255-408.

Hippe H., Andreesen J. R. and Gottschalk G. [1992]. The Genus *Clostridium*-Nonmedical. In: *The Prokaryotes. A Handbook on the biology of bacteria: ecophysiology, isolation, identification, applications.* (Balows A, Truper H. G., Dworkin M., Harder W., Schleifer K-H., eds.). Vol. 2, Chapter 81, Springer-Verlag New York Inc., New York, N.Y., U.S.A., p. 1800-1866.

Ho, T. [1997]. Joint project defines sewage biosolids stabilization criteria. *Environ. Sci. Eng.*, **10** (5), 34-36.

Hobson P. N., Bousfield S., Summers R and Mills P. J. [1980]. Anaerobic digestion of piggery and poultry wastes. In: *Anaerobic digestion.* (Stafford D., Wheatley B. and Hughes D. E., eds), Applied Science Publishers, London, UK, 237-250.

Hotez P. J., Zheng F., Xu L. G., Chen M.-G., Xiao S.-H, Liu S.-X., Blair D. McManus D. P. and Davis G. M. [1997]. Emerging and reemerging helminthiases and the public health of China. *Emerg. Infect. Dis.*, **3**, 303-317.

Huang J. J. H. and Shih J. C. H. [1981]. The potential of biological methane generation from chicken manure. *Biotechnol. Bioeng.*, **23**, 2307-2314.

Hunik J. H., Hamelers H. V. M. and Koster I. W. [1991]. Growth-rate inhibition of acetoclastic methanogens by ammonia and pH in poultry manure digestion. *Biol. Wastes*, **32** (4), 285-298.Jones P. W. [1980]. Health hazards associated with the handling of animal wastes. *The Veterinary Record*, **106**, 4-7.

International Energy Agency [1997]. *IEA bioenergy AD activity system providers list.* A report available from IEA, U.S.A.

Jantrania A. R. and White R. K. [1985]. High-solids anaerobic fermentation of poultry manure. In: *Proceedings of the Fifth International Symposium on Agricultural Wastes, Agricultural Waste Utilization and Management*, Dec. 16-17, Chicago, IL, ASAE Publication 13-85, St. Joseph, MI, 73-80.

Jarrel K. F. and Saulnier M. [1987]. Inhibition of methanogenesis in pure cultures by ammonia, fatty acids, and heavy metals, and protection against heavy metal toxicity by sewage sludge. *Can. J. Microbiol.*, **33**, 551-554.

Jeris J. S. and McCarty P. L. [1965]. The biochemistry of methane fermentation using ^{14}C-tracers. *J. Wat. Pollut. Control Fed.*, **37**, 178-192.

Jewell W. J. and Loehr R. C. [1977]. Energy recovery from animal wastes: anaerobic digestion, pyrolysis, hydrogenation. In: *Animal Wastes.* (Taiganides E. P., ed.), Applied Science Publishers Ltd., Barking, Essex, England, 273-294.

Kaneshiro E. S. and Stern G. [1986]. Survival of parasite eggs in stored sludge. Project Summary. U.S. EPA Office of Research and Development, *EPA/600/S2-85/142*, Cincinnati, OH, U.S.A., p. 1-5.

Kaspar H. F. and Wuhrmann K. [1978]. Kinetic parameters and relative turnovers of some important catabolic reactions in digesting sludge. *Appl. Environ. Microbiol.*, **36**, 1-7.

Kayhanian M. [1999]. Ammonia inhibition in high-solids biogasification: an overview and practical solutions, *Environ. Technol.*, **20**, 355-365.

Kayhanian M., Tchobanoglous G. and Mata-Alvarez J. [1996]. Development of a mathematical model for the simulation of the biodegradation of organic substrates in a high-solids anaerobic digestion process, *J. Chem. Tech. Biotechnol.*, **66**, 312-322.

Kayhanian M. [1995]. Biodegradability of the organic fraction of municipal solid waste in a high-solids anaerobic digester. *Waste Manage. Res.*, **13**, 123-136.

Kayhanian M., Lindenauer K., Hardy S. and Tchobanoglous G. [1991]. The recovery of energy and production of compost from the biodegradable organic fraction OF-MSW using the high-solids anaerobic digestion/ aerobic biodrying process. A report prepared for the Prison Industry Authority of the State of California. University of California at Davis, CA, USA.

Kearney T. E., Larkin M. J. and Levett P. N. [1993]. The effect of slurry storage and anaerobic digestion on survival of pathogenic bacteria. *J. Appl. Bacteriol.*, **74**, 86-93.

Kenner B. A. and Clark H. P. [1974]. Detection and enumeration of *Salmonella* and *Pseudomonas aeruginosa*. *J. Wat. Pollut. Control Fed.*, **46** (9), 2163-2171.

Kiely G. Tayfur G., Dolan C. and Tanji K. [1997]. Physical and mathematical modeling of anaerobic digestion of organic wastes. Wat. Res., **31** (3), 534-540.

Kleiner D., Traglauer A. and Domm S. [1998]. Does ammonia production by Klebsiella contribute to pathogenesis? *Bulletin Del Institut Pasteur*, **96** (4), 257-265.

Kondo F. and Tateyama S. [1990]. In vitro activity of chloropolysporin-C, a new glycopeptide-group antibiotic, on *Clostridium perfringens* isolates and its in vivo activity against *C. perfringens* and other intestinal microflora of the caeca of broiler chickens. *Res. Vet. Sci.*, **48** (2), 175-179.

Koster I. W. and Koomen E. [1988]. Ammonia inhibition of the maximum growth rate (μ_m) of the hydrogenotrophic methanogens at various pH levels and temperatures. *Appl. Microbiol. Biotechnol.*, **28**, 500-505.

Koster I. W. [1986]. Characteristics of the pH-influenced adaptation of methanogenic sludge to ammonium toxicity. *J. Chem. Tech. Biotechnol.*, **36**, 445-455.

Koster I. W. and Lettinga G. [1988]. Anaerobic digestion at extreme ammonia concentrations. *Biol. Wastes*, **25**, 51-59.

Kroeker E. J., Schulte D. D., Sparling A. B. and Lapp H. M. [1979]. Anaerobic treatment process stability. *J. Wat. Pollut. Control Fed.*, **51** (4), 718-727.

Krogdahl A. and Dalsgard B. [1981]. Estimation of nitrogen digestibility in poultry: content and distribution of major urinary nitrogen compounds in excreta. *Poultry Sci.*, **60**, 2480-2485.

Krylova N. I., Khabiboulline R. E., Naumova R. P. and Nagel M. A. [1997]. The influence of ammonium and methods for removal during the anaerobic treatment of poultry manure. *J. Chem. Technol. Biotechnol.*, **70** (1), 99-105.

Kudva I. T., Blanch K. and Hovde C. J. [1998]. Analysis of Escherichia coli O157:H7 survival in ovine or bovine manure and manure slurry. *Appl. Environ. Microbiol.*, **64** (9), 3166-3174.

Kugelman I. J. and Chin K. K. [1971]. Toxicity, synergism and antagonism in anaerobic waste treatment processes. In: *Anaerobic biological treatment processes.* Advanced Chem. Series 105 (Gould R. F., ed.), American Chemical Society, New York, NY, USA, 55-90.

Kugelman I. J. and McCarty P. L. [1965]. Cation toxicity and stimulation in anaerobic waste treatment. *J. Wat. Pollut. Control Fed.*, **37**, 97-116.

Lacey R. E., Ross I. J., Taraba J. L. and Walton L. R. [1981]. Nutrient changes in poultry manure during batch liquid phase anaerobic fermentation. In*: Livestock Waste: A Renewable Resource.* Proceedings of the Fourth International Symposium on Livestock Wastes- 1980, ASAE Publication, Amarillo, Texas, USA, 31-33.

Lange M. Macario A. J. L., Ahring B. K. and deMacario E. C. [1997]. Increased transcripts of the dnaK locus genes in Methanosarcina mazei S-6 exposed to supraoptimal concentrations of ammonia. *FEMS Microbiol. Letters*, **152** (2), 379-384.

Lapp H. M., Schulte D. D., Kroeker E. J., Sparling A. B. and Topnik B. H. [1975]. Start-up of pilot scale swine manure digesters for methane production. In: *Managing Livestock Wastes: Proceedings Third International Symposium on Livestock Wastes*, ASAE Publication no. PROC-275, St. Joseph, Michigan, USA, 234-237 and 243.

Lay J. J., Li Y. Y. and Noike T. [1998]. The influence of pH and ammonia concentration on the methane production in high-solids digestion processes, *Water Environ. Res.*, **70** (5), 1075-1082

Lewis-Jones R. and Winkler M. [1991]. *Sludge parasites and other pathogens.* Ellis Horwood Limited, Chichester, West Sussex, England.

Llabres P., Pavan P., Battistioni P., Cecchi F. and Mata-Alvarez J. [1999]. The use of organic fraction of municipal solid waste hydrolysis products for biological nutrient removal in wastewater treatment plants, *Wat. Res.*, **33**(1), 214-222.

Logan, T. J. and Faulmann E.. [1999]. Pasteurization of chicken and hog manure with alkaline stabilization. In: *Proceedings of WEF/AWWA Joint Residuals and Biosolids Management Conference*. Charlotte, NC, USA, Feb., 9-.

Lusk P., Wheeler P. and Rivard C. [1996]. *Deploying anaerobic digesters: current status and future possibilities.* A report prepared by the National Renewable Energy Laboratory of the U.S. Department of Energy, available from NTIS, Springfield, VA, USA.

Lee D. L. and Atkinson H. J. [1976]. *Physiology of nematodes.* Chapter 6: reproductive physiology and hatching, 2nd edition, Macmillan Press Ltd., Great Britain, p. 127

Lee K. M., Brunner C. A., Farrell J. B. and Eralp A. E. [1989]. Destruction of enteric bacteria and viruses during two-phase digestion. *J. Wat. Pollut. Control Fed.*, **61** (8), 1421-1429.

Lee M.-R. and Shih J. C. H. [1988]. Effect of anaerobic digestion on oocysts of the protozoan *Eimeria tenella*. *Appl. Environ. Microbiol.*, **54** (10), 2335-2341.

Little M. D. [1986]. The detection and significance of pathogens in sludge: Parasites. In: *Control of sludge pathogens.* (Sorber C. A., ed.), Water Pollution Control Federation, Washington, DC, USA.

Little M. D. [1999]. Procedure for determination of helminth eggs in sewage sludge. Tulane University School of Public Health and Tropical Medicine, New Orleans, LA, U.S.A.

Liu C. [2000]. *Pathogen inactivation in biosolids with lime and fly ash addition.* A thesis submitted to the Faculty of Graduate Studies in partial fulfilment of the requirements for the degree of M.Sc., University of Manitoba, Winnipeg, MB, Canada.

Mah R. A., Xun L. Y., Boone D. R. and Ahring B. [1990]. Methanogenesis from propionate in sludge and enrichment systems. In: *FEMS Symposium No. 54.* Plenum Press, New York, NY, USA.

Marquardt R. R. [1983]. A simple spectrophotometric method for the direct determination of uric acid in avian excreta. *Poultry Sci.*, **62**, 2106-2108.

McCartney D. M. and Oleszkiewicz J. A. [1993]. Competition between methanogens and sulfate reducers: effect of COD:sulfate ratio and acclimation. *Wat. Environ. Res.*, **65**, 655-664.

McCarty P. L. [1964a]. Anaerobic waste treatment fundamentals: Part III: Toxic materials and their control. *Public Works*, **95**, 91-94.

McCarty P. L. [1964b]. The methane fermentation. In*: Principles and application in aquatic microbiology.* (Henkelkian H. and Dondero N. C., eds), John Wiley and Sons, Inc., New York, NY, USA.

McCarty P. L. and Brosseau M. H. [1963]. Effect of high concentrations of individual volatile acids on anaerobic treatment. In: *Proceedings, 18th Industrial Waste Conference.* Purdue University, Butterworth Publishers, Boston, MA, USA, 283-296.

McCarty P. L. and McKinney R. [1961]. Salt toxicity in anaerobic digestion. *J. Wat. Pollut. Contr. Fed.*, **33**, 399-415.

McCarty P. L. and Smith D. P. [1986]. Anaerobic wastewater treatment. *Environ. Sci. Technol.*, **20**, 1200-1206.

Melbinger N. R. and Donnellon J. [1971]. Toxic effects of ammonia-nitrogen in high-rate digestion. *J. Wat. Pollut. Control Fed.*, **43**, 1658-1670.

Metcalf and Eddy, Inc. [1991]. *Wastewater engineering; treatment, disposal and reuse.* McGraw-Hill, Inc., New York, NY, USA.

Morris G. R., Jewell W. J. and Casler G. L. [1975]. Alternative animal waste anaerobic fermentation designs and their costs. In*: Proceedings of Agricultural Waste Management Conference.* Cornell University, Ithaca, NY, USA, 317-335.

Mackey B. M. [1984]. Lethal and sublethal effects of refrigeration, freezing and freeze-drying on microorganisms. In: *The revival of injured microbes.* Academic Press, Inc., Orlando FL, U.S.A. Chapter 3, p. 45-75.

Mallory L. M., Yuk Ch-S, Liang L-N and Alexander M. [1983]. Alternative prey: a mechanism for elimination of bacterial species by protozoa. *Appl. Environ. Microbiol.*, **46** (5), 1073-1079.

Martinko J. M. [2000]. Clinical and diagnostic microbiology and immunology. In: *Brock biology of microorganisms.* (Madigan, M. T., Martinko J. M., Parker J., eds). Chapter 21, Prentice-Hall, Inc., Upper Saddle River, N.J., U.S.A., 854-890.

Mazur P. [1966]. Physical and chemical basis of injury in single-celled microorganisms subjected to freezing and thawing. In: *Cryobiology* (Meryman H. T., ed.), Academic Press, Inc., London, U.K., p. 213-315 (after Mackey, 1984).

Meekings H. J., Stentiford E. I. and Lee D. L. [1996]. The effect of sewage sludge compost on the viability of the eggs of a parasitic nematode. *Compost Sci. and Utiliz.*, **4** (2), 46-54.

Minister of Public Works and Government Services (MPWGS) [1999]. Canadian Environmental Protection Act. Canadian Government Publishing, Ottawa, Canada, *Canada Gazette*, **22** (3).

Ministry of the Environment [1998]. 'Draft' guidelines for aerobic composting facilities and compost use. Document released for discussion purposes in May 1998, Ontario, Canada.

Montruccoli M. [1996]. Disinfection of sewage sludge by lime treatment. *ZKG-International*, **49** (12), A26-A26.

Munch B., Larsen H. E. and Aalbaek B. [1987]. Experimental studies on the survival of pathogenic and indicator bacteria in aerated and non-aerated cattle and pig slurry. *Biol. Wastes*, **22**, 49-65.

Naundorf G. and Aumen N. G. [1989]. Ammonia-induced injury in pure cultures and natural populations of coliform bacteria. *FEMS Microbiol. Letters*, **65**, 65-70.

Neil A. and Webster P.E. [1994]. VOC Emissions from an Alkaline Stabilization (N-Viro) Facility. In Proceedings of the WEF Specialty Conference on Emission Control. Jacksonville, FL, USA, April, 11-.

Noike T., Endo G., Chang J.-E., Yaguchi J.-I. and Matsumoto J.-I. [1985]. Characteristics of carbohydrate degradation and the rate-limiting step in anaerobic digestion, *Biotechnol. Bioengineer.*, **27**, 1482-1489.

O'Brien T. A. and Barker A. V. [1998]. Acidification of lime-stabilized biosolids in formulation of synthetic topsoil. *Communic. Soil Sci. Plant Anal.*, **29** (9-10), 1107-1114.

O'Donnell, C. J., Meyer K. B., Jones J. V., Benton T., Kaneshiro E. S., Nichols J. S. and Schaefer F. W. III [1984]. Survival of parasite eggs upon storage in sludge. *Appl. Environ. Microbiol.*, **48**, 618.

Oerke D. W. [1999]. Alkaline stabilization of biosolids can save money, space. *WaterWorld*, March issue, 14 & 18-21.

Oleszkiewicz J., Bujoczek G. and Barnard J. [2000]. Biosolids management in biological nutrient removal plants. In: *Proceedings of the International Seminar: Design Philosophy versus Plant Operation*, Krakow, Poland, June 28-30, Lem Projekt s.c., p. 191-223, (in Polish).

Oleszkiewicz J. A. and Poggi-Varaldo H. M. [1997]. High-solids anaerobic digestion of mixed municipal and industrial waste. *J. Environ. Engng.*, November issue, 1087-1092 (1997).

Olsen J. E. and Nansen P. [1987]. Inactivation of some parasites by anaerobic digestion of cattle slurry. *Biol. Wastes*, **22**, 107-114.

Omil F., Mendez R. and Lema J. M. [1995]. Anaerobic treatment of saline wastewaters under high sulphide and ammonia content. *Bioresource Technol.*, **54** (3), 269-278.

Ottolenghi A. C. and Hamparian V. V. [1987]. Multiyear study of sludge application to farmland: prevalence of bacterial enteric pathogens and antibody status of farm families. *Appl. Environ. Microbiol.*, **53** (5), 1118-1124.

Outwater A. B. [1994]. *Lime stabilized sludge*. In: Reuse of sludge and minor wastewater residuals. Chapter 8, CRC Press, Inc., Boca Raton, FL, U.S.A., p. 101-111.

Owen W. F., Stuckey D. C., Healy J. B., Young L. Y. and McCarty P. L. [1979]. Bioassay for monitoring biochemical methane potential and anaerobic toxicity. *Wat. Res.*, **13**, 485-492.

Ozturk M. [1991]. Conversion of acetate, propionate and butyrate to methane under thermophilic conditions in batch reactors. *Wat. Res.*, **25**, 1509-1513.

Parkin G. F., Lynch N. A., Kuo W. C., Van Keuren E. L. and Bhattacharya S. K. [1990]. Interaction between sulfate reducers and methanogens fed acetate and propionate. *J. Wat. Pollut. Control Fed.*, **62**, 780-788.

Passey R. F. and Fairbairn D. [1955]. The respiration of *Ascaris lumbricoides* eggs. *Can. J. Biochem. Physiol.*, **33**, 1033-1046.

Paulsrud B. and Eikum A. S. [1984]. Experiences with lime stabilisation and composting of sewage sludge. In: *Sewage Sludge Stabilisation and Disinfection*. Chapter 13, 261-277. (Bruce A. M., ed.), Chichester, Ellis Horwood, England.

Pavan P., Battistoni P., Traverso P., Musacco A. and Cecchi F. [1998]. Effect of addition of anaerobic fermented OFMSW (organic fraction of municipal solid waste) on biological nutrient removal (BNR) process: preliminary results, *Wat. Sci. Tech.*, **38(1)**, 327-334.

Plachy P., Juris P., Placha I. and Venglovsky J. [1996]. Use of hydrated lime for disinfection of model pathogens *Salmonella typhimurium* and *Ascaris suum* in sewage sludges. *Veterinarni Medicina*, (in English), **41** (8), 255-259.

Poggi-Varaldo H. M. and Oleszkiewicz J. A. [1992]. Anaerobic co-composting of municipal solid waste and waste sludge at high total solids levels, *Environ. Technol.*, **13**, 409-421.

Poggi-Varaldo H. M., Tingley J. and Oleszkiewicz J. A. [1991]. Inhibition of growth and acetate uptake by ammonia in batch anaerobic digestion. *J. Chem. Tech. Biotechnol.*, **52**, 135-143.

Ponugoti P. R., Dahab M. F. And Surampalli R. [1997]. Effects of different biosolids treatment systems on pathogen and pathogen indicator reduction. *Wat. Environ. Res.*, **69** (7), 1195-1206.

Qasim S. R. [1994]. *Wastewater treatment plants; planning, design, and operation.* Technomic Publishing Company, Inc., Lancaster, Pennsylvania, USA.

Quinn J. J., Hall J. E. [1984]. Lime stabilisation of undigested sewage sludge. In: *Sewage Sludge Stabilisation and Disinfection*. Chapter 16, 540-546, (Bruce A. M, ed.), Chichester, Ellis Horwood, England.

Ramasamy E. V. and Abbasi S. A. [2000]. High-solids anaerobic digestion for the recovery of energy from municipal solid waste (MSW), *Environ. Technol.*, **21**, 345-349.

Reimers R. S., Little M. D., Akers T. G., Henriques W. D., Badeaux R. C., McDonnell D. B. and Mbela K. K. [1989]. Persistence of pathogens in lagoon-stored sludge. Project Summary. *EPA 600/S2-89/015*, U.S. EPA Office of Research and Development, Cincinnati, OH, U.S.A., p. 1-7.

Reimers R. S., Little M. D., Englande A. J., Leftwich D. B. Bowman D. D. and Wilkinson R. F. [1982]. Parasites in southern sludges and disinfection by standard sludge treatment. *Wat. Sci. Technol.*, **14**, 1568.

Rich D., Kayhanian M., Hardy S. and Tchobanoglous G. [1994]. *Co-management of municipal solid waste and wastewater treatment plant sludges using an anaerobic*

composting process, a report prepared for the California Integrated Waste Management Board and West County Wastewater District, West County Agency, Richmond Sanitary Service, and Delta Diablo Sanitation District. University of California at Davis, CA, USA.

Rintala J. A. and Jarvinen K. T. [1996]. Full-scale mesophilic anaerobic co-digestion of municipal solid waste and sewage sludge: methane production characteristics. *Was. Manage. Res.*, **14**, 163-170.

Ripley L. E., Kmet N. M., Boyle W. C. and Converse J. C. [1985]. The effects of ammonia nitrogen on the anaerobic digestion of poultry manure. In: *Proceedings, 39th Purdue Industrial Waste Conference, Purdue University.* Butterworth Publishers, Boston, MA, USA, 73-80.

Rivard, J. C. [1993]. Anaerobic bioconversion of municipal solid wastes using a novel high-solids reactor design: Maximum organic loading rate and comparison with low-solids reactor systems, *Appl. Biochem. Biotechnol.*, **39/40**, 71-82.

Rivard C. J., Nagle N. J., Adney W. S. and Himmel M. E. [1993]. Anaerobic bioconversion of municipal solid wastes: Effects of total solids levels on microbial numbers and hydrolytic enzyme activities, *Appl. Biochem. Biotechnol.*, **39/40**, 107-117.

Rivard J. C., Himmel M. E., Vinzant T. B., Adney W. S., Wyman C. E. and Grohmann K. [1990]. Anaerobic digestion of processed municipal solid waste using a novel high solids reactor: maximum solids levels and mixing requirements, *Biotechnol. Letters*, **12** (3), 235-240.

Rivard C. J., Adney W. S., Vinzant T. B. and Grohmann K. [1989 a] Waste to energy: nutrient requirements for aerobic and anaerobic digestion. *J. Environ. Health*, **52** (2), 96-99.

Rivard C. J., Himmel M. E., Vinzant T. B., Adney W. S., Wyman C. E. and Grohmann K. [1989 b]. Development of a novel laboratory scale high solids reactor for anaerobic digestion of processed municipal solid wastes for the production of methane." *Appl. Biochem. Biotechnol.*, **20/21**, 461-478.

Roediger H. [1987]. Using quicklime - hygienization and solidification of dewatered sludge. *Operations Forum*, **4** (3).

Rothberg, Tamburini&Winsor, Inc. [1998]. *Biosolids treatment: comparing add-on stabilization processes*. National Lime Association, Arlington, VA, U.S.A.

Russ C. F. and Yanko W. A. [1981]. Factors affecting Salmonellae repopulation in composted sludges. *Appl. Environ. Microbiol.*, **41** (3), 597-602.

Russell A. D., Furr J. R. and Maillard J-Y [1997]. Microbial susceptibility and resistance to biocides. *ASM News*, **63** (9), 481-487.

Safley L. M., Vetter R. L. and Smith D. [1985]. Managing a poultry manure anaerobic digester. In: *Proceedings of the Fifth International Symposium on Agricultural Wastes, Agricultural Waste Utilization and Management*. Dec. 16-17, Chicago, IL, ASAE Publication 13-85, St. Joseph, MI, USA, 491-499.

Salanitro J. P., Blake I. G., Muirhead P. A., Maglio M., and Goodman J. R. [1978]. Bacteria isolated from the duodenum, ileum, and cecum of young chicks. *Appl. Environ. Microbiol.*, **35** (4), 782-790.

Sanin F. D., Vesilind P. A. and Martel C. J. [1994]. Pathogen reduction capabilities of freeze/thaw sludge conditioning. *Wat. Res.*, **28** (11), 2393-2398.

Schnaeider M., Marison I. W. and Von Stockar U. [1996]. The importance of ammonia in mammalian cell culture. *J. Biotechnol.*, **46** (3), 161-185.

Schwartzbrod J., Gaspard P. and Thiriat L. [1998]. Pathogenic micro-organisms in sludge and effects of various treatment processes for their removal. European Wat. Manage., 2 (1), 64-69.

Scully F. E., Hogg P. A., Kennedy G., Lewicki C., Rule A. M., and Soffriti J. G. [1999]. Development of disinfection-resistant bacteria during wastewater treatment. *Wat. Environ. Res.*, **71** (3), 277-281.

Smith P H. and Hungate R. E. [1958]. Isolation and characterization of *Methanobacterium* ruminantium N. SP. *J. Bacteriol.*, **75**, 713-718.

Smith P. H. and Mah R. A. [1966]. Kinetics of acetate metabolism during sludge digestion. *Appl. Microbiol.*, **14**, 368-371.

Sparling R. R. M. [1988]. Diazotrophy in methanogens and formate metabolism in *Methanococcus thermolithotrophicus*, Ph.D. Thesis, University of Iowa, Iowa City, IA, USA.

Speece R. E. [1996]. *Anaerobic biotechnology for industrial wastewaters*, Archae Press, Nashville, TN, USA.

Spillmann S. K., Traub F., Schwyzer M. and Wyler R. [1987]. Inactivation of animal viruses during sewage sludge treatment. *Appl. Environ. Microbiol.*, **53** (9), 2077-2081.

Sprott G. D. and Patel G. B. [1986]. Ammonia toxicity in pure cultures of methanogens. *System. Appl. Microbiol.*, **7**, 358-363.

Sprott G. D., Shaw K. M. and Jarrel K. F. [1985]. Methanogenesis and the K^+ transport system are activated by divalent cations in ammonia-treated cells of *Methanospirillum hungatei*. *J. Biol. Chem.*, **260** (16), 9244-9250.

Sprott G. D., Shaw K. M. and Jarrel K. F. [1984]. Ammonia/potassium exchange in methanogenic bacteria. *J. Biol. Chem.*, **259** (20), 12602-12608.

Stroot P. G., McMahon K. D., Mackie R. I. and Raskin L. [2001]. Anaerobic codigestion of municipal solid waste and biosolids under various mixing conditions - I. Digester performance. *Wat. Res.*, **35** (7), 1804-1816.

Switzenbaum M. S., Moss L. H., Epstein E., Pincince A. B. and Donovan J. F. [1997]. Defining biosolids stability. *J. Environ. Engng.*, **123** (12), 1178-1184.

Taylor J. M., Epstein E., Burge W. D., Chaney R. L., Menzies J. D. and Sikora L. J. [1978]. Chemical and biological phenomena observed with sewage sludges in simulated soil trenches. *J. Environ. Quality*, **7**, 477-482.

ThomazSoccol V., Paulino R. C., Castro E. A., Andreoli C. V. [1997]. Helminth eggs viability in sewage and biosolids sludge in Curitiba, Parana, Brazil. *Arquivos de Biologia e Tecnologia*, (in English), **40** (4), 829-836.

