Alkaline Inactivation of Microbial Indicator Species in Digested and Dewatered Biosolids

BY

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A Thesis
Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements for the Degree
of

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ABSTRACT

The purpose of this research was to further the knowledge on the disinfection of digested and dewatered biosolids by investigating the disinfectant power of various alkaline substances and by monitoring various microbial indicator species. The research was conducted in three separate studies. The first study, built upon previous bench-scale testing at the University of Manitoba, was based on full-scale testing, while the other two studies were at a bench-scale level.

Full-scale anoxic disinfection of dewatered and digested biosolids from Winnipeg, Manitoba, using low lime doses and lagoon fly ash was investigated to determine if it was feasible to produce a class A product. Lime doses of 50g, 100g, and 200g per kg of biosolids (dry) were used along with fly ash doses of 500g, 1000g, and 1500g per kg of biosolids (dry). This mixed product was buried in eight-10 cubic metre trenches at the West End Water Pollution Control Centre in Winnipeg. The trenches were backfilled with dirt and tarped to simulate anoxic conditions. Sampling cages were packed with the mixed product and pathogens that were non-indigenous to Winnipeg's biosolids. The non-indigenous pathogens spiked in the laboratory were the helminth *Ascaris suum* and the enteric virus reovirus. The cages were buried amongst the mixed product in the trenches.

The samples were removed at days 12, 40, 69, 291, and 356 and were tested for the presence of fecal coliform, *Clostridium perfringens* spores, *Ascaris suum* eggs, and reovirus. The pH, total solids, and free ammonia content of the mixed product were also determined for each sample.

The anoxic low lime dose (ALLD) process was effective at inactivating fecal coliform and reovirus to class A levels in 12 days. Lime doses as low as 50g CaO/kg TS (dry biosolids) in combination with fly ash were effective. A 5-log reduction in *C. perfringens* spores was achieved when the lime treated biosolids – with lime doses as low as 50g/kg TS - was amended with fly ash at doses as low as 500g fly ash per kg biosolids (dry). Lime doses as low as 50g lime – amended with 1000g fly ash - per kg biosolids (dry) were effective in reducing *Ascaris suum* eggs to class A levels after 291 days of anoxic storage.

The second study investigated the ability of a proprietary alkaline material (PAM) both alone and in combination with lime, to disinfect both digested-dewatered and undigested-dewatered biosolids in accordance with the US EPA's rule 503 alternative 2 (US EPA, 1993). The PAM is a by-product of producing slag from stainless steel, while the dewatered-digested and dewatered-undigested biosolids were obtained from Winnipeg and Oklahoma City wastewater treatment facilities, respectively. The investigation first determined if the disinfectant ability of the PAM improved (increased pH) when moisture was added to it. Moisture contents of 12, 15, 20, and 25 percent were tested and it was determined that additional moisture had little effect on the final pH once the PAM was mixed with the biosolids. The dry PAM was then used for the rest of the study.

Treatments involved lime alone, lime in combination with PAM, and PAM alone. Doses were initially based on previous bench scale tests, while subsequent treatments were based on trial and error. Mixing an appropriate dose of lime and/or the PAM with biosolids in a Hobart mixer created each treatment. The mixed product

was stored in 2.5L sealed buckets. The class A treatments were stored in 2.5L insulated buckets in a temperature control chamber set at 35°C. The temperature and pH of the biosolids were monitored throughout, while the initial and final fecal coliform concentrations were recorded for treatments that successfully produced a class A or B product.

Lime doses as low as 80g and 115g per kg of dry digested and undigested biosolids, respectively, were able to produce a class B product in accordance with the US EPA's time-pH criteria. PAM doses of 1200g and 1600g per kg of digested and undigested biosolids, respectively, were able to lower the amounts of lime required to produce a class B product to 60g and 80g per kg digested and undigested biosolids, respectively. The PAM alone was not able to meet the time-pH criteria to produce a class B product. Lime doses of 500g and 600g per kg of digested and undigested biosolids, respectively, were needed to elevate the temperature to that required to produce a class A product, but maintaining the temperature was dependant on insulation. The PAM was not able to reduce the amount of lime required to produce a class A product.

The final phase of this research involved a study on indicators of enteric virus contamination. The resistance of reovirus to inactivation by mesophillic and thermophillic temperatures was investigated and compared to existing inactivation patterns for poliovirus, the most commonly used enteric virus indicator. The resistance of reovirus to alkaline condition resulting from the addition of quicklime (CaO) to biosolids was also examined in time phases. Phase one involved the incubation of reovirus in a buffered solution for a 28-day period at different

temperatures ranging from 4°C to 50°C. This phase also involved reovirus incubation at thermophillic temperatures ranging from 50°C to 55°C for a 30-minute period. The second phase looked at reovirus survival in biosolids mixed with lime doses ranging from 0 to 100g lime per kg dry biosolids over a 23-day period.

Reovirus was more resistant to inactivation in long-term storage under mesophillic temperature conditions than poliovirus. Reovirus also was more resistant to inactivation caused by short-term storage under thermophillic temperature conditions than poliovirus. Treatment doses of 80g lime per kg of dry biosolids and greater were able to achieve class A levels for enteric viruses over the 23 day storage period.

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NOMENCLATURE AND ABBREVIATIONS

ALLD = alkaline low lime dose

CaO = quick lime, calcium oxide

CEC = Council of European Communities

CFU = colony forming units

DD = dewatered and digested

DU = dewatered and undigested

FA = fly ash

L = lime

MC = moisture content

MPN = most probable number

NEWPCC = North End Water Pollution Control Centre

NH_3^F = fraction of free ammonia

NH_3^C = concentration of free ammonia

PAM = proprietary alkaline material

PFRP = process that further reduces pathogens

PFU = plaque forming unit

PSRP = process that significantly reduces pathogens

TAN = total ammonia nitrogen

TS = total solids

US EPA = United States Environmental Protection Agency

WEWPCC = West End Water Pollution Control Centre

1.0 INTRODUCTION

Sewage sludge is the partial solid, partial liquid residual by-product of wastewater treatment and is referred to as biosolids when it has the potential for beneficial use. There are a variety of existing process trains for sludge treatment and disposal, encompassing a wide range of costs and benefits. The purpose of the treatment stage is to stabilize and disinfect the sludge, while the disposal option is usually a factor of geographic location, existing regulations, and public awareness.

Sewage sludge treatment and disposal is an extremely important part of wastewater treatment as it accounts for approximately half of the costs that a wastewater treatment facility must bear. The cost has the potential to escalate as effluent qualities are made more stringent by regulating authorities. With the elimination of ocean disposal, heightened environmental concerns regarding incineration, and increasing difficulty in citing landfills, turning sludge into a useful product has become of greater importance.

1.1 Land Application of Biosolids

The nutrients available in the biosolids make it a valuable resource. When biosolids are land-applied and incorporated into the soil they can act as a fertilizer and soil conditioner. The fertilizing ability comes from the nitrogen and phosphorus content. Biosolids typically contain 4% and 2.5% nitrogen and phosphorus, respectively, on a dry weight basis, with approximately 50% of this being available the year the biosolids are applied (Black *et al.*, 1984). The biosolids' ability as a soil

conditioner can be attributed to the organic matter it contains (Black *et al.*, 1984). The increase in organic matter increases the soil's ability to retain water.

1.2 Risks Associated with Land Application

Land application of biosolids is not always the win-win situation that has been depicted thus far; it is also accompanied by risks to human health. These risks come in the form of toxic metals and disease-carrying microorganisms, known as pathogens.

1.21 Toxic Metals

Monitoring the metal concentration of land-applied biosolids is very important as the accumulation of metals can render the soil unproductive. Metals are usually of concern when there are industries discharging into the sewer system. Wastewater treatment does remove a high percentage of metals that end up in the bisolids, while chemicals can be added to precipitate out metals, as they are only toxic when they are soluble (Metcalf and Eddy, 1991; Rabosky and Banerjee, 1992). Generally, high metal concentrations are targeted at the source. Legislation governs what can be discharged to the sewer. Some metals are also immobilized by the soil and the sludge through an absorptive matrix of complex hydrous ions and manganese-oxides coated with phosphate that tie up the contaminants and render them unavailable to plants (Renner, 2000).

1.22 Pathogens

The major pathogens source in sewage sludge is fecal matter from humans (Black *et al.*, 1984). The three major categories of organisms found in sewage sludge responsible for spreading diseases are bacteria, viruses, and parasitess.

1.221 Bacteria

Pathogenic bacteria in wastewater is estimated by monitoring for the presence of indicator species. The most commonly used indicator species are fecal coliform and *Salmonella* bacteria. Fecal coliform bacteria are defined as all coliform bacteria that can grow at 44.5 ± 0.5 °C, while total coliform bacteria are all aerobic and facultative anaerobic, gram-negative, non-spore-forming, rod-shaped bacteria that can ferment lactose with gas formation with in 48 hours at 35°C (APHA *et al.*, 1998). Fecal coliforms are from the family *Enterobacteriaceae* and include the bacteria species *Escherichia coli*, *Klebsiella, Enterobacter,* and *Citrobacter* (APHA *et al.*, 1998). *E. coli* is found in the gut of humans and is responsible for causing diarrhoea in children and gastroenteritis and urinary tract infections in adults (Black *et al.*, 1984).

Salmonella is used to indicate fecal contamination because they are also commonly found at high concentrations in wastewater. They are a rod shaped, gram-negative pathogenic bacteria responsible for typhoid and enteric fever in humans (Burnett and Schuster, 1973).

1.222 Viruses

High concentrations of a wide range of enteric viruses can be found in sewage sludge since viruses tend to combine with sludge solids during wastewater treatment (Sano *et al.*, 2001). Pathogenic viruses that may be found in sewage sludge include poliovirus, caxsackie virus, echovirus, and enterovirus from the family *Picornaviridae*, as well as viruses from the families *Asteroviruses, Caliciviruses, and Reoviridae* (Sano *et al.*, 2001; Cann, 1998). Viruses tend to be more resistant to inactivation than fecal coliforms. Black *et al.* (1984) claim that while viruses are for the most part not affected by mesophillic digestion, heat treatment and composting can effectively inactivate them.

The concentration of enteric viruses is one of the more difficult regulations to monitor. Enteric viruses are often concentrated in sewage sludge because they have a tendency to combine with sludge solids (Sano *et al.*, 2001). It is because of this tendency that they are difficult to enumerate (Hurst *et al.*, 1978). Spiking (the process of adding predefined quantities of a specific microorganism prior to testing inactivation conditions) is often necessary to show a reduction of enteric viruses. Poliovirus is the virus that is most often spiked into biosolids and used as an indicator (US EPA, 1992). A problem with using poliovirus as an indicator is that it is highly pathogenic to humans.

1.223 Helminths

Helminths refer to parasitic intestinal worms and can be divided into two groups. One group is the nematodes, or round worms, and the second group is the

cestodes, or tape worms. The helminth that is often regarded as the best indicator of human parasite contamination is the nematode *Ascaris lumbricoides.* *Ascaris lumbricoides* is an excellent indicator because its eggs are extremely resistant (Black *et al.*, 1984). Another *Ascaris* species that is often used as an indicator of disinfection is the pig parasite *Ascaris suum.* There has been no morphological or physiological difference observed between the eggs of *Ascaris lumbricoides* and *Ascaris suum*, but because *Ascaris suum* is not a human parasite it is safer to work with (Nelson and Darby, 2001).

1.3 US EPA Regulations

There are currently no regulations in Canada concerning the pathogen content of land-applied biosolids, but it is anticipated that they will be implemented in the near future. It is also expected that the regulation will resemble those implemented in the United States by the US EPA. The US EPA classifies biosolids as either a class A or class B product based on the pathogen content and the processes used to treat the biosolids.

1.31 Class B Biosolids

A biosolids product is considered class B when the fecal coliform bacteria content is less than $2x10^6$ most probable number (MPN) per gram of total solids (TS) in the sludge. The US EPA (1993) has also designated processes that significantly reduce pathogens (PSRP) as processes that can produce a class B product. PSRP include anaerobic (mesophillic) and aerobic digestion, air-drying, composting, and

lime treatment. The mean cell residence times and temperatures for anaerobic and aerobic digestion are 15 days at 35°C (or 60 days at 20°C) and 40 days at 20°C (or 60 days at 15°C), respectively. The minimum duration for air drying biosolids is three months, at which two of those three months the temperature must be greater than 0°C. Composting can occur using either the within-vessel, static aerated pile, or windrow method as long as the temperature is greater than 40°C for five days and is greater than 55°C for four hours during the five-day period. In lime treatment the pH must be maintained at or above 12 for two hours.

Land that has had class B biosolids applied to it is limited in its use as there may still be pathogens present. There are restriction as to the time after application that food crops or turf can be harvested, animals are allowed to graze, or the public is permitted access (US EPA, 1993).

1.32 Class A Biosolids

Class A biosolids are not limited in their use. They can be land-applied as a fertilizer or soil conditioner without any restrictions. They can also be sold for profit, as is the case with Miligoranite™, a popular organic fertilizer that is essentially treated Milwaukee sludge. Processes that can produce a class A product are referred to as processes that further reduce pathogens (PFRP). The six alternatives that can produce a class A product are summarized in the Table 1 below.

Table 1: Treatment alternatives capable of producing a class A biosolids product (US EPA, 1993).