Turpin P. E., Maycroft K. A., Rowlands C. L., Wellington E. M. H. [1993]. Viable but non-culturable salmonellas in soil. *J. Appl. Bacteriol.*, **74**, 421-427.

U.S. Environmental Protection Agency [1999]. Standards for the use or disposal of sewage sludge; final rule (amendments to Round I, Phase I). *Federal Register*, **54** (149), 42551-42573.

U.S. Environmental Protection Agency [1994]. A plain English guide to the EPA Part 503 biosolids rule. *EPA/832/R-93/003*, Office of Wastewater Management – U.S. EPA, Washington, DC, U.S.A., 176 p.

U.S. Environmental Protection Agency [1993]. Part 503 - Standards for the use and disposal of sewage sludge. Rules and regulations. *Federal Register*, **58** (32), 9248-9415.

U.S. Environmental Protection Agency [1992]. Environmental regulations and technology. Control of pathogens and vector attraction reduction in sewage sludge. *EPA-625/R-92/013*, Office of Research and Development – U.S. EPA, Washington, DC, U.S.A., 152 p.

Vinzant T. B., Adney W. S., Grohmann K. and Rivard C. J. [1990]. Aerobic and anaerobic digestion of processed municipal solid waste: Effects of retention time on cellulose degradation, *Appl. Biochem. Biotechnol.*, **24/25**, 765-771.

Vogels and Van Der Drift [1976]. Degradation of purines and pyrimidines. *Bacteriol. Rev.*, 40, 429-468.

Ward R. L. and Ashley C. S. [1977]. Identification of virucidal agent in wastewater sludge. *Appl. Environ. Microbiol.*, **33** (4), 860-864.

Ward A., Stensel H. D., Ferguson J. F., Ma G. and Hummel S. [1999]. Preventing growth of pathogens in pasteurized digester solids. *Water Environ. Res.*, **71** (2), 176-182.

Water Environment Federation and American Society of Civil Engineers [1998]. Design of municipal wastewater treatment plants. 4th ed. *WEF manual of practice no. 8, ASCE manual and report on engineering practice; no. 76*; 22-170.

Water Quality Management Library [1998]. *Municipal sewage sludge management: a reference text on processing, utilization and disposal.* **Vol. 4**, Second edition. Edited by Lue-Hing C, Zenz D R, Tata P, Kuchenrither R, Malina J F Jr., Sawyer B, Technomic Publishing Co., Inc., Lancaster, PA, USA

Weast R. C. (ed) [1987]. *Handbook of chemistry and physics*, (1st student ed.), CRC Press Inc., Boca Raton, FL, USA, D103-D104.

Webb A. R. and Hawkes F. R. [1985]. The anaerobic digestion of poultry manure: variation of gas yield with influent concentration and ammonium-nitrogen levels. Agricultural Wastes, **14**, 135-156.

Wiegant W. M. and Zeeman G. [1986]. The mechanism of ammonia inhibition in the thermophilic digestion of livestock wastes. *Agric. Wastes*, **16**, 243-253.

World Health Organization [1989]. *Health guidelines for use of wastewater in agriculture and aquaculture.* Technical Report Series No. 778, WHO, Geneva, Switzerland.

Wu N. and Smith J. E. [1999]. Reducing pathogen and vector attraction for biosolids. *BioCycle*, **40** (11), 59-61.

Wujcik W. J. and Jewell W. J. [1980] Dry anaerobic fermentation. *Biotechnol. Bioeng. Symp.*, **10**, 43-65.

Yanko W. A., Walker A. S., Jackson J. L., Libao L. L. and Garcia A. L. [1995]. Enumerating *Salmonella* in biosolids for compliance with pathogen regulations. *Wat. Environ. Res.*, **67**, 364-370.

Yanko W. A. [1988]. Occurrence of pathogens in distribution and marketing municipal sludges- Project summary. US EPA, Research and Development, EPA/600/S1-87/014.

Van Den Bosch M. [1999]. Environmental regulation for treatment and disposal of biosolids in Manitoba. In: *Proceedings of the Biosolids Seminar: Biosolids regulatory, process and utilization technologies.* (Western Canada Water and Wastewater Association and Western Canada Water Environment Association WEF, org.), Chapter 1, p. 1-11, January 26-27, Winnipeg, Canada.

APPENDICES (A THROUGH E)

APPENDIX A

STOICHIOMETRY OF URIC ACID BREAKDOWN UNDER ANAEROBIC CONDITION

Uric acid breakdown proceeds in II stages:

Stage I

$$8\ C_5H_4O_3N_4 + 10\ H_2O \rightarrow 12\ CH_3COOH + 28\ CO_2 + 32\ NH_3$$

Stage II

$$12\ CH_3COOH \rightarrow 6CH_4 + 6\ CO_2 + 12\ H_2O$$

The overall balance is:

$$8\ C_5H_4O_3N_4 \rightarrow 6\ CH_4 + 34\ CO_2 + 32\ NH_3 + 2\ H_2O$$

or

$$C_5H_4O_3N_4 \rightarrow ¾\ CH_4 + 4¼\ CO_2 + 4\ NH_3 + ¼\ H_2O$$

COD EQUIVALENCY OF METHANE

Methane oxidation to carbon dioxide and water proceeds in accordance with the following stoichiometric relation:

$$\begin{array}{llll} (16) & (2*32) & (44) & (18) \qquad\qquad\qquad g/mol \\ CH_4 + 2O_2 & \rightarrow & CO_2 + 2H_2O & \end{array}$$

From the above equation it may be determined that for each mole of methane consumed (22.4 L @ 0°C), two moles of oxygen equivalent are destroyed (64 g). Thus 0.35 L (22.4 L/64 g) of CH_4 at 0°C and 760 mm Hg pressure (STP) is equivalent to 1 g COD destruction. To compensate for higher temperature, the CH_4 equivalence is 0.395 L at 35°C and one atmosphere.

APPENDIX B

TABLE B-1. MEAN METHANE PRODUCTION IN ANAEROBIC DIGESTERS DURING 17-WEEK INCUBATION

Experiment	CH_4 production (mL @ STP) on day: 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Fresh Manure (FM) alone																		
100% FM (undil., TS 21.7%)	3.1	1.9	1.0	1.3	1.3	0.7	0.9	0.6		1.2		1.1		0.7			1.1	
100% FM (dil., TS 10%)	8.6	2.0	1.0	1.2	0.8	0.8	0.6	0.7	0.9	0.6	0.9	0.6		2.2		2.7		2.3
100% FM (dil., TS 5%)	8.2	1.7	0.9	1.0	0.6	0.6	0.4	0.7	1.0	0.7	0.8	0.5		1.5		1.5		0.8
FM and Pit Manure (PM) mixture																		
90% FM + 10% PM (TS 20.7%)	4.1	2.5	1.3	1.5	1.4	0.9	0.8	0.6		1.4		1.3		0.8			1.3	
40% FM + 60% PM (TS 15.7%)	7.4	3.6	0.7	2.0	1.3	1.4	1.2	1.1	0.9	0.9	0.7		1.8		1.8		1.2	
90% FM (dil. 1:2) + 10% PM (TS 10.9%)	9.5	3.3	1.6	1.5	1.1	0.9	0.8	0.8	1.1	0.7	1.1	0.7		1.9		2.1		1.5
FM and anaer. digested sludge (ADS) mixture																		
90% FM + 10% ADS (TS 19.8%)	5.2	2.8	1.6	1.2	1.1	0.6	0.8	0.5		1.1		1.1		0.7			1.2	
40% FM + 60% ADS (TS 10.3%)	5.4	2.1	1.2	1.4	1.0	1.5	1.2	1.5	1.3	2.0	1.6		3.4		2.6		1.6	
90% FM (dil.1:2) + 10% ADS (TS 10.0%)	8.5	2.2	1.1	1.3	0.8	0.8	0.6	0.8	0.9	0.7	1.1	0.8		2.6		2.9		2.2
Controls																		
100% PM (TS 11.7%)	2.6	1.9	1.5	1.0	0.7	0.9	0.9	1.1	0.9	0.7	0.5		1.6		1.8		1.1	
100% ADS (TS 2.7%)	3.9	2.1	1.3	1.3	0.9	1.4	1.3	1.9	1.4	1.2	0.7		3.3		3.0		1.1	

Experiment	CH_4 production (mL @ STP) on day: 20	21	23	24	26	27	29	30	32	33	35	36	38	39	41	42	44	45
Fresh Manure (FM) alone																		
100% FM (undil., TS 21.7%)	0.9		0.6		1.2		0.7		0.9		0.6		0.6		0.7		0.5	
100% FM (dil., TS 10%)		4.6		4.7		6.1		11.6		21.3		36.4		42.3		59.5		58.6
100% FM (dil., TS 5%)		2.2		1.9		2.9		7.5		12.5		23.0		30.7		48.5		46.7
FM and Pit Manure (PM) mixture																		
90% FM + 10% PM (TS 20.7%)	1.1		0.8		1.4		0.8		1.1		0.9		0.8		0.9		0.9	
40% FM + 60% PM (TS 15.7%)	2.0		1.4		1.1		2.3		1.4		1.9		1.9		2.2		2.8	
90% FM (dil. 1:2) + 10% PM (TS 10.9%)		3.2		3.5		4.7		8.9		13.5		24.0		29.8		46.6		50.9
FM and anaer. digested sludge (ADS) mixture																		
90% FM + 10% ADS (TS 19.8%)	1.0		1.0		1.7		1.0		1.1		0.8		0.7		0.7		0.7	
40% FM + 60% ADS (TS 10.3%)	2.6		2.8		4.2		9.9		17.2		32.8		50.2		70.4		64.5	
90% FM (dil.1:2) + 10% ADS (TS 10.0%)		4.2		4.6		6.6		13.2		24.3		40.4		47.4		64.8		61.9
Controls																		
100% PM (TS 11.7%)	2.5		2.3		2.9		5.8		7.7		13.8		19.0		37.8		38.5	
100% ADS (TS 2.7%)	2.8		1.6		0.7		4.2		1.1		2.1		1.8		1.6		1.7	

% - fraction (percent) of 50 g of the digester content (wet weight basis)
TS - initial level of solids in digester

TABLE B-1. CONTINUED...

Experiment	CH_4 production (mL @ STP) on day: 47	48	50	51	53	54	55	58	59	61	64	65	68	71	72	75	78	79
Fresh Manure (FM) alone																		
100% FM (undil., TS 21.7%)	0.4		0.8				0.5			0.5			1.1			1.1		
100% FM (dil., TS 10%)		59.6		45.8		31.4			23.5			18.8			21.1			20.5
100% FM (dil., TS 5%)		45.1		33.2		22.8			19.3			20.4			23.2			16.6
FM and Pit Manure (PM) mixture																		
90% FM + 10% PM (TS 20.7%)	0.7		1.1				0.8			0.9			1.7			1.4		
40% FM + 60% PM (TS 15.7%)	3.3		4.4		6.7			3.7			28.7			58.3			77.5	
90% FM (dil. 1:2) + 10% PM (TS 10.9%)		64.7		65.6		56.2			59.4			44.5			32.4			30.3
FM and anaer. digested sludge (ADS) mixture																		
90% FM + 10% ADS (TS 19.8%)	0.5		1.0				0.7			0.7			1.6			1.5		
40% FM + 60% ADS (TS 10.3%)	48.2		30.5		21.6			20.3			23.6			29.9			32.2	
90% FM (dil.1:2) + 10% ADS (TS 10.0%)		52.0		35.9		25.0			24.4			28.3			36.6			33.5
Controls																		
100% PM (TS 11.7%)	51.5		59.2		63.9			109.0			120.3			85.2			59.4	
100% ADS (TS 2.7%)	1.1		1.0		2.4			1.2			1.1			2.7			1.5	

Experiment	CH_4 production (mL @ STP) on day: 81	84	85	88	91	92	94	97	98	105	108	109	119
Fresh Manure (FM) alone													
100% FM (undil., TS 21.7%)	1.3			1.4			1.2			1.6			1.7
100% FM (dil., TS 10%)			15.7			15.8			15.6			15.8	13.4
100% FM (dil., TS 5%)			11.9			14.3			13.2			11.7	12.2
FM and Pit Manure (PM) mixture													
90% FM + 10% PM (TS 20.7%)	1.7			2.1			1.7			1.9			2.1
40% FM + 60% PM (TS 15.7%)		62.3			51.1			45.4			45.9		45.1
90% FM (dil. 1:2) + 10% PM (TS 10.9%)			23.8			23.2			23.5			20.7	18.1
FM and anaer. digested sludge (ADS) mixture													
90% FM + 10% ADS (TS 19.8%)	1.9			2.0			1.7			1.5			1.4
40% FM + 60% ADS (TS 10.3%)		28.7			40.0			47.1			52.2		50.5
90% FM (dil.1:2) + 10% ADS (TS 10.0%)			22.6			22.8			21.3			18.4	21.6
Controls													
100% PM (TS 11.7%)		49.0			41.7			32.1			27.5		24.8
100% ADS (TS 2.7%)		2.8			2.8			2.5			2.4		2.5

% - fraction (percent) of 50 g of the digester content (wet weight basis).
TS - initial level of solids in digesters

TABLE B-2. INITIAL AND FINAL CHARACTERISTICS (MEAN VALUES) OF DIGESTER CONTENTS

Experiment	Initial TS		Initial TVS		VS of FM in mixture	VS of seed in mixture	Final TS		Final TVS		Initial TKN	
	%	mg/Kg	%TS	mg/Kg	mg/Kg	mg/Kg	%	mg/Kg	%TS	mg/Kg	mg/Kg	% TS
Fresh Manure (FM) alone												
100% FM (undil., TS 21.7%)	21.7	216667	61.8	134000	134000	-	18.0	180000	47.2	85000	14883	6.9
100% FM (dil., TS 10%)	10.0	99846	61.8	61751	61751	-	5.7	57200	37.8	21600	7881	7.9
100% FM (dil., TS 5%)	5.0	49923	61.8	30876	30876	-	3.7	37100	39.9	14800	4159	8.3
FM and Pit Manure (PM) mixture												
90% FM + 10% PM (TS 20.7%)	20.7	206740	62.2	128690	120600	8090	19.0	190100	48.0	91200	14375	7.0
40% FM + 60% PM (TS 15.7%)	15.7	157107	65.0	102140	53600	48540	14.0	140000	46.4	65000	11574	7.4
90% FM (dil. 1:2) + 10% PM (TS 10.9%)	10.9	109240	62.6	68390	60300	8090	7.0	70000	46.6	32600	8526	7.8
FM and anaer. digested sludge (ADS) mixture												
90% FM + 10% ADS (TS 19.8%)	19.8	197725	61.6	121750	120600	1150	18.6	186000	45.7	85000	13862	7.0
40% FM + 60% ADS (TS 10.3%)	10.3	103017	58.7	60500	53600	6900	6.5	65300	46.9	30600	7780	7.6
90% FM (dil.1:2) + 10% ADS (TS 10.0%)	10.0	100225	61.3	61450	60300	1150	5.5	54500	42.9	23400	7854	7.8
Controls												
100% PM (TS 11.7%)	11.7	117400	68.9	80900	-	80900	9.0	90000	45.5	40909	9019	7.7
100% ADS (TS 2.7%)	2.7	27250	42.2	11500	-	11500	2.2	21860	41.3	9018	1740	6.4

Experiment	Final TKN		Initial Organic N		Final Organic N		Init. Norg of FM in mixture	Init. Norg of seed in mixture	Final net Norg of FM
	mg/Kg	%TS	mg/Kg	% TKN	mg/Kg	% TKN	mg/Kg	mg/Kg	mg/Kg
Fresh Manure (FM) alone									
100% FM (undil., TS 21.7%)	16132	9.0	9714	65.3	2758	17.1	9714	-	2758
100% FM (dil., TS 10%)	7799	13.6	5144	65.3	1597	20.5	5144	-	1597
100% FM (dil., TS 5%)	4508	12.2	2715	65.3	609	13.5	2715	-	609
FM and Pit Manure (PM) mixture									
90% FM + 10% PM (TS 20.7%)	15502	8.2	9249	64.3	2709	17.5	8743	439	2512
40% FM + 60% PM (TS 15.7%)	12367	8.8	6698	57.9	2558	20.7	3886	2636	1381
90% FM (dil. 1:2) + 10% PM (TS 10.9%)	8001	11.4	5414	63.5	1116	14.0	4371	439	920
FM and anaer. digested sludge (ADS) mixture									
90% FM + 10% ADS (TS 19.8%)	14472	7.8	9016	65.0	2423	16.7	8743	75	2365
40% FM + 60% ADS (TS 10.3%)	7403	11.3	4864	62.5	1645	22.2	3886	450	1298
90% FM (dil.1:2) + 10% ADS (TS 10.0%)	7505	13.8	5091	64.8	1529	20.4	4371	75	1471
Controls									
100% PM (TS 11.7%)	9298	10.3	4393	48.7	1962	21.1	-	4393	-
100% ADS (TS 2.7%)	1629	7.5	750	43.1	579	35.5	-	750	-

TABLE B-2. CONTINUED...

Experiment	Initial T-COD	Final T-COD	Init. T-COD of FM	Init. T-COD of seed	Final net T-COD of FM	Initial S-COD	Final S-COD	Initial C-COD	Final C-COD
Fresh Manure (FM) alone	mg/Kg	mg/Kg	mg/Kg	mg/Kg	mg/Kg	mg/L	mg/L	mg/kg	mg/kg
100% FM (undil., TS 21.7%)	206564	188835	206564	-	188835	149571	126868	138528	115089
100% FM (dil., TS 10%)	95191	62670	95191	-	62670	59982	24260	59162	27016
100% FM (dil., TS 5%)	47595	35708	47595	-	35708	28415	16406	28582	15101
FM and Pit Manure (PM) mixture									
90% FM + 10% PM (TS 20.7%)	199200	178549	185908	13292	169668	139866	119645	133486	107682
40% FM + 60% PM (TS 15.7%)	162379	106753	82626	79753	53468	94771	67303	109471	50219
90% FM (dil. 1:2) + 10% PM (TS 10.9%)	106246	71859	92954	13292	62979	65367	29823	67270	35281
FM and anaer. digested sludge (ADS) mixture									
90% FM + 10% ADS (TS 19.8%)	187826	170369	185908	1919	168867	131574	100497	124458	104210
40% FM + 60% ADS (TS 10.3%)	94139	53369	82626	11514	44356	52991	11638	58575	19529
90% FM (dil.1:2) + 10% ADS (TS 10.0%)	94873	61650	92954	1919	60148	58720	25075	58970	27342
Controls									
100% PM (TS 11.7%)	132922	88808	-	132922	-	62347	35202	91693	46304
100% ADS (TS 2.7%)	19189	15023	-	19189	-	1142	136	11235	7574

Experiment	Time to reach 60% CH4 in biogas	Duration of phase II		CH4 production		
		t=0	t=end	during ph. I	during ph. II	during ph. III
Fresh Manure (FM) alone	d	d	d	ml/g VS fed	ml/g VS fed	ml/g VS fed
100% FM (undil., TS 21.7%)						
100% FM (dil., TS 10%)	37	36	51	36	122	62
100% FM (dil., TS 5%)	38	36	51	46	178	107
FM and Pit Manure (PM) mixture						
90% FM + 10% PM (TS 20.7%)						
40% FM + 60% PM (TS 15.7%)	68	64	97	18	75	18
90% FM (dil. 1:2) + 10% PM (TS 10.9%)	40	39	59	34	134	63
FM and anaer. digested sludge (ADS) mixture						
90% FM + 10% ADS (TS 19.8%)						
40% FM + 60% ADS (TS 10.3%)	35	35	47	32	109	125
90% FM (dil.1:2) + 10% ADS (TS 10.0%)	35	33	48	26	113	95
Controls						
100% PM (TS 11.7%)	45	44	64	36	136	79
100% ADS (TS 2.7%)	1	1	15	7	41	74

APPENDIX C

TABLE C-1. AVERAGE NET (FROM DEGRADATION OF OF-MSW ALONE) METHANE PRODUCTION

Experiment	cumulative net CH_4 production (mL @ STP) on day: 1	2	4	6	8	10	12	14	16	18	20	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	41	42	43	45	46	47	49	50
85% MSW+15% PS in feed*																																				
SA 0.17 cm^2	0	0	0	0	1	2	3	4	5	6	8	9	9	10	11	12	12	14	14	16	16	18	19	20	21	22	23	24	26	28	28	31	32	33	35	36
SA 0.39 cm^2	0	0	0	0	0	1	2	3	4	5	6	7	8	9	10	11	11	13	14	15	16	17	18	19	20	21	22	24	26	27	28	30	31	33	34	36
SA 1.13 cm^2	0	0	0	0	0	1	2	3	4	5	5	6	7	8	9	10	11	12	12	14	14	16	17	18	19	20	21	22	24	26	27	29	30	31	33	34
90% MSW+10% PS in feed*																																				
SA 0.17 cm^2	0	0	0	0	1	2	3	4	5	6	8	9	9	10	11	12	13	14	15	16	17	18	19	20	21	22	24	25	27	28	29	31	32	33	35	36
SA 0.39 cm^2	0	0	0	0	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	25	26	27	29	30	31	33	34
SA 1.13 cm^2	0	0	0	0	0	1	1	3	4	5	6	7	8	9	10	11	11	12	13	14	15	16	17	18	19	20	21	22	25	26	27	29	30	31	33	34
95% MSW+5% PS in feed*																																				
SA 0.17 cm^2	0	0	0	0	1	2	3	4	6	7	8	10	10	11	12	13	14	15	16	17	18	19	20	21	22	24	25	26	28	29	30	32	33	35	37	38
SA 0.39 cm^2	0	0	0	0	0	1	2	3	4	5	7	8	9	10	11	12	13	14	15	16	17	18	19	20	20	22	23	24	26	27	28	30	31	32	34	35
SA 1.13 cm^2	0	0	0	0	0	1	2	3	4	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	26	27	28	30	31	32	34	35
100% MSW+0% PS in feed*																																				
SA 0.17 cm^2	0	0	0	0	1	2	3	4	6	7	9	10	10	11	12	13	14	15	16	17	18	18	19	20	21	21	22	23	24	25	26	27	28	28	29	30
SA 0.39 cm^2	0	0	0	0	0	1	2	3	4	5	7	8	9	10	10	12	12	13	14	15	16	17	17	18	19	19	20	21	22	23	23	24	25	25	26	27
SA 1.13 cm^2	0	0	0	0	0	1	2	3	4	6	7	9	9	10	11	12	13	14	15	16	16	17	18	19	19	20	21	21	22	23	23	25	25	26	26	27

Experiment	cumulative net CH_4 production (mL @ STP) on day: 52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	78	79	80	81	82	83	84	85	86	87	88	89
85% MSW+15% PS in feed*																																				
SA 0.17 cm^2	39	40	41	42	43	44	45	47	48	49	51	52	53	55	56	58	59	60	61	63	64	65	67	68	71	72	73	74	76	77	78	79	80	82	83	84
SA 0.39 cm^2	38	39	40	41	42	43	44	46	47	48	50	51	52	54	55	56	58	59	60	62	63	64	65	67	70	71	72	73	74	75	76	77	78	79	80	82
SA 1.13 cm^2	36	37	38	39	40	42	43	44	45	47	48	49	50	52	53	54	55	57	58	59	60	61	63	64	67	67	68	70	71	72	73	74	75	76	77	78
90% MSW+10% PS in feed*																																				
SA 0.17 cm^2	39	40	41	42	43	44	46	47	48	49	51	52	53	54	55	57	58	59	60	61	63	64	65	67	69	70	71	72	73	74	76	77	78	79	80	81
SA 0.39 cm^2	36	37	39	40	41	42	43	44	45	46	48	49	50	51	53	54	55	56	58	59	60	61	62	64	66	67	68	69	70	72	73	74	75	76	77	78
SA 1.13 cm^2	36	37	38	39	40	42	43	44	45	46	47	49	50	51	52	54	55	56	57	58	59	60	61	63	65	66	67	68	69	70	72	73	74	75	76	77
95% MSW+5% PS in feed*																																				
SA 0.17 cm^2	40	41	42	43	44	45	46	47	48	50	51	51	52	53	54	55	56	57	58	59	60	61	62	63	65	66	67	68	68	69	70	71	72	72	73	74
SA 0.39 cm^2	37	38	39	40	41	43	44	45	46	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	62	63	63	64	65	66	67	67	68	69	69	70
SA 1.13 cm^2	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	58	59	61	62	63	64	65	65	66	67	67	68	69	69
100% MSW+0% PS in feed*																																				
SA 0.17 cm^2	31	32	32	33	33	33	34	34	35	35	36	36	37	37	38	38	38	39	39	40	40	41	41	41	42	43	43	43	44	44	44	44	45	45	45	46
SA 0.39 cm^2	28	28	29	29	29	30	30	30	31	31	32	32	32	33	33	33	34	34	35	35	35	36	36	36	37	38	38	38	39	39	39	40	40	40	40	41
SA 1.13 cm^2	28	28	29	29	30	30	30	31	31	31	32	32	33	33	33	34	34	35	35	35	36	36	36	37	37	38	38	38	39	39	39	40	40	40	40	41

SA: surface area of paper components of the OF-MSW

* % contribution from OF-MSW and PS based on dry solids

TABLE C-1. CONTINUED...

		cumulative net CH_4 production (mL @ STP) on day:																								
51	Experiment	91	92	96	99	102	103	104	106	108	109	110	112	113	114	116	118	120	122	123	124	125	126	128	130	132	133	134
	85% MSW+15% PS in feed*																											
38	SA 0.17 cm^2	86	87	90	92	94	95	96	98	100	101	102	104	105	106	107	109	111	113	114	115	116	117	119	121	122	123	124
36	SA 0.39 cm^2	84	85	87	89	92	93	94	96	98	99	100	102	103	103	105	107	109	110	112	112	114	114	116	118	120	121	122
34	SA 1.13 cm^2	80	81	83	85	87	88	89	91	93	94	95	97	98	99	101	102	104	106	107	108	109	110	112	114	116	117	118
	90% MSW+10% PS in feed*																											
38	SA 0.17 cm^2	83	84	86	88	89	90	91	93	94	95	96	97	98	98	100	101	102	104	104	105	106	106	108	109	110	111	111
35	SA 0.39 cm^2	80	81	83	84	86	87	88	89	91	92	92	94	95	95	97	98	99	100	101	102	102	103	104	105	107	107	108
35	SA 1.13 cm^2	79	79	82	83	85	86	87	89	90	91	92	93	94	95	96	97	98	100	100	101	101	102	103	104	106	106	107
	95% MSW+5% PS in feed*																											
39	SA 0.17 cm^2	76	76	78	79	80	81	81	82	83	84	84	85	85	86	86	87	88	88	88	89	89	89	90	90	91	91	91
36	SA 0.39 cm^2	71	72	73	75	76	77	77	78	79	79	80	80	81	81	82	83	83	84	84	84	85	85	85	86	86	87	87
35	SA 1.13 cm^2	71	71	73	74	76	76	77	78	79	79	80	80	81	81	82	82	83	84	84	84	84	85	85	86	86	87	87
	100% MSW+0% PS in feed*																											
30	SA 0.17 cm^2	46	46	47	47	47	47	47	48	48	48	48	48	48	48	48	48	48	48	49	49	49	49	49	49	49	49	49
27	SA 0.39 cm^2	41	41	42	42	43	43	43	43	43	43	43	44	44	44	44	44	44	44	44	44	44	44	44	44	44	44	44
27	SA 1.13 cm^2	41	41	42	42	43	43	43	43	43	44	44	44	44	44	44	44	44	44	44	44	44	44	44	44	44	44	44

		cumulative net CH_4 production (mL @ STP) on day:																		Std.	
90	Experiment	135	136	138	140	142	144	146	148	150	152	154	156	158	160	162	165	167	169	171	Dev.
	85% MSW+15% PS in feed*																				
85	SA 0.17 cm^2	125	126	127	129	131	132	134	136	137	139	140	142	143	145	146	148	150	151	153	
83	SA 0.39 cm^2	123	123	125	127	128	130	131	133	134	135	137	138	140	141	143	145	146	148	149	
79	SA 1.13 cm^2	119	120	121	123	125	126	128	129	131	132	134	135	137	138	140	142	143	145	146	3
	90% MSW+10% PS in feed*																				
82	SA 0.17 cm^2	111	112	113	114	114	116	116	117	118	119	120	121	122	123	124	125	126	127	128	
79	SA 0.39 cm^2	109	109	110	111	112	113	114	115	116	117	117	118	119	120	121	122	123	124	125	
78	SA 1.13 cm^2	108	108	109	110	111	112	113	114	115	116	117	118	119	120	121	121	122	123	124	2
	95% MSW+5% PS in feed*																				
75	SA 0.17 cm^2	91	92	92	92	93	93	94	94	94	95	95	96	96	96	97	97	98	98	99	
70	SA 0.39 cm^2	87	87	88	88	89	89	89	89	90	90	90	91	91	91	92	92	93	93	94	
70	SA 1.13 cm^2	87	87	88	88	88	89	89	89	90	90	91	91	91	92	92	92	93	93	94	3
	100% MSW+0% PS in feed*																				
46	SA 0.17 cm^2	49	49	49	49	49	49	49	49	49	49	49	49	49	49	49	49	49	49	49	
41	SA 0.39 cm^2	44	44	44	44	44	44	44	44	44	44	44	44	44	44	44	44	44	44	44	
41	SA 1.13 cm^2	44	44	44	44	44	44	44	44	44	44	44	44	44	44	44	44	44	44	44	3

PS-15

PS-10

PS-5

PS-0

SA: surface area of paper components of the OF-MSW

* % contribution from OF-MSW and PS based on dry solids

There was no significant difference in the final cumulative net methane production among the reactors fed feed with same level of PS solids (see Std. Dev. on day 171; even though three different surface areas (SA) of the paper component were used to prepare the high-solid feed. These reactors were treated as triplicates for data processing, with the following character representation: PS-15 (15% primary sludge solids in feed to reactor), PS-10, PS-5, and PS-0

TABLE C-2. SPECIFIC WEIGHT OF (OF-MSW + PS) FEED FED TO DIGESTERS

Experiment	Specific Weight, kg/m³					
	1	2	3	4	Mean	Std. Dev.
85% MSW+15% PS in feed*						
SA 0.17 cm²/	476	494	470	478	480	10
SA 0.39 cm²/	460	474	466	482	471	9
SA 1.13 cm²/	476	475	470	481	476	5
90% MSW+10% PS in feed*						
SA 0.17 cm²/	438	441	434	430	436	5
SA 0.39 cm²/	450	448	435	438	443	7
SA 1.13 cm²/	432	429	436	451	437	10
95% MSW+5% PS in feed*						
SA 0.17 cm²/	402	396	393	407	400	6
SA 0.39 cm²/	401	394	393	399	397	4
SA 1.13 cm²/	405	391	400	392	398	10
100% MSW+0% PS in feed*						
SA 0.17 cm²/	375	367	371	366	370	4
SA 0.39 cm²/	363	361	362	354	360	4
SA 1.13 cm²/	353	348	346	354	350	4

SA: surface area of paper components of the OF-MSW

* % contribution from OF-MSW and PS based on dry solids

TABLE C-3. CARBON-TO-NITROGEN RATIO OF (OF-MSW + PS) FEED FED TO DIGESTERS

Experiment	Carbon, %			Carbon adjusted for AIL, %			Nitrogen, %			C/N		
	1	2	Mean	1	2	Mean	1	2	Mean		Mean	Std. Dev.
PS-15												
1	41.0	41.1	41.0	37.1	37.2	37.1	1.0	0.9	1.0	37.7		
2	41.1	40.8	40.9	37.1	36.9	37.0	0.9	1.0	0.9	39.6		
3	41.0	41.2	41.1	37.1	37.2	37.2	0.9	0.8	0.9	42.5	39.9	2.4
PS-10												
1	41.0	40.8	40.9	36.4	36.3	36.3	0.9	0.9	0.9	40.4		
2	40.8	40.9	40.9	36.2	36.4	36.3	0.9	0.8	0.9	42.4		
3	41.1	40.9	41.0	36.5	36.3	36.4	0.9	0.8	0.8	44.4	42.4	2.0
PS-5												
1	41.2	41.2	41.2	36.1	36.1	36.1	0.8	0.7	0.7	49.1		
2	41.1	41.1	41.1	36.0	36.0	36.0	0.7	0.7	0.7	49.6		
3	41.2	41.3	41.2	36.1	36.1	36.1	0.7	0.6	0.7	54.7	51.1	3.1
PS-0												
1	41.0	41.1	41.0	35.6	35.7	35.7	0.6	0.5	0.6	62.5		
2	41.4	41.5	41.5	36.0	36.1	36.0	0.6	0.6	0.6	61.1		
3	41.4	41.3	41.4	36.0	35.9	35.9	0.6	0.5	0.6	65.3	63.0	2.2

Assume that all Carbon is in VS form

FEED TYPE	*AIL, %VS*
0% PS	13.1
5% PS	12.4
10% PS	11.2
15% PS	9.5

TABLE C-4. MEAN METHANE CONCENTRATION (%) IN REACTOR HEADSPACE

Experiment	Mean methane content (%, volume/volume) in biogas on day 8	14	22	28	34	46	57	65	73	85	93	101	113	121	133	141	150	159	168
PS-15																			
1	62.7	57.2	52.2	51.3	50.5	53.1	52.0	53.5	50.8	54.0	50.2	51.1	51.9	50.7	50.4	49.6	49.3	49.3	49.0
2	62.3	57.4	52.9	52.8	51.8	51.2	52.0	51.8	51.0	52.7	51.6	51.9	49.8	48.8	50.9	50.4	48.4	48.9	49.2
3	62.1	56.6	51.6	51.0	51.8	50.7	52.0	50.5	49.3	50.8	49.2	49.8	51.1	51.0	52.0	50.3	50.9	49.1	51.1
PS-10																			
1	61.4	56.5	50.5	50.5	50.8	51.9	52.3	49.6	49.9	52.1	46.1	46.0	47.4	43.1	43.7	37.5	38.7	39.6	39.3
2	61.8	58.3	51.6	49.1	49.6	49.9	50.3	49.7	49.2	50.3	46.7	42.9	44.4	41.4	41.1	39.6	39.3	37.4	38.6
3	62.7	57.9	52.8	49.1	51.2	50.1	50.5	48.6	47.5	49.8	48.0	48.4	43.9	39.5	44.3	39.5	41.1	37.8	39.6
PS-5																			
1	61.7	59.1	52.1	50.2	48.4	49.8	51.5	49.9	44.7	41.9	39.5	39.2	34.9	30.7	28.2	25.8	27.3	26.2	23.3
2	61.5	57.2	52.7	48.2	49.4	49.8	50.3	47.5	44.2	39.7	36.4	35.8	33.1	28.9	27.6	25.2	22.4	24.8	25.2
3	61.4	56.8	51.3	48.4	48.4	49.3	49.7	47.2	43.4	38.1	40.0	37.8	31.6	29.8	25.5	27.4	25.9	23.5	24.0
PS-0																			
1	61.5	55.5	50.3	44.6	43.2	42.6	38.3	34.2	32.5	26.0	24.6	20.9	18.6	16.2	10.2	8.6	8.8	10.2	9.6
2	61.0	55.3	49.5	47.8	44.7	42.4	37.8	32.5	31.6	27.0	25.6	23.3	21.5	16.0	11.5	9.8	8.2	10.2	9.1
3	60.3	59.0	51.3	46.1	42.9	42.4	37.7	37.5	31.1	28.0	26.4	20.7	20.5	12.6	10.4	8.6	9.1	8.1	6.2
CONTROL	66.0	60.6	60.3	62.6	61.5	63.5	60.2	60.4	60.3	61.2	60.3	60.3	60.0	61.9	58.9	62.3	60.4	61.7	61.7

TABLE C-5. MEAN TOTAL SOLID (TS) CONCENTRATION IN DIGESTATE

Experiment	Mean total solid (TS) concentration (mg/Kg) on day 2	10	16	22	30	38	51	60	71	82	92	99	106	114	122	130	140	152	162	171
PS-15																				
1	2.2	4.2	5.3	7.5	9.3	9.9	12.3	14.9	17.0	19.9	24.1	26.4	25.3	25.0	26.2	25.6	24.9	26.0	25.8	26.7
2	2.1	4.3	5.5	7.4	9.0	10.3	12.4	15.4	18.8	20.7	24.4	25.8	25.1	26.5	25.9	25.0	25.7	25.8	26.7	26.0
3	2.1	4.3	5.4	7.5	9.3	10.0	12.6	15.2	18.5	20.8	23.9	25.7	24.8	25.2	26.7	24.9	25.6	26.5	26.6	27.4
PS-10																				
1	2.2	4.2	6.0	7.6	9.3	10.4	12.8	16.5	18.7	23.3	26.0	27.9	27.3	27.4	27.9	27.6	26.9	28.4	27.5	27.7
2	2.2	4.3	6.3	7.6	9.4	10.7	13.5	17.1	19.7	23.1	26.6	27.8	28.0	28.2	27.5	27.2	28.5	28.3	28.0	28.0
3	2.2	4.3	5.0	7.6	9.2	10.4	13.1	16.3	19.6	24.0	26.4	27.1	27.6	27.7	27.8	26.9	29.4	28.0	27.6	27.8
PS-5																				
1	2.2	4.3	5.9	7.5	9.3	11.2	13.8	17.3	21.8	25.6	27.1	28.4	29.8	28.0	29.0	26.4	29.6	29.6	28.2	29.0
2	2.2	4.5	5.6	7.6	9.4	11.7	13.9	17.3	21.3	27.0	28.5	29.9	29.5	28.9	28.6	28.4	29.0	28.9	28.6	28.5
3	2.3	4.3	5.7	7.8	9.4	11.3	14.0	16.6	21.7	25.5	27.7	29.4	28.0	29.4	28.9	28.4	29.3	28.6	28.0	29.0
PS-0																				
1	2.3	4.4	6.4	7.7	9.3	12.2	15.7	20.5	25.6	29.0	30.1	30.4	30.5	29.4	29.4	29.7	30.3	30.5	31.4	29.9
2	2.3	4.4	5.8	7.8	9.5	12.2	15.7	19.8	24.5	29.4	30.9	30.2	29.9	30.2	30.3	30.2	29.8	30.0	31.0	30.2
3	2.3	4.5	5.6	7.8	9.5	12.6	16.2	19.9	26.0	29.7	29.7	30.2	30.3	30.5	31.0	29.9	30.2	30.3	30.6	30.5

Initial TS in all reactors was 2% TS

TABLE C-6. MEAN TOTAL AMMONIA CONCENTRATION IN DIGESTATE

	Mean total ammonia concentration (mg N/L) on day																			
Experiment	0	10	16	22	30	38	51	60	71	82	92	99	106	114	122	130	140	152	162	171
PS-15																				
1	359	562	763	831	955	1006	1222	1313	1413	1466	1609	1722	1641	1755	1758	1807	1605	1737	1767	1816
2	359	585	749	810	944	1016	1144	1235	1367	1389	1728	1603	1660	1698	1928	1705	1897	1867	1628	1770
3	359	572	756	809	840	948	1116	1264	1239	1330	1531	1770	1611	1495	1899	1946	1676	1607	1798	1983
PS-10																				
1	359	558	732	780	903	919	1082	1198	1347	1294	1328	1429	1477	1428	1549	1257	1347	1460	1398	1485
2	359	539	721	787	883	900	1078	1199	1211	1333	1319	1359	1242	1379	1458	1247	1419	1429	1268	1408
3	359	572	673	749	808	842	1019	1198	1104	1138	1279	1290	1231	1358	1358	1307	1360	1308	1468	1430
PS-5																				
1	359	538	678	731	856	823	1000	965	1175	1032	1009	877	953	1046	1007	1065	967	1038	976	988
2	359	518	690	716	831	824	1049	936	861	925	1104	987	849	997	956	856	866	987	1007	1131
3	359	533	675	696	739	746	903	1013	979	1101	975	878	946	1038	1057	986	982	866	986	877
PS-0																				
1	359	518	651	664	744	766	827	636	757	671	484	553	563	484	605	605	605	464	555	433
2	359	511	637	674	773	785	837	850	677	602	642	494	504	655	524	383	534	645	606	544
3	359	510	620	635	704	621	818	753	629	652	523	543	632	494	474	479	550	509	383	533

TABLE C-7. MEAN UN-IONIZED AMMONIA CONCENTRATION IN DIGESTATE

	Mean un-ionized ammonia (NH_3) concentration (mg N/L) on day:																			
Experiment	0	10	16	22	30	38	51	60	71	82	92	99	106	114	122	130	140	152	162	171
PS-15																				
1	6	9	10	13	15	15	24	69	135	109	193	133	163	130	186	152	181	169	146	209
2	6	8	10	15	13	11	9	48	63	117	78	119	91	155	95	126	232	154	165	166
3	6	9	10	11	9	16	25	100	41	99	102	106	127	97	144	122	170	127	125	144
PS-10																				
1	6	9	11	8	14	14	15	75	43	52	41	37	31	115	151	84	72	82	54	61
2	6	8	9	10	10	12	22	53	46	97	72	68	35	137	120	61	55	29	42	36
3	6	8	10	13	9	13	18	69	43	96	45	74	46	31	63	70	68	50	41	69
PS-5																				
1	6	9	8	7	11	12	9	16	32	14	14	6	21	13	4	8	7	7	5	7
2	6	8	6	8	7	9	14	18	8	10	13	22	9	10	5	40	13	10	8	8
3	6	7	6	9	8	11	10	45	10	11	8	29	10	15	9	13	12	7	6	8
PS-0																				
1	6	7	8	7	6	4	7	6	5	4	3	3	3	3	4	3	2	2	2	1
2	6	7	7	7	10	3	7	7	3	3	3	2	2	3	2	3	2	2	2	1
3	6	7	7	7	5	3	7	4	3	3	4	3	6	6	5	3	2	1	1	1

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TABLE C-8. MEAN TOTAL VOLATILE FATTY ACID (VFA) CONCENTRATION IN DIGESTATE

	Mean total VFA concentration (mg/L as acetic acid or HAc) on day																		
Experiment	10	16	22	30	38	51	60	71	82	92	99	106	114	122	130	140	152	162	171
PS-15																			
1	31	55	38	128	216	65	458	435	265	748	913	907	677	955	875	1236	864	839	1213
2	26	54	56	267	143	118	408	740	341	873	877	952	972	876	1301	1218	972	1145	1166
3	40	104	60	298	303	195	422	659	270	653	976	678	1031	766	965	970	768	1021	1089
PS-10																			
1	16	41	44	135	236	134	400	851	743	830	1378	1121	1728	1650	2222	2901	2710	2734	2374
2	69	34	55	283	164	153	792	872	685	1096	1221	1666	1725	1961	2390	2195	2097	2856	2066
3	68	106	57	424	327	93	869	835	467	805	1256	1233	1515	1410	2452	2401	2442	2293	2455
PS-5																			
1	15	43	34	486	322	783	731	1079	1119	1413	3089	2888	5528	4596	6390	8907	7769	8558	9201
2	64	39	55	220	486	132	1331	1206	2133	2735	3149	2099	4351	5032	6367	9326	7790	8589	10823
3	64	124	94	365	356	341	1572	1940	2244	3148	2405	3507	4128	5213	5802	7908	7100	8406	8711
PS-0																			
1	41	155	65	2994	2367	10779	9197	9916	7898	14086	15292	21663	20725	22195	21915	24437	27100	25421	26322
2	26	161	41	1360	3067	9496	8472	8571	8422	13629	9633	11928	14260	11131	13160	13994	17198	19071	20293
3	21	220	43	2460	2283	10244	10277	11359	9384	12550	14226	13090	12462	11679	14423	17367	18229	21055	20824

TABLE C-9. MEAN UN-IONIZED VFA CONCENTRATION IN DIGESTATE

	Mean un-ionized VFA concentration (mg/L as acetic acid or HAc) on day:																		
Experiment	10	16	22	30	38	51	60	71	82	92	99	106	114	122	130	140	152	162	171
PS-15																			
1	0	0	0	1	1	0	1	0	0	0	1	1	1	1	1	1	1	1	1
2	0	0	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1
3	0	1	0	2	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1
PS-10																			
1	0	0	0	1	1	1	0	2	1	2	4	4	1	1	2	4	3	5	4
2	0	0	0	2	1	1	1	2	1	1	2	4	1	2	3	4	7	6	6
3	0	1	0	3	2	0	1	2	0	2	2	2	5	2	3	3	4	6	4
PS-5																			
1	0	0	0	3	2	7	3	3	6	7	31	10	33	75	64	95	89	122	94
2	0	0	0	2	3	1	5	10	15	16	10	14	31	67	9	44	58	82	110
3	0	1	1	3	2	2	2	13	16	29	5	24	20	42	31	45	67	107	71
PS-0																			
1	0	1	0	28	31	87	65	108	96	143	195	340	253	242	314	439	583	669	633
2	0	1	0	8	66	82	75	123	115	200	198	175	223	209	140	235	463	383	598
3	0	1	0	23	33	87	154	152	141	125	199	104	78	88	180	319	783	542	600

TABLE C-10. MEAN SOLUBLE CHEMICAL OXYGEN DEMAND (S-COD) CONCENTRATION IN DIGESTATE

	Mean S-COD concentration (mg O_2/L) on day:																		
Experiment	10	16	22	30	38	51	60	71	82	92	99	106	114	122	130	140	152	162	171
PS-15																			
1	532	1162	2169	5627	5985	3780	5113	5531	6460	11206	9804	10134	10960	10025	10752	10862	10873	11954	13048
2	554	1143	2047	5977	5278	7020	5658	5677	7494	10490	11216	10978	10746	8799	11966	10220	11953	12832	11957
3	563	1219	1702	5979	5277	5739	4989	5006	8589	9338	9704	10129	11194	10029	10256	11085	12180	12397	11973
PS-10																			
1	509	1239	2246	6481	6343	5538	6342	6356	7301	9505	11030	12697	15618	13226	13222	13702	16763	15880	15883
2	574	933	2597	6648	5724	6961	7159	7178	7791	13183	11704	15037	15863	13221	15664	15895	17412	17624	16322
3	622	1200	2342	6160	5545	5471	5799	5818	8535	13474	12841	14184	16550	13469	12968	14607	15454	14146	15450
PS-5																			
1	518	1169	2015	6526	7146	8856	5398	5422	12077	14816	13925	17853	18640	18114	21031	21128	21129	21438	21795
2	604	1034	2866	6588	5818	6355	6076	6101	10131	16442	15367	17848	16571	17755	18134	22134	22810	22126	22463
3	532	1303	2841	6952	6260	7775	8240	8829	12822	15253	15049	17510	19596	17057	21320	21802	20769	22451	21457
PS-0																			
1	572	1016	2745	18513	7331	22998	26601	24655	20830	35089	32502	36863	35376	41344	42061	42206	39155	39191	39130
2	491	1118	2060	9996	6354	23921	23002	22733	24750	34276	25336	32794	31756	29761	33281	30981	35399	30679	31671
3	586	1264	2278	12187	6890	24487	24324	19732	22081	32379	33120	28761	29939	31194	30806	35746	33372	33383	33380

TABLE C-11. MEAN *pH IN* DIGESTATE

	Mean pH on day:																		
Experiment	10	16	22	30	38	51	60	71	82	92	99	106	114	122	130	140	152	162	171
PS-15																			
1	7.11	7.02	7.11	7.09	7.09	7.20	7.64	7.92	7.80	8.03	7.82	7.94	7.80	7.97	7.86	8.00	7.93	7.85	8.01
2	7.04	7.01	7.17	7.04	6.93	6.79	7.50	7.58	7.86	7.57	7.80	7.66	7.90	7.61	7.80	8.04	7.85	7.95	7.91
3	7.09	7.04	7.05	6.94	7.14	7.25	7.83	7.43	7.80	7.75	7.70	7.83	7.74	7.81	7.72	7.95	7.83	7.77	7.79
PS-10																			
1	7.13	7.08	6.91	7.10	7.09	7.05	7.72	7.41	7.52	7.40	7.32	7.23	7.84	7.93	7.75	7.65	7.67	7.50	7.53
2	7.06	7.00	6.99	6.96	7.02	7.22	7.56	7.49	7.79	7.66	7.62	7.36	7.94	7.85	7.61	7.50	7.22	7.43	7.31
3	7.05	7.07	7.13	6.97	7.09	7.16	7.68	7.50	7.86	7.46	7.68	7.49	7.26	7.58	7.65	7.62	7.50	7.35	7.60
PS-5																			
1	7.14	6.97	6.89	7.02	7.05	6.83	7.13	7.35	7.05	7.06	6.75	7.24	6.98	6.54	6.76	6.73	6.70	6.60	6.75
2	7.07	6.87	6.95	6.84	6.94	7.04	7.20	6.86	6.92	6.98	7.25	6.92	6.90	6.63	7.59	7.09	6.89	6.78	6.75
3	7.02	6.85	7.01	6.92	7.06	6.95	7.56	6.93	6.91	6.80	7.43	6.93	7.07	6.85	7.03	7.00	6.78	6.65	6.85
PS-0																			
1	7.06	7.01	6.94	6.78	6.64	6.85	6.91	6.72	6.67	6.75	6.65	6.56	6.67	6.72	6.60	6.50	6.42	6.33	6.37
2	7.06	6.94	6.92	7.01	6.42	6.82	6.81	6.60	6.62	6.59	6.44	6.59	6.56	6.48	6.73	6.53	6.32	6.45	6.28
3	7.05	6.96	6.96	6.79	6.59	6.83	6.58	6.63	6.58	6.76	6.61	6.86	6.96	6.88	6.66	6.49	6.11	6.34	6.29

APPENDIX D

TABLE D-1. Most Probable Number (MPN) of organisms with three tubes per dilution (a five-tube MPN index can be obtained from Standard Methods [APHA, AWWA, WEF, 1992])

Combination of Positives	MPN Index	95% Confidence limits	
		Lower	Upper
0/0/0	<0.3	-	-
0/0/1	0.3	0.064	1.4
0/1/0	0.3	0.084	1.4
0/1/1	0.6	0.13	2.8
0/2/0	0.6	0.13	2.8
1/0/0	0.4	0.085	1.9
1/0/1	0.7	0.15	3.3
1/0/2	1.1	0.24	5.2
1/1/0	0.7	0.15	3.3
1/1/1	1.1	0.24	5.2
1/2/0	1.1	0.24	5.2
1/2/1	1.5	0.32	7.0
1/3/0	1.6	0.34	7.5
2/0/0	0.9	0.19	4.2
2/0/1	1.4	0.30	6.6
2/0/2	2.0	0.45	9.4
2/1/0	1.5	0.21	7.0
2/1/1	2.0	0.43	9.4
2/1/2	3.0	0.64	14
2/2/0	2.0	0.43	9.4
2/2/1	3.0	0.64	14
2/2/2	3.5	0.75	16
2/2/3	4.0	0.85	19
2/3/0	3.0	0.64	14
2/3/1	3.5	0.75	16
2/3/2	4.0	0.86	19
3/0/0	2.5	0.53	12
3/0/2	6.5	1.4	30
3/1/0	4.5	0.96	21
3/1/1	7.5	1.6	35
3/1/2	11.5	2.5	54
3/1/3	16.0	3.4	75
3/2/0	9.5	2.0	44
3/2/1	15.0	3.2	70
3/2/2	20.0	4.3	94
3/2/3	30.0	6.4	140
3/3/0	25.0	5.3	120
3/3/1	45.0	7.6	200
3/3/2	110.0	24	520
3/3/3	140.0	30	660

Source: Finstein M. S. [1972]. *Pollution microbiology: a laboratory manual.* Marcel Dekker, Inc., New York, NY, U.S.A.

TABLE D-2. FECAL COLIFORM, SALMONELLA , AND CLOSTRIDIUM PERFRINGENS DENSITY IN SLUDGE TREATED WITH LIME AT 20-22°C

Sludge TS (%):	31.36	*Additional solids due to H2O removal (g):*	2.01
Lime dose (g/kg TS):	20	Overall TS after lime addition (%):	31.99
Lime dose (g/kg wet sludge):	6.27	(assuming complete reaction of CaO and H2O)	
Sludge TS after lime addition (%):	31.79	Min density (CFU/g TS):	3126

	FECAL COLIFORM S													
Sample	dil. factor	CFU	Final CFU/mL	TS %	CFU/g	Mean CFU/g	MPN comb.	MPN in 100 mL	at Conf. Limit 95% lower	upper	largest vol., mL	*MPN/g*	*MPN/g* lower	upper
RAW SLUDGE	1000	206	2060000	31.36	6.57E+06									
	1000	238	2380000		7.59E+06	7.08E+06		MPN TEST NOT DONE						
AT START	*1000*	*206*	*2060000*	*31.79*	6.48E+06									
	1000	238	2380000		7.49E+06	6.98E+06								
1 h	1000	121	1210000	31.99	3.78E+06									
	1000	120	1200000		3.75E+06	3.77E+06								
2 h	1000	85	850000	31.99	2.66E+06									
	1000	*76*	*760000*		*2.38E+06*	*2.52E+06*								
9 h	10	28	2800	31.99	8.75E+03									
	10	18	1800		5.63E+03	7.19E+03								
1 d	10	3	300	31.99	9.38E+02									
	10	3	300		9.38E+02									
	10	1	100		3.13E+02	7.29E+02								
6 d	10	0	0	31.44	0.00E+00									
	10	0	0		0.00E+00									
	10	1	100		3.18E+02	1.06E+02								
44 d	10	0	0	31.44	0.00E+00									
	10	0	0		0.00E+00	0.00E+00								

***Salmonella* sp. and *Clostridium perfringens* were not tested in sludge treated with this dose.**

TABLE D-2. CONTINUED...