Alternative	Treatment	Requirements
1	Thermal	a) D = 131,700,000/10 EE 0.14t, for all biosolids except those covered by b) b) D = 50,070,000/10 EE 0.14t, biosolids with < 7% TS, t > 50%, and D > 30 min where D = time (days), t = temperature (°C), TS = total solids - fecal coliform bacteria < 1000 MPN per gram total solids (dry) or *salmonella* bacteria < 3 MPN per 4 grams of total solids (dry)
2	High pH	- pH > 12 for 72 hours - temperature > 52°C for 12 hours while the pH is > 12 - air dry to a solids content of > 50% after the 72 hour period of pH > 12 - fecal coliform bacteria < 1000 MPN per gram total solids (dry) or *salmonella* bacteria < 3 MPN per 4 grams of total solids (dry)
3	Known Process	- demonstrate the presence of viruses and helminth ova prior to treatment - after treatment: - enteric viruses reduced to < 1 plaque-forming unit (PFU) per 4 grams TS - helminths reduced to < 1 viable helminth per 4 grams of total solids - fecal coliform bacteria < 1000 MPN per gram total solids (dry) or *salmonella* bacteria < 3 MPN per 4 grams of total solids (dry)
4	Unknown Process	- the same pathogen requirements as alternative three must be met for each batch of biosolids that is used or disposed of - fecal coliform bacteria < 1000 MPN per gram total solids (dry) or *salmonella* bacteria < 3 MPN per 4 grams of total solids (dry)
5	Processes that Further Reduce Pathogens (PFRP)	- see Table 2 - fecal coliform bacteria < 1000 MPN per gram total solids (dry) or *salmonella* bacteria < 3 MPN per 4 grams of total solids (dry)
6	Equivalent PFRP	- processes determined to be equivalent to a PFRP by the permitting authority - fecal coliform bacteria < 1000 MPN per gram total solids (dry) or *salmonella* bacteria < 3 MPN per 4 grams of total solids (dry)

Table 2: Processes that further reduce pathogens (PFRP) (US EPA, 1993).

Treatment	Requirements
Composting	a) within vessel or static aerated pile method - temperature > 55°C for 3 days b) windrow method - temperature > 55°C for 15 days and turned five times
Heat Drying	- direct or indirect contact with hot gases - moisture content reduced to 10% or lower - temperature > 80°C
Heat Treatment	- liquid biosolids heated at 180°C for 30 minutes
Thermophilic Aerobic Digestion	- liquid biosolids are aerated and heated at a temperature > 55°C
Beta Ray Irradiation	- irradiation with beta rays at dosages of at 1.0 megarad - temperature = room temperature
Gamma Ray Irradiation	- irradiation with gamma rays - temperature = room temperature
Pasteurization	- temperature > 70°C for at least 30 minutes

1.4 Alkaline Disinfection

While there are numerous options available that can produce a class A product, as demonstrated in Table 2, the focus of this paper will be on producing a class A product using alkaline treatment.

1.41 Lime

The material most often associated with alkaline treatment is quicklime, or CaO. Currently this method is recognised by the US EPA as a process that significantly reduces pathogens to class B levels. The lime dose that is generally accepted as the disinfecting dose is 150 to 250 grams of lime per kilogram of biosolids on a dry basis (Lue-Hing et al, 1998). Lime works as a disinfectant by increasing the temperature, pH, and free ammonia content of the biosolids. Quicklime reacts with the water in the sludge to produce heat (Equation 1).

$$CaO + H_2O \Leftrightarrow Ca(OH)_2 + 65.2kJ \text{ (heat)} \quad (1)$$

The majority of pathogenic organisms are mesophiles, as the ultimate temperature for their growth is the 37°C that persists inside the human body. The optimal temperature for mesophile growth ranges from 20-45°C (Prescott *et al.*, 1996). Temperatures greater than the upper range can result in the denaturing of the enzymes, transport carriers, and other proteins, as well as the disruption of microbial membranes (Prescott *et al.*, 1996). Organisms will persist in a dormant form when the temperature is below their lower limit for growth (Prescott *et al.*, 1996).

The increase in pH is a result of calcium hydroxide dissociating into calcium ions and hydroxyl ions (Equation 2).

$$Ca(OH)_2 \Leftrightarrow Ca^{2+} + 2OH^- \quad (2)$$

An increase in pH can reduce nutrient availability to the organism, disrupt the plasma membrane and inhibit enzyme and membrane transport protein activity (Prescott *et al*, 1996).

The free ammonia content of the sludge is increased with the addition of lime because of the increase in hydroxyl ions (Equation 3).

$$NH^{4+} + OH^- \Leftrightarrow NH_3 + H_2O \quad (3)$$

Both ammonia ion and free ammonia are already present in sludge, but an increase of hydroxyl increases the production of free ammonia. Free ammonia is considered to be toxic to most microbial organisms. It can diffuse through the membrane and upset the internal pH balance thereby forcing the cell to draw in protons from outside the cell to balance the pH. This creates a positive charge in the cell, which is countered by the release of potassium ions. This results in the death of the cell (Lui *et al*, 2000).

The addition of quicklime to biosolids has other benefits besides disinfection. When lime is added to the biosolids the resulting chemical reaction increases the bearing capacity of the biosolids (Roediger, 1987). An increase in bearing capacity – the ability to withstand stress - is beneficial when utilizing the limed biosolids for landfill cover. Advantages of using a limed biosolids product for landfill cover is the fixing of heavy metals and decrease in total porosity (Roediger, 1987; Smith *et al.*, 1998). Smith *et al.* (1998) also concluded that an increase in the lime dose improved the density and physical handling characteristics of the biosolids.

1.42 Other Alkaline Products

Other alkaline materials have been used to disinfect sludge. These products include fly ash, cement kiln dust, lime kiln dust, and Portland cement. Fly ash is an alkaline coal combustion waste product and is another waste product that can be put to good use. The alkalinity in fly ash is attributed to calcium, sodium, and magnesium (Bilinski et al, 1995). According to Adriano *et al.* (1980) the elements contained in the ash can be beneficial to the soil when the biosolids mixture is land-

applied because they can provide elements that are lacking in the soil. El-Mogazi *et al.* (1988) also cite lower bulk density, increased water holding capacities, and lower hydraulic conductivities as additional advantages of adding fly ash to the soil.

The N-Viro process is a patented process that involves the use of cement kiln dust, lime kiln dust, and quicklime (Burham *et al.*, 1991). The process that uses cement kiln dust alone is classified as a PSRP, while the other processes that involve the use of cement kiln dust, lime kiln dust, and quicklime with or without additional heat are classified as PFRP (MacConnell *et al.*, 1992).

Another patented process called the Chemfix process involves the use of Portland cement (Reimer *et al.*, 1991). Portland cement is a sodium silicate settling agent that is mixed with the sludge resulting in a process can be classified as either a PSRP or a PFRP (MacConnell *et al.*, 1992)

2.0 BACKGROUND

2.1 Biosolids Disposal in Winnipeg

The source of the land-applied biosolids in Winnipeg is the North End Water Pollution Control Centre (NEWPCC). The NEWPCC provides treatment of primary and waste activated sludge produced at Winnipeg's three. The method of treatment involves mesophillic anaerobic digestion followed by dewatering by centrifugation. The total solids content of the biosolids after centrifugation must be at least 20 percent, but is often greater depending on the season (Manitoba Conservation, 2000). Most of the biosolids are disposed of onto agricultural lands throughout rural Manitoba under the WINGRO program. Some of the biosolids are also co-disposed with municipal solid waste at the Brady Road Landfill.

2.11 WINGRO Program

The WINGRO program handles 10,500 metric tonnes of dry biosolids (based on a average total solids content of 25%) annually (City of Winnipeg, 2002). The majority of the biosolids are deposited on farmland at no cost to the farmers. Any remaining biosolids are deposited at the City of Winnipeg's Brady landfill. Winnipeg's biosolids act as a good fertilizer due to the nitrogen and phosphorus content and the valuable nutrients they contain (City of Winnipeg, 1990). One application can provide sufficient nutrients to last up to four years (City of Winnipeg, 1990). The biosolids can also act as a soil conditioner because of their ability to retain water (City of Winnipeg, 1990).

The WINGRO program is regulated under the Manitoba Environment Act and therefore the program must operate in accordance with the licence conditions. The licence allows the disposal of 56 tonnes of biosolids on a dry weight basis per hectare (Manitoba Conservation, 2000). The biosolids must also be incorporated into the land as soon as it is practicable and a crop other than a fruit or vegetable must be planted by the start of the next growing season to utilize the available phosphorus and nitrogen (Manitoba Conservation, 2000).

2.12 Metal Content

Under existing licence conditions, the metal concentration in Winnipeg's sludge is considered a non-issue. As can be seen in Tables 3 and 4, the metal concentrations in Winnipeg's sludge is far below the ceiling limits set by the US EPA and the stricter standards set by the Council of European Communities (CEC). Pathogen content is a major concern when it comes to Winnipeg's sludge, although currently it is only monitored and not regulated.

Table 3: The metal concentrations in Winnipeg's sludge compared with the ceiling concentrations listed by the US EPA in 1992 for metal concentration in sludge applied to land (Ross and Hemphill, 1993).

Metal	EPA Ceiling Concentrations (mg/kg ds)	Winnipeg (mg/kg ds)
Arsenic	75	3.8
Cadmium	85	11
Chromium	3000	1049
Copper	4300	999
Lead	840	167
Mercury	57	1.9
Molybdenum	75	8.9
Nickel	420	41
Selenium	100	2.3
Zinc	7500	1230

Table 4: The metal concentrations in Winnipeg's sludge compared to the Council of European Communities (CEC) standards (Ross and Hemphill, 1993).

Metal	CEC Sludge Standards (mg/kg ds)	Winnipeg (mg/kg ds)
Cadmium	20-40	11
Copper	1000-1750	999
Nickel	300-400	41
Zinc	2500-4000	1230
Lead	750-1200	167
Mercury	16-25	1.9

If regulations resembling those imposed by the US EPA regarding pathogens in land-applied biosolids are imposed in Canada, the City of Winnipeg would have to reduce the pathogens in their biosolids if they want to continue with the WINGRO land application program. To adhere to the US EPA's class A criteria Winnipeg would have to reduce the fecal coliform bacteria in its biosolids from approximately 80 million MPN/g TS to less than 1000 MPN/g TS. They would also have to

demonstrate that if enteric viruses and helminths were persistent in the biosolids there levels could be reduced to less than 1 PFU/4g TS and less than 1 egg/4g TS, respectively.

2.2 Lime Treatment

Lime treatment is a low-tech, low-cost method that can potentially reduce the pathogen content in biosolids. Lime treatment can be added to existing wastewater treatment plants without major changes to the existing plants. The costs associated with lime treatment include installation and operation of mixing machines, from the purchase of the lime itself, and for odour control. The lime cost is dependent on the lime dose that can effectively reduce the pathogen content of the biosolids. As discussed previously, to produce a class B product the dose that is generally accepted ranges from 150-250 gram of lime per kilogram of total solids on a dry basis (Lue-Hing et al, 1998). Previous research at the University of Manitoba has demonstrated that significantly lower lime doses have the potential to produce a class A product when stored under the proper conditions.

2.21 Previous Research on Alkaline Disinfection

When temperature alone is the criteria used to determine whether a class A product is produced the accepted dose ranges from 200 to 500 grams lime per kilogram biosolids on a dry basis (Smith *et al.*, 1998). A lime dose in this range is able to satisfy Alternative 1 of the US EPA's Part 503 rule that states that a

temperature of 70°C would have to be maintained for 30 minutes to result in a class A product.

Bujoczek, Lui, and Oleszkiewicz (2000) completed a study at the University of Manitoba on the effects of alkaline low-lime doses (ALLD) on pathogen content. They used anaerobically digested and dewatered sludge from Winnipeg's NEWPCC. The sludge was maintained under anoxic conditions in buckets that ranged in temperature from 20-22°C. They determined that lime doses as low as 30g/kg TS could reduce fecal coliform bacteria and *Salmonella* to class A levels. It was concluded that the anoxic storage conditions enabled the high pH to be maintained, and as was discussed previously, a high pH is partially responsible for pathogen inactivation. This study also looked at the disinfection capabilities of fly ash. Fly ash is a waste product produced at Manitoba Hydro thermal generating stations in Brandon and Selkirk, MB. It was determined that while twenty times the amount of fly ash as lime was needed to have an equal effect on fecal coliform bacteria, there was a synergistic effect when fly ash and lime were combined (Bujoczek *et al.*, 2000). A higher pH was achieved when lime and fly ash were combined with the biosolids than when either of the components was used on their own.

Bujoczek (2001) completed another study involving the storage of digested, dewatered, and limed biosolids at 4-6°C under anoxic conditions. It was determined that the storage temperature does play a significant role in the mortality of fecal coliforms and *Salmonella*. A two to three log reduction of fecal coliforms was reported after nine months at 4-6°C, while class A levels were achieved after 83 days of storage at 20-22°C for non-limed, digested, and dewatered biosolids.

The study by Bujoczek (2001) also looked at the inactivation of *Ascaris suum* ova and *Clostridium perfreingens* spores along with fecal coliform bacteria. Complete inactivation of *C. perfringens* was achieved in ten to eleven weeks when a quicklime dose of 80g/kg biosolids (dry) was applied at 20-22°C. A one-log reduction in *Ascaris suum* egg was achieved after approximately nine months of storage at 4-6°C, although it is possible that this reduction was achieved in a shorter duration as *Ascaris suum* egg viability was only tested on the first day and then after nine months of storage. *Ascaris suum* egg inactivation was not studied at elevated temperatures.