Sludge TS (%):	31.74	Additional solids due to H2O removal (g):	3.05	Lowest MPN/100mL in a 3-tube setup:	0.4
Lime dose (g/kg TS):	**30**	Overall TS after lime addition (%):	32.69	Min. density (MPN/g TS):	0.12
Lime dose (g/kg wet sludge):	9.52	(assuming complete reaction of CaO and H2O)		Lowest MPN/100ml in a 5-tube setup:	2.0
Sludge TS after lime addition (%):	32.38	Min density (CFU/g TS):	3059	Min. density (MPN/g TS):	0.61

	FECAL COLIFORMS													CLOSTRIDIUM PERFRINGENS				
Sample	dil. factor	CFU	Final CFU/mL	TS %	CFU/g	Mean CFU/g	MPN comb.	MPN in 100 mL	at Conf. Limit 95% lower	upper	largest vol., mL	MPN/g	MPN/g lower	upper	dil. factor	CFU	Final CFU/mL	CFU/g	Mean CFU/g
RAW SLUDGE	10000	60	6000000	31.74	1.89E+07										10000	68	680000	2.14E+06	
	10000	*74*	*7400000*		*2.33E+07*	*2.11E+07*	5/1/0	30.0	10.0	120.0	1.00E-07	9.45E+07	3.15E+07	3.78E+08	10000	60	600000	1.89E+06	**2.02E+06**
AT START	10000	60	6000000	32.38	1.85E+07										10000	68	680000	2.10E+06	
	10000	74	7400000		2.29E+07	2.07E+07	5/1/0	30.0	10.0	120.0	1.00E-07	9.26E+07	3.09E+07	3.71E+08	10000	60	600000	1.85E+06	**1.98E+06**
15 min	1000	143	1430000	32.69	4.38E+06										10000	63	630000	1.93E+06	
	1000	165	1650000		5.05E+06	4.71E+06	5/4/0	130.0	50.0	390.0	1.00E-05	3.98E+06	1.53E+06	1.19E+07	10000	60	600000	1.84E+06	**1.88E+06**
1 h	10	30	3000	32.69	9.18E+03										10000	70	700000	2.14E+06	
	10	43	4300		1.32E+04	1.12E+04	5/2/0	50.0	20.0	70.0	1.00E-03	1.53E+04	6.12E+03	2.14E+04	10000	63	630000	1.93E+06	**2.03E+06**
2 h	10	0	0	32.69	0.00E+00	less than									10000	76	760000	2.33E+06	
	10	0	0		0.00E+00	3.06E+03	5/0/0	23.0	9.0	86.0	1.00E-02	7.04E+02	2.75E+02	2.63E+03	10000	45	450000	1.38E+06	**1.85E+06**
4 h	10	0	0	32.69	0.00E+00	less than						less than			10000	74	740000	2.26E+06	
	10	0	0		0.00E+00	3.06E+03	0/0/0	<2	-	-	1.00E+00	6.12E-01	-	-	10000	65	650000	1.99E+06	**2.13E+06**
1 d	10	0	0	32.62	0.00E+00	less than						less than			10000	116	1160000	3.56E+06	
	10	0	0		0.00E+00	3.06E+03	0/0/0	<2	-	-	1.00E+00	6.12E-01	-	-	10000	127	1270000	3.89E+06	**3.72E+06**
15 d	*10*	*0*	*0*	32.50	*0.00E+00*	*less than*						less than			10000	64	640000	1.97E+06	
	10	0	0		0.00E+00	3.06E+03	0/0/0	<0.3	-	-	1.00E+00	1.22E-01	-	-	10000	64	640000	1.97E+06	**1.97E+06**
26 d	10	0	0	32.26	0.00E+00	less than						less than			10000	35	350000	1.08E+06	
	10	0	0		0.00E+00	3.06E+03	0/0/0	<0.3	-	-	1.00E+00	1.22E-01	-	-	10000	30	300000	9.23E+05	**1.00E+06**
83 d	10	0	0	32.41	0.00E+00	less than						less than			10000	26	260000	8.02E+05	
	10	0	0		0.00E+00	3.06E+03	0/0/0	<0.3	-	-	1.00E+00	1.22E-01	-	-	10000	23	230000	7.10E+05	**7.56E+05**
120 d	-	-	-	32.41	-							less than			1000	180	180000	5.55E+05	
	-	-	-		-	-	0/0/0	<0.3	-	-	1.00E+00	1.22E-01	-	-	1000	180	180000	5.55E+05	**5.55E+05**
189 d	-	-	-	32.63	-							less than			1000	80	80000	2.45E+05	
	-	-	-		-	-	0/0/0	<0.3	-	-	1.00E+00	1.22E-01	-	-	1000	200	200000	6.13E+05	**4.29E+05**

	SALMONELLA												
Sample	dil. factor	CFU	Final CFU/mL	TS %	CFU/g	Mean CFU/g	MPN comb.	MPN in 100 mL	at Conf. Limit 95% lower	upper	largest vol., mL	MPN/g	MPN/g lower	upper
RAW SLUDGE		DIRECT PLATING NOT DONE					3/1/1	7.5	1.6	35	1.00E-02	2.36E+02	5.04E+01	1.10E+03
AT START							2/1/0	7.5	1.6	35	1.00E-02	2.32E+02	4.94E+01	1.08E+03
15 min							2/1/0	1.5	0.21	7.0	1.00E-02	4.59E+01	6.42E+00	2.14E+02
1 h							0/0/0	<2	-	-	1.00E+00	less than 6.12E-01	-	-

Salmonella sp. were not detected on any further sampling occasion

Sludge TS (%):	31.51	Additional solids due to H2O removal (g):	6.05	Lowest MPN/100mL in a 3-tube setup:	0.4
Lime dose (g/kg TS):	60	Overall TS after lime addition (%):	33.37	Min. density (MPN/g TS):	0.12
Lime dose (g/kg wet sludge):	18.91	(assuming complete reaction of CaO and H2O)		Lowest MPN/100ml in a 5-tube setup:	2.0
Sludge TS after lime addition (%):	32.78	Min density (CFU/g TS):	2996	Min. density (MPN/g TS):	0.60

	FECAL COLIFORM S													CLOSTRIDIUM PERFRINGENS				
Sample	dil. factor	CFU	Final CFU/mL	TS %	CFU/g	Mean CFU/g	MPN comb.	MPN in 100 mL	at Conf. Limit 95% lower	at Conf. Limit 95% upper	largest vol., mL	MPN/g	MPN/g lower	MPN/g upper	dil. factor	CFU	Final CFU/mL	CFU/g	Mean CFU/g
RAW SLUDGE	10000	49	4900000	31.51	1.56E+07										10000	118	1180000	3.74E+06	
	10000	35	3500000		1.11E+07	1.33E+07	2/1/1	9.0	3.0	24.0	1.00E-07	2.86E+07	9.52E+06	7.62E+07	10000	134	1340000	4.25E+06	**4.00E+06**
AT START	10000	49	4900000	32.78	1.49E+07										10000	118	1180000	3.60E+06	
	10000	35	3500000		1.07E+07	1.28E+07	2/1/1	9.0	3.0	24.0	1.00E-07	2.75E+07	9.15E+06	7.32E+07	10000	134	1340000	4.09E+06	**3.84E+06**
15 min	100	48	48000	33.37	1.44E+05										10000	59	590000	1.77E+06	
	100	48	48000		1.44E+05	1.44E+05	5/3/0	80.0	30.0	250.0	1.00E-03	2.40E+04	8.99E+03	7.49E+04	10000	61	610000	1.83E+06	**1.80E+06**
1 h	10	0	0	33.37	0.00E+00	less than									10000	35	350000	1.05E+06	
	10	0	0		0.00E+00	3.00E+03	5/2/0	50.0	20.0	170.0	1.00E+00	1.50E+01	5.99E+00	5.09E+01	10000	40	400000	1.20E+06	**1.12E+06**
2 h	10	0	0	33.37	0.00E+00	less than									10000	30	300000	8.99E+05	
	10	0	0		0.00E+00	3.00E+03	3/1/0	11.0	4.0	29.0	1.00E+00	3.30E+00	1.20E+00	8.69E+00	10000	42	420000	1.26E+06	**1.08E+06**
1 d	10	0	0	33.69	0.00E+00	less than						less than			10000	20	200000	5.94E+05	
	10	0	0		0.00E+00	3.00E+03	0/0/0	<2	-	-	1.00E+00	5.99E-01	-	-	10000	30	300000	8.90E+05	**7.42E+05**
2 d	10	0	0	33.69	0.00E+00	less than						less than			1000	25	25000	7.42E+04	
	10	0	0		0.00E+00	3.00E+03	0/0/0	<0.3	-	-	1.00E+00	1.20E-01	-	-	1000	30	30000	8.90E+04	**8.16E+04**
16 d	10	0	0	34.15	0.00E+00	less than						less than			1000	10	10000	2.93E+04	
	10	0	0		0.00E+00	3.00E+03	0/0/0	<0.3	-	-	1.00E+00	1.20E-01	-	-	1000	11	11000	3.22E+04	**3.07E+04**
24 d	10	0	0	33.38	0.00E+00	less than						less than			100	57	5700	1.71E+04	
	10	0	0		0.00E+00	3.00E+03	0/0/0	<0.3	-	-	1.00E+00	1.20E-01	-	-	100	44	4400	1.32E+04	**1.51E+04**
81 d	10	0	0	33.88	0.00E+00	less than						less than			100	1	100	**2.95E+02**	less than
	10	0	0		0.00E+00	3.00E+03	0/0/0	<0.3	-	-	1.00E+00	1.20E-01	-	-	100	1	100	**2.95E+02**	3.00E+03
118 d	-	-	-	33.88	-							less than			10	5	50	**1.48E+02**	less than
	-	-	-		-	-	0/0/0	<0.3	-	-	1.00E+00	1.20E-01	-	-	10	5	50	**1.48E+02**	3.00E+03
187 d	-	-	-	33.92	-							less than			100	1	100	**2.95E+02**	less than
	-	-	-		-	-	0/0/0	<0.3	-	-	1.00E+00	1.20E-01	-	-	100	1	100	**2.95E+02**	3.00E+03

	SALMONELLA													
Sample	dil. factor	CFU	Final CFU/mL	TS %	CFU/g	Mean CFU/g	MPN comb.	MPN in 100 mL	at Conf. Limit 95% lower	at Conf. Limit 95% upper	largest vol., mL	MPN/g	MPN/g lower	MPN/g upper
RAW SLUDGE		DIRECT PLATING NOT DONE												
AT START							3/2/1	15	3.2	70	1.00E-02	4.58E+02	9.76E+01	2.14E+03
15 min							0/0/0	<2	-	-	1.00E+00	less than 5.99E-01	-	-
1 h							0/0/0	<2	-	-	1.00E+00	less than 5.99E-01	-	-

Salmonella sp. were not detected on any further sampling occasion

Sludge TS (%):			31.51	Additional solids due to H2O removal (g):			12.10	Lowest MPN/100mL in a 3-tube setup:			0.4										
Lime dose (g/kg TS):			120	Overall TS after lime addition (%):			35.17	Min. density (MPN/g TS):			0.11										
Lime dose (g/kg wet sludge):			37.81	(assuming complete reaction of CaO and H2O)				Lowest MPN/100ml in a 5-tube setup:			2.0										
Sludge TS after lime addition (%):			34.01	Min density (CFU/g TS):			2843	Min. density (MPN/g TS):			0.57										
	FECAL COLIFORM S															CLOSTRIDIUM PERFRINGENS					
Sample	dil. factor	CFU	Final CFU/mL	TS %	CFU/g	Mean CFU/g	MPN comb.	MPN in 100 mL	at Conf. Limit 95% lower	at Conf. Limit 95% upper	largest vol., mL	MPN/g	MPN/g lower	MPN/g upper		dil. factor	CFU	Final CFU/mL	CFU/g	Mean CFU/g	
RAW SLUDGE	10000	56	5600000	31.51	1.78E+07											10000	68	680000	2.16E+06		
	10000	45	4500000		1.43E+07	1.60E+07	5/2/0	50.0	20.0	170.0	1.00E-06	1.59E+07	6.35E+06	5.40E+07		10000	69	690000	2.19E+06	2.17E+06	
AT START	10000	56	5600000	34.01	1.65E+07											10000	68	680000	2.00E+06		
	10000	45	4500000		1.32E+07	1.49E+07	5/2/0	50.0	20.0	170.0	1.00E-06	1.47E+07	5.88E+06	5.00E+07		10000	69	690000	2.03E+06	2.01E+06	
15 min	10	0	0	35.17	0.00E+00	less than										1000	60	60000	1.71E+05		
	10	2	200		5.69E+02	2.84E+03	5/2/0	50.0	20.0	170.0	1.00E-01	1.42E+02	5.69E+01	4.83E+02		1000	79	79000	2.25E+05	1.98E+05	
1 h	10	0	0	35.17	0.00E+00	less than										-	-	-	-		
	10	0	0		0.00E+00	2.84E+03	5/0/0	23.0	9.0	86.0	1.00E-01	6.54E+01	2.56E+01	2.45E+02		-	-	-	-	-	
2 h	10	0	0	35.17	0.00E+00	less than										1000	67	67000	1.90E+05		
	10	0	0		0.00E+00	2.84E+03	5/0/0	23.0	9.0	86.0	1.00E+00	6.54E+00	2.56E+00	2.45E+01		1000	60	60000	1.71E+05	1.81E+05	
1 d	-	-	-	35.17	-							less than				1000	40	40000	1.14E+05		
	-	-	-		-	-	0/0/0	<2	-	-	1.00E+00	5.69E-01	-	-		1000	60	60000	1.71E+05	1.42E+05	
2 d	-	-	-	35.17	-							less than				-	-	-	-		
	-	-	-		-	-	0/0/0	<0.3	-	-	1.00E+00	1.14E-01	-	-		-	-	-	-	-	
22 d	10	0	-	35.02	0.00E+00	less than						less than				100	0	-	-	less than	
	10	0	-		0.00E+00	2.84E+03	0/0/0	<0.3	-	-	1.00E+00	1.14E-01	-	-		100	0	-	-	2.84E+03	
79 d	10	0	-	35.70	0.00E+00	less than						less than				100	0	-	-	less than	
	10	0	-		0.00E+00	2.84E+03	0/0/0	<0.3	-	-	1.00E+00	1.14E-01	-	-		100	0	-	-	2.84E+03	
116 d	-	-	-	35.67	-							less than				10	0	-	-	less than	
	-	-	-		-	-	0/0/0	<0.3	-	-	1.00E+00	1.14E-01	-	-		10	0	-	-	2.84E+03	
185 d	-	-	-	35.90	-							less than				10	0	-	-	less than	
	-	-	-		-	-	0/0/0	<0.3	-	-	1.00E+00	1.14E-01	-	-		10	0	-	-	2.84E+03	

	SALMONELLA													
Sample	dil. factor	CFU	Final CFU/mL	TS %	CFU/g	Mean CFU/g	MPN comb.	MPN in 100 mL	at Conf. Limit 95% lower	at Conf. Limit 95% upper	largest vol., mL	MPN/g	MPN/g lower	MPN/g upper
RAW SLUDGE														
		DIRECT PLATING NOT DONE					3/2/0	9.5	2.0	44	1.00E-02	3.01E+02	6.35E+01	1.40E+03
AT START														
							3/2/0	9.5	2.0	44	1.00E-02	2.79E+02	5.88E+01	1.29E+03
15 min												less than		
							0/0/0	<2	-	-	1.00E+00	5.69E-01	-	-
1 h												less than		
							0/0/0	<2	-	-	1.00E+00	5.69E-01	-	-

Salmonella sp. were not detected on any further sampling occasion

Sludge TS (%):	31.51	Additional solids due to H2O removal (g):	15.12	Lowest MPN/100mL in a 3-tube setup:	0.4
Lime dose (g/kg TS):	**150**	Overall TS after lime addition (%):	36.05	Min. density (MPN/g TS):	0.11
Lime dose (g/kg wet sludge):	47.27	(assuming complete reaction of CaO and H2O)		Lowest MPN/100ml in a 5-tube setup:	2.0
Sludge TS after lime addition (%):	*34.60*	*Min density (CFU/g TS):*	2774	Min. density (MPN/g TS):	0.55

	FECAL COLIFORM S													CLOSTRIDIUM PERFRINGENS				
Sample	dil. factor	CFU	Final CFU/mL	TS %	CFU/g	Mean CFU/g	MPN comb.	MPN in 100 mL	at Conf. Limit 95% lower	upper	largest vol., mL	MPN/g	MPN/g lower	upper	dil. factor	CFU	Final CFU/mL	CFU/g	Mean CFU/g
RAW SLUDGE	10000	96	9600000	31.51	3.05E+07										10000	80	800000	2.54E+06	
	10000	67	6700000		2.13E+07	2.59E+07	5/4/1	170.0	70.0	480.0	1.00E-06	5.40E+07	2.22E+07	1.52E+08	10000	74	740000	2.35E+06	**2.44E+06**
AT START	10000	96	9600000	34.60	2.77E+07										10000	80	800000	2.31E+06	
	10000	67	6700000		1.94E+07	2.36E+07	5/4/1	170.0	70.0	480.0	1.00E-06	4.91E+07	2.02E+07	1.39E+08	10000	74	740000	2.14E+06	**2.23E+06**
15 min	10	0	0	36.05	0.00E+00	less than									1000	101	101000	2.80E+05	
	10	0	0		0.00E+00	2.77E+03	5/2/2	90.0	40.0	250.0	1.00E+00	2.50E+01	1.11E+01	6.94E+01	1000	99	99000	2.75E+05	**2.77E+05**
1 h	10	0	0	36.05	0.00E+00	less than									1000	21	21000	5.83E+04	
	10	0	0		0.00E+00	2.77E+03	5/1/0	30.0	10.0	120.0	1.00E+00	8.32E+00	2.77E+00	3.33E+01	1000	32	32000	8.88E+04	**7.35E+04**
2 h	10	0	0	36.05	0.00E+00	less than						less than			1000	30	30000	8.32E+04	
	10	0	0		0.00E+00	2.77E+03	0/0/0	<2	-	-	1.00E+00	5.55E-01	-	-	*1000*	*20*	*20000*	*5.55E+04*	***6.94E+04***
1 d	-	-	-		-							less than			100	143	14300	3.97E+04	
	-	-	-		-	-	0/0/0	<2	-	-	1.00E+00	5.55E-01	-	-	100	120	12000	3.33E+04	**3.65E+04**
2 d	-	-	-		-							less than			-	-	-	-	
	-	-	-		-	-	0/0/0	<0.3	-	-	1.00E+00	1.11E-01	-	-	-	-	-	-	-
19 d	10	0	-	35.90	0.00E+00	less than						less than			100	1	100	2.79E+02	less than
	10	0	-		0.00E+00	2.77E+03	0/0/0	<0.3	-	-	1.00E+00	1.11E-01	-	-	100	1	100	2.79E+02	**2.77E+03**
76 d	10	0	-	35.99	0.00E+00	less than						less than			100	0	-	-	less than
	10	0	-		0.00E+00	2.77E+03	*0/0/0*	*<0.3*	-	-	*1.00E+00*	*1.11E-01*	-	-	100	0	-	-	**2.77E+03**
113 d	-	-	-	36.20	-							less than			10	0	-	-	less than
	-	-	-		-	-	0/0/0	<0.3	-	-	1.00E+00	1.11E-01	-	-	10	0	-	-	**2.77E+03**
182 d	-	-	-	36.54	-							less than			10	0	-	-	less than
	-	-	-		-	-	0/0/0	<0.3	-	-	1.00E+00	1.11E-01	-	-	10	0	-	-	**2.77E+03**

	SALMONELLA													
Sample	dil. factor	CFU	Final CFU/mL	TS %	CFU/g	Mean CFU/g	MPN comb.	MPN in 100 mL	at Conf. Limit 95% lower	upper	largest vol., mL	MPN/g	MPN/g lower	upper
RAW SLUDGE		DIRECT PLATING NOT DONE					3/2/1	15	3.2	70	1.00E-02	4.76E+02	1.02E+02	2.22E+03
AT START							3/2/1	15	3.2	70	1.00E-02	4.34E+02	9.25E+01	2.02E+03
15 min												less than		
							0/0/0	<2	-	-	1.00E+00	5.55E-01	-	-
1 h												less than		
							0/0/0	<2	-	-	1.00E+00	5.55E-01	-	-

Salmonella sp. were not detected on any further sampling occasion

VI

Sludge TS (%):			31.36	Additional solids due to H2O removal (g):			24.08	Lowest MPN/100mL in a 3-tube setup:			0.4											
Lime dose (g/kg TS):			240	Overall TS after lime addition (%):			38.40	Min. density (MPN/g TS):			0.10											
Lime dose (g/kg wet sludge):			75.26	(assuming complete reaction of CaO and H2O)				Lowest MPN/100ml in a 5-tube setup:			2.0											
Sludge TS after lime addition (%):			36.16	Min density (CFU/g TS):			2604	Min. density (MPN/g TS):			0.52											
	FECAL COLIFORM S															**CLOSTRIDIUM PERFRINGENS**						
Sample	dil. factor	CFU	Final CFU/mL	TS %	CFU/g	Mean CFU/g	MPN comb.	MPN in 100 mL	at Conf. Limit 95% lower	upper	largest vol., mL	MPN/g	MPN/g lower	upper		dil. factor	CFU	Final CFU/mL	CFU/g	Mean CFU/g		
RAW SLUDGE	10000	98	9800000	31.36	3.13E+07											10000	69	690000	2.20E+06			
	10000	101	10100000		3.22E+07	3.17E+07	5/2/2	90.0	40.0	250.0	1.00E-06	2.87E+07	1.28E+07	7.97E+07		10000	78	780000	2.49E+06	2.34E+06		
AT START	10000	98	9800000	36.16	2.71E+07											10000	69	690000	1.91E+06			
	10000	101	10100000		2.79E+07	2.75E+07	5/2/2	90.0	40.0	250.0	1.00E-06	2.49E+07	1.11E+07	6.91E+07		10000	78	780000	2.16E+06	2.03E+06		
15 min	10	0	0	38.40	0.00E+00	less than										1000	90	90000	2.34E+05			
	10	1	100		2.60E+02	2.60E+03	5/4/0	130.0	50.0	390.0	1.00E+00	3.39E+01	1.30E+01	1.02E+02		1000	90	90000	2.34E+05	2.34E+05		
1 h	10	0	0	38.40	0.00E+00	less than						less than				1000	12	12000	3.12E+04			
	10	0	0		0.00E+00	2.60E+03	0/0/0	<2	-	-	1.00E+00	5.21E-01	-	-		1000	12	12000	3.12E+04	3.12E+04		
2 h	-	-	-	38.40	-							less than				-	-	-	-			
	-	-	-		-	-	0/0/0	<2	-	-	1.00E+00	5.21E-01	-	-		-	-	-	-	-		
1 d	10	0	0	38.65	0.00E+00	less than						less than				100	78	7800	2.03E+04			
	10	0	0		0.00E+00	2.60E+03	0/0/0	<2	-	-	1.00E+00	5.21E-01	-	-		100	78	7800	2.03E+04	2.03E+04		
2 d	-	-	-	38.75	-							less than				-	-	-	-			
	-	-	-		-	-	0/0/0	<0.3	-	-	1.00E+00	1.04E-01	-	-		-	-	-	-	-		
12 d	10	0	-	38.08	0.00E+00	less than						less than				100	0	-	-	less than		
	10	0	-		0.00E+00	2.60E+03	0/0/0	<0.3	-	-	1.00E+00	1.04E-01	-	-		100	0	-	-	2.60E+03		
69 d	10	0	-	38.52	0.00E+00	less than						less than				100	0	-	-	less than		
	10	0	-		0.00E+00	2.60E+03	0/0/0	<0.3	-	-	1.00E+00	1.04E-01	-	-		100	0	-	-	2.60E+03		
106 d	-	-	-	38.95	-							less than				10	0	-	-	less than		
	-	-	-		-	-	0/0/0	<0.3	-	-	1.00E+00	1.04E-01	-	-		10	0	-	-	2.60E+03		
175 d	10	0	-	39.19	0.00E+00	less than						less than				10	0	-	-	less than		
	10	0	-		0.00E+00	2.60E+03	0/0/0	<0.3	-	-	1.00E+00	1.04E-01	-	-		10	0	-	-	2.60E+03		

	SALMONELLA													
Sample	dil. factor	CFU	Final CFU/mL	TS %	CFU/g	Mean CFU/g	MPN comb.	MPN in 100 mL	at Conf. Limit 95% lower	upper	largest vol., mL	MPN/g	MPN/g lower	upper
RAW SLUDGE														
		DIRECT PLATING NOT DONE					3/2/0	9.5	2.0	44	1.00E-02	3.03E+02	6.38E+01	1.40E+03
AT START														
							3/2/0	9.5	2.0	44	1.00E-02	2.63E+02	5.53E+01	1.22E+03
15 min												less than		
							0/0/0	<2	-	-	1.00E+00	5.21E-01	-	-
1 h												less than		
							0/0/0	<2	-	-	1.00E+00	5.21E-01	-	-

Salmonella sp. were not detected on any further sampling occasion

Sludge TS (%):	31.36	Additional solids due to H2O removal (g):	48.17	Lowest MPN/100mL in a 3-tube setup:	0.4																
Lime dose (g/kg TS):	480	Overall TS after lime addition (%):	44.53	Min. density (MPN/g TS):	0.09																
Lime dose (g/kg wet sludge):	150.53	(assuming complete reaction of CaO and H2O)		Lowest MPN/100ml in a 5-tube setup:	2.0																
Sludge TS after lime addition (%):	40.34	Min density (CFU/g TS):	2246	Min. density (MPN/g TS):	0.45																
	FECAL COLIFORM S														**CLOSTRIDIUM PERFRINGENS**						
Sample	dil. factor	CFU	Final CFU/mL	TS %	CFU/g	Mean CFU/g	MPN comb.	MPN in 100 mL	at Conf. Limit 95% lower	at Conf. Limit 95% upper	largest vol., mL	MPN/g	MPN/g lower	MPN/g upper	dil. factor	CFU	Final CFU/mL	CFU/g	Mean CFU/g		
RAW SLUDGE	10000	52	5200000	31.36	1.66E+07										10000	57	570000	1.82E+06			
	10000	52	5200000		1.66E+07	1.66E+07	5/4/0	130.0	50.0	390.0	1.00E-06	4.15E+07	1.59E+07	1.24E+08	10000	67	670000	2.14E+06	1.98E+06		
AT START	10000	52	5200000	40.34	1.29E+07										10000	57	570000	1.41E+06			
	10000	52	5200000		1.29E+07	1.29E+07	5/4/0	130.0	50.0	390.0	1.00E-06	3.22E+07	1.24E+07	9.67E+07	10000	67	670000	1.66E+06	1.54E+06		
15 min	10	0	0	44.53	0.00E+00	less than									1000	67	67000	1.50E+05			
	10	0	0		0.00E+00	2.25E+03	1/1/0	4.0	1.0	15.0	1.00E-01	8.98E+00	2.25E+00	3.37E+01	1000	74	74000	1.66E+05	1.58E+05		
1 h	10	0	0	44.53	0.00E+00	less than						less than			100	50	5000	1.12E+04			
	10	0	0		0.00E+00	2.25E+03	0/0/0	<2	-	-	1.00E+00	8.98E-02	-	-	100	1	100	2.25E+02	5.73E+03		
2 h	-	-	-	44.53	-							less than			-	-	-	-			
	-	-	-		-	-	0/0/0	<2	-	-	1.00E+00	8.98E-02	-	-	-	-	-	-	-		
1 d	10	0	0	44.39	0.00E+00	less than						less than			100	1	100	2.25E+02	less than		
	10	0	0		0.00E+00	2.25E+03	0/0/0	<2	-	-	1.00E+00	8.98E-02	-	-	100	2	200	4.49E+02	2.25E+03		
2 d	-	-	-	44.39	-							less than			-	-	-	-			
	-	-	-		-	-	0/0/0	<0.3	-	-	1.00E+00	8.98E-02	-	-	-	-	-	-	-		
10 d	10	0	-	45.20	0.00E+00	less than						less than			100	2	200	4.42E+02	less than		
	10	0	-		0.00E+00	2.25E+03	0/0/0	<0.3	-	-	1.00E+00	8.98E-02	-	-	100	0	0	0.00E+00	2.25E+03		
67 d	10	0	-	45.35	0.00E+00	less than						less than			100	0	-	-	less than		
	10	0	-		0.00E+00	2.25E+03	0/0/0	<0.3	-	-	1.00E+00	8.98E-02	-	-	100	0	-	-	2.25E+03		
103 d	-	-	-	45.69	-							less than			10	0	-	-	less than		
	-	-	-		-	-	0/0/0	<0.3	-	-	1.00E+00	8.98E-02	-	-	10	0	-	-	2.25E+03		
173 d	10	0	-	46.29	0.00E+00	less than						less than			10	0	-	-	less than		
	10	0	-		0.00E+00	2.25E+03	0/0/0	<0.3	-	-	1.00E+00	8.98E-02	-	-	10	0	-	-	2.25E+03		

	SALMONELLA													
Sample	dil. factor	CFU	Final CFU/mL	TS %	CFU/g	Mean CFU/g	MPN comb.	MPN in 100 mL	at Conf. Limit 95% lower	at Conf. Limit 95% upper	largest vol., mL	MPN/g	MPN/g lower	MPN/g upper
RAW SLUDGE		DIRECT PLATING NOT DONE					3/1/1	7.5	1.6	35	1.00E-02	2.39E+02	5.10E+01	1.12E+03
AT START							3/1/1	7.5	1.6	35	1.00E-02	1.86E+02	3.97E+01	8.68E+02
15 min							0/0/0	<2	-	-	1.00E+00	less than 4.49E-01	-	-
1 h							0/0/0	<2	-	-	1.00E+00	less than 4.49E-01	-	-

Salmonella sp. were not detected on any further sampling occasion

Sludge TS (%):	31.74	Additional solids due to H2O removal (g):	0.00	Lowest MPN/100mL in a 3-tube setup:	0.4
Lime dose (g/kg TS):	0	Overall TS after lime addition (%):	31.74	Min. density (MPN/g TS):	0.13
Lime dose (g/kg wet sludge):	0.00	(assuming complete reaction of CaO and H2O)		Lowest MPN/100ml in a 5-tube setup:	2.0
Sludge TS after lime addition (%):	31.74	Min density (CFU/g TS):	3151	Min. density (MPN/g TS):	0.63

	FECAL COLIFORM S														CLOSTRIDIUM PERFRINGENS				
Sample	dil. factor	CFU	Final CFU/mL	TS %	CFU/g	Mean CFU/g	MPN comb.	MPN in 100 mL	at Conf. Limit 95% lower	at Conf. Limit 95% upper	largest vol., mL	MPN/g	MPN/g lower	MPN/g upper	dil. factor	CFU	Final CFU/mL	CFU/g	Mean CFU/g
AT START	10000	60	6000000	31.74	1.89E+07										10000	68	680000	2.14E+06	
	10000	74	7400000		2.33E+07	2.11E+07									10000	60	600000	1.89E+06	2.02E+06
2 d	10000	126	12600000	31.51	4.00E+07										-	-	-	-	
	10000	102	10200000		3.24E+07	3.62E+07		MPN TEST NOT DONE							-	-	-	-	-
4 d	10000	104	10400000	31.51	3.30E+07										-	-	-	-	
	10000	83	8300000		2.63E+07	2.97E+07									-	-	-	-	-
6 d	10000	125	12500000	31.51	3.97E+07										-	-	-	-	
	10000	108	10800000		3.43E+07	3.70E+07									-	-	-	-	-
8 d	10000	124	12400000	31.36	3.95E+07										-	-	-	-	
	10000	96	9600000		3.06E+07	3.51E+07									10000	56	560000	1.79E+06	
15 d	10000	128	12800000	31.36	4.08E+07										10000	68	680000	2.17E+06	1.98E+06
	10000	106	10600000		3.38E+07	3.73E+07									10000	79	790000	2.47E+06	
26 d	1000	42	420000	31.95	1.31E+06										10000	76	760000	2.38E+06	2.43E+06
	1000	48	480000		1.50E+06	1.41E+06									-	-	-	-	
64 d	10	52	5200	32.79	1.59E+04										-	-	-	-	-
	10	49	4900		1.49E+04	1.54E+04									10000	62	620000	1.94E+06	
83 d	10	0	-	33.21	0.00E+00	less than						less than			10000	56	560000	1.75E+06	1.85E+06
	10	0	-		0.00E+00	3.06E+03	0/0/0	<0.3	-	-	1.00E+00	6.02E-01	-	-	10000	42	420000	1.31E+06	
120 d	-	-	-	34.01	-							less than			10000	42	420000	1.31E+06	1.31E+06
	-	-	-		-	-	0/0/0	<0.3	-	-	1.00E+00	5.88E-01	-	-	10000	55	550000	1.55E+06	
189 d	-	-	-	35.53	-							less than			10000	35	350000	9.85E+05	1.27E+06
	-	-	-		-	-	0/0/0	<0.3	-	-	1.00E+00	5.63E-01	-	-					

	SALMONELLA													
Sample	dil. factor	CFU	Final CFU/mL	TS %	CFU/g	Mean CFU/g	MPN comb.	MPN in 100 mL	at Conf. Limit 95% lower	at Conf. Limit 95% upper	largest vol., mL	MPN/g	MPN/g lower	MPN/g upper
RAW SLUDGE		DIRECT PLATING NOT DONE					3/1/2	11.5	2.5	54	1.00E-02	3.62E+02	7.88E+01	1.70E+03
AT START							3/1/2	11.5	2.5	54	1.00E-02	3.65E+02	7.93E+01	1.71E+03
2 d							3/0/0	2.5	0.53	12	1.00E+00	7.93E-01	1.68E-01	3.81E+00
15 d							1/0/1	0.7	0.15	3.3	1.00E+00	2.22E-01	4.76E-02	1.05E+00
64 d							0/0/0	<0.3	-	-	1.00E+00	less than 1.26E-01	-	-

Salmonella sp. were not detected on any further sampling occasion

TABLE D-3. FECAL COLIFORM, SALMONELLA , AND CLOSTRIDIUM PERFRINGENS DENSITY IN SLUDGE TREATED WITH LIME AT 20-22°C

	Treatment, lime dose in g CaO/Kg TS							
	Control	20 g/Kg	30 g/Kg	60 g/Kg	120 g/Kg	150 g/Kg	240 g/Kg	480 g/Kg
START	7.61	7.50	7.61	7.12	7.01	7.54	7.32	7.50
15 min			10.67	12.30	12.55	12.52	12.26	12.52
1 h		9.40	10.65	12.23	12.43	12.31	12.39	12.54
2 h		9.44	10.47	12.25	12.52	12.51	12.59	12.55
1 d	7.10	9.10	10.02	12.12	12.42	12.51	12.59	12.55
2 d	7.12		10.10	12.08				
4 d	7.10							
6 d	7.12	8.46						
10 d								12.28
12 d							12.31	
15 d	7.75		9.60					
16 d				11.60				
19 d						12.31		
22 d					12.26			
24 d				11.11				
26 d	7.65		9.70					
44 d		7.70						
49 d								12.62
51 d							12.69	
58 d						12.56		
61 d					12.46			
63 d				11.28				
65 d	7.54		9.48					
67 d		7.63						12.55
69 d							12.57	
76 d						12.57		
79 d					12.44			
81 d				11.21				
83 d	7.62		9.44					
173 d								12.62
175 d							12.75	
182 d						12.58		
185 d					12.56			
187 d				10.86				
189 d	6.75		8.52					

TABLE D-4. SAMPLE CALCULATION OF HEAVY METAL CONCENTRATIONS IN SOIL APPLIED WITH FLY ASH-TREATED SLUDGE

Application rate (dry tonnes/ha):	55
average Total Solids (%):	25
TS with 600 g fly ash/kg (%):	34.75
TS fraction of biosolids in mixture (%):	62.6
TS fraction of fly ash in mixture (%):	37.4
Dry mass of biosolids in mixture (kg):	34430
Dry mass of fly ash in mixture (kg):	20570

Heavy metal	Biosolids mg/Kg	Fly ash mg/Kg	Mixture mg/Kg
Barium	239	0.33	149.74
Copper	1482	0.005	927.73
Nickel	53	0.002	33.18
Vanadium	14	0.0058	8.77
Boron	11	0.0001	6.89

in 55000 kg dry weight of biosolid&fly ash mixtu	Biosolids	Fly ash	Mixture
Barium	239	0.33	52.03
Copper	1482	0.005	322.34
Nickel	53	0.002	11.53
Vanadium	14	0.0058	3.05
Boron	11	0.0001	2.39

Area of application (m^2):	10000
Depth of application (m):	0.15
Volume of soil in one ha (m^3):	1500
Bulk density of soil (Kg/m^3):	1200
Mass of dry soil (Kg):	1800000

Mass of biosolids&fly ash (kg): 158046 kg

Final concentration of pollutant in sludge-soil mixture (mg/Kg):

Heavy metal	Mixture, mg/Kg
Barium	4.5
Copper	27.6
Nickel	1.1
Vanadium	0.36
Boron	0.30

TABLE D-5. ISOLATION OF SALMONELLA SP. FROM WASTEWATER AND SLUDGE FOR SPIKING STUDIES

	Salmonella Isolate:							
Time, h	S-PS8	S-PS9	S-PS10	E-RW12	E-RW13	E-PS19	E-PS20	E-DS25
0	0.035	0.055	0.065	0.055	0.065	0.055	0.055	0.050
0.5	0.080	0.115	0.135	0.115	0.135	0.120	0.115	0.110
1	0.135	0.185	0.210	0.190	0.220	0.200	0.185	0.180
1.5	0.245	0.330	0.345	0.320	0.380	0.350	0.335	0.315
2	0.390	0.500	0.520	0.480	0.560	0.540	0.520	0.490
2.5	0.600	0.710	0.710	0.690	0.780	0.750	0.710	0.710
3	0.800	0.910	0.910	0.900	0.950	0.940	0.880	0.950

PS: primary sludge

RW: raw wastewater

S: no non-selective pre-enrichment

E: non-selective pre-enrichment

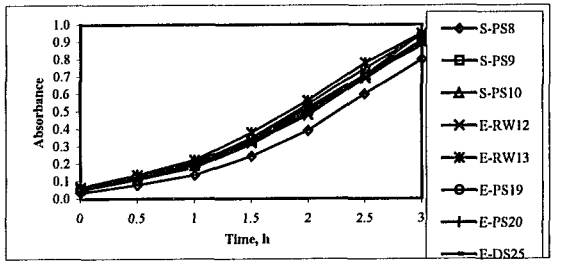

Plate counts for isolate E-DS25:

	Dilution				CFU/mL			
Time, h	-4	-5	-6	-7	-6	-7		
0	TNTK	315	25	0	2.5E+08		0.0	2.5E+08
0.5	TNTK	380	32	0	3.2E+08		0.5	3.2E+08
1	TNTK	570	65	2	6.5E+08		1.0	6.5E+08
1.5	TNTK	TNTK	98	8	9.8E+08		1.5	9.8E+08
2	TNTK	TNTK	248	31		3.1E+09	2.0	3.1E+09
2.5	TNTK	TNTK	495	43		4.3E+09	2.5	4.3E+09
3	TNTK	TNTK	750	76		7.6E+09	3.0	7.6E+09

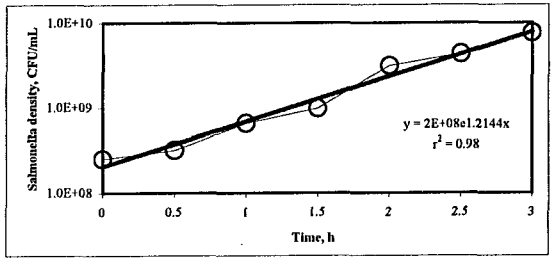

Plate counts for isolate E-RW13 at dilution 10^{-7}:

Time, h	1	2	3	CFU/mL
3	159	156	146	4.6E+10

TABLE D-6. pH DATA IN LIME AND/OR FLY ASH APPLIED SLUDGE STORED AT 20-22°C AND 4-6°C

treatment	d_0		d_1				d_8				d_31			
	4-6 and 20-22degrC		20-22degrC		4-6degrC		20-22degrC		4-6degrC		20-22degrC		4-6degrC	
	pH	avg. pH	pH	avg. pH	pH	avg. pH	pH	avg. pH	pH	avg. pH	pH	avg. pH	pH	avg. pH
L0	7.46		7.36		6.92		6.78		7.01		7.23		7.13	
	7.64		7.34		6.90		6.78		7.06		7.19		7.16	
	7.51	7.54	7.29	7.33	6.96	6.93	6.75	6.77	7.01	7.03	7.18	7.20	7.20	7.16
L20	9.61		9.45		9.43		8.36		9.18		7.50		8.68	
	9.66		9.45		9.39		8.34		9.20		7.56		8.66	
	9.66	9.64	9.45	9.45	9.44	9.42	8.40	8.37	9.25	9.21	7.55	7.54	8.65	8.67
L40	10.45		10.34		10.24		9.49		9.72		9.04		9.38	
	10.45		10.34		10.31		9.52		9.81		9.06		9.47	
	10.54	10.48	10.33	10.34	10.32	10.29	9.50	9.50	9.83	9.79	9.02	9.04	9.41	9.42
FA300	8.73		9.01		9.14		8.83		8.87		8.25		8.89	
	8.74		9.00		9.12		8.85		8.94		8.25		8.80	
	8.72	8.73	9.04	9.02	9.11	9.12	8.85	8.84	8.88	8.89	8.24	8.25	8.83	8.84
FA600	8.50		9.14		9.38		9.33		9.09		9.34		8.95	
	8.50		9.15		9.40		9.29		9.15		9.40		9.08	
	8.50	8.50	9.15	9.15	9.41	9.39	9.30	9.31	9.14	9.12	9.38	9.37	9.04	9.02
FA900	8.60		9.55		9.81		9.62		9.45		9.80		9.64	
	8.68		9.58		9.83		9.67		9.41		9.82		9.45	
	8.63	8.64	9.57	9.57	9.83	9.83	9.68	9.66	9.47	9.44	9.85	9.82	9.49	9.53
L20+FA300	9.70		9.97		9.91		9.64		9.54		9.46		9.77	
	9.78		9.98		9.94		9.63		9.58		9.46		9.58	
	9.82	9.77	10.00	9.98	9.95	9.93	9.60	9.62	9.58	9.57	9.46	9.46	9.64	9.66
L20+FA600	10.01		10.36		10.18		9.94		9.93		9.85		10.05	
	10.08		10.33		10.19		9.95		10.02		9.90		9.94	
	10.08	10.06	10.35	10.35	10.18	10.18	9.95	9.95	9.95	9.96	9.90	9.88	9.83	9.94
L20+FA900	10.25		10.60		10.26		10.28		10.18		10.11		9.96	
	10.27		10.58		10.22		10.24		10.25		10.08		10.07	
	10.28	10.27	10.64	10.61	10.29	10.25	10.24	10.25	10.24	10.22	10.09	10.09	10.04	10.03

L: lime Doses in g/Kg TS (dry weight)

FA: Fly Ash

TABLE D-6. CONT'D...

L40+FA300	10.72		10.57		10.38		10.10		10.09		9.65		9.90	
	10.73		10.53		10.45		10.15		10.08		9.68		10.01	
	10.70	10.72	10.60	10.57	10.44	10.43	10.10	10.12	10.12	10.10	9.64	9.66	9.95	9.95
L40+FA600	11.00		10.72		10.51		10.40		10.28		10.15		10.25	
	11.02		10.75		10.47		10.45		10.34		10.18		10.22	
	11.03	11.02	10.72	10.73	10.49	10.49	10.42	10.42	10.35	10.33	10.15	10.16	10.31	10.26
L40+FA900	11.03		10.92		10.69		10.65		10.49		10.52		10.54	
	10.96		10.90		10.73		10.69		10.47		10.55		10.53	
	10.99	10.99	10.91	10.91	10.71	10.71	10.70	10.68	10.49	10.48	10.53	10.53	10.46	10.51

L: lime
FA: Fly Ash
Doses in g/Kg TS (dry weight)

APPENDIX E

STANDARD OPERATING PROCEDURE (SOP)
FOR ENUMERATION OF FECAL COLIFORM BACTERIA
IN WASTEWATER AND WASTEWATER SOLIDS

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Introduction

The following SOP describes two analytical techniques used to enumerate fecal coliforms and one qualitative technique to confirm the presence or absence of the organisms.

Enumeration techniques:

1. Direct-plate count- the procedure is based on the Fecal Coliform Membrane Filter Procedure described in Part 9000, Section 9222, of the Standard Methods (SM) (APHA *et al.*, 1998).
2. Multiple-tube fermentation- the procedure is based on the Standard Total Coliform Fermentation Technique in Part 9000, Section 9221 B (SM) and the Fecal Coliform Procedure in Part 9000, Section 9221 E (SM).

The coliform group consists of several genera of bacteria belonging to the family *Enterobacteriaceae*. The definition of this group is based on the method used for detection rather than on systematic bacteriology.

Total coliforms:
When the fermentation technique is used, this group is defined as all facultative anaerobic, gram-negative, non-spore-forming, rod-shaped bacteria that ferment lactose with gas and acid production within 48 h at $35^{o}C$.
The total coliform group includes genera *Escherichia*, *Citrobacter*, *Enterobacter*, and *Klebsiella*.
The fraction of up to 4% of this group is estimated to be the pathogenic *E. coli* species [US Congress, 1987].

Fecal coliforms:
When the direct-plate count technique is used, bacteria that produce colonies with various shades of blue within 24 h at $44.5 \pm 0.2^{o}C$ on m-FC medium (containing lactose) are considered fecal coliforms.
When the fermentation technique is used, the fecal coliform group is defined as those facultative anaerobic, gram-negative, non-spore-forming, rod-shaped bacteria that ferment lactose with gas production within 24 h at $44.5 \pm 0.2^{o}C$.
The fecal coliform group normally contains *Escherichia coli*, and thermo tolerant *Klebsiella* (*K. pneumonae*), *Enterobacter*, and *Citrobacter*. *E. coli* is the most common representative of the fecal coliform group and is a member of the indigenous fecal flora of warm-blooded animals. The occurrence of *E. coli* is considered a specific indicator of fecal contamination and the possible presence of enteric pathogens. Nonfecal coliform colonies are gray to cream-colored. Normally, few nonfecal coliform colonies will be observed on m-FC agar (containing lactose) because of selective action of the elevated temperature and addition of rosolic acid salt reagent (a bile salt that inhibits the growth of nonintestinal organisms). Non-coliform colonies that appear on the surface of the agar are excluded by inability to ferment lactose, resulting in a color other than various shades of blue.

Materials and culture media

Fecal coliform cultures (to serve as a positive control culture):
Fecal coliform cultures were isolated from sewage or sewage sludge by means of plating on m-FC agar and serve as: 1) positive control cultures.

(Size of culture media containers [test tubes, flasks, and Petri dishes] are specified in preparation of each medium. All media containers must have covers, caps, or plugs that prevent contamination but maintain aerobic conditions unless otherwise directed.)

Buffered Dilution Water (BDW):

First, prepare stock phosphate buffer solution. Dissolve 34.0 g potassium dihydrogen phosphate (KH_2PO_4), in 950 ml sterile, deionized water, adjust to pH 7.2 ± 0.1 with sodium hydroxide (NaOH). First, add about 8-10 ml 6*N* NaOH to reach the pH of around 7.0. Use 1*N* NaOH to reach the final pH of 7.2. Dilute to 1 L and mix thoroughly.

To prepare BDW for dilutions, dilute 1.25 ml stock phosphate buffer solution and 5.0 ml magnesium chloride solution (81.1 g $MgCl_2 \times 6H_2O$/L deionized water) in deionized water in 1L volumetric flask. Mix thoroughly and transfer to five 300 ml beakers. Cover loosely with aluminum foil and autoclave for 15 min at 121°C. Dispense in 9-ml quantities into sterilized 16×150-mm test tubes. The tubes are ready to prepare serial dilutions.

m-FC Agar (direct-plate count procedure):

Rehydrate 52 g of m-FC agar (Difco) in 1L dilution water and boil to dissolve completely. Add 10 ml of 1% solution of rosolic acid (Sigma) in 0.2*N* NaOH (see below) and continue heating for another minute or to near boiling. Promptly remove from heat, and cool to below 50°C. Do not autoclave the agar.

Rosolic acid solution- 1g of rosolic acid is dissolved in 100 ml of 0.2*N* NaOH.

1) weigh 1 g of rosolic acid and add to a 250-ml volumetric flask,

2) add 100 ml of 0.2*N* NaOH from a 100 ml volumetric flask and mix to dissolve.

Do not autoclave as rosolic acid reagent will decompose if sterilized by autoclaving.

Refrigerate stock solution in the dark and discard after 2 weeks or sooner if its color changes from dark red to muddy brown.

Note: This is the procedure recommended by Difco (the manufacturer of m-FC agar). Standard Methods (1998) recommends adding the rosolic acid solution to the dilution water prior to suspending the dehydrated agar in it. Prolonged heating could alter the properties of the rosolic acid solution and, therefore, the manufacturer's procedure is preferred over the SM procedure.

Final pH of the agar should be 7.4 ± 0.2. Dispense 15-mL quantities to 50 x 12-mm Petri plates and let solidify. Dry the plates in an inverted position for 2 to 3 hours. Refrigerate finished medium in sealed plastic bags to reduce moisture loss, and discard unused agar after 2 weeks. Compare quantitative recoveries of new lots with previously acceptable ones. Include sterility and positive and negative control culture checks on all media.

Sterility checks: check sterility of the medium, dilution water, glassware, and pipets as a minimum at the end of each series of samples using sterile reagent water as the sample. Incubate a representative portion of each batch at an appropriate temperature for 24 h and observe for growth. If any contamination is indicated, determine the cause and reject analytical data from samples tested with these materials. Re-sample and re-analyze.

Control cultures: for each lot of medium check analytical procedures by testing with known positive and negative control cultures of the organism under test. For Fecal coliforms use *E. coli* as a positive and *E. aerogenes* or *Streptococcus faecalis* (*Enterococcus faecalis* as an alternative) as a negative.

Duplicate analyses: perform duplicate analyses on at least 10% of samples and on at least one sample per test run.

Culture dishes (Petri plates): Use loose-lidded 50- x 12-mm plastic petri plates. Incubate the plates in inverted position in the 44.5 ± 0.2°C air incubator with water-filled container to prevent moisture evaporation with resultant drying of medium and to maintain a humid environment for optimum colony

development. Alternatively, place petri plates in plastic bags (4/bag) and add water (~3-5 ml) to cover bottom of the bag. Do not overfill as water may get into the plates.

Incubators: Use static air incubators to provide temperature of 44.5 ± 0.2°C and to maintain a humid environment (60% relative humidity).

EC Broth (confirmed phase of the direct-plate count procedure and multiple-tube fermentation technique):
Add 37 g dehydrated medium to 1 L deionized water, mix thoroughly, and heat slightly (not to exceed 40°C) to dissolve. Dispense in fermentation tubes, each with an inverted Durham vial, sufficient medium to cover the inverted vial with at least one-half to two-thirds after sterilization. Usually, 10 ml of the broth per tube is enough. Close tubes loosely (!) with plastic caps and autoclave at 121°C not exceeding 15 min. Final pH (after sterilization) should be 6.9 ± 0.2.

Lauryl Sulphate Broth (presumptive phase of the multiple-tube fermentation technique):
Suspend 35.6 g of the powder in 1 L deionized water, mix thoroughly, and heat slightly (not to exceed 40°C) to dissolve. Add bromcresol purple solution (pH indicator to determine acid production; 3.4 ml of 0.25 g/L bromcresol purple solution per 1 L of the broth). Dispense into test tubes containing inverted Durham vials, in 10 ml amounts for testing 1 ml or less of samples. For testing 10 ml quantities of samples, dissolve 71.2 g of the powder in 1 L deionized water and distribute in 10 ml amounts. The concentration of the medium should be varied according to the size of the test samples. Autoclave at 121°C not exceeding 15 min. After autoclaving cool the broth as quickly as possible. Final pH should be 6.8 ± 0.2.
Note: Alternatively to Lauryl Sulphate Broth, use Lauryl Tryptose Broth. It is a very similar medium that contains Tryptose instead Pancreatic Digest of Casein.

Sample preparation

Selection of sample size: Use such dilution of wastewater or sludge sample to be examined that will yield counts between 20 and 60 fecal coliform colonies per plate (and not more than 200 colonies of all types per plate) (APHA *et al.*, 1998). The following information is based on the U.S. EPA's "Environmental regulations and technology. Control of pathogens and vector attraction reduction in sewage sludge." *EPA-625/R-92/013*, Office of Research and Development- US EPA, Washington, DC, Appendix F (1992).

Liquid samples (total solid content of less than 7%)-

1. Use a sterile pipet to transfer 1.0 mL of well-mixed sample to 9 mL of sterile buffered dilution water in a sterile, capped test tube, and mix vigorously using vortex mixer. This is dilution "A." Mark the dilution on side of the tube. A volume of 1.0 mL of this mixture is 0.10 mL (or 10^{-1} mL) of the original sample.

2. Use a sterile pipet to transfer 1.0 mL of dilution "A" to a second test tube containing 9 mL of sterile buffered dilution water, and mix as before. This is dilution "B." Mark the dilution on side of the tube. A volume of 1.0 mL of this mixture is 0.010 mL (or 10^{-2} mL) of the original sample. Use a sterile pipet to transfer 1.0 mL of dilution "B" to a third test tube containing 9 mL of sterile buffered dilution water, and mix as before. This is dilution "C." Mark the dilution on side of the tube. A volume of 1.0 mL of this mixture is 0.0010 mL (or 10^{-3} mL) of the original sample.

When the bacterial density of the sample is unknown, prepare several dilutions to achieve a countable density. Estimate the dilution expected to yield a countable plate and select two additional quantities representing one tenth and ten times this volume, respectively.

Semi-solid and solid samples (total solid content of 7% or higher)-

1. In a sterile dish weigh out 50.0 g of well-mixed sample. Transfer the sample to a sterile blender. Use 450 mL of sterile buffered dilution water to rinse any remaining sample into the blender. Cover and blend on high speed for 2 min.

2. Use a sterile pipette to transfer about 10 mL of the blender contents to a sterile, capped test tube, and mix once again using vortex mixer. This is dilution "A." A volume of 1.0 mL of this mixture is 0.10 mL (or 10^{-1} mL) of the original sample.

3. Follow the procedures as for fluid samples starting at step 2.

Direct count plate procedure

1. Presumptive Phase.

Inoculation of Petri plates: Aseptically transfer 0.1-0.2 mL of each dilution onto surface of the m-FC agar in corresponding plates, use spreader stick to distribute seeded material on the surface of the agar and mark the dilutions on plate covers. Let the sample be absorbed in the agar prior to incubation but do not let the plates be exposed for more than 30 min after inoculation.

Incubation: Place prepared plates in plastic bags (up to 4 plates/ bag) and pour some water to the bottom of the bag. The water helps to keep a relative humidity of about 60%. Place sealed bags with plates in a static air incubator and incubate for 24 h at 44.5 ± 0.2°C.

Counting: Colonies produced by fecal coliforms are various shades of blue. Count colonies with a Quebec colony counter. The counter illuminates the petri plates uniformly from the slide, and the plate is magnified for easier counting of small colonies. Use marker pen to mark each colony and record the count.

2. Confirmed Phase.

Verification: Verify typical blue colonies and any atypical gray to green colonies as described below (SM, Section 9020B.9.b.2).

a) Positives:

Verify positives monthly by picking at least 10 blue colonies from one positive sample. Verify in EC broth as in Method 9221E (SM, Fecal Coliform Procedure). Adjust counts based on percent verification.

Procedure: Using a sterile 3- or 3.5- mm-diam loop or sterile wooden applicator stick, select transfer growth (at least 10 blue colonies- presumptive fecal coliforms) from a presumptive plate with the positive sample to 10 fermentation tubes containing EC broth. Each fermentation tube is seeded with one colony from the plate. Tap and incline the fermentation tube, insert end of the loop into the liquid in the tube. Gently shake or rotate the tube to re-suspend the colony. Incubate inoculated EC broth tubes at 44.5 ± 0.2°C for 24 ± 2h.