Wong *et al.* (2001) also conducted a bench scale study on the disinfection capabilities of low lime doses when combined with coal fly ash collected from the China Light and Power Co. Ltd. It was determined in this study that a dose of 85 grams of lime ammended with 100 grams of fly ash per kilogram of dried biosolids was necessary to reduce fecal coliforms and *Salmonella* bacteria to class A levels. This dose is greater than that found at the University of Manitoba by Bujoczek *et al.* (2000). The difference could be the storage conditions employed at the University of Manitoba. Wong *et al.* (2001) also concluded that due to the high population of surviving indigenous bacterial flora in the limed biosolids, it would be suitable for land application as this characteristic is necessary for sustaining biological activity in the soil.

The previous research demonstrated the potential for low lime doses to result in a class A product. It would be beneficial to perform the tests at full-scale and subject to naturally-occurring environmental conditions, as there is concern that a

large scale mixer would not be able provide the intimate mixing provided by the small scale mixer used during the bench scale testing. Intimate mixing is important as it eliminates the potential for pockets of low pH to be created in the biosolids.

It has already been determined that low lime doses can reduce fecal coliform and *Salmonella* bacteria to class A levels. According to the US EPA's Part 503 rule, class A criteria must also be demonstrated for enteric viruses and helminths. While *Ascaris* ova survival under alkaline conditions has been investigated and has been discussed in the previous section, in general, very little research has been completed on the effect of alkaline conditions on enteric viruses.

2.3 Previous Research on Enteric Virus Inactivation

The available information primarily focuses on the inactivation of the pathogenic poliovirus under a variety of temperature conditions. Previous research by Ward *et al* (1976) demonstrated the resistance of poliovirus type-1 strain CHAT to a variety of temperatures when contained in various media. Poliovirus experienced a 3.5-log reduction in 200 minutes at 43°C, a 5-log reduction in 50 minutes at 47°C, and a 4.5-log reduction in 20 minutes at 51°C when in phosphate-buffered saline. Poliovirus behaved similarly in digested sludge but was protected against heat in the raw sludge. The reductions in raw sludge were less than 1-log after 200 minutes at 43°C, 1-log after 50 minutes at 47°C, and 4-log after 50 minutes at 51°C. Yeager and O'Brien (1979) determined the effect of lower temperatures on the inactivation of polioviruses type 1 present in sandy soils saturated with wastewater. They found that poliovirus persisted for 180, 36, and 11 days at 4°C, 22°C, and 37°C respectively.

An inherently serious problem with using poliovirus in such studies is that it is a human pathogen. Thus, its use is expected to be severely limited in the near future. It would be beneficial to use a non-pathogenic virus indicator that is equal to or greater than poliovirus in terms of resistance to inactivation.

Reovirus is an enteric virus that is very environmentally stable and is not a human pathogen. Reovirus belongs to the virus family *Reoviridae* (genus Orthoreovirus). While reovirus is not a human pathogen, many species of the family Reoviridae (ex. rotavirus and coltivirus) are pathogenic in humans. Reovirus is highly resistant to common disinfectants such as Lysol and formalin and can persist for long periods of time at or below room temperature as aerosols (Coombs, 2002).

OBJECTIVES

- Determine if low lime doses – alone, and in combination with fly ash - reduces the pathogen content of digested and dewatered biosolids to class A levels when mixed at full-scale and stored under naturally-occurring environmental conditions.

- Determine if there is any correlation in the inactivation patterns of *Ascaris suum* eggs and spores of the thermophillic bacteria *Clostridium perfringens* when subjected to alkaline conditions.

- Determine if a proprietary alkaline material (PAM) – alone and in combination with lime – produces class A and class B products in accordance with US EPA's pH-temperature criteria outlined in rule 503 (US EPA, 1993).

- Investigate if reovirus is an effective indicator of enteric virus contamination by comparing the resistance of reovirus to temperature and lime induced pH/ammonia conditions to that of poliovirus – the most commonly used indicator of enteric virus contamination .

4.0 METHODOLOGY

4.1 Full-scale Biosolids Disinfection with Lime and Fly Ash

The study was preformed in part at the West End Water Pollution Control Centre (WEWPCC) in Winnipeg, Manitoba, and partially at the University of Manitoba's Environmental Engineering Laboratories. Eight large-scale treatments were selected based on the results from bench-scale tests. The treatments were expressed as grams of lime (L) and/or fly ash (FA) per kilogram of dry biosolids. The treatments were as follows: Control (biosolids only), L200, L100, FA1500, L50 + FA500, L100 + FA500, L50 + FA1000, and L10 + FA1000.

4.11 Laboratory Preparation

Prior to the start of full-scale mixing, packs of the indicator species not naturally occurring in Winnipeg's biosolids had to be prepared. These indicator species were *Ascaris* suum ova and the reovirus. Batches of biosolids for Ascaris spiking and reovirus spiking were prepared separately using the same four-step procedure outlined below.

1. Enough biosolids to make 8 packets (one extra to account for biosolids that would be lost on the mixing apparatus) and to fill 7 sentinel chambers were mixed using a standard Kitchen Aid mixer.
2. Approximately 800,000 *Ascaris suum* eggs were added per batch. The eggs were contained in a suspension of buffered dilution water. 1.5ml at 2.25 x 10^{11} Plaque Forming Units (PFU)/ml of the reovirus was provided for all eight

treatments and therefore 0.188ml was allocated for each batch. Mixing continued as the eggs or reovirus were added.

3. Appropriate lime and fly ash quantities were added according to the total solids content of the biosolids and the specific treatment being mixed.
4. Proper quantities of the mixed and spiked product were weighed and wrapped in garden cloth. Seven packets containing *Ascaris suum* ova and seven packets containing the reovirus were prepared for each treatment. Seven sentinel chambers where also packed with mixed biosolids spiked with *Ascaris suum* ova. The purpose of the sentinel chambers is to get a second set of data on the *Ascaris suum* egg viability. When the chambers were removed they were shipped to Cornell University in Ithaca, N.Y. for enumeration. The sentinel chambers and garden cloth packets can be seen in Figure 1.

Figure 1: Sentinel chambers containing *Ascaris suum* ova on the left and garden cloth packets containing reovirus on the right.

4.12 On-site Mixing and Sample Burial

A mixer provided by Lodige, a mixing company out of Germany, was set up on-site. The mixer, pictured in Figure 2, is designed to specifically mix sewage sludge with lime or fly ash. Eight trenches were dug with each trench being designed to hold one-half truck of biosolids (or $10m^3$). Biosolids were delivered to the site by the truckload as needed. The biosolids were trucked from the NEWPCC and the total solids were in the 28-30 percent range. The mixer was calibrated to mix the appropriate quantities of lime and or fly ash with the biosolids. As there was only one hopper for the biosolids and one hopper for the lime and fly ash, the treatments involving lime and fly ash had to be run through the mixer twice. This was accomplished by first mixing all the biosolids with the fly ash. The fly ash/biosolids mixture was then run through the mixer again, this time mixing it with lime. The fly ash was mixed first as it has a lesser effect than the lime in terms of disinfection.

Figure 2: The Lodige sludge mixer set up at the WEWPCC.

The mixed product was collected in bins as it came out of the mixer on a conveyor belt. The bins were transported and dumped in the trenches by a forklift as seen in Figure 3. Two packets (one *ascaris suum* and one reovirus) and one chamber were packed into a permeable cage containing the mixed product. The cages (seven per trench) were then placed in the trench already containing the mixed product, as seen in Figure 4. One cage per treatment contains a StowAway™ Data Logger to monitor the temperature. The Data Loggers were programmed to record the temperature every four hours for one year. They were required to be removed to recover that data at the end of the testing period.

Figure 3: The mixed product being dumped into the trench

Figure 4: Cages loaded with the mixed product and the *Ascaris suum* ova and reovirus packets are deposited into the trench partially filled with the mixed product.

The cages were then covered with more of the mixed product until the trench was full. A layer of dirt and a tarp was placed on top of the full trench to promote anoxic conditions and to prevent water from leaching through the biosolids. A finished trench can be seen in Figure 5.

Figure 5: Photo of a completed treatment. The trench has been filled with the mixed product, cages inserted, covered with dirt and tarped.

4.13 Sample Recovery

Samples were recovered on three occasions over the first 69 days of storage in the fall and then on days 291 and 356 in the spring and summer, respectively. The first set of samples was removed twelve days after that specific treatment had been buried. The second and third sampling periods corresponded with day 40 and day 69 respectively. Samples were recovered according to the procedure outlined below.

1. The tarp was pulled back to expose the top layer of dirt which, was then removed with a front-end loader.
2. The chain attached to the sampling cage was attached to the bucket of the front-end loader.
3. The bucket was lifted to remove the sampling cage from the trench.

4. The dirt was replaced and the tarp was pulled back over the trench and secured with sand bags.

4.14 Sample Analysis

The sample cages were transported to the Environmental Engineering Laboratories at the University of Manitoba for analysis. They were analysed for:

1. Fecal coliform content – The multiple-tube most probable number (MPN) procedure was followed as described by "Standard Methods for the Examination of Water and Wastewater" (APHA *et al.*, 1998).
2. *Cl. perfringen* spore content – This method was based on the "Standard Operating Procedure for Enumeration of Bacterial Spores of *Clostridium perfringens* in Wastewater and Wastewater Solids" (Bujoczek, 2001). This method can be seen in Appendix D.
3. *Ascaris suum* egg viability – This method was based on the "Standard Operating Procedure for the Enumeration of Helminth Eggs in Wastewater and Wastewater Solids" (Bujoczek, 2001). This method can be seen in Appendix D. Bujoczek's method is a modified version of the Tulane University method (Little, 1999). The sentinel chambers spiked with *Ascaris suum* ova were shipped to Dr. Dwight Bowman at Cornell University in Ithaca, New York for enumeration. The Tulane University method is also practiced at Cornell University. The sentinel chambers will act as a replication of the viability results determined at the University of Manitoba.

4. Reovirus enumeration – This method was preformed by Dr. Kevin Coombs of the Medical Microbiology Department at the Banatynne Campus of the University of Manitoba. The viruses were shipped to Dr. Coombs at the end of each sampling period.
5. Total Solids analysis – This method was performed using a 105°C drying oven.
6. pH analysis – determined using the Fisher Scientific Accumet 50 pH meter and following Method 4500-H^+ B in SM (APHA *et al.*, 1998)
7. Ammonia analysis - Total ammonia nitrogen was calculated using the Method 4500-NH_3 G in SM (APHA *et al.*, 1998). The concentration of free ammonia was calculated according to the sample equations seen below.

$$TAN = AA \times \left\{ \frac{9 + \left(\frac{100 - TS}{100} \right)}{\left(\frac{100 - TS}{100} \right)} \right\} \quad (4)$$

where: TAN = Total Ammonia Nitrogen (mg/L)
AA = Auto Analyzer value (mg/L)
TS = Total Solids (%)

$$NH_3^F = \frac{100}{1 + 10^{9.263 - pH}} \quad (5)$$

where: NH_3^F = Fraction of Free Ammonia (%)
pH = pH of the biosolids
9.263 = pK when temperature is 25°C

$$NH_3^C = TAN \times \frac{NH_3^F}{100} \quad (6)$$

where: NH_3^C = Concentration of Free Ammonia (mg/L)

8. Temperature analysis – StowAway™ Data Logger were used to record the temperature every four hours for the duration of the experiment.

4.2 Bench-scale Biosolids Disinfection with Lime and a PAM

This study was performed at the University of Manitoba's Environmental Engineering Laboratories. Two biosolids types were investigated. A dewatered and digested sludge with a total solids content of 31.5% was taken from the North End Water Pollution Control Centre (NEWPCC) in Winnipeg, MB. The second biosolids type was a dewatered and undigested sludge shipped from Oklahoma City, OK. The total solids content of the Oklahoma City sludge was 26.9%. The lime used in the study was Mississippi Quick Lime (CaO). The proprietary alkaline material used in this study is a by-product of producing stainless steel from slag. The resulting product is dried to a powder (99% TS) and has cementitious properties. It is currently used as an agricultural liming agent and fertilizer additive (Banerjee and Blumenschein, 2002). The PAM is largely made up of calcium compounds, silicon dioxide, and magnesium oxide and therefore is alkaline in nature (Banerjee and Blumenschein, 2002). When added to biosolids, disinfection occurs due to an increased in pH and the resulting increase in free ammonia production.

4.21 Optimal Moisture Determination

The first step was to determine the optimal moisture content for the PAM. This was done by adding deionised water to the PAM to increase the moisture content of varying treatments to 12, 15, 20, and 25 percent. The pH of these treatments was

taken at time zero and then after 24 hours. The highest two moisture contents (20% and 25%) were eliminated because there was no evident difference in pH between them and the 15% moisture content treatment.

The PAM of varying moisture contents (0%, 12%, and 15%) was then mixed with the biosolids to make up a total of six treatments. The treatments were as follows (in grams of dry PAM per kg of dry biosolids and where MC = moisture content of the PAM):

1. PAM 500 (MC = 0%)
2. PAM 500 (MC = 12%)
3. PAM 500 (MC = 15%)
4. PAM 1000 (MC = 0%)
5. PAM 1000 (MC = 12%)
6. PAM 1000 (MC = 15%)

The pH of these treatments was measured after 0, 12, 22, 48, and 77 hours.

4.22 Mixing and Monitoring Stage

The second part of the study used the optimal moisture content determined in Part One to create treatments in each of the following categories (DD = dewatered and digested, DU = dewatered and undigested):

1. DD Control

2. DD Lime (class A) – L250, L350, L500, L600
3. DD Lime (class B) – L80
4. DD PAM/lime (class A) – L500+PAM1000
5. DD PAM (class B) – PAM800, PAM2000
6. DD PAM/lime (class B) – L40+PAM600, L60+PAM1200
7. DU Control
8. DU Lime (class A) – L250, L350, L500, L600
9. DU Lime (class B) – L60, L80, L90, L115
10. DU PAM/lime (class A) – L500+PAM1000
11. DU PAM (class B) – PAM1500
12. DU PAM/lime (class B) – L60+PAM1200, L80+PAM1600

The initial lime doses were based on previous research performed at the University of Manitoba (Lui *et al*, 2000). The initial PAM doses were based on preliminary tests involving PAM, water, and biosolids. The results for the preliminary PAM testing can be seen in Table 7 of the Appendix. Additional doses were supplemented on a trial and error basis as necessary.

The alkaline products were mixed with the biosolids in a Hobart mixer. A photograph of the mixer can be seen in Figure 1. The class B treatments were mixed for 5 minutes and then stored under anoxic conditions in 2.5L buckets. The class A treatments were only mixed for 1 minute because heat loss can occur with excessive mixing (Lui *et al*, 2000). The initial treatments were then stored at room temperature

in insulated 2.5L buckets to prevent heat loss to the environment. Subsequent trails were stored in at 35°C in an environmental chamber and in insulated buckets to further prevent heat loss.

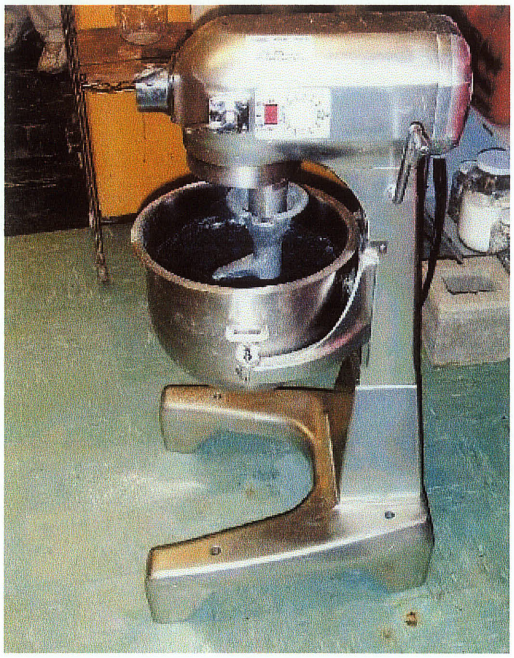

Figure 6: The Hobart mixer that was used to mix the biosolids with the lime and or PAM.

The pH of the mixed product was monitored as needed to ensure that time-pH regulations were met. The pH was determined using the Fisher Scientific Accumet 50 pH meter with a solids pH probe and following Method 4500-H^+ B in SM (APHA *et al.*, 1998). The temperature was monitored using StowAway Tidbit Temp

Loggers™, seen in Figure 2. The loggers were buried in the biosolids and programmed to record the temperature every 30 minutes.

Figure 7: StowAway Tidbit Temp Loggers™ that was buried in the biosolids to record temperature (Onset Computer Corporation, 1996).

The fecal coliform content of each treatment was determined immediately after mixing and after either a class A or B product was produced. Final fecal coliform content was not determined for the treatments that failed to meet the class A or B criteria. The multiple-tube most probable number method was used to estimate the fecal coliform concentration. It was performed according to "Standard Methods for the Examination of Water and Wastewater"(SM) (APHA *et al.*, 1998).

4.3 Bench-scale Reovirus Inactivation

This study was preformed in part at the University of Manitoba's Environmental Engineering Laboratories and partially at the University of Manitoba's Medical Microbiology Laboratories at the Banatynne Campus. The study was comprised of two phases. The first phase examined the effect of temperature on the inactivation of reovirus, while the second phase focuses on the ability of ALLD to inactivate reovirus.

4.31 Temperature Phase

The first stage of this phase of the study involved the incubation of reovirus in a buffered solution (2xSSC: 300mM NaCl, 30mM sodium citrate, pH 7.0) at a variety of temperatures for 28 days. The temperatures investigated included 22°C (control), 37°C, 39.5°C, 42°C, 45°C and 50°C. Percent survival of reovirus was determined by standard plaque assay; serial 1:10 dilutions of virus samples were made, adsorbed to L929 cell monolayers for 1 hour, and overlaid with agar/medium 199. Plates were incubated at 37°C for 3 days, fed with additional agar/199, and stained with 0.04% neutral red 6 days after adsorption to allow plaque enumeration the next day.

The second stage of this phase involved the incubation of reovirus in the same buffer at thermophillic temperatures of 50°C, 52.5°C, and 55°C. The incubation periods remained short, as percent survival was determined after 2, 5, 10, 15, and 30 minutes. Percent survival of reovirus was determined by plaque assay, as described above.

4.32 ALLD phase

The second phase of the study focused on the ability of the ALLD process to inactivate reovirus spiked into biosolids. Anaerobically digested and dewatered biosolids from Winnipeg's North End Water Pollution Control Centre (NEWPCC) were used, with a total solids content of 23%TS. The lime doses used were (expressed as grams lime (L) per kg of dry biosolids): Control (L0), L10, L20, L40, L80, and L100 g/kg TS. Reovirus had to be spiked into the biosolids, as it was not present at high enough concentrations in Winnipeg's biosolids to act as an appropriate indicator.

Spiking involved adding 0.0167ml of the purified virus to 175g of biosolids and mixing in a Kitchen Aid™ mixer for 5 minutes. The appropriate lime dose was then added and mixed for another 5 minutes. The final product was stored under anoxic conditions in sealed containers at 20-22°C. Treatments were prepared on days 0, 13, 16, 20, and 22 so that they were all ready to be processed at the same time. This translates into determining the percent of reovirus survival after 1, 3, 7, 10, and 23 days of storage.

Reovirus recovery from the biosolids involved placing approximately 10ml of the biosolids sample in a 50ml centrifuge tube with 8-10 sterile glass beads and 25ml of sterile water. The tubes were then placed on a vortex mixer to break up clumps before centrifuging at low speeds for 15 minutes to pellet large aggregates. A pipette was then used to remove 4ml of supernatant. This was then combined with 3.5 ml of Vertrel-XF® in a 15ml "snap cap" polypropylene tube and virus was extracted by

emulsification of the organic and aqueous phases (Mendez *et al.,* 2000). Extracted virus was then titered by plaque assay, as described above.

5.0 RESULTS AND DISCUSSION

5.1 Full-Scale ALLD Results

5.11 Total Solids

There was little change in the total solids of the treatments involving lime only or biosolids only, as can be seen in Figure 8. This indicates that the mixing was successful in equally distributing the lime throughout the biosolids. This also implies that anoxic conditions persisted in the trench as little to no drying occurred while the biosolids were in the trench. There are some inconsistencies with the treatments involving fly ash. This became evident after examining the biosolids product after mixing with the fly ash. Due to the granular nature of the fly ash some chunks remained visible following mixing, indicating that the fly ash was not evenly distributed throughout the biosolids. There was no lime visible in the biosolids after mixing.

5.12 pH and Ammonia

A general decrease in the pH of each treatment over time can be seen in Figure 9. This trend was not as obvious with the highest lime dose. In the bench-scale testing Bujoczek (2001) also observed a greater decrease in pH for lower lime doses. This decrease could be due to the bacterial action of resistant bacteria. Bacteria persisting in the biosolids can produce organic acids and carbon dioxide that would lead to the pH decrease (Bujoczek, 2001). This argument is supported by the presence of significant concentrations of *C. perfringen* spores in all the limed treatments where this gradual decrease occurred.

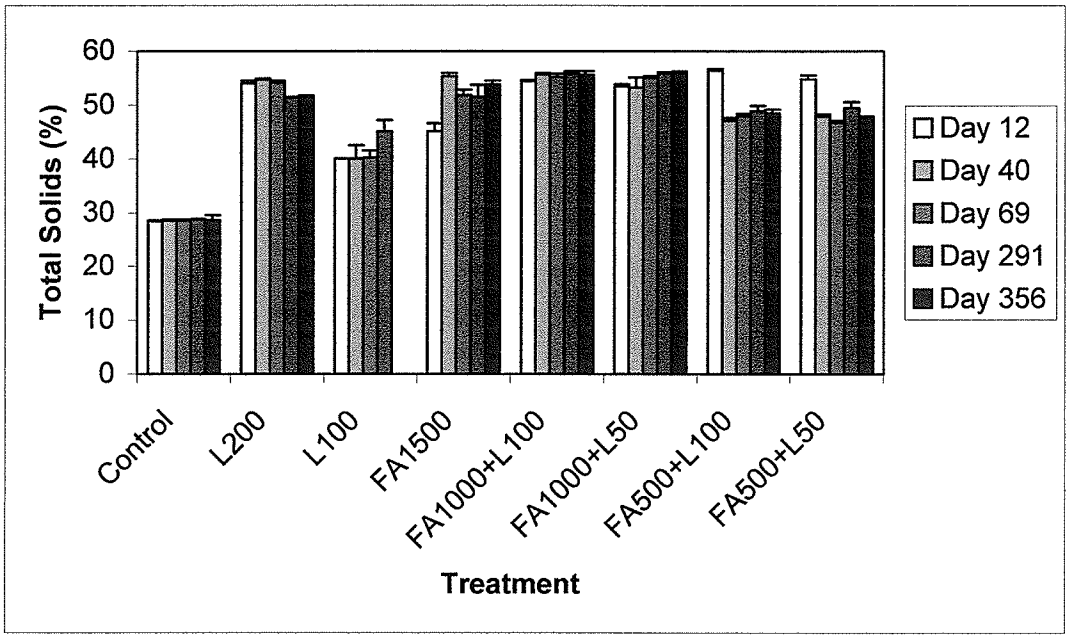

Figure 8: The variation in total solids for each treatment over 356 days.

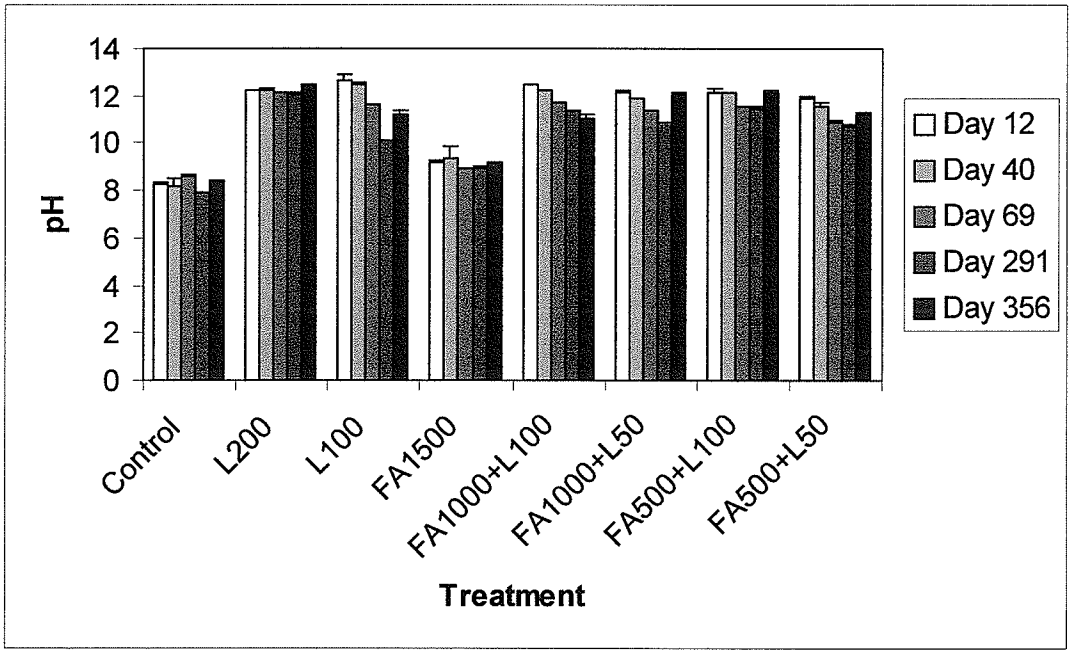

Figure 9: The variation in pH for each treatment over 356 days.

The decrease in free ammonia remaining over time is likely a result of volatilization. There was a slower decrease in free ammonia remaining in the L100 treatments that were mixed with fly ash then the L100 treatment without fly ash. This can be explained by the slow pH increase in fly ash treated biosolids over time, while limed biosolids experience a fast initial pH increase followed by a gradual decrease (Bujoczek *et al.*, 2000).

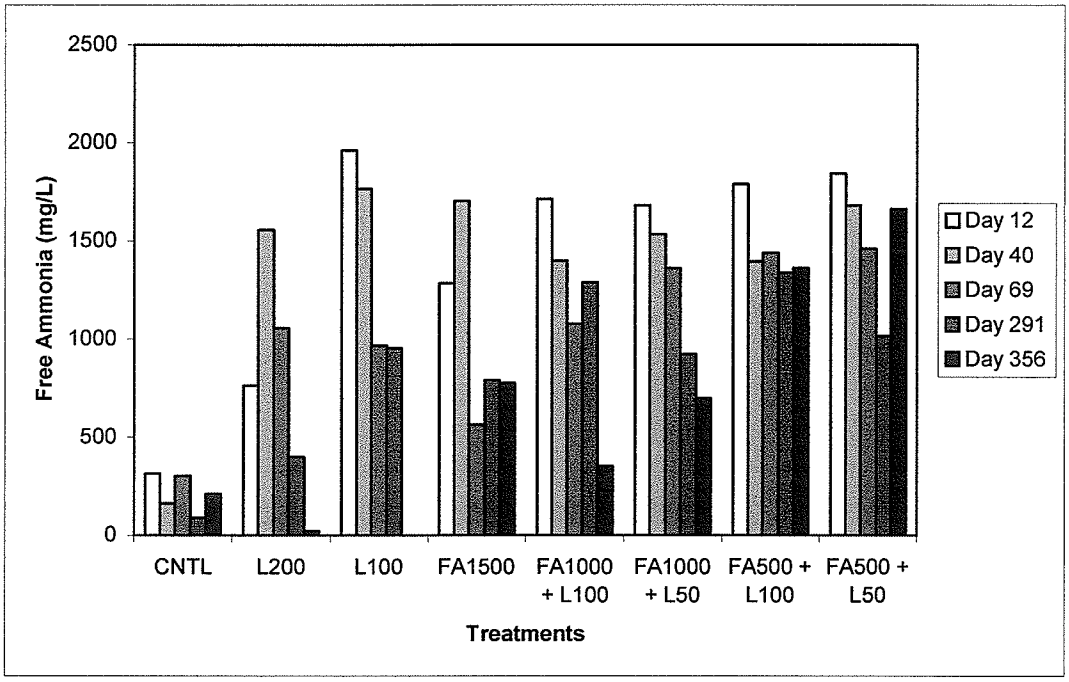

Figure 10: The variation in free ammonia over time for each treatment.