Interpretation: Gas production with growth in an EC broth culture within 24 ± 2 h or less is considered a positive fecal coliform reaction. Failure to produce gas (with little or no growth) constitutes a negative reaction

b) Negatives:

To determine false negatives, pick representative atypical colonies (gray to green) of different morphological types and verify as above. Failure to produce gas (with little or no growth) constitutes negative reaction.

3. Calculation of fecal coliform density.

Compute the density from the sample quantities that produced bacterial counts within the desired range of *20 to 60 fecal coliform colonies and not more than 200 colonies of all types per plate. This colony density* range is more restrictive than the 20 to 80 total coliform range because of larger colony size on M-FC medium. Adjust density based on the results of the confirmed phase. When using only one tube for sub-culturing from a single presumptive plate, report as presence or absence of fecal coliforms.

Multiple tube MPN procedure

1. Presumptive phase.

Inoculation: Arrange fermentation tubes containing Lauryl Sulphate Broth (or Lauryl Tryptose Broth) in rows of five or three tubes each in a test tube rack. The number of rows and the sample volumes selected depend upon the quality of the sample to be examined. Refer to the "Sample preparation" part of this document. Inoculate each tube in a set of five or three with replicate sample volumes (in increasing decimal dilutions). Mix the contents of each tube on a Vortex mixer.

Incubation and interpretation: Incubate inoculated tubes at 35 ± 0.5°C. After 24 ± 2 h swirl each tube gently and examine it for growth, gas, and acidic reaction (shades of yellow color) and if no gas or acidic reaction is evident, re-incubate and re-examine at the end of 48 ± 3 h h. Growth with acidity and gas signifies a positive presumptive reaction. Submit tubes with a positive presumptive reaction to the confirmed phase.

2. Confirmed phase.

Procedure: Using a sterile loop or sterile wooden stick, transfer growth from each presumptive fermentation tube to EC broth. Incubate inoculated EC broth tubes in a water bath at 44.5 ± 0.2°C for 24 ± 2 h. Place all EC tubes in water bath within 30 min after inoculation. Maintain a sufficient water depth in water bath incubator to immerse all tubes to upper level of the medium.

Interpretation: Gas production with growth in an EC broth culture within 24 ± 2 h or less is considered a positive fecal coliform reaction. Failure to produce gas (with little or no growth) constitutes a negative reaction. Calculate MPN (Most Probable Number) from the number of positive EC broth tubes as described in Section 9221C (SM). Calculate fecal coliform density using the appropriate MPN tables (for 3-tube or for 5-tube test).

The presence-absence (P-A) coliform procedure

The occurrence (qualitative) of fecal coliform bacteria is determined by the presence-absence test. The P-A test is a simple modification of the multiple-tube procedure.

1. Presumptive phase.

The presumptive phase of the test is performed according to SM Method 9221 D (Presence-Absence Coliform Test).

Culture medium- Lauryl Tryptose Broth: Preparation of Lauryl Tryptose broth is done according to Table 9221:I (SM). *For the inoculum size of 100 ml the amount of medium in bottle can be either 50 ml* or 20 ml. For the 20-ml volume the amount of dehydrated Lauryl Tryptose broth required is 213.6 g/L (6 ×), and for the 50-ml volume the amount of broth powder is 106.8 g/L (3 ×). The size of milk dilution bottle, or P-A culture bottle, is 150 mL and 250 mL, respectively. Make Lauryl Tryptose broth the required strength. Add 3.4 ml of 0.25 g/l bromcresol purple solution (pH indicator to determine acid production) to one liter of the broth. A Durham tube insert (fermentation tube) is not necessary (bromcresol purple is an alternative) but can be included to confirm the reading following incubation. Suspend the powder in deionized water

and mix on a magnetic stirrer plate with heating to dissolve. Dispense prepared medium into a screw-cap milk dilution bottle. Autoclave for 15 minutes at 121°C. pH should be 6.8 ± 0.2 after sterilization.

Procedure: Shake sample vigorously and inoculate 100 ml of sample into a P-A culture bottle. Mix thoroughly by inverting bottle few times to achieve even distribution of the medium throughout the sample. Incubate at 37 ± 0.5°C and inspect after 24 h for acid reactions.

Interpretation: A distinct yellow color forms in the medium when acid conditions exist following lactose fermentation. If gas is also being produced, gently shaking the bottle will result in a foaming reaction. Any amount of gas and/or acid constitutes a positive presumptive test requiring confirmation.

2. Confirmed Phase.

The confirmed phase of the test is performed according to SM Method 9221 E (Fecal Coliform Procedure). Culture media and procedure are like above (see "Confirmed phase" for the direct-count plate technique or for the multiple-tube fermentation technique).

References

U.S. Congress [1987]. *Wastes in the marine environment.* 07A-0334, U.S. GPO, Office of Technology Assessment, April, Washington, DC, U.S.A.

NOTE: The information in this document has been adopted in a revised form from Standard Methods- the publication of American Public Health Association; American Water Works Association; and Water Environment Federation [1998]. *Standard Methods for the Examination of Water and Wastewater* (Clesceri L. S., Greenberg A. E., Eaton A. D. - Editors), 20th Edition, Washington, DC, U.S.A.

STANDARD OPERATING PROCEDURE (SOP) FOR ENUMERATION OF *Salmonella* BACTERIA IN WASTEWATER AND WASTEWATER SOLIDS

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Introduction

Most methods for detection and identification of *Salmonella* are adapted from the field of food or clinical microbiology. The AOAC Official Methods of Analysis (1995) presents a concise approach to the methods for food products. However, there is no specific protocol or standard method available for reliable detection of *Salmonella* in wastewater or biosolids. There are limitations and variations in both the sensitivity and selectivity of accepted *Salmonella* procedures for the detection of the more than 2300 *Salmonella* serotypes currently recognized (APHA *et al.*, 1998). There are reports on superior properties of one method over another (Yanko et al., 1995) but none is regarded as a standard method. The extensive literature search led to selection of a method for the use in our laboratory. The method is based on the work of Kenner and Clark (1974) recommended by US EPA (EPA/625/R-92/103, 1992), and has been modified following findings of others (Thomason et al., 1977; Cox, 1993).

Differentiation of the genus *Salmonella* into five subgroups is based on biochemical characteristics, e.g. carbohydrate fermentation, and origins of isolation, e.g. from animal sources or from human clinical specimens (Farmer *et al.*, 1985; Farmer *et al.*, 1984).

Differences in differential characteristics of available enrichment and isolation media for the detection of *Salmonella* were subject to extended testing and research in recent years. Generally, methods for the detection and enumeration of *Salmonella* include enrichment, isolation, and identification. Since *Salmonella* species found in wastewater and biosolids (being hostile environments) are subject to environmental stress, they have to be recovered prior to detection. A pre-enrichment process involves incubation of the sample in a nonrestrictive medium to allow the growth of the pathogen and other bacteria. This is followed by secondary enrichment in a selective medium restricted mainly to *Salmonella* species. After that the material from the enrichment medium is streaked or plated onto a solid medium for bacterial isolation, following selection of typical colonies for further identification by means of biochemical and serological tests.

Principle of the method and background information

The pre-enrichment process designed to non-selectively enrich stressed *Salmonella* species is required in cases where expected levels of *Salmonella* are low. Such cases include treated wastewater, activated sludge and digested sludge (MPN of zero to 400 /g dry solids; Yanko et al., 1995), or composted sludge (MPN of 21 /g dry solids; Hussong et al., 1985). The buffered peptone water (BPW) has proven to be reliable (Thomason et al., 1977) and is used in this method as the pre-enrichment step. In some cases the level of *Salmonella* organisms can be as high as 10^5/ g TS (Russ and Yanko, 1981). Use of a nonselective resuscitation step may be of value in combination with an isolation system that can better suppress *Proteus* populations.

Selective enrichment chosen for this method is selenium based. The conditions presented in Dulcitol Selenite (DS) medium do not present optimum growth conditions for *Salmonella* but suppress the growth of coliform bacteria. Dulcitol, a carbohydrate, can be utilized by almost all *Salmonella* strains from subgroups 1, 2, and 5. Arizona paracolons (subgenus III salmonellas) cannot. Selenite broth is good for fresh samples but if the samples are stored the tetrathionate broth is significantly better (Harvey and Price, 1979).

Selective growth on XLD agar is obtained with sodium desoxycholate (bile salt) as inhibitor. The addition of this bile salt selects intestinal species since these alone are accustomed to this substance. It provides

optimum suppression of coliforms while permitting good recovery of the pathogenic group. The medium contains three sugars, lactose, saccharose, and xylose, with the last at a lesser concentration than the first two. The pH indicator used is phenol red, which is yellow in acid. Coliform colonies will be yellow because of fermentation of all of the carbohydrates present. Nonpathogenic non-lactose fermenters, such as *Proteus* species, will be detected by the fermentation of the other carbohydrates present, while Salmonella species, which can ferment xylose, will neutralize the acid produced through the decarboxylation of lysine, resulting in a colony of red color. The presence of excess lactose and saccharose prevents lysine decarboxylase-active coliforms from neutralizing the acid produced. The production of hydrogen sulfide is indicated by reaction with ferric salts in the medium to produce red-black or yellow-black colonies, the former being common to *Salmonella* species (Cooper and Danielson, 1997).

Cox (1993) developed a modified form of XLD agar, the Lysine-Mannitol-Glycerol agar. The XLD agar is the base for LMG agar and, therefore, is referred to in this procedure as Modified Xylose Lysine Deoxycholate agar. Glycerol addition (as a carbon source) to the medium allows for the differentiation of *Salmonella* and *Citrobacter* spp. Inclusion of proteose peptone and increased content of yeast extract in the agar serves to supplement *S. typhi* and allows for its detection as it can only produce H_2S from organic sulfur compounds. Fermentation of mannitol in the first stage of incubation is to produce initial acidification of the medium followed by alkaline reversion due to decarboxylation of lysine and because of utilization of the proteinaceous substrates (proteose peptone and yeast extract) in the medium.

Further identification and confirmation of Salmonella species relies on streaking of TSI agar (and/or LI agar, the use of LI agar is optional) with the colonies from XLD agar. Following this, the colonies from TSI agar are transferred to urea broth and to lysine decarboxylase (LD) broth for subsequent identification. This part falls into biochemical confirmation stage of the procedure.

- TSI agar contains three carbohydrates (glucose, lactose and sucrose), phenol red for detecting carbohydrate fermentation and ferrous sulfate for detection of hydrogen sulfide production (indicated by blackening in the butt of the tube). Glucose is the only carbohydrate used by organisms of all *Salmonella* serotypes. To facilitate the detection of organisms that only ferment glucose, the glucose (dextrose) concentration in TSI agar is one-tenth the concentration of lactose and sucrose- the other two carbohydrates. The small amount of acid produced in the slant of the tube during dextrose fermentation oxidizes rapidly, causing the medium to remain red or revert to an alkaline pH. In contrast, the acid reaction (yellow) is maintained in the butt of the tube because it is under lower oxygen tension.
- Urease test is conducted to exclude urease positive *Proteus* spp. organisms. Urease is an enzyme which hydrolyzes urea (which is the principal form in which excess nitrogen is excreted by mammals): $NH_2.CO.NH_2 + H_2O \xrightarrow{urease} CO_2 + 2NH_3$. Since urea is a neutral substance the ammonia released by its hydrolysis causes media to become alkaline; urease activity is therefore detected by a change in color of the pH indicator (phenol red) included in urea broth.
- Lysine decarboxylase test allows exclusion of *Citrobacter* sp. that are lacking lysine decarboxylase activity (*Citrobacter* sp. can also be differentiated from *Salmonella* by their typical fecal odor and growth in potassium cyanide (KCN) broth; Balows et al., 1992).

Cultural isolation and at least preliminary biochemical differentiation must precede any serological examination, and final identification cannot be made without biochemical and serological characterization. This is particularly true for of members of Enterobacteriaceae. Members of this family are antigenetically related and, cross-reactions can occur, therefore identification of genera within the family cannot be based on serological data alone. Final serological identification must be preceded by biochemical identification. Members of the genus Salmonella are genetically related to Citrobacter and Arizona. Antigens of these organisms are identical with, or similar to, each other. Further, species within the Salmonella may have similar or identical antigenic formulae.

Successful *Salmonella* isolation involves a combination of subjective and objective qualities. The former is concerned with recognizing the *Salmonella* colony. Surface appearance, translucency, ease of removal from a selective agar, and ease of emulsification in saline determine selection of a suspicious colony for slide

agglutination (serological testing). Polyvalent O antiserum cannot be used toward confirmation of the presence of *S. typhi* since the organism has the Vi type capsular antigen that blocks the activity of O antigens against the polyvalent O antiserum. Use polyvalent O antisera that contain Vi antiserum. Positive slide agglutination with Vi antiserum indicates that the suspect culture is *S. typhi* or *S. paratyphi C.*

As mentioned above, the similarity of some non-pathogens to pathogens requires that serological examination is necessary to identify a suspect culture positively as a pathogen. Serology is the study of the interactions of *antigens* and *antibodies*. An antigen is any substance (but usually a protein) which, when injected into an animal, causes the formation of an antibody that is capable of reacting specifically and exclusively with the antigen causing its (i.e. antibody) formation. Antibodies are proteins that occur in the fluid fraction of blood (serum). Sera that contain an antibody specific for a single bacterial group (bacterial proteins are antigenic) are termed the antisera for that group; the bacterial group is comprised of strains and species, which are the *n said to form a serotype*. Bacterial antigens are commonly of two types, *somatic* (occurring in the body of the cell) and *flagellar* (occurring in flagella; therefore only motile bacteria have flagellar antigens). These types are called O and H antigens, respectively. If the culture is a member of the suspected species or genus, the polyvalent antiserum causes the cells to clump together; the agglutinated cells settle to leave a clear supernatant liquid.

Agglutination: this type of reaction uses specific antibodies, agglutinins that are formed in response to the introduction of particulate antigens into host tissues. When these particulate antigens combine with homologous antiserum, a three-dimensional mosaic complex occurs. This is called an agglutination reaction and can be visualized microscopically and in some cases macroscopically.

Agglutination is a clumping of microbial cells due to the antibody-antigen reaction, which occurs after antiserum has been added to a cell suspension on a slide. Place a drop of cell suspension on a slide, add a small drop of antiserum, and mix with a clean toothpick. Agglutination is often easily observed against a black background, but visualization may be improved by tilting the slide back and forth. A negative control should be performed using serum that has no antibodies against the cells being tested. This negative control allows distinction between a true agglutination reaction and a small amount of cell clumping that may occur as the drop loses moisture.

Materials and culture media

Stock *Salmonella* cultures: *Salmonella* cultures were isolated from sewage or sewage sludge and serve as: 1) positive control cultures or 2) material for spiking biosolids. Isolated cultures are maintained on slopes of Nutrient agar at 4°C after overnight growth at 37°C (Turpin *et* al., 1993).

(Size of culture media containers [test tubes, flasks, and Petri dishes] are specified in preparation of each medium. All media containers must have covers, caps, or plugs that prevent contamination but maintain aerobic conditions unless otherwise directed.)

Sterile deionized water: Pour deionized water into 300 ml beaker, cover with aluminum foil and autoclave at 121 °C for 15 min.

Buffered Dilution Water (BDW): First, prepare stock phosphate buffer solution. Dissolve 34.0 g potassium dihydrogen phosphate (KH_2PO_4), in 950 ml sterile, deionized water, adjust to pH 7.2 ± 0.1 with sodium hydroxide (NaOH). First, add about 8-10 ml 6*N* NaOH to reach the pH of around 7.0. Use 1*N* NaOH to reach the final pH of 7.2. Dilute to 1 L and mix thoroughly.
To prepare BDW for dilutions, dilute 1.25 ml stock phosphate buffer solution and 5.0 ml magnesium chloride solution (81.1 g $MgCl_2 \times 6H_2O$/L deionized water) in deionized water in 1L volumetric flask. Mix thoroughly and transfer to five 300 ml beakers. Cover loosely with aluminum foil and autoclave for 15 min at 121°C. Dispense in 9-ml quantities into sterilized 16×150-mm test tubes. The tubes are ready to prepare serial dilutions.

Buffered Peptone Water (BPW) (non-selective pre-enrichment): Suspend 10 g of peptone, 5 g of sodium chloride, 9 g of disodium hydrophosphate ($Na_2HPO_4 \times 12\ H_2O$), and 1.5 g potassium dihydrogen phosphate

(KH_2PO_4) in 1 L sterile, deionized water and mix on magnetic stirrer unit with the use of sterile, magnetic bar. Sterilize using a sterilization filter unit (cold sterilization) with a 0.2 µm membrane filter (Gelman™ brand or equivalent). non-selective pre-enrichment Dispense in 9.0 ml amounts in 16×150-mm sterilized test tubes. The tubes are ready for seeding and serial dilutions.

Dulcitol Selenite (DS) broth (selective-enrichment): Suspend 23 g of dehydrated selenite broth (SB) and 4 g of dulcitol in 1 L sterile, deionized water. After adding the substrates insert a sterile magnetic stirrer bar into the flask, cover with foil, and heat to 88 °C on a stirrer-heater unit to obtain a clear medium. The medium does not require adjustment of pH. Dispense 9-ml portions into sterile 16×150-mm test tubes. *Do not autoclave*. Medium is not sterile. Use the same day as prepared.

Dulcitol Strontium Hydrogen Selenite broth (DSHS) is optional to the use of DS broth. Prepare the broth according to Iveson and Mackay-Scollay (1969). Enhances recovery of *Salmonella typhi*.

Xylose Lysine Deoxycholate (XLD) agar (isolation): Rehydrate 55 g of XLD agar powder in 1 L sterile, deionized H_2O and mix thoroughly. Do not autoclave. Heat with frequent agitation just until medium boils (up to 88 °C). Avoid overheating and consequent precipitation. Cool in a water bath to 45-50 °C and pour 15-ml portions into 15 × 100-mm Petri dishes as it is cooled. Let dry about 2 h with covers partially removed; then close plates. Final pH, 7.5 ± 0.2.

Modified Xylose Lysine Deoxycholate (M-XLD) agar (based on Cox, 1993) is optional to the use of XLD agar and is mainly used to include enumeration of *Salmonella typhi* species and atypical strains of *Salmonella spp.* Rehydrate 55 g of dehydrated XLD agar in 1 L sterile, deionized H_2O and mix thoroughly. Add 5.0 g of glycerol, 5.0 g of mannitol, and 3.0 g of proteose peptone. Follow steps as above. The pH of the medium is adjusted to 7.4 with 0.1 M NaOH, if necessary.

Triple Sugar Iron (TSI) agar (presumptive stage): Suspend 59.4 g of dehydrated TSI agar in 1 L sterile, deionized H_2O, mix thoroughly, and heat with occasional agitation (turn the stirrer knob on and off intermittently). Boil about 1 min until the powder dissolves. Fill sterile 16×150-mm test tubes 1/3 full and cap or plug so that aerobic conditions are maintained during use. Autoclave for 12 min at 121 °C. The total sterilization time (from the moment the autoclave is turned on, until the end of the cycle) should not exceed 45 min. Remove tubes and cool promptly. Before medium solidifies, place tubes in slanted position so that deep butts (~3 cm) and adequate slants (~ 5 cm) are formed on solidification. Final pH, 7.3 ± 0.2.

Lysine Iron (LI) agar (optional to or for use with TSI agar): Suspend the required amount of dehydrated LIA agar in 1 L sterile, deionized water, heating until dissolved. Dispense 4 ml portions into 13×100 mm sterile test tubes and cap or plug so that aerobic conditions are maintained during use. Autoclave 12 minutes at 121 °C. Before medium solidifies, place tubes in slanted positions so that 4 cm butts and 2.5 cm slants are formed on solidification. Final pH, 6.7 ± 0.2.

Urea broth (presumptive stage): Dissolve 20 g urea, 1 g yeast extract, 9.1 g KH_2PO_4, 9.5 g Na_2HPO_4, and 4.0 ml 0.25 % phenol red (10 mg) solution in 1 L sterile, deionized H_2O. Do not heat. Sterilize by filtration and aseptically dispense 1.5-3 ml portions into 13×100 mm sterile test tubes. Final pH, 6.8 ± 0.2.

Lysine Decarboxylase (LD) broth (presumptive stage): Dissolve 5.0 g Gelysate or peptone, 3.0 g yeast extract, 1.0 g glucose, 5.0 g L-lysine (or 5.0 g L-lysine hydrochloride), and 8 ml 0.25% g bromocresol purple solution in 1 L H_2O, heating until dissolved. Dispense 5 ml portions into 16×125-mm screw-cup test tubes. Autoclave, loosely capped, 15 minutes at 121 °C. Screw caps on tightly for storage and after inoculation. Final pH, 6.5-6.8.

Optionally, order dehydrated broth (Difco product) from Fisher Scientific, Canada.

Salmonella O Antiserum Poly A-I and Vi (confirmation stage): Obtain from Fisher Scientific, Canada. Follow manufacturer's instructions to prepare the antiserum.

Sample preparation

Selection of sample size: Use such dilution of wastewater or sludge sample to be examined that will yield counts between 20 and 60 fecal coliform colonies per plate (and not

Procedure

1. Non-selective pre-enrichment

This step is required when expected number of *Salmonella* species in the material is low and there is evidence that the bacteria are stressed.

The first row of the preenrichment MPN (five-tube MPN setup) using BPW (Edel and Kampelmacher, 1973) is prepared by adding 100 ml of blended sample suspension (total solids content of less than 7 %) into a sterile wide-mouth milk dilution bottle. Prior to that add 1.0 g of peptone and 0.5 g of NaCl to each bottle (these quantities provide the same concentrations of the chemicals in 100 ml of the sample as in 1 L of BPW). Peptone/NaCl is dissolved in the sample-buffered dilution water suspension using a sterile stir bar on magnetic stir plate. The 10-ml row of the MPN should consist of 10-ml sterile 16×150-mm test tubes of this sample suspension.

Inoculate tubes filled with 9.0 ml of sterile BPW in second row by adding 1ml of the buffered peptone sample suspension. Make higher dilutions by transferring 1ml of the preceding tube to the following tube, until all tubes in each row are inoculated.

Prepare solid and semi-solid samples by weighing 50.0 g of well-mixed sample and mixing with 450 ml of sterile BPW in a sterile blender on high speed at 4 ^{o}C for 15 minutes (Edel and Kampelmacher, 1973; after Hussong et al., 1985). Then, transfer 10 ml of the blender contents to the first tube and make serial dilutions as for liquid samples. BPW is also used for dilution blanks. Incubate the tubes at 37 ^{o}C for 24 h (optionally, try 2-18 hour incubation).

Growth is demonstrated by turbidity. Usually all dilutions exhibit growth, hence the same number of tubes has to be used in the selective enrichment stage. Following incubation vortex each tube and transfer 1 ml of the mixture to 9 ml of the enrichment broth in the corresponding tube of the selective enrichment MPN set up.

2. Selective enrichment

Upon addition of 1.0 ml BPW culture, vortex the enrichment tubes. When expected number of *Salmonella* species is high, the pre-enrichment step (resuscitation) is omitted. Transfer 10 ml of sample suspension to each sterile 16×150-mm test tube in the first row of the MPN setup into 10 ml of double-strength DS (double the amount of dehydrated substrates while preparing the broth). Transfer 1 ml of sample suspension into 9 ml of single-strength DS in the second row, and so on (Kenner and Clark, 1974). Incubate the tubes at 40 ± 0.2 ^{o}C for 24 h.

Use 0.8 % (w/v) sodium selenite (double strength DS or 8 g of sodium selenite) for isolation of *S. typhi*. This will increase the isolation rate of *S. typhi* particularly if the organism was in cultural competition with other *Salmonella* serotypes. Incubate the tubes at 37 ^{o}C for 24 h, or longer (after 48 h).

3. Isolation

After primary incubation, streak surface of XLD (or MXLD) agar plates with the surface content of each DS multiple-tube culture (by means of a 10 µl loop). Following the MPN set up, each selective enrichment tube has its own respective XLD agar plate. Optionally, streaking duplicate plates from the same selective enrichment tube, one heavily and one lightly, often aids in recognition of enteric pathogens in the presence of large numbers of interfering organisms. Number the plates and incubate at 37 ^{o}C for 22 to 24 h.
Outline colony characteristics such as size, form, color, elevation, margin, etc. Typical *Salmonella* colonies on XLD agar are pink with or without black centers. Many *Salmonella* may have large, glossy black centers or may appear as almost completely black colonies. Atypically, a few *Salmonella* cultures produce yellow colonies with or without black centers. When MXLD agar is used colony morphologies are described in the table in Appendix. If MXLD agar plates are used following 0.8 % selenite enrichment for *S. typhi* examine the plates after 48 h of incubation too.

Multiple subculture from enrichment broth is recommended when the number of *Salmonella* is small in the inoculum (Harvey and Price, 1979). It is well known that *Salmonella* species are not uniformly distributed throughout contaminated material, even in liquid enrichment cultures. In the above case, up to four plates should be used at a single time after incubation. If samples contain multiple *Salmonella* serotypes subcultures from enrichment broth should be done after different times of incubation (e.g. after 18, 24, 48 h). One serotype may be dominant in the mixture at one time of subculture and another 24 h, or more, later.

4. Presumptive identification- treatment of typical and suspicious colonies

Presumptive identification involves use of triple sugar iron agar followed by urease test and lysine decarboxylase test.

Inoculation of TSI agar- Pick with needle 1 typical or suspicious colony, if present, from MXLD plate having growth. Inoculate TSI slant with portion of the colony by streaking slant and stabbing butt. Optionally, use 2 or more TSI agar tubes to seed 2 or more suspicious colonies. Store picked selective (MXLD agar) plates at 5-8 ^{o}C or at room temperature (unless the plates should be kept for additional 24 h at 37 ^{o}C for isolation of *S. typhi*). Incubate TSI slants at 37 ^{o}C for 24 ± 2 h. Cap tubes loosely to maintain aerobic conditions while incubating slants to prevent excessive H_2S production. *Salmonella* cultures typically have alkaline (red) slant and acid (yellow) butt, with or without H_2S (blackening of agar) in TSI. Retain all presumptive positive *Salmonella* cultures on TSI (alkaline slant and acid butt) agar for biochemical and serological tests. Use Table 1 at end of the document to identify the presumptive positive *Salmonella* spp.

Note that *S. enteritidis* bioser Paratyphi A, *S. choleraesuis* (diphasic), some cultures of *S. typhi*, *S. enteritidis* bioser Typhisuis, *S. enteritidis* bioser Sendai, *S. enteritidis* ser Berta and a few rare types fail to produce H_2S on TSI agar slants.

Selection for identification- Apply subsequent biochemical and serological identification tests to the presumptive positive TSI cultures picked from selective agar plates.

Urease test (exclusion of *Proteus* sp.)- Subculture small amount of growth from presumptive positive TSI agar culture to urea broth, and incubate 24 ± 2 h at 37 ^{o}C. Discard all cultures that give positive test (purple-red color). *Salmonella spp.* are urease negative (no change in orange color of medium). The urease reaction insures differentiation of urease positive *Proteus* spp. from *Salmonella* spp.

Lysine decarboxylase test (exclusion of *Citrobacter* sp.)- This test is used in parallel with the urease test or to check urease-negative cultures. Inoculate tube of lysine decarboxylase broth with small amount of growth from presumptive positive TSI agar culture. Close tube cap tightly after inoculation and incubate 48 ± 2 h at 37 ^{o}C. Examine at least every 24 hours. *Salmonella* spp. Give purple color of alkaline reaction throughout broth (final color is slightly darker than original purple color of medium). Sometimes tubes that have yellow color after 8-12 h of incubation change to purple later. Negative test is permanent yellow color throughout broth. If medium appears to be discolored (neither purple nor yellow) add few drops of 0.2 % bromocresol purple dye (dissolve 0.2 g in sterile, deionized H_2O and dilute to 100 ml), and reread tube reactions.