5.13 Fecal Coliform Bacteria and Reovirus Inactivation

Fecal coliform bacteria and reovirus were completely inactivated in all treatments involving lime by the first sampling period. Fecal coliform inactivation was consistent with the results obtained by Bujoczek (2001) in the bench-scale study.

In the bench scale study fecal coliform bacteria were reduced well below class A levels in 4 hours when lime doses of 30g/kg TS and greater.

Fecal coliform bacteria were no longer present in the fly ash treatment by the 69^{th} day, while reovirus persisted in this treatment for the entire duration of the study. Both fecal coliform bacteria and reovirus were present in the control treatment after 356 days of storage although there was a significant decline in fecal coliform concentration, while the reovirus remained relatively stable. This demonstrates that reovirus is more resistant than the fecal coliform bacteria to alkaline inactivation. Fecal coliform and reovirus log reductions can be seen in Figures 11 and 12, respectively.

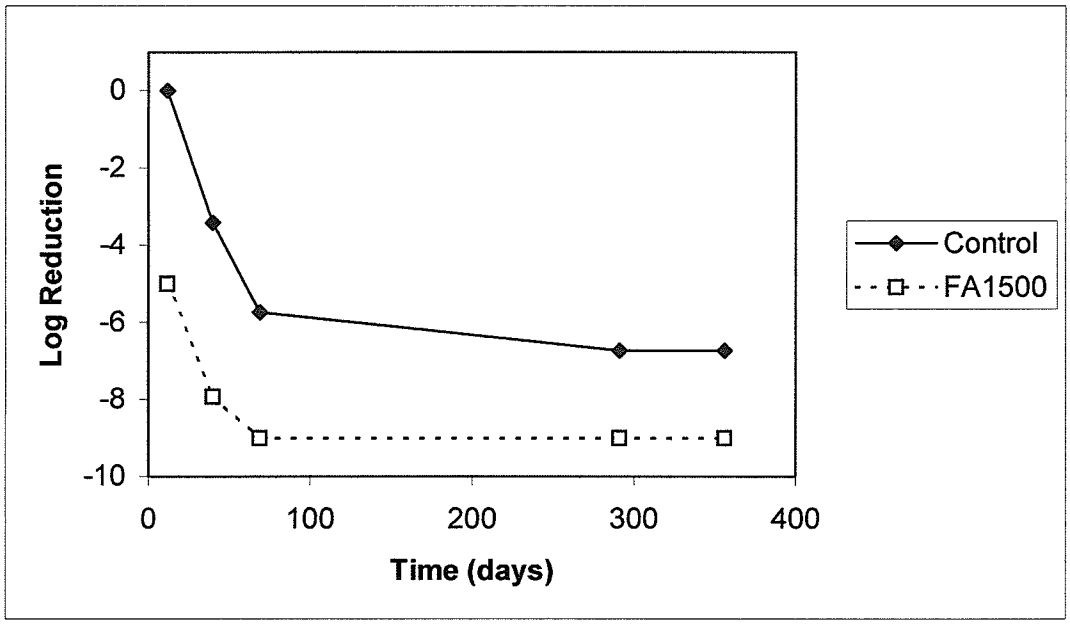

Figure 11: The log reduction in fecal coliform bacteria in the control and fly ash only treatments. All of the fecal coliform bacteria in the **other** treatments were eliminated by the first sampling period (Day 12).

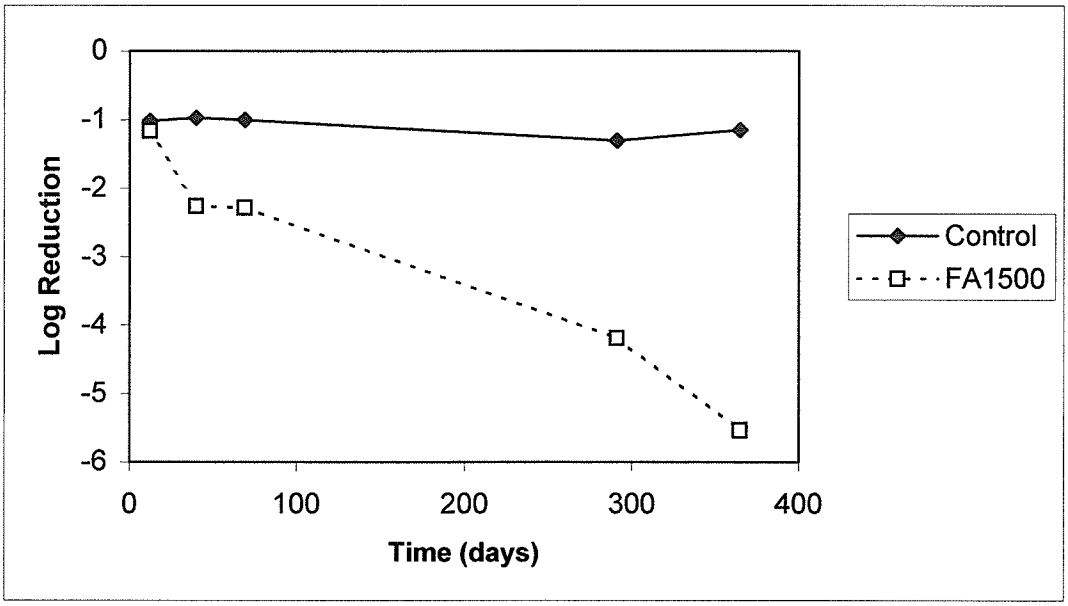

Figure 12: The log reduction in reovirus in the control and fly ash only treatments. Reovirus was completely inactivated in all the **other** treatments by the first sampling period (Day 12).

5.14 *Clostriduim perfringen* Bacteria Inactivation

The inactivation of *C. perfringens* spores provides a good understanding of the effectiveness of elevated pH and free ammonia, as the spores are highly resistant to high temperatures. There was very little decrease in the viability of the control. The fly ash-only treatment has been reduced slightly, but not nearly to the extent of the combination treatments involving both lime and fly ash. All of the treatments involving lime and fly ash experienced a 5-log reduction in CFU/g dry biosolids by the 69th day of storage, as can be seen in Figure 13. The only lime/fly ash treatment with any remaining significant *C. perfringens* CFU by the 291st day of storage

consisted of a lime dose of 50g – amended with 1000g fly ash – per kg dry biosolids. The spores of *C. perfringens* were completely inactivated in all combined lime/fly ash treatments by the 356th day of storage.

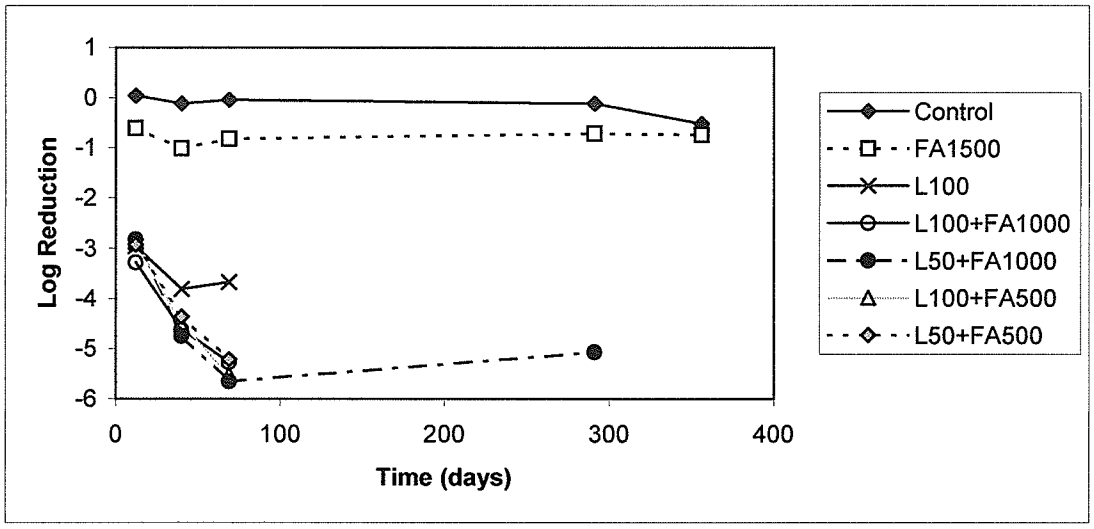

Figure 13: The log reduction in *C. perfringens* spores over 356 days of storage.

5.15 *Ascaris suum* Egg Inactivation

The *Ascaris suum* eggs found in the control treatment were relatively unaffected throughout the duration of the experiment as the decrease in viability through 356 days was only 11.5%. The fly ash-only treatment also had a minimal impact on the viability of the *Ascaris* eggs. The decrease in egg viability in the fly ash only treatment after 356 days was 19%. All of the treatments involving lime experienced a 2-log reduction or greater in Ascaris egg viability by day 40. The only treatment to have all of the eggs in both the sentinel chambers and the packets inactivated by day 69 was the L100+FA500 treatment. All of the eggs in the limed

treatments – except L50+FA500 – were completed inactivated by day 291, as demonstrated in Figure 14. The L50+FA500 treatment contained three viable eggs out of 6282 eggs recovered in the five-gram packet. By day 356 all of the eggs from the limed treatments were inactivated.

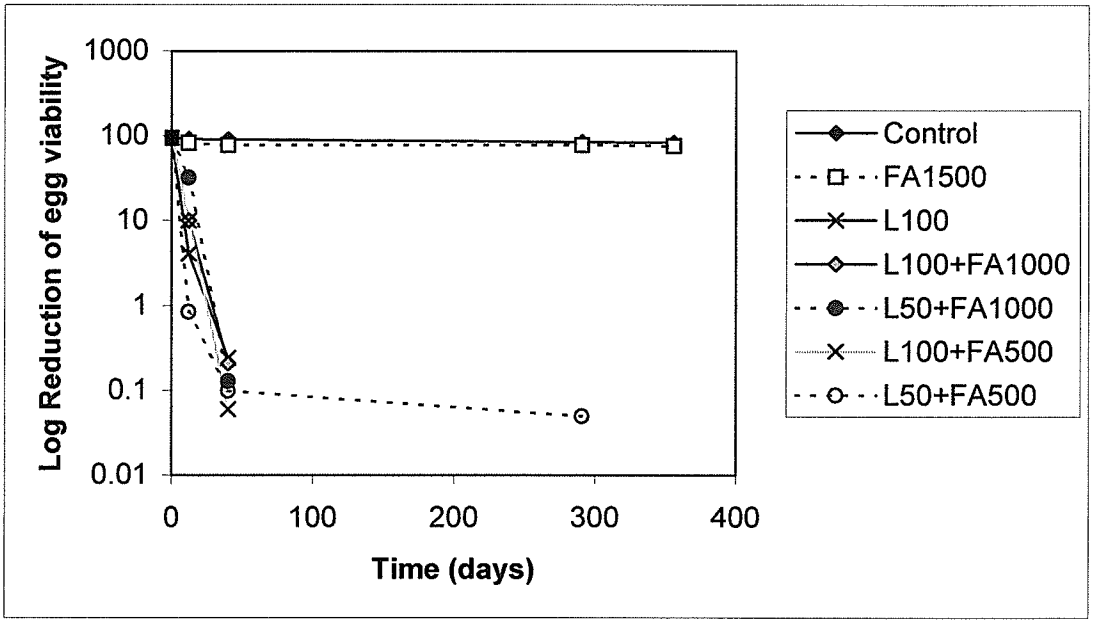

Figure 14: The log reduction in *Ascaris* egg viability in all treatments for the entire duration of the full-scale study.

The results from the third sampling period (69 days) were eliminated. They contrasted with the enumeration results from the back-up sentinel chambers. The error is believed to be from allowing the eggs to remain in the storage solution too long after incubation but prior to enumeration. A comparison between the sentinel chamber results and the packet results can be seen in Table 5. The general decrease in viability in the sentinel chamber results is believed to be the combined result of the

shipping of the eggs to Cornell University for enumeration and the additional time that the eggs were exposed to alkaline conditions. The recovery of eggs from the L200 treatment was low and it was suspected that this was a result of the eggs being completely destroyed. Due to the lack of recovery, or in other words the small sample size, the variability for this treatment was quite large.

Table 5: A comparison between the enumeration results for the packets and the sentinel chambers.

Treatments	*Ascaris suum* ova viability (%)					
	Day 12		Day 40		Day 69	
	packets	sentinels	packets	Sentinels	Packets	sentinels
Control	92.3 ± 1.4	74.7 ± 5.8	90.3 ± 1.3	62.2 ± 6.7	43.1 ± 2.4	60.2 ± 6.3
L200	0 + 19.3	n/a	0 + 45.1	13.2 ± 6.2	0 + 77.6	0 + 63.2
L100	4.1 ± 1.1	0 + 1.5	0.24 + 1.8	0.5 + 3.9	0 ± 0.1	0.5 + 3.9
FA1500	83.2 ± 1.9	n/a	78.1 ± 3.0	65.6 ± 6.1	4.4 ± 4.9	60.35 ± 6.6
FA1000+L100	10.1 ± 1.3	5.85 ± 3.2	0.21 + 1.5	0.97 + 6.8	0 ± 0.1	4.5 ± 2.9
FA1000+L50	32.4 ± 2.5	30.4 ± 6.1	0.13 + 1.0	0 ± 1.5	.0007 + 0.5	2.5 ± 2.2
FA500+L100	10.1 ± 0.9	3.75 ± 2.6	0.06 + 0.4	0 ± 1.2	0 ± 0.2	0 ± 1.5
FA500+L50	0.85 + 8.6	0 + 1.4	0.1 + 0.8	0 ± 1.5	.0002 + 0.2	0.5 + 3.9

It is believed that class A levels could have been achieved in a shorter duration in the full-scale experiment if higher temperatures were maintained throughout the study, as storage temperature is a major factor in *Ascaris* egg inactivation. The temperature in the L100+FA1000 treatment trench – at sampling cage depth - can be seen in Figure 15. The average temperature over the first 69 days was 17.5°C, while the average temperature between days 69 and 291 was 2.9°C. It is possible that a class A product was produced between sampling periods 3 and 4, but sampling was impractical during this stage because the ground was frozen.

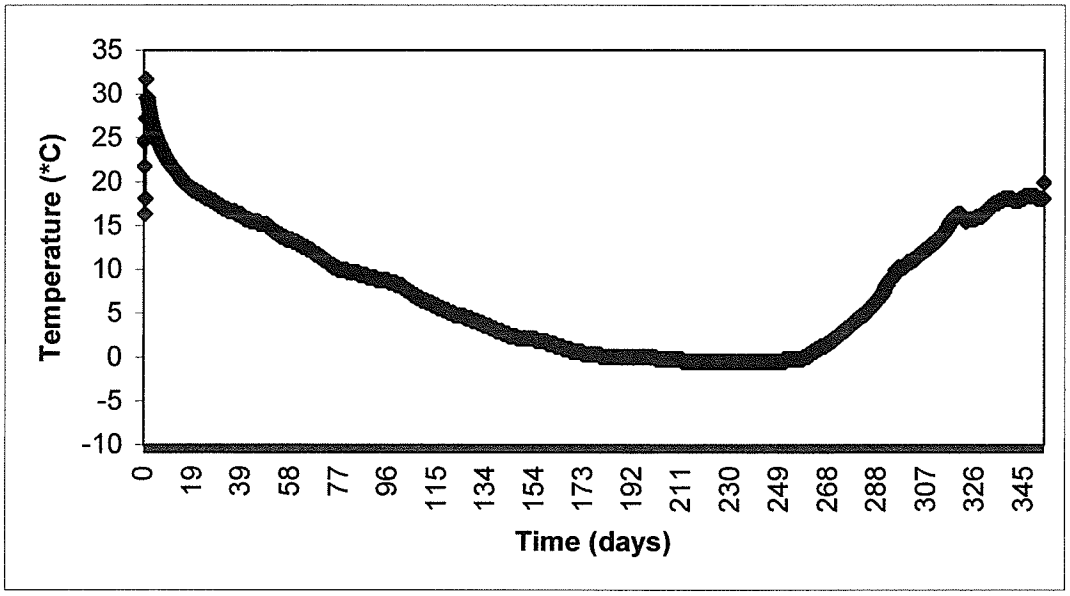

Figure 15: Temperatures at the depth of the sampling cages in the L100+FA1000 treatment.

The effect of storage temperature on *Ascaris* egg viability can be seen in Figure 16. In a bench-scale study where the biosolids were incubated at 4-6°C, it took over 400 days to achieve a 2-log reduction at a lime dose of 120g per kg dry biosolids. In the full-scale study the average temperature over the first 69-day period was 17.5°C and a lime dose of 100g per kg dry biosolids was able to achieve a 2-log reduction in 40 days. The ineffectiveness of *Ascaris* egg inactivation at cold temperatures would have an impact on the feasibility of this process, as inactivation during winter storage would be difficult. This problem could be rectified by burying the sludge at greater depths to avoid freezing or by storing them in a heated building. However, the latter option would significantly increase the costs of storage.

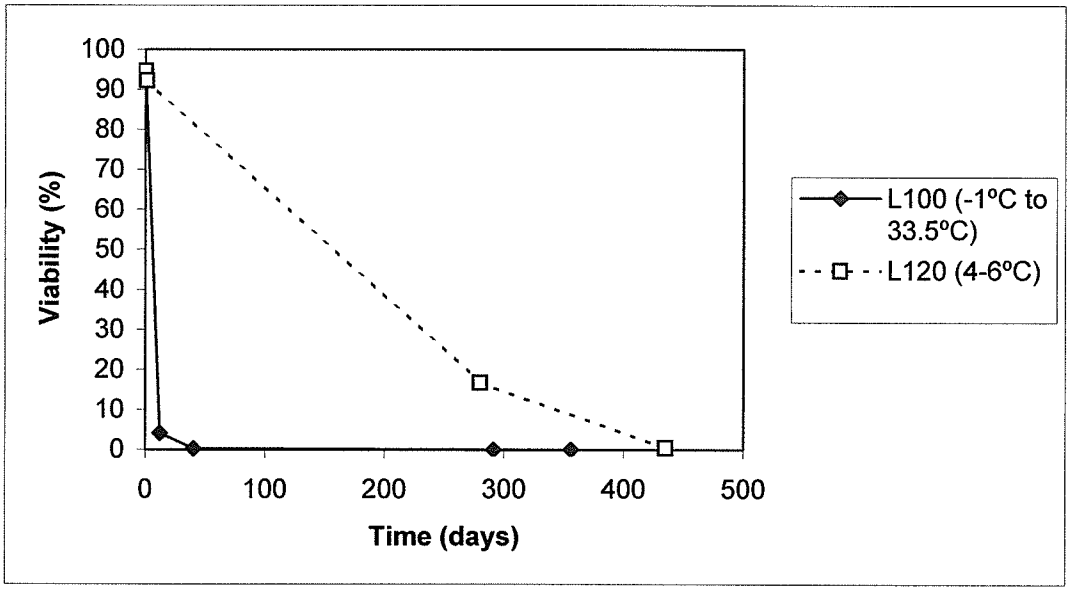

Figure 16: The effect of temperature on *Ascaris* egg viability: comparing the viability of the L100 treatment from the full-scale study with the results from a bench-scale study where the biosolids were stored at 4-6°C.

5.16 *Clostriduim perfringen* Inactivation vs. *Ascaris suum* Egg Inactivation

Figures 13 and 14 demonstrate that there is a similar pattern in the log reduction of *C. perfringen* spores and the log reduction of *Ascaris* egg viability. With both microorganisms the control treatment had little to no effect over the duration of the study. The fly ash-only treatment also had little effect on the viability of *Ascaris* eggs, but did have a minimal effect on the *C. perfringen* spores. The remaining treatments all followed similar patterns. The L200 treatment never produced any significant colony forming units of *C. perfringen* spores nor were any viable *Ascaris* eggs found. The last period at which significant colony forming units were produced

for the remaining treatments – with exception of the L50+FA1000 treatment - was the third sampling period. The last period that a viable egg was found in the remaining treatments – with the exception of the L50+FA500 treatment – was also the third sampling period. A greater log reduction was achieved for *C. perfringen* spores, but if more *Ascaris* eggs were spiked and recovered it is likely that the log reduction in *Ascaris* eggs would also have increased.

The *C. perfringen* spore enumeration procedure is far less arduous and time consuming than the procedure for enumerating *Ascaris* eggs. Due to the similarity in the time required to inactivate the two microorganisms, it would be beneficial to use the *C. perfringen* spore enumeration procedure to indicate the level of disinfection achieved by an alkaline agent. However, *C. perfringen* spores are thermophillic and therefore are resistant to the higher temperature that would destroy *Ascaris* eggs so this would only be applicable to disinfection methods where temperature is not the primary disinfectant agent.

5.2 Bench-Scale PAM study

5.21 Comparison of Various Moisture Contents

The initial phase of Part One of the study demonstrated that an increase in the moisture content of the PAM resulted in an increase in the pH up to a moisture content of 15%. There was no additional benefit to increasing the moisture content above 15%, as there was no additional increase in pH. The effect of moisture content on the pH of the PAM can be seen in Figure 17.

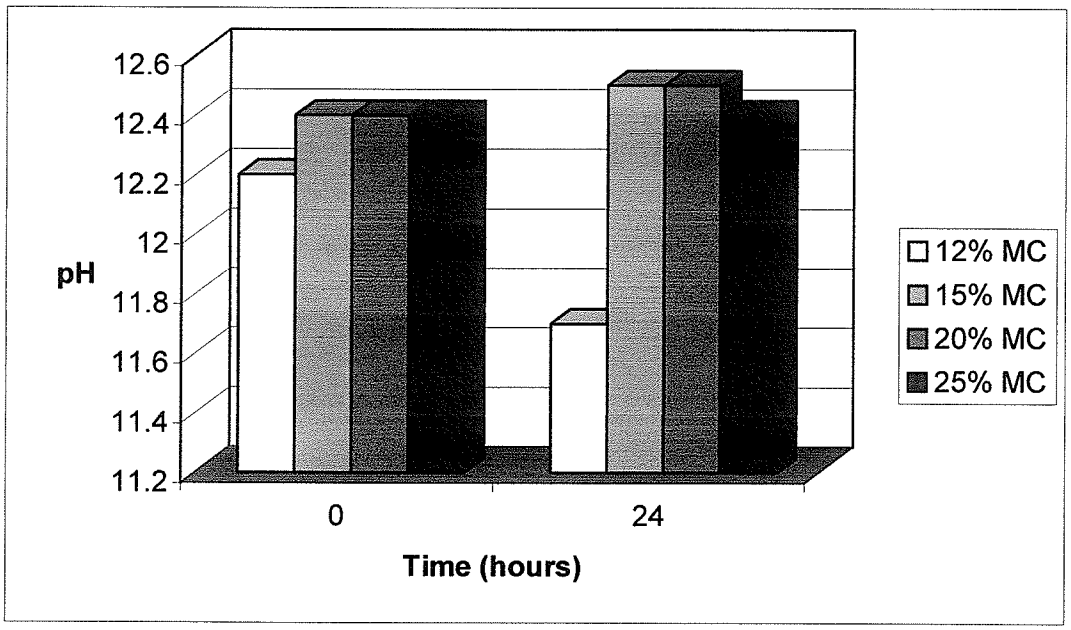

Figure 17: The resulting pH of varying moisture contents of the PAM (not mixed with biosolids) initially and after 24 hours.

The change in moisture content had little effect on the pH obtained when mixed with the biosolids. Figure 18 demonstrates that while the treatments with moisture added to the PAM initially had a greater pH, they all eventually levelled off at the same pH. Figure 19 also demonstrates that there was little difference in the resulting pH at varying moisture contents of the PAM.

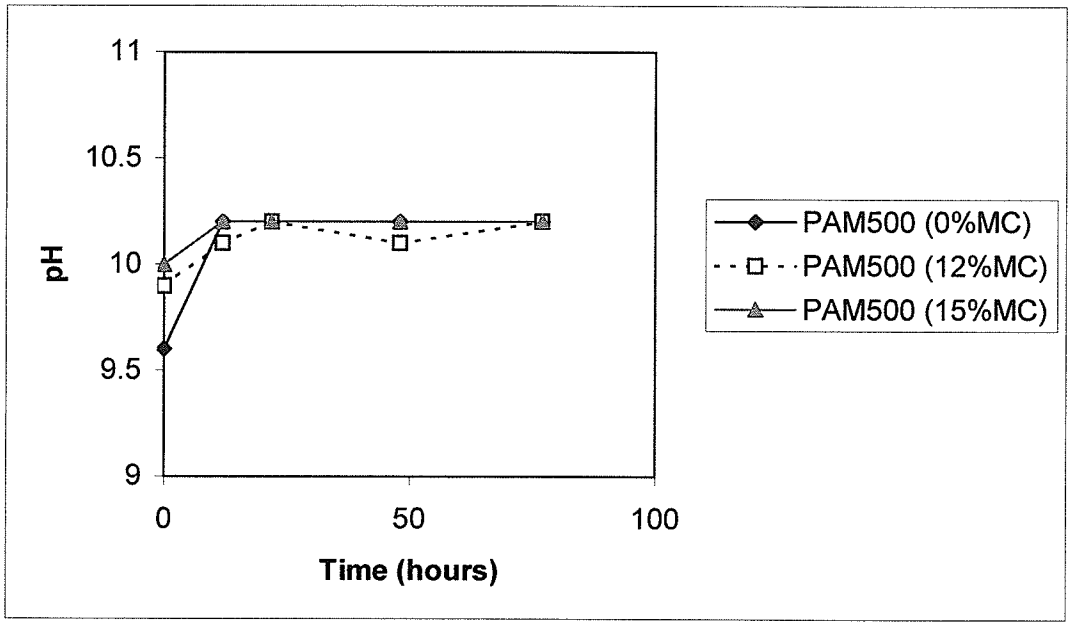

Figure 18: The pH of PAM doses of 500g per kg dry biosolids at varying moisture contents.