5. Confirmation stage- final serological identification- agglutination test using *Salmonella O* Antisera *Poly A-I and Vi*

(Based on manufacturer directions (Difco) and Standard Methods, 1998)

a) Introduction

Preliminary serological identification can be accomplished after differential biochemical tests have been performed.

Reagents: *Bacto-Salmonella O, H and Vi* are stable desiccated absorbed, (when necessary), single factor or whole group antisera. To rehydrate add 3 ml of 0.85% NaCl solution and rotate gently to dissolve completely. The resultant solution contains sufficient Merthiolate® to render the antisera in a bacteriostatic condition when stored at 2-8 ^{o}C. In most cases the final concentration is 1:10,000.

Check the purity (bacterial) and pH of the saline used in rehydration if the antiserum rehydrates cloudy. Discard any serum that is cloudy and/or has a precipitate unless it has been clarified and shown to react properly with known control cultures.

When rehydrated, the protein content of Bacto- Salmonella "O" Antisera approximates that of a glycerinated serum and therefore should be considered as a 1:2 dilution. The somatic (O) sera at this dilution are to be considered at the RTD (Routine Test Dilution).

Do not expose rehydrated serum to room temperature for prolonged periods of time. Discard any antiserum that becomes cloudy during storage. They should not be subject to repeated freezing and thawing. Such treatment is detrimental to the antibody content.

b) Precautions

1. In the slide test, al materials and equipment must be at room temperature at the time of test performance.
2. Adhere strictly to the time limitations.
3. Exposure of the organism or plate to heat from external sources (a hot bacteriological loop, burner flame, light source, etc.) may result in either a culture which cannot be suspended readily or evaporation and/or precipitation of the test mixture which may result in false positive reactions.
4. The test culture must be checked in a saline control for smoothness. Often stock cultures and sometimes isolated cultures are rough and will agglutinate spontaneously. Therefore it is necessary to select more smooth colonies for serological testing. It is recommended that more than one colony be tested from both assay and control cultures.
5. In salmonella serology known positive and negative control cultures should be employed to ascertain the validity of test results.
6. In the slide test, all equipment and materials (glass plates, loop, culture) must be sterilized after use by autoclave since living organisms are used as antigen.
7. The antisera must be stored at 2-8 ^{o}C when not in actual use.

c) Procedure- Somatic O Antigen Analysis

Cultures of suspected *Salmonella*, as indicated by results from differential tests (TSI agar, urease test, lysine decarboxylase test), should be subjected to serological procedures using *Bacto- Salmonella O* Antisera *Poly A-I and Vi*.

For preliminary serological identification growth may be taken directly from the TSI agar slant and used in the procedure outlined below.

1. Mark off a glass plate into 2.5 cm square sections with a wax pencil.
2. Place a small drop of the appropriate Bacto-Salmonella O Antiserum on the ruled section of the plate using the supplied dropper.
3. To the square next to the one containing the antiserum, place one drop of 0.85% NaCl solution. This will serve as a negative control of the bacterial suspension.
4. Transfer a portion of a loopful of growth from the appropriate medium to the \section containing the NaCl solution, and suspend thoroughly.
5. Similarly, transfer a portion of a loopful of growth to the square containing the antiserum and suspend thoroughly.
6. Rock the slide for 1 minute and avoid excessive evaporation.
7. Record agglutination as follows:

++++ agglutination = all of the cells agglutinate
+++ agglutination = 75 % of the cells agglutinate
++ agglutination = 50 % of the cells agglutinate

+	agglutination = 25 % of the cells agglutinate
±	agglutination = <25 % of the cells agglutinate
–	agglutination = none of the cells agglutinate

A +++ reaction or greater should be considered as the end-point at the Routine Test Dilution.

When a negative reaction is obtained, the organisms can only be considered presumptively negative for *Salmonella* belonging to serogroups A-I. Biochemical tests should be performed to confirm this negative result. If biochemical tests prove the organism to be a *Salmonella*, a serogroup beyond serogroup A-I is probably involved (detectable by use of Salmonella O Antisera Poly C, D, E, F or G).

Calculation

Positive tubes in the MPN set up (either from the preenrichment stage or, in case of more concentrated samples believed to have high *Salmonella* counts, from the enrichment stage) are identified to estimate the *Salmonella* MPN with the tables from the Standard Methods (APHA *et al.*, 1998). Adjustments are made based on the outcome of the identification stage (on XLD agar plates, on TSI agar slants, in urease test, and in lysine decarboxylase test). A tube is considered positive if, during the identification stage, there was at least one positive colony isolated from the growth transferred from that tube. Any false positive colonies from this stage are included in the final calculation. Presumptive *Salmonella* spp. can be confirmed by means of serological testing following biochemical identification.

Preparation of *Salmonella* sp. for spiking sludge

Growth curve tests: Add 3 mL of the log phase *Salmonella* culture to the milk dilution bottle containing 60 mL of Tryptic Soy Broth (TSB). Mix and establish the initial absorbance (A_{init}). The A_{init} should be 0.05 to 0.1 at 600 nm. Determine t=0 h and prepare dilutions of the culture for XLD agar direct plate counts. Incubate TSB cultures at 37°C on a shaker table. At each 30 min interval, transfer about 5 mL of the culture to a small tube and determine its absorbance. Also transfer 1 mL of the culture into a tube with 9 mL of BDW and prepare serial dilutions for seeding XLD agar plates. Stop incubations when the absorbance reading approaches 0.95 to 1.00.

Harvesting of cells: Transfer 3 mL of *Salmonella* sp. culture to 60 mL of TSB and incubate shaken at 37°C overnight. Repeat transfers next morning and continue incubation for another 3-4 h. Such produced cells are ready for spiking.

References

APHA, AWWA, WEF (**1998**). Standard methods for the examination of water and wastewater (Greenberg A E, Clesceri L S, Eaton A D; Editors), 20th Edition, APHA, Washington, DC

Cooper R C and Danielson R E (**1997**). "Detection of bacterial pathogens in wastewater and sludge." *In: Manual of environmental microbiology* (Hurst C J, Knudsen G R, McInerney M J, Stetzenbach L D, Walter M V; Editors), American Society for Microbiology, ASM Press, Washington, DC

Cox J M (**1993**). "Lysine-mannitol-glycerol agar, a medium for the isolation of *Salmonella* spp., including *S. typhi* and atypical strains." *Applied and Environmental Microbiology*, **59** (8), 2602-2606

Edel W and Kampelmacher E H (**1973**). "Comparative studies on the isolation of 'sublethally injured" salmonellae in nine European laboratories." Bulletin of the World Health Organization., **48**, 167-174

Farmer J J III, Davis B R, Hickman-Brenner F W, McWhorter A C, Huntley-Carter G P, Ashbury M A, Riddle C, Wathen-Grady H G, Elias C, Fanning G R, Steigerwalt A G, O'Hara C M, Morris G K, Smith P B, and Brenner D J (**1985**). "Biochemical identification of new species and biogroups of *Enterobacteriaceae* isolated from clinical specimens." *Journal of Clinical Microbiology*, **21**, 46-76

Farmer J J III, McWhorter A C, Brenner F W, Morris G K, (**1984**). "The *Salmonella*-Arizona group of *Enterobacteriaceae*: nomenclature, classification and reporting." *Clinical Microbiol. Newsletter*, **6**, 63-66

Harvey R W S, and Price T H (1979). "Principles of *Salmonella* isolation."

Hussong D, Burge W D, Enkiri N K (**1985**). "Occurrence, growth, and suppression of salmonellae in composted sewage sludge." *Applied and Environmental Microbiology*, **50** (4), 887-893

Iveson J B and Mackay-Scollay E M (**1969**). "Strontium chloride and strontium selenite enrichment media in the isolation of *Salmonella*." *Journal of Hygiene, Cambridge*, **67**, 457-464
Kenner B A and Clark H P (**1974**). "Detection and enumeration of *Salmonella* and *Pseudomonas aeruginosa*." *J. Water Pollution Control Federation*, **46** (9), 2163-2171
Russ C F, and Yanko W A (**1981**). "Factors affecting *Salmonellae* repopulation in composted sludges." *Applied and Environmental Microbiology*, **41** (3), 597-602
Balows A, Truper H G, Dworkin M, Harder W, Schleifer K-H, Eds. (**1992**) "The Prokaryotes. A handbook on the biology of bacteria: ecophysiology, isolation, identification, applications." 2nd ed., Volume II, Chapter 146, Springer-Verlag, New York, NY, USA
Thomason B M, Dodd D J, Cherry W B (**1977**). "Increased recovery of salmonellae from environmental samples enriched with buffered peptone water." *Applied and Environmental Microbiology*, **34**, 270-273
Turpin P E, Maycroft K A, Rowlands C L, and Wellington E M H (1993). "Viable but non-culturable salmonellas in soil." *Journal of Applied Bacteriology*, **74**, 421-427.
US EPA (**1992**). Environmental regulations and technology, control of pathogens and vector attraction in sewage sludge (including domestic septage) under 40 CFR Part 503. Report No.: EPA/ 625/R-92/013. US EPA Office of Research and Development; Office of Science, Planning and Regulatory Evaluation; Center for Environmental Research following biochemical identification Information; Cincinnati, OH
Yanko W A, Walker A S, Jackson J L, Liabo L L, Garcia A L (**1995**). "Enumerating *Salmonella* in biosolids for compliance with pathogen regulations." *Water Environment Research*, **67**, 364-370

APPENDIX

Table 1. Colony appearance on modified xylose lysine deoxycholate agar (Cox, 1993).

Organism	**Colony morphology**
Typical, lactose positive and sucrose-positive Salmonella spp.	Colonies have black centers, ranging in size from small bull's-eyes to zones extending almost to the edges of the colonies; each colony is surrounded by a zone varying in color from yellow-orange to pink; after 48 hours, the colonies are almost entirely black, and each colony is surrounded by a pink zone
S. typhi	Yellow colonies are surrounded by yellow zones; after 48 hours, a small black center in each colony is surrounded by a yellow zone; typical colonies occur after 24 hours following selenite enrichment
H_2S-negative Salmonella spp.	Colonies are pink or colorless and are surrounded by pink zones in the medium
Lysine decarboxylase negative Salmonella sp. serotype Derby	Colonies are yellow with yellow zones in the medium; after more than 48 hours of incubation, slight alkaline reversion in the medium occurs and the colonies have small black centers
Citrobacter spp. (most strains)	Yellow colonies occur with yellow zones in the medium; no alkaline reversion or blackening occurs after prolonged incubation (up to 7 days)
Proteus spp.	Colonies have dark centers; pink zones surround colonies; dark centers are generally smaller than those of Salmonella spp. And greenish gray or black

STANDARD OPERATING PROCEDURE (SOP) FOR ENUMERATION OF BACTERIAL SPORES OF *Clostridium perfringens* IN WASTEWATER AND WASTEWATER SOLIDS

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Introduction

The genus *Clostridium* includes psychrophilic, mesophilic, and thermophilic species. The major role of these organisms in nature is the degradation of organic material to acids, alcohols, CO_2, H_2, and minerals. Four criteria characterize organism belonging to the genus *Clostridium*: it forms spores, relies solely on anaerobic energy metabolism, is unable to carry out a dissimilatory sulfate reduction, has a Gram-positive cell wall structure. The ability to form spores that resist dryness, heat, and aerobic conditions make the clostridia ubiquitous in the environment [Hippe *et al.*, 1992a]. Common habitats of these bacteria include soil, water, air (dust), mud, marine sediments, intestinal tract of man and animals, and have frequent and universal geographic distribution.

Clostridial strains of the species *perfringens* are of particular interest. Strains of *Cl. perfringens* type A, one of the five types (A to E) distinguished on the basis of lethal toxin production, are principally found in the soil and intestines of humans, animals, and birds. They are able to produce the "alpha" toxin or enterotoxin (referred to by its properties) causing pathogenicity in humans, and are both proteolytic and saccharolytic organisms. The habitat of other four types (B, C, D, and E) seems to be limited to the intestine of animals only. Strains of types A, D, and E have the optimal temperature for the growth of 44 to 45^{o}C, whereas strains of types B and C have their optimum at 37 to 40^{o}C. Unlike most organisms of the genus *Clostridium*, all strains of the *perfringens* species are motile [Smith, 1992]. Overall, *Cl. perfringens* (type A) is considered a mesophilic bacterium [Hippe *et al.*, 1992].

C. perfringens is probably the easiest of all obligate anaerobes to isolate. Its short generation time under anaerobic conditions at 43 to 45 ^{o}C enables it to outgrow most accompanying organisms and *C. perfringens* is easily isolated from plates streaked from such rapidly growing cultures. Its lack of susceptibility to low concentrations of oxygen and its relative resistance to such compounds as sodium sulfite, sulfadiazine, polymyxin, neomycin, kanamycin, and cycloserine have enabled several media to be devised for its isolation and enumeration [Smith and Williams, 1984].

Cl. perfringens produces characteristic round, smooth colonies while other clostridia produce small, ill – defined colonies with irregular peripheries. Colonies have usually zones of beta-hemolysis; many strains of *Cl. perfringens* produce a characteristic zone of double hemolysis [Carter, 1973].

Principle of the method

Heat-resistant spores are produced in a sample by eliminating vegetative cells of *C. perfringens* during sample incubation at 75+ ^{o}C for 20 minutes. The activated spores germinate and transform into vegetative cells. For spore activation and destruction of the vegetative cells, the tubes with lowest sludge dilution are kept in a water bath at 75 ^{o}C for 20 minutes, cooled in water, and higher dilutions (using buffered dilution or peptone water, BDW or BPW) are made prior to plating.

The spore counts of *C. perfringens* are generally preferred over total counts. The former are incorrectly defined. Because the spores usually constitute a significant portion of the total *C. perfringens* (vegetative cells + spores), but vary in dormancy, the "total count" is usually incomplete and may even be lower than the spore count [Hobbs and Sutton, 1967; Sutton and Hobbs, 1968]. In addition, most of the potentially interfering intestinal microorganisms are killed during heat activation of the spores.

Presumptive phase:

Selective media have been devised for isolating *C. perfringens* from material contaminated with other bacteria. Many of them contain sulfite and some iron salt. Although sulfite is inhibitory for other bacteria, it is only slightly so for the clostridia. Most clostridia reduce sulfite to sulfide, giving rise to black colonies or black zones around colonies in media containing iron. The most common solid media for the presumptive enumeration of *C. perfringens* include SPS (sulfite-polymyxin-sulfadiazine), TSN (tryptose-sulfate-neomycin), SFP (Shahidi-Ferguson perfringens), TSC (tryptose-sulfite-cycloserine), and OPSP (Oleandomycin-polymyxin-sulfadiazine perfringens). Of these media, TSC was found to be the most satisfactory [Hauschild and Hilsheimer, 1974, Harmon, 1976] with $_{D}$-Cycloserine at concentration of 200 to 400ug/ml. This agar (TSC) was originally proposed by Harmon *et al.* [(1971b]. Pour plates rather than surface-inoculated plates should be used to reduce the impact of atmospheric oxygen during sample handling (e.g. agar solidification) prior to making the agar anaerobic.

Most frequently an indicator is incorporated into the medium, to detect the reaction. The most common are the various pH indicators to detect acidity or alkalinity. Ferrous salts are used to detect the reduction of sulfate (by sulfate-reducing bacteria) or sulfite (by clostridia and other sulfite reducers) to sulfide which then reacts with the ferrous salt to form the black insoluble compound ferrous sulfide.

The $_{D}$-Cycloserine antibiotic is added from a frozen stock solution (10 mg/ml) to a final concentration of 400 µg/ml in the TSC agar. Final *$_{D}$-Cycloserine* concentration in the medium should be 0.04% or 400 µg/ml [Harmon *et al.*, 1971b].

Facultative anaerobes are more inhibited in the TSC agar as compared to the oleandomycin-polymyxin-sulfadiazine perfringens (OPSP) agar. Therefore, the use of TSC agar is preferred over the OPSP agar.

For alkali-treated sludge, the use of lysozyme supplemented TSC agar is recommended. A treatment with alkali removes a soluble protein layer from the exterior of the spore. Spores so altered are incapable of germination and outgrowth in complex media. However, such spores will germinate if they are treated with lysozyme or an initiation protein produced during the vegetative growth of *C. perfringens* [Smith and Williams, 1984]. Apparently, the alteration of the spores by heat, reducing agents, or alkali inactivates a lytic system that normally is responsible for degradation of the cortex of the spore [Franceschini and Labbe, 1979; found in: Smith and Williams, 1984]. The working concentration of 0.001% or 10 mg lysozyme/l TSC agar should be used.

Confirmed phase:

C. perfringens are non-motile, sulfide, and nitrite producing (or sulfite, and nitrate reducing), gelatin liquefying bacteria.

Five or 10 presumptive *C. perfringens* colonies from each enumeration agar per sludge sample are stab-inoculated into supplemented buffered nitrate-motility (S-BNM) medium [Hauschild and Hilsheimer, 1974a; Harmon and Kautter, 1978] and into lactose gelatin (LG) medium [Hauschild and Hilsheimer, 1974b].

The S-BNM medium [Harmon and Kautter, 1978] is prepared by including 0.5 % galactose and 0.5 % glycerol in the basic buffered motility-nitrate (BNM) medium [Hauschild and Hilsheimer, 1974a]. The basic BNM medium is not recommended. Traces of nitrite in basic BNM medium are evidenced by a faint red color. The negative tubes show no growth. The tubes with S-BNM medium show good growth and produce positive nitrate reactions. Most of these reactions are very intense, in contrast to the reactions in basic BNM medium (Hauschild and Hilsheimer, 1974a). The S-BNM medium can be stored up to 5 weeks at 4 ^{o}C without losing its freshness whereas basic BNM medium deteriorates rapidly. The practical limit however should be no longer than 2 weeks. S-BNM agar should be de-aerated before stabbing.

Materials and culture media

Size of culture media containers (test tubes, flasks, Petri dishes) is specified in preparation of each medium. All media containers must have covers, caps, or plugs that prevent contamination.

Buffered Dilution Water (BDW):

First, prepare stock phosphate buffer solution. Dissolve 34.0 g potassium dihydrogen phosphate (KH_2PO_4), in 950 ml sterile, deionized water, adjust to pH 7.2 ± 0.1 with sodium hydroxide (NaOH). First, add about 8-10 ml 6*N* NaOH to reach the pH of around 7.0. Use 1*N* NaOH to reach the final pH of 7.2. Dilute to 1 L and mix thoroughly.

To prepare BDW for dilutions, dilute 1.25 ml stock phosphate buffer solution and 5.0 ml magnesium chloride solution (81.1 g $MgCl_2 \times 6H_2O$/L deionized water) in deionized water in 1L volumetric flask. Mix thoroughly and transfer to five 300 ml beakers. Cover loosely with aluminum foil and autoclave for 15 min at 121°C. Dispense in 9-ml quantities into sterilized 16×150-mm test tubes. The tubes are ready to prepare serial dilutions of heat-shocked samples.

Buffered Peptone Water (BPW)- 0.1% peptone solution:

(Optional to the use of buffered dilution water)

Suspend 10 g of peptone, 5 g of sodium chloride, 9 g of disodium hydrophosphate ($Na_2HPO_4 \bullet 12 H_2O$), and 1.5 g potassium dihydrogen phosphate (KH_2PO_4) in 1 L deionized water and mix on magnetic stirrer unit with the use of magnetic bar. Sterilize using a sterilization filter unit (cold sterilization) with a 0.2-µm membrane filter. Dispense in sterilized culture tubes as above.

Tryptose-Sulfate-Cycloserine (TSC) Agar (presumptive phase):

TSC agar should be prepared prior to each experiment to avoid hydrogen peroxide formation [Harmon and Kautter, 1976; Juneja *et al.*, 1993]. The agar is prepared using Shahidi Ferguson Perfringens (SFP) agar base (Difco, obtained through Fisher Scientific Canada). Suspend 47 g of the SFP agar powder in 960 ml deionized water, heat to boiling with frequent agitation to dissolve the powder and dispense into two 500 ml Erlenmeyer flasks. Cover flasks loosely with aluminum foil and autoclave for 15 min at 121°C. Transfer the agar from both flasks into sterilized 1 L or 2 L beaker and cool down to about 50°C. Avoid further loss of heat as the agar will solidify. Once the agar is at 50°C add *D-Cycloserine* solution. Do not add the antibiotic when agar temperature is >50°C. The antibiotic solution is stored in vials in 5-ml quantities. Eight tubes with 5 ml of 10 mg/ml *D-Cycloserine* solution per 960 ml of the SFP agar base are needed to produce the final working *D-Cycloserine* concentration of 400 µg/ml or 400 mg/L of the agar (or use 28.2 g agar in 575 ml H_2O and add 25 ml *D-Cycloserine* i.e. 5 tubes). Mix thoroughly to ensure same antibiotic concentration throughout medium.

> *D-Cycloserine solution (D-CS)*– prepare stock solution (10 mg/ml or g/L) by dissolving 2 g *D-Cycloserine* in 200 ml sterile deionized water. Weigh the antibiotic powder directly into 200 ml volumetric flask placed on the scale. Fill with water to the mark and mix on a magnetic stirrer unit to dissolve completely. Do not heat. Sterilize by filtering through a 0.22-µm membrane filter. Dispense in 5-ml quantities into sterilized 100×13-mm screw-cup test tubes. Screw tubes tightly and place in freezer at –18°C for storage. Prior to TSC agar preparation remove the required number of tubes from freezer and let thaw.
>
> Optional to the use of *D-Cycloserine* solution in water, dissolve 1 g D-Cycloserine without heating in 200 ml 0.05 M phosphate buffer (pH, 8.0 ± 0.1). This is 0.5% *D-Cycloserine* solution. Sterilize by filtering through a 0.22-µm membrane filter. Before plating, add 20 ml 0.5% filter-sterilized solution of *D-Cycloserine* to each 250 ml sterile SFP medium at 50°C.

When TSC agar is supplemented with *lysozyme* to repair alkali-injured spores, add the enzyme stock solution after addition of *D-Cycloserine*. The appropriate volume of stock lysozyme solution added to TSC agar to reach the working concentration of 0.001% or 10 mg lysozyme/L agar is 6 ml stock solution per 0.6 L agar (this quantity will produce 40 plates with 15 ml agar per plate).

Lysozyme (*LYS*) - Prepare stock solution (1 g/L) by dissolving 0.2 g lysozyme in 200 ml 0.01*N* HCl (using 200-ml volumetric flask) and sterilize with 0.22µm membrane filter. Alternatively, use 200 ml distilled water. Dispense in 5-ml quantities into sterilized 100×13-mm screw-cup test tubes. Screw tubes tightly and place in freezer at −18°C for storage. Prior to TSC agar preparation remove the required number of tubes from freezer and let thaw.

Supplemented Buffered motility-nitrate (S-BMN) medium (confirmed phase):

(The basic buffered motility-nitrate (BMN) medium is prepared by dissolving 3.0 g beef extract, 5.0 g peptone, 5.0 g KNO_3, 2.5 g Na_2HPO_4, 3.0 g agar, in 1 L H_2O. The supplemented BMN medium should be used due to increased sensitivity and storage period prior to use)

Prepare supplemented BMN (S-BMN) by dissolving 3.0 g beef extract, 5.0 g peptone, 5.0 g KNO_3, 2.5 g Na_2HPO_4, 3.0 g agar, 5.0 g D-galactose, and 5.0 g glycerol in 1L deionized water. Adjust to pH 7.3 ± 0.1. Dispense 5 ml portions into 100×13-mm screw-cup test tubes. Autoclave loosely capped for15 minutes at 121°C. Medium not used within 4 h after preparation must be de-aerated. Heat test tubes for 10 minutes in 103 °C oven to expel O_2. Cool the tubes rapidly in tap water without agitation just before stabbing/use. Store refrigerated at 4°C.

Nitrite test reagents-

Solution A: Dissolve 8 g of sulfanilic acid in 1 L of 5*N* CH_3COOH (or 1 g sulfanilic acid in 125 ml of 5*N* acetic acid).

Solution B: Dissolve 5 g of α-naphtol or N-(1-naphthyl) ethylene diamine dihydrochloride in 1 L of 5*N* CH_3COOH (or 1 g in 200 ml of 5*N* acetic acid).

Prepare 5 *N* acetic acid from concentrated (99.5%, 17.4 *N*) acid.

Lactose-gelatin (LG) medium (confirmed phase):

Dilute 15 g tryptose, 10 g yeast extract, 10.0 g lactose, 5.0 g Na_2HPO_4, 0.05 g phenol red, and 120 g gelatin in 950 ml deionized water. First, add water to 2 L beaker and heat to 50°C. Add gelatin in small quantities to allow dissolution. Cool down the solution and add tryptose yeast extract and disodium hydrogen phosphate. Mix thoroughly to dissolve and adjust pH to 7.5 ± 0.1 before adding lactose and phenol red. Usually, 1-2 mL of 6 *N* NaOH are sufficient. Dispense 12-ml portions into 100×18 mm culture tubes and sterilize 15 min at 121°C. Store refrigerated at 4°C.

Fluid Thioglycollate (FTG) medium (for spore production and maintenance of cultures)

Dilute 29.5 g of the powder (BBL dehydrated medium available from Fisher Scientific Canada) in 1,000 ml de-ionized water. Mix thoroughly and heat with frequent agitation to completely dissolve the powder. For culture maintenance, place about 0.1 g of $CaCO_3$ in each test tube. Fill half full with the medium. Autoclave at 121°C for 15 min. Cool before use. Store at room temperature, not in the refrigerator.

Duncan and Strong (DS) medium (for spore production):

Suspend 15.0 g proteose peptone, 4.0 g yeast extract, 1.0 g sodium thioglycollate, 4.0 g soluble starch or 4.0 g raffinose [Labbe and Rey, 1979], and 10 g sodium phosphate (dibasic heptahydrate).

Procedure

Presumptive phase:

The antibiotic is added as a 4% filter-sterilized solution in water. It is added to the autoclaved medium once it cools down to 50 °C (Hauschild and Hilsheimer, 1974a).

For enumeration of *C. perfringens* spores, 5-7 ml samples at dilution 10^{-1} in test tubes (16 by 150 mm) are incubated for 20 min in a water bath at 75°C with the entire sample submerged. Cool immediately in cold water (Hauschild and Hilsheimer, 1974b). Prepare higher dilutions from heat-shocked sample.

When agar has solidified, place plates in upright position in anaerobic jar. Produce anaerobic conditions (80% N_2 balanced with CO_2) using the purge-vacuum system (located in cottage room next to Room 404), and incubate jar for 20-24 hours at 35°C. Incubation for up to 24 hours prevents from including other clostridia in the count. Some of the other clostridia require at least 48 hours of incubation at 35-38°C in

TSC agar to obtain appreciable growth. After incubation, remove plates from jar and observe macroscopically for growth and black colony production.

All *C. perfringens* colonies produce apparent halos (luminous radiance, crown of light).

Select plates showing estimated 20-200 black colonies. Using Quebec colony counter with piece of white tissue paper over counting area, count black colonies and calculate number of *C. perfringens* spp. / g solids. Pick 5 colonies (Harmon *et al.*, 1971a) for confirmation in S-BMN medium and LG medium.

Confirmed phase:

Simultaneously transfer presumptive colonies from TSC agar to supplemented nitrate motility medium and to lactose gelatin medium.