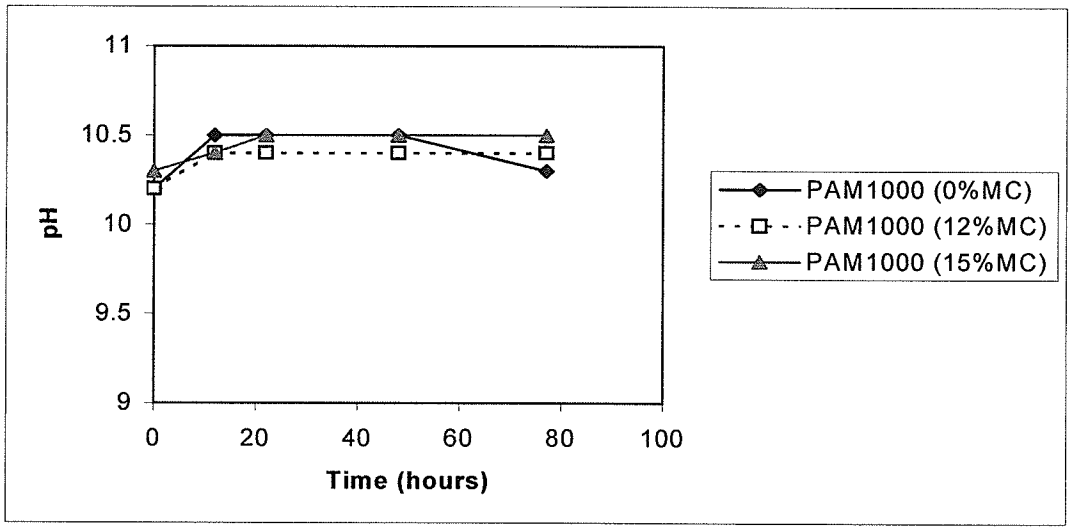

Figure 19: The pH of PAM doses of 1000g per kg dry biosolids at varying moisture contents.

It is likely that the effect of the additional moisture content is negligible when combined with the biosolids as the additional moisture is relatively small when compared to the moisture contained in the biosolids. For example, at a dose of 500g PAM per kg of dry biosolids, the water contributed by the biosolids is 10 times that contributed by the PAM at a moisture content of 15%. As a result of this data no moisture was added to the PAM in the second phase of this study.

5.22 Class B Treatments

The average pH for the dewatered and digested sludge from the NEWPCC in Winnipeg was 7.5, while the average pH of the dewatered and undigested sludge from Oklahoma City was 5.7. Due to the lower initial pH of the Oklahoma City biosolids, more of the alkaline material was necessary to obtain the same results as for the NEWPCC biosolids. This was seen to be true when determining the doses necessary to produce a class B product, as seen in Figure 20. A lime dose of 80g per kg dry biosolids was able to satisfy rule 503's time-pH regulations for a class B product for the digested sludge, while a higher dose of lime (115g per kg dry biosolids) was necessary for the undigested sludge.

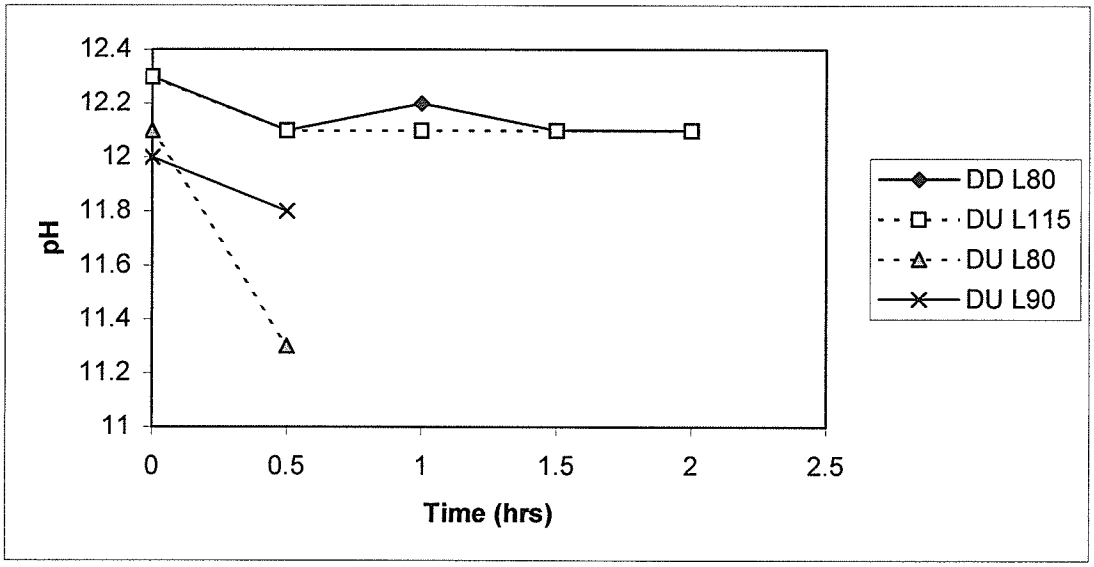

Figure 20: Treatments that attempted to meet class B time-pH regulations. Successful treatments maintained a pH above 12 for at least a 2-hour duration.

The PAM alone was unable to attain a pH of 12 at doses as high as 2000g per kg dry biosolids. The treatments with the PAM alone generally took three to four days before the maximum pH was reached. When lime was added the pH quickly increased, but also quickly dropped off when the lime dose was too low. Figure 21 demonstrates the effect of combining low lime doses and the PAM together with the biosolids. Initially the lime is responsible for the increase in pH, but over time it is the PAM that is responsible for maintaining the pH when the effect of the low lime is lost.

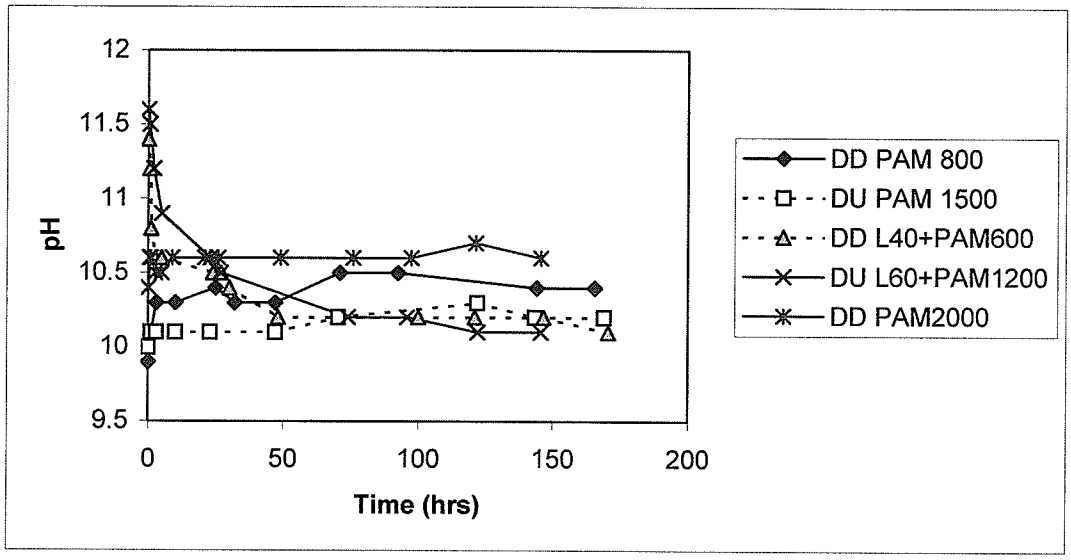

Figure 21: The effect of amending the PAM with low lime doses when mixing with biosolids.

The PAM, combined with low lime doses, produced a class B product. The lime doses were lower than those that were able to produce a class B product without PAM. The successful digested sludge treatment contained a PAM dose of 1200g - amended with 60g lime - per kg dry biosolids, while the undigested treatment required a PAM dose of 1600g –amended with 80g lime – per kg dry biosolids. The lime-only treatments required 80g and 115g to meet class B regulations for the digested and undigested sludges, respectively.

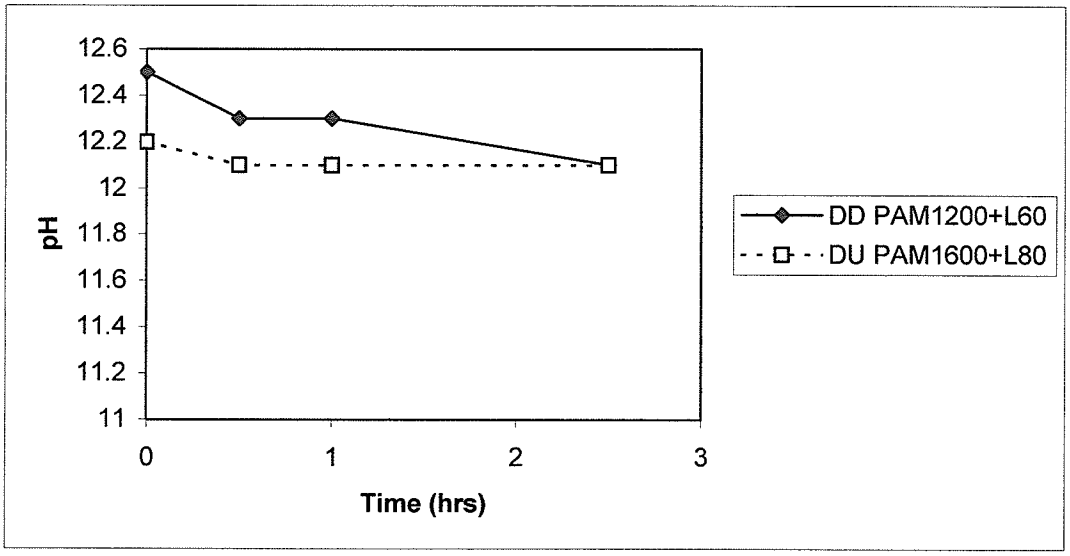

Figure 22: PAM/lime combination treatments that were able to meet class B time-pH regulations

The fecal coliform reductions for the treatments meeting the class B time-pH regulations were all below the required 1000MPN/g, as seen in Table 6.

Table 6: The fecal coliform concentration initially and after meeting the time-pH criteria for a class B biosolids product.

Treatment	Fecal Coliform Concentration MPN/g	
	initial (t=0hr)	final (t=2hr)
DD L80	744	0
DU L115	45.4	0
DD PAM1200+L60	1911.5	1.8
DU PAM1600+L80	13.5	5.2

- Control DD = 79,365,079 MPN/g & Control DU = 9,293,680 MPN/g

5.23 Class A Treatments

Attaining a class A product proved to be far more difficult than obtaining a class B product. This was due in large part to the temperature requirement, which was deemed to be the limiting factor. Initially, the alkaline materials were mixed with the biosolids for five minutes. It was later determined that mixing times greater than one minute could result in heat loss (Lui *et al.*, 2000). All treatments were repeated with a one minute mixing time. Limed treatments as low as 250g per kg dry biosolids (lowest dose attempted) were able to achieve the pH goal of a pH greater than 12 for 72 hours. The time-pH results for the class A trials can be seen in the appendix. A lime dose of 600g per kg dry undigested biosolids and a lime dose of 500g per kg digested biosolids was needed to elevate the temperature to the 52°C required. Keeping the temperature of the biosolids above 52°C was dependent on insulation, as was concluded by Lui (2000).

The PAM is not capable of inducing a temperature increase and therefore the addition of PAM can do little to reduce the amount of lime necessary to produce a class A product. Figure 23 shows that while there was a slightly higher maximum temperature when the L500 DD treatment was amended with 1000g of PAM per kg dry biosolids, there was a slightly lower maximum temperature when the L500 DU treatment was amended with 1000g PAM per kg dry biosolids.

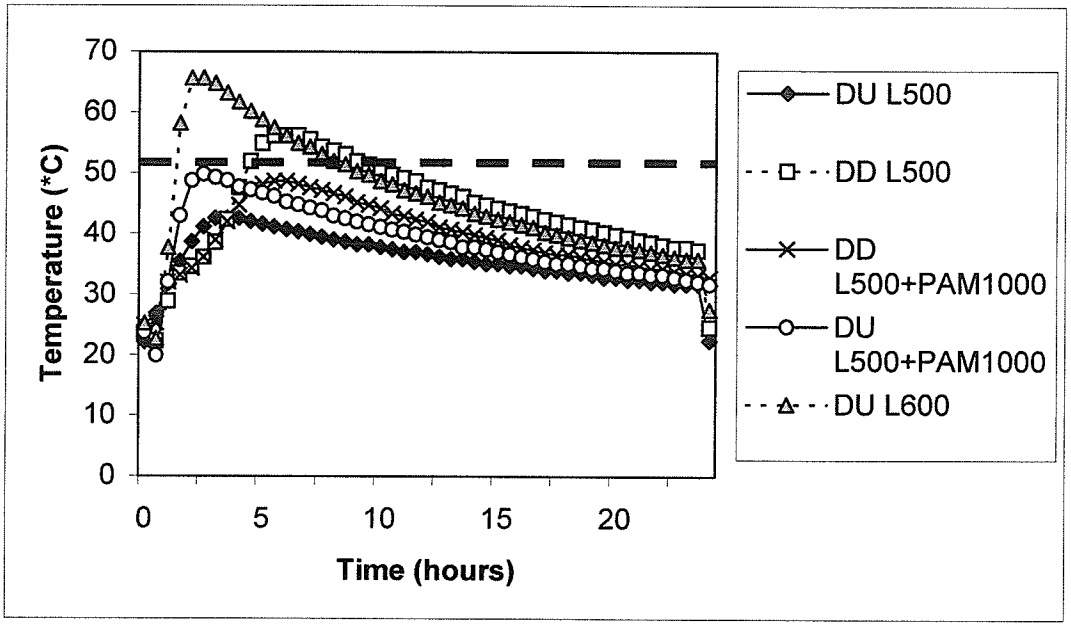

Figure 23: The effect of varying lime doses on the temperature of both digested and undigested biosolids.

5.3 Enteric Virus Indicator Study

5.31 Mesophillic Heat Treatment of Reovirus vs. Poliovirus

The stability of reovirus at temperatures up to and including 50°C is demonstrated in Figure 24. A temperature of 42°C had little effect on the virus for the first 21 days but a decline in virus titre was apparent between days 21 and 28. A temperature of 45°C caused a gradual decline in reovirus survival over the 28-day period. While this temperature resulted in a 5-log reduction in reovirus survival, there was still reovirus present and therefore class A criteria was not achieved. The only temperature that was able to meet class A criteria was 50°C. Reovirus was completely inactivated by the 10^{th} day in this treatment. Reovirus seems to be a better indicator than poliovirus for long-term storage at mesophillic temperatures, as it was

unaffected at 37°C while poliovirus was completely inactivated at 37°C by the 11th day in the study by Yeager and O'Brien (1979).

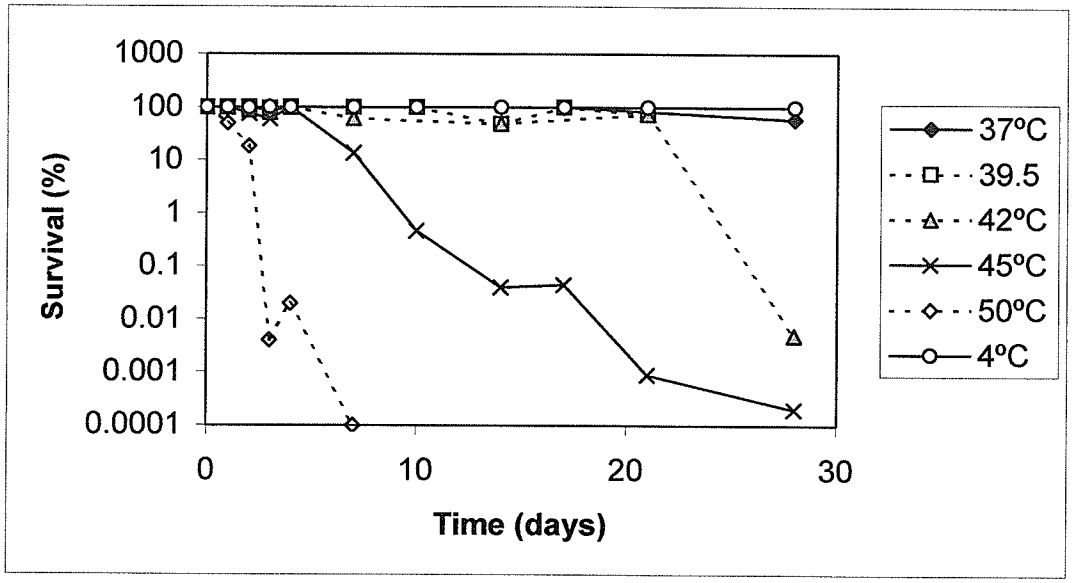

Figure 24: The effect of various temperatures on the survival of reovirus over a 28-day period.

5.32 Thermophillic Heat Treatment of Reovirus vs. Poliovirus

Short-term survival of reovirus and poliovirus at thermophillic temperatures can be seen in Figure 25. Reovirus was stable for the 30-minute study when stored at 50°C. This is in agreement with the results from the long-term study. Temperatures of 52.5°C and 55°C affected the survival of reovirus, but were not able to completely inactivate the virus in the short incubation period and therefore class A levels were not achieved. Reovirus experienced a 3-log reduction in 15 minutes at 52.5°C and a 5-log reduction in 30 minutes at 55°C. On the other hand, poliovirus experienced a 7-

log reduction in 50 minutes when subjected to a temperature of 51°C (Ward *et al.*, 1976). Reovirus also seems to be a better indicator than poliovirus for short-term survival at thermophillic temperatures.

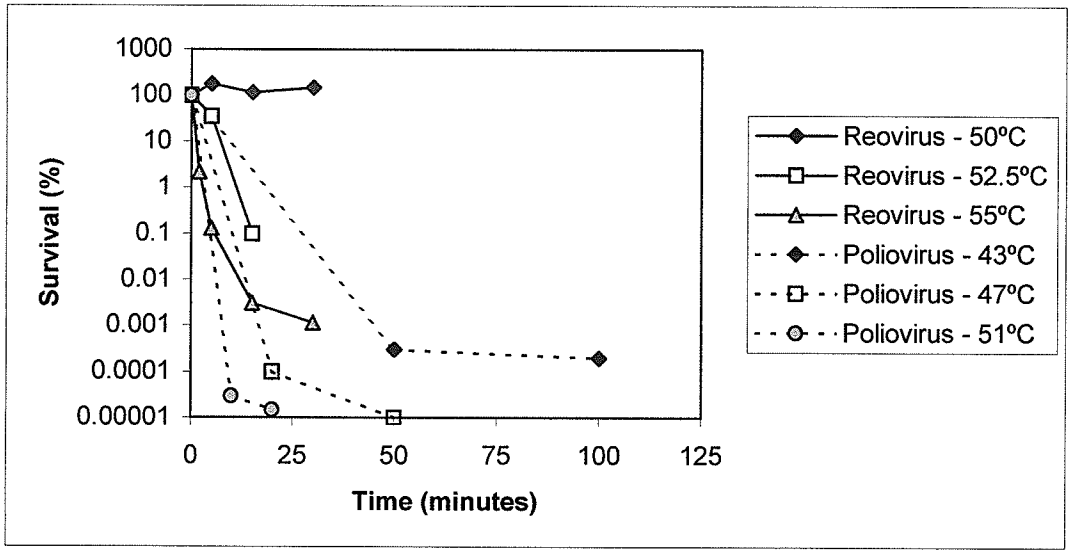

Figure 25: The effect of various thermophillic temperatures on reovirus over a short duration. Comparison values for the survival of poliovirus in phosphate-buffered saline are taken from Ward *et al.* (1976).

5.33 ALLD Treatment of Reovirus

A lime dose of at least 40 g/kg TS was necessary to achieve any sort of reduction in reovirus survival, as seen in Figure 26. This treatment resulted in more than a 2-log reduction in reovirus survival after 10 days of storage, but reovirus was still detected after 23 days of storage so class A levels were not achieved. Lime doses of 80/kg TS and greater, contained no detectable reovirus after one day of storage and therefore a class A product – in terms of enteric virus criteria - was produced in these

treatments. Earlier studies by Bujoczek (2001) have shown that the dose of 80 g/kg TS did not increase the temperature significantly so the main inactivation mechanism is increased pH and released ammonia from the digested solids.

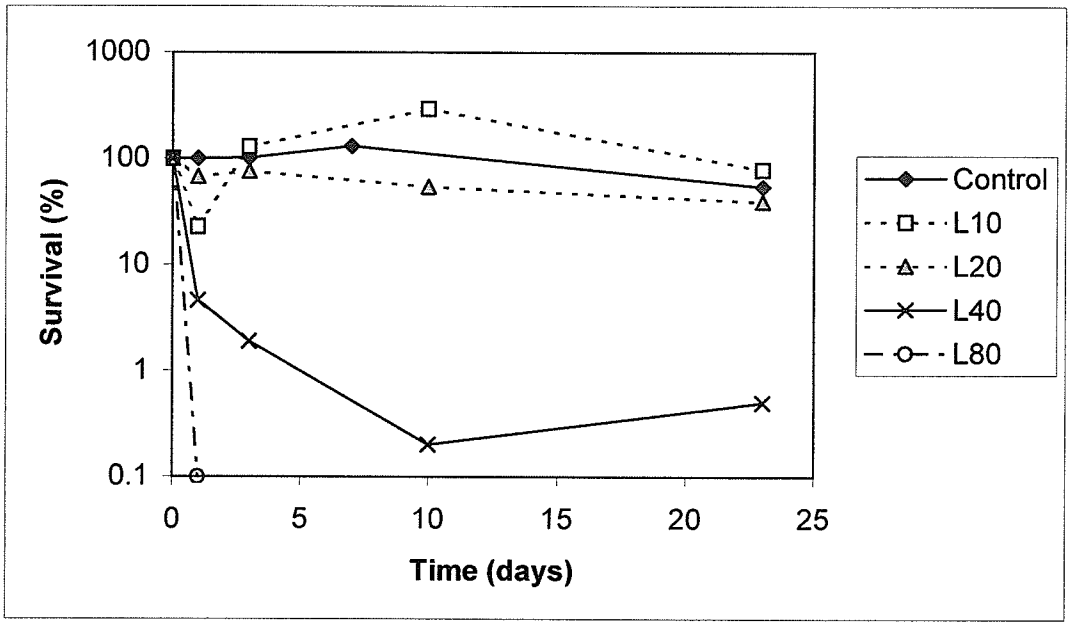

Figure 26: The effect of various low-lime doses on reovirus survival over a 23-day period. Lime doses are expressed as grams of lime (L) per kg of dry biosolids (g/kg TS).

6.0 CONCLUSIONS

6.1 Full-scale ALLD Study

1) The ALLD process was effective at inactivating fecal coliform and reovirus to class A levels in 12 days. Lime doses as low as 50g CaO/kg TS (dry biosolids) in combination with fly ash were effective.

2) A 5-log reduction in *C. perfringens* spores was achieved when the lime treated biosolids – with lime doses as low as 50g/kg TS - was amended with fly ash at doses as low as 500g fly ash per kg biosolids (dry).

3) Lime doses as low as 50g lime – amended with 1000g fly ash - per kg biosolids (dry) were effective in reducing *Ascaris suum* eggs to class A levels after 291 days of anoxic storage. 50 g/kg TS corresponds to 14 g CaO/kg wet biosolids at 28%TS. By comparison a dose needed for certification by US EPA of the Class A product based on temperature/pH alkaline treatment would be 500 g CaO/kg/TS or 140 g CaO/kg wet solids.

4) When stored under anoxic conditions for at least 291 days a dose of 50g lime – amended with 1000g fly ash - per kg biosolids can meet all the pathogen reductions required for a class A biosolids product.

5) The storage period required to achieve a class A product was extended by the low storage temperature in the middle part of the study. Storage temperature was demonstrated to be the limiting factor in achieving a class A product by alkaline disinfection.

6) *C. perfringen* spore enumeration is an effective method for indicating the level of *Ascaris* egg inactivation for ALLD, as long as temperature is not the primary disinfection factor.

6.2 Bench-scale PAM Study

1) An increase in the moisture content of the PAM will not result in an increase in pH of the biosolids it is mixed with.

2) Lime doses as low as 80g and 115g per kg of dry digested and undigested biosolids, respectively, were able produce a class B product in accordance with the US EPA's time-pH criteria.

3) PAM doses of 1200g and 1600g per kg of digested and undigested biosolids, respectively, were able to lower the amount of lime required to produce a class B product to 60g and 80g per kg digested and undigested biosolids, respectively.

4) The PAM alone was not able to meet the time-pH criteria to produce a class B product.

5) Lime doses of 500g and 600g per kg of digested and undigested biosolids, respectively, were needed to elevate the temperature to that required to produce a class A product, but maintaining the temperature is dependent on insulation.

6) The PAM was not able to reduce the amount of lime required to produce a class A product.

6.3 Enteric Virus Indicator Study

1) Reovirus is more resistant than poliovirus to inactivation caused by exposure to long-term mesophillic storage conditions.

2) Reovirus is more resistant than poliovirus to inactivation caused by exposure to short-term thermophillic conditions.

3) Reovirus is a better indicator than poliovirus of enteric virus inactivation resulting from exposure to temperature.

4) A lime dose of at least 80g per kg of dry biosolids - stored for at least one day - is needed to meet class A levels for enteric viruses.

7.0 ENGINEERING SIGNIFICANCE AND FUTURE WORK

7.1 Full-scale ALLD Study

The ALLD process was shown to be a very inexpensive method of generating class A biosolids. The process has all the elements of sustainable development technology:

a) Drastically reduces (as much 10 times) the use of chemicals for disinfection

b) Utilizes waste material – fly ash, by activating the alkaline agents within fly ash.

However, prior to the implementation of the ALLD process for inactivation of pathogens to Class A biosolids several factors must still be considered.

1) The soil characteristics and agricultural value of the product must be determined so as to find a suitable end-use. Possible end-uses include: i) agricultural product (ie. soil conditioner or fertilizer), ii) landfill cover, or iii) fill for soil remediation projects (ie. contaminated or acidic soils, mine site remediation).

2) The odours released by this process must be investigated, as odour is usually the limiting factor when it comes to public acceptance of any land application program.

3) A cost analysis must also be performed to compare this method with others on the market. The factors that must be considered in a cost analysis include: i) capital costs (mixing equipment and installation), ii) liming costs (minimizing the amount of lime with the amount of fly ash available), and iii) storage and transportation costs.

7.2 Bench-scale PAM study

The PAM was able to reduce the amount of lime required to produce a class B product in accordance with the US EPA's time-pH guidelines and therefore has the ability to decrease the cost of obtaining a class B product. This process is also environmentally sustainable in that a waste product is reused in an effective manner.

Prior to the implementation of the PAM material the soil characteristics and agricultural value of the end product must be determined so as to find a suitable