Nitrate-Motility test

Test S-BMN medium for presence of nitrite by adding 0.5 ml of Solution A and 0.2 ml of Solution B to test tube. Orange color that develops within 15 minutes indicates presence of nitrites. If no color develops, add a few grains of powdered Zn metal, and let stand 10 minutes. No color change after addition of Zn indicates that organism is incapable of reducing nitrates.

Lactose-Gelatin test

Examine LG (lactose-gelatin) medium for gas and color change from red to yellow, indicating that lactose is fermented with production of acid. Chill tubes for 1 hour at 5 ^{o}C and check for gelatin liquefaction. If medium solidifies, re-incubate additional 24 hours at 35 ^{o}C and repeat test for gelatin liquefaction. *C. perfringens* produces acid and gas from fermentation of lactose and liquefies gelatin in 20 to 24 hours. Gelatin liquefaction is also determined after 44 hours.
NOTE: Non-motile, Gram-positive bacilli that produce black colonies in TSC agar, reduce nitrates to nitrites, produce acid and gas from lactose, and liquefy gelatin within 48 hours are identified as *C. perfringens*.

Calculation of spore density

Calculate number of *C. perfringens* in sample on basis of % colonies tested that are confirmed as *C. perfringens*. For example, if average plate count of 10^{-4} dilution was 85, and 8 of 10 colonies tested were confirmed as *C. perfringens*, number of *C. perfringens* spores or Colony Forming Units (CFU) per g solids is $85 \times (8 / 10) \times 10{,}000 = 680{,}000$.

Presence-Absence test

The test is performed using the lowest sludge dilution (10^{-1}). One ml of this dilution, previously heat-treated, is transferred to a 125-ml Erlenmeyer flask. The TSC agar (~75 ml) is poured into the flask and contents mixed to provide even distribution of sludge in agar. Once the agar has solidified, the flask is placed in an anaerobic chamber for incubations at 35-37°C. The presence of black growth following overnight incubation constitutes a positive reaction.

Isolation of cultures for growth and spore production

Once confirmed as *C. perfringens*, a loop of growth from the lactose-gelatin medium was transferred into 10 ml of fluid thioglycollate medium (FTG) and dispersed by gentle shaking. The medium was heated at 75°C for 20 minutes, cooled, and incubated at 37°C for 16-18 hours (or overnight). The plastic caps of the culture tubes were kept on but not too tight to permit evolved gas to escape. A 1-ml portion from this culture was transferred to 10 ml of deoxygenated (freshly heated at 103°C in laboratory oven for 10 min to repel oxygen, and cooled to 37°C) FTG medium and incubated overnight. The culture was added at 1% concentration to Duncan-Strong sporulation medium (0.75 ml FTG culture to ~75 ml DS medium in a milk dilution bottle) and incubated at 37°C for 24 h. No special precautions were followed to maintain anaerobic conditions other than inclusion of sodium thioglycollate and stationary incubation of the culture. Heat-

resistant spore levels were determined after incubation in the sporulation medium by heating 5 ml of a (24-h) sporulating culture for 20 minutes at 75°C in a capped tube, followed by cooling, dilution and enumeration in TSC agar. Plates were counted after 24 hours of incubation at 35-37°C in an anaerobic jar.

References

Carter G. R. [1973]. Clostridium. In: *Diagnostic procedures in veterinary microbiology*. Chapter 20, p. 125-137, 2nd ed., C. C. Thomas (Publisher), Springfield, IL.

Duncan C L and Strong D H [1968]. Improved medium for sporulation of *Clostridium perfringens*. *Appl. Microbiol.*, **16** (1), 82-89.

Harmon S. M., Kautter D. A. and Peeler J. T. [1971a]. Comparison of media for the enumeration of *Clostridium perfringens*. *Appl. Microbiol.*, **21** (5), 922-927.

Harmon S. M., Kautter D. A., and Peeler J. T. [1971b]. Improved medium for enumeration of *Clostridium perfringens*. *Appl. Microbiol.*, **22** (4), 688-692.

Harmon S. M. and Kautter D. A. [1976]. Beneficial effects of catalase treatment on growth of *Clostridium perfringens*. *Appl. Environ. Microbiol.*, **32**, 409-416.

Harmon S. M. and Kautter, D. A. [1978]. Media for confirming *Clostridium perfringens* from food and feces. *J. Food Protect.*, **41** (8), 626-630.

Hauschild A. H. W. and Hilsheimer R. [1974a]. Evaluation and modifications of media for enumeration of *C. perfringens*. *Appl. Microbiol.*, **27** (1), 78-82.

Hauschild A. H. W. and Hilsheimer R. [1974b]. Enumeration of food-borne *C. perfringens* in egg yolk-free tryptose-sulfite-cycloserine agar. *Appl. Microbiol.*, **27** (3), 521-526.

Hippe H., Andreesen J. R. and Gottschalk G. [1992]. The Genus *Clostridium*-Nonmedical. In: *The Prokaryotes. A Handbook on the biology of bacteria: ecophysiology, isolation, identification, applications*. (Balows A, Truper H. G., Dworkin M., Harder W., Schleifer K-H., eds.). Vol. 2, Chapter 81, Springer-Verlag New York Inc., New York, N.Y., U.S.A., p. 1800-1866.

Hobbs B. C. and Sutton R. G. A. [1967]. Characteristics of spores of *Clostridium welchii* in relation to food hygiene. In: *The anaerobic bacteria* (V. Fredette, ed.), University of Montreal, Quebec, Canada, p. 51-59.

Juneja V. K., Call J. E. and Miller A. J. [1993]. Evaluation of methylxanthines and related compounds to enhance *Clostridium perfringens* sporulation using a modified Duncan and Strong medium. *J. Rapid Methods and Automation in Microbiol.*, **2**, 203-218.

Labbe R G and Rey D K [1979]. Raffinose increases sporulation and enterotoxin production by *Clostridium perfringens* type A. *Appl. Environ. Microbiol.*, **37** (6), 1196-1200.

Smith L. D.S. [1992]. The Genus *Clostridium*-Medical. In: *The Prokaryotes. A Handbook on the biology of bacteria: ecophysiology, isolation, identification, applications*. (Balows A, Truper H. G., Dworkin M., Harder W., Schleifer K-H. eds.). Volume 2, Chapter 82, p. 1867-1878, Springer-Verlag New York Inc., New York, N.Y.

Smith L. D.S. and Williams B. L. [1984]. *Clostridium perfringens*. In: *The pathogenic anaerobic bacteria*. Chapter 7, 3rd ed., C. C. Thomas (Publisher), Springfield, IL, U.S.A., p. 101-136.

Sutton R. G. A., and Hobbs B. C. [1968]. Food poisoning caused by heat-sensitive *Clostridium welchii*. A report of five recent outbreaks. *J. Hygiene*, **66**, 135-146.

STANDARD OPERATING PROCEDURE (SOP) FOR ENUMERATION OF HELMINTH EGGS (Including Genus *Ascaris*) IN WASTEWATER AND WASTEWATER SOLIDS

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1 INTRODUCTION

Helminth eggs (ova) are commonly found in municipal wastewater and wastewater sludge. They are highly resistant to these hostile environments and therefore create a potential risk to exposed humans and animals. Most prevalent helminth genera are the roundworms of *Ascaris*, *Trichuris*, *Toxocara* (also *Capillaria*) and the tapeworms of *Taenia* and *Hymenolepis*.

In the final rule published by the U.S. Environmental Protection Agency (40 CFR 503; EPA, 1993), sludge/biosolids are designated as Class A if there are no site restrictions to apply the biosolids onto agricultural land, forest, a public contact site, a reclamation site, or give away for home garden or lawn use. The specific pathogen reduction criteria for biosolids prior to their application include the requirement of **less than one helminth ova per 4 g of dry solids** (i.e. total solids, TS). The biosolids is Class A with respect to bacteria, viruses, helminth eggs, when the following criteria are met: *Salmonella* bacteria < 3 MPN / 4 g TS or fecal coliform bacteria < 1,000 MPN / g TS, enteric viruses < 1 PFU / 4 g TS, and viable helminth ova < 1 egg / 4 g TS.

Storage is a very common practice of reducing pathogenic levels in biosolids. Factors that influence the effectiveness of this method are storage time, temperature, type of biosolids (aerobic, anaerobic). Storage temperature was shown to have the greatest influence on helminth egg inactivation (O'Donnell *et* al., 1984). The eggs of various helminths, including *Ascaris* spp., *Toxocara* spp., *Trichuris* spp., and *Hymenolepis* spp., stored in aerobically digested wastewater sludge, anaerobically digested sludge, and soil, lost their viability and were inactivated faster at 25 ^{o}C storage temperature than at 4 ^{o}C. Also, viability reductions of the *Ascaris* eggs were faster in anaerobically digested sludge than in aerobically treated sludge (10 and 16 months at 25 ^{o}C, respectively). The viability of *Toxocara* eggs stored in aerobically digested sludge showed slower decrease than the one in anaerobically digested sludge.

Black *et* al. (1982) demonstrated a higher potential for helminth egg survival once the eggs passed through either aerobic or anaerobic digestion. This suggests that the eggs that survived were those more resistant to adverse environmental conditions. Prolonged storage over several months was required to reduce egg viability.

Helminth eggs are a good indicator of sludge/biosolids disinfection performance. Despite a number of successful research programs there is still need to resolve several problems. Highly efficient and reliable method of recovery of helminth (*Ascaris*) eggs from environmental samples has to be selected or compiled from other available methods. Commonly used method of Yanko (1987) recommended by US EPA suffers from several inadequacies. The acid alcohol-ether technique affects the viability of environmentally stressed eggs. The procedure yields only decorticated, dead eggs (killed with Lugol's iodine) and the efficacy of this procedure in recovering viable eggs from sludge samples is unknown.
Enumeration of recovered eggs is another important issue. For example, a Sedgwick-Rafter cell could lead to erroneous results, as the eggs pipetted in suspension onto a slide for counting are not uniformly distributed across the slide.
Some form of control has to be provided as well. Hardiness of the eggs used in spiking biosolids has to be known to validate die-off patterns during subsequent experimental work. Differentiation between the natural die-off of the eggs and that which is due to other factors as a result of biosolids treatment, is needed.

2 MATERIALS, REAGENTS AND APPARATUS

2.1 Materials

Beaker, 1000 mL and 2000 mL, tall form, graduated, glass, Pyrex brand;
Beaker, 400 mL, graduated, glass, Pyrex brand;
Funnel, 100 mm diameter, 140 mm stem, glass;
Funnel, 250 mm diameter, 80 mm stem, glass;
Erlenmeyer flasks, 400 mL, glass;
Centrifuge tubes, 50 mL, conical, with caps, polypropylene;
Centrifuge tubes, 15 mL, conical, glass;
Sieve, 600 µm (28 mesh, no. 30), 210 mm diameter, metal;
Sieve, 4 mm (5 mesh, no. 5), 210 mm diameter, metal;
Sieve, 20 µm, Spectra/Mesh nylon (the screen is glued onto glass ring inserted in plastic base), 65 mm diameter;
Rods, 200 mm long, glass;
Wash bottles, 500 mL, plastic;
Spray bottles, 500 mL, plastic;
Cylinder, 45 mm diameter, 320 mm height, for use with hydrometer, glass;
Microscope slides, size 25×75×1 mm; glass;
Cover glass, thickness 1, size 22×22 mm and higher;
Culture dishes (Petri plates), size 60×15 mm, glass;
Pipettes, Pasteur type, 5 ¾ in., Kimble brand or similar;
Pipette, automatic, with 1-200 mL capacity large orifice disposable plastic tips (yellow);
Parafilm®, 4 in. wide roll.

2.2 Reagents

2.2.1 Tween 80, 1% vol/vol (surfactant)

1% Tween 80 solution – dilute 10 mL of the Tween 80 concentrated liquid up to 1 L in de-ionized water, in 2 L tall graduated beaker. First, pour some 0.7-0.8 L de-ionized water to the beaker with magnetic bar inside, and place the beaker on a stirrer unit. While stirring (high speed), gradually add 10 mL of Tween 80 concentrated liquid from a pipette in three portions of 4 mL, 4 mL and 2 mL. Do not immerse the pipette tip in the water, as it can easily clog. Let the remainder of the Tween 80 liquid drain to the beaker. It is always convenient to use an insignificantly larger volume of the Tween 80 liquid, to account for the portion that gets stack to the sides of the pipette tip. Following preparation, store bottled at room temperature until use.

2.2.2 Phosphate Buffered Water (PBW containing 0.1% Tween 80, vol/vol)

First, prepare stock phosphate buffer solution. Dissolve 34.0 g potassium dihydrogen phosphate (KH_2PO_4), in 950 mL sterile, de-ionized water, adjust to pH 7.2 ± 0.1 with sodium hydroxide (NaOH). First, add about 8-10 mL 6*N* NaOH to reach the pH of around 7.0. Use 1*N* NaOH to reach the final pH of 7.2. Dilute to 1 L and mix thoroughly.

To prepare PBW containing 0.1% Tween 80, dilute 1.25 mL stock phosphate buffer solution and 5.0 mL magnesium chloride solution (81.1 g $MgCl_2 \times 6H_2O$/L de-ionized water) in 0.7-0.8 L de-ionized water in a 1 L volumetric flask. Mix the solution by swirling, by hand. Add 100 ml of 1% Tween 80 and fill up to 1 L level with de-ionized water. Insert magnetic bar and place on a stirrer unit. Mix until homogenous mixture results. Adjust the pH to 7.2±0.1 with 1*N* NaOH. Store bottled in fridge until use.

2.2.3 Magnesium sulphate, $MgSO_4$ (floatation liquid)

Weigh ~220 g of the magnesium sulphate powder into weighing dish. Measure 1000 mL of de-ionized water using volumetric flask and pour some 800 mL into a 1000 mL graduate beaker. Insert magnetic bar and place on stirrer unit. While stirring (high speed), gradually add the powder to dissolve. Take breaks in between to allow complete dissolution of the powder. Continue adding in small batches as not to allow formation of large crystals. Once all powder is dissolved, transfer the solution to the remaining 200 mL in the volumetric flask and mix thoroughly on the stirrer unit. In case of preparation of a larger volume of $MgSO_4$, collect the contents to a larger container and mix thoroughly. Pour some of this solution to glass cylinder provided with hydrometer and check specific gravity (SG). The SG should be 1.20. If necessary, adjust SG by adding water (when SG>1.20) or $MgSO_4$ powder (when SG<1.20).

2.2.4 Formalin, 0.5% (culture fluid)

Add 50 mL of 10% phosphate buffered formalin solution to a 1 L volumetric flask and fill to the line with de-ionized water. Insert magnetic bar and mix on a stirrer unit until solution is complete. Store bottled in fridge until use.

2.2.5 Dimethyl-dichlorosilane in 1,1,1-trichloroethane, 2% vol/vol (coating solution)

This solution is used to treat glassware (beakers, funnels, 15 mL centrifuge tubes, rods, etc.), plastic ware (50 mL centrifuge tubes with caps, magnetic bars, pipette tips, etc.), and other items in direct contact with tested material.

Prepare the solution under the fume-hood and using extreme care not to spill the contents or breathe in the vapors (dimethyl-dichlorosilane is corrosive, 1,1,1-trichloroethane is toxic and highly volatile). Place some 900 mL of 1,1,1-trichloroethane in a 1 L volumetric flask. While swirling by hand, gradually add 20 mL of dimethyl-dichlorosilane liquid from a pipette. Fill up with 1,1,1-trichloroethane to the 1 L mark, insert the magnetic bar and place on a stirrer unit. Mix for 1 min and transfer to brown bottle for storage. Do not close too tight as temperature changes will cause vapor build-up inside (and not too loose because of evaporation). Store away from sunlight and from heat.

Use always under fume-hood. When treating glassware or other ware, immerse treated object(s) in the solution for 2-4 min, pour off excess liquid, and allow drying. Immerse in water for at least 5 min, changing water occasionally (2-3 times). Then, rinse thoroughly and allow drying.

2.2.6 Propanol-2 (alcohol to disinfect contaminated benches etc.)

Use commercially available 2-Propanol or Ethyl alcohol. Spray or place soaked paper towels over contaminated area. Keep wetted for at least 30 min.

2.3 Apparatus

Blender, Waring brand with 40 oz. glass container;
Centrifuge, bench top, with rotor head for 15 mL tubes and with rotor head and cups for 50 mL tubes;
Platform shaker, New Brunswick Scientific;
Hydrometer, range 1.000-1.600 specific gravity (SG);
Stirrer unit (magnetic);
Vortex mixer;
Microscope, compound, binocular, with 100× and 400× magnification;
Balance, range 0-1000 g and higher;
Oven.

3 PROCEDURE

The U.S. EPA Class A biosolids requirement for helminth eggs is less than 1 viable egg/4 g total solids (TS). An adequate amount, preferably containing about 5 g TS, of tested sludge solids has to be used to demonstrate the lack of viable eggs in the entire sample taken for testing. The corresponding appropriate amounts of wet sludge needed are:
liquid sludge - 500 mL if sludge at 1% TS, 250 mL – 2% TS, 170 mL – 3% TS, 125 mL – 4% TS, 100 mL – 5% TS, 85 mL – 6% TS, 75 mL – 7% TS.
dewatered sludge - 25 g if sludge at 20% TS, 20 g – 25% TS, 17 g – 30% TS.

3.1 INITIAL DILUTION

Number all containers accordingly to avoid cross-contamination among samples tested. Make sure that all sieves are disinfected and thoroughly rinsed with water before next sample is processed.

3.1.1 Liquid sludge (TS content of less than 7%)

- Sieve the sludge through a 600 µm standard sieve (28 mesh, no. 30) held on a large glass funnel over a 1000 mL beaker. Wash the sample through the sieve with BDW from spray bottle. Make sure that larger objects that remain on the sieve are well rinsed. This allows eliminating any loss of eggs due to their attachment to these objects. Sieving through a smaller sieve may be necessary if smaller objects were not caught on the first sieve.
- Take an amount (volume) that will contain about 5 g total solids and place in a blender. Add about 200 mL of fridge-stored phosphate buffered water and blend for 1 minute at high speed.
- Transfer the blender contents into a 1000 mL tall form graduated beaker with a magnetic bar and, using wash bottle, thoroughly rinse the blender container with PBW into beaker.
- Fill with 1% Tween *80 up to about 700 mL mark and stir on a stirrer unit for 5-10 min. Place the beaker in fridge and let settle for 4 h. While settling, the solution may need to be stirred occasionally with a glass rod to ensure that any material floating on the surface will settle.

NOTE: In case of alkali-treated sludge, neutralize the sludge immediately after blending and transferring the contents to the beaker. Immerse magnetic bar and pH probe and start neutralizing with 6*N* H_2SO_4. Re-check for the pH after 2 min of further mixing following neutralization to see if pH is stable (i.e. neutralization is complete). Place the beaker in fridge and let settle for 4 h.

3.1.2 Solid and semi-solid sludge (TS higher than 7%)

- Weigh the appropriate amount of well-mixed sample in a 1000 mL tall form graduated beaker, add about 200-300 mL of fridge-stored PBW, insert a magnetic bar and place on stirrer unit. Mix for 15-30 min, place the beaker in the fridge and let soak overnight.
- Transfer sample to blender and blend for 1 min at high speed. Transfer the blender contents back into the beaker and, using wash bottle, thoroughly rinse the blender container with PBW into beaker.
- Fill with 1% Tween *80 up to about 700 mL mark and stir on a stirrer unit for 5-10 min. Place the beaker in fridge and let settle for 4 h. While settling, the solution may need to be stirred occasionally with a glass rod to ensure that any material floating on the surface will settle.

NOTE: In case of alkali-treated sludge, neutralize the sludge immediately. Weigh the appropriate amount of well-mixed sample in a blender container, add 200-300 mL of the PBW and blend for 1 min at high speed. Transfer the contents to a 1000 mL graduate beaker, insert magnetic bar and place on a stirrer unit. While stirring, immerse a pH probe and start neutralizing with 6N H_2SO_4. Re-check for the pH after 2 min of further mixing following neutralization to see if disintegration of sludge clumps is complete and no more alkalinity is being released to solution. Fill with 1% Tween *80 up to about 700 mL mark and stir on a stirrer unit for 5-10 min. Place in fridge and let settle for 4 h.

3.2 Preliminary separation and concentration of eggs from sludge

- After settling, pour off the supernatant onto a 20 µm Spectra/Mesh nylon sieve placed over a glass funnel (10 cm diameter) drained to a 400 mL graduated beaker. Discard the filtrate, rinse the residue collected on the sieve back into the 1000 mL beaker by directing a stream of PBW from a wash bottle and then from a spray bottle over the entire surface of the inverted sieve. Transfer the sediment from the beaker to blender, thoroughly rinse beaker with PBW into blender container and add more phosphate buffered water to 300 mL mark, and blend again for 1 min at high speed. Transfer back to the beaker and thoroughly rinse the blender container with PBW into beaker.
- Add 1% Tween 80 solution to the 800-900 mL mark. Let settle for 4 h. While settling, stir occasionally.
- After settling, pour off the supernatant onto a 20 µm sieve and discard the filtrate. Transfer the residue back to the beaker and the sediment from the beaker to blender, thoroughly rinse beaker with PBW into blender container and add more PBW to 300 mL mark, and blend again for 1 min at high speed.
- Transfer the sample back to the beaker, add 1% Tween 80 solution to 800-900 mL mark and allow to settle for 2 h. While settling, stir occasionally. Pour off supernatant onto a 20 µm sieve and discard the filtrate. Transfer the residue back to the beaker.
- Add 300 mL of 1% Tween *80 solution to the sediment and stir for 5 minutes on a magnetic stirrer.
- Stain homogenized sample through a 28-mesh sieve into a beaker. The sieve is placed on a funnel over a 2000 mL tall beaker and the sample washed through the sieve with aid of spray of 1% Tween 80 solution from a spray bottle.
- Add 1% Tween 80 solution to 800-900 mL mark and allow to settle for 1 to 2 h.
- Decant supernatant onto a 20 µm sieve and discard the filtrate. Transfer the residue back to the beaker. Mix sediment by swirling and distribute equally to eight 50 mL centrifuge tubes. Thoroughly wash any sediment in beaker in tubes with the aid of a wash bottle. Bring volume in each tube to about 50 mL with phosphate buffered water. Place each tube on a balance and adjust to same weight. Make sure that the difference in weight among the tubes does not exceed 0.1 g. The number of tubes required will depend upon the amount of sediment. Usually at least 8 tubes are required.
- Centrifuge for 5 minutes at setting 6 (or 800×g), pour off supernatant from each tube onto a 20 µm sieve and discard the filtrate. Transfer the residue to a 400 mL beaker. NOTE: The packed sediment in each tube should not exceed 5 mL; if so, add water and distribute evenly among additional tubes, repeat centrifugation and decant supernatant.

3.3 Further concentration of eggs by floatation

- Add $MgSO_4$ solution to each tube to 30-40 mL mark, add cap and place on a platform shaker in slanted position. Secure each tube and shake for 18 min (0.3 h) at 250 rpm.
- Un-screw the cap and rinse the residue on the inside back to the tube by directing a stream of $MgSO_4$ from a wash bottle. Mix the contents with a glass rod, rinse the rod while adding additional $MgSO_4$ solution to bring the volume to about 50 mL, adjust the weight of each tube if necessary, and centrifuge tubes for 5 min at setting 6.
- After centrifuge has come to a complete stop (without use of brake), pour the top 25 to 35 mL of supernatant from each tube through a 20 µm sieve over the 400 mL beaker.
- Using the PBW wash bottle, wash the excess floatation liquid and fine particles through the sieve. Make sure that the sediment is thoroughly rinsed, i.e. there is no $MgSO_4$ solution residue left.
- Rinse the sediment collected on the sieve into another 400 mL beaker by directing a stream of PBW from a wash bottle and then from a spray bottle onto the surface of the inverted sieve.
- Repeat the floatation procedure by adding more $MgSO_4$ solution to the sediment in tubes and processing the sample as above. The shaking time can be reduced to 12 min (0.2 h).

3.4 Final centrifugation

- After thoroughly washing the sediment from the sieve, transfer the suspension to the required number of 15 mL centrifuge tubes, taking care to rinse the beaker into the tubes. Make sure to adjust the weight of each tube so that the difference among the tubes does not exceed 0.1 g.

- Centrifuge the tubes for 3 min at setting 6, then discard supernatant to a 400 mL Erlenmeyer flask.
- If more than two tubes have been used for the sample, distribute the sediment between two tubes. Mix the sediment by adding 2-3 mL of culture fluid (0.5% formalin) and placing tube on a Vortex mixer. Make necessary transfers remembering to rinse the residue with culture fluid to the tube(s) to be further used. Fill with culture fluid to 15 mL mark, adjust the weight if necessary, and repeat the centrifugation.
- Centrifuge for 3 minutes at setting 6, then pour off supernatant to the Erlenmeyer flask. Autoclave the flask to destroy the ova that could remain in the discarded supernatant. The presence of the ova is possible but the number of them eventually lost rather insignificant.

3.5 Viability test

- Add a few drops of culture fluid to sediment, mix on a Vortex mixer, and transfer to culture dish. Rinse tube with culture fluid into culture dish. Add culture fluid, if needed, to have fluid completely cover bottom of dish (a depth of 3-4 mm, or about 30 mL). Place a sheet of Parafilm* over Petri dish bottom to seal, then cover with Petri dish top.
- Store culture in dark or subdued light at 25-28°C. Dishes should be agitated every few days to mix sediment and aerate fluid.
- After a minimum of 28 d, the cultured sediment is transferred to one or more 15 mL centrifuge tubes, depending on the volume of the sample, by placing a small glass funnel into the tube and rinsing the suspension into the tube.
- Centrifuge for 3 min at setting 6, then pour off supernatant to the Erlenmeyer flask.
- Add 8-10 mL of 5% commercial bleach to the sediment and mix thoroughly. Allow to stand for 5 min mixing occasionally (this decolorizes the outer shell of the eggs of *Ascaris* and permits the contents of the eggs to be observed more easily).
- Centrifuge for 3 min at setting 6, then discard supernatant.
- Add 10-15 mL of culture fluid, mix, and centrifuge again. Discard supernatant.
- With Pasteur type pipette, mix sediment with the small amount of water remaining, immediately remove and transfer a portion of sediment to a microscope slide, and cover with an appropriate size of cover glass (fluid should not extend beyond edges of cover glass; if this happens observe for presence of ova in that part of the slide too).
- Systematically examine each preparation under at least 100-x magnification of the microscope. Be sure to look for eggs in any liquid around the edge of the cover glass. If necessary, check identity of objects under 400-x magnification.
- Count all of each type of parasitic helminth eggs present (*Ascaris*, *Trichuris*, *Toxocara*, etc.) and record whether they contain a developed, motile larvae (viable egg), or are underdeveloped (dead or non-viable egg).

3.6 Calculation

- Report the numbers of viable eggs present on a dry weight basis, i.e. number of eggs per gram (or kg) of solids (TS- total solids, or TSS- total suspended solids).

(Some of the unfertile eggs inoculated into sludge disappear, presumably due to cell lysis and breakdown by the indigenous sludge microorganisms. Another reason is that these eggs are usually heavier than the fertile eggs and cannot be included in the count using the floatation method).

3.7 References

- Little M. D. [1999]. Procedure for determination of helminth eggs in sewage sludge. Tulane University School of Public Health and Tropical Medicine, New Orleans, LA, U.S.A.
- Yanko W. [1987]. Analytical method for viable helminth ova. The method referenced in Part 503 Regulation by the U.S. EPA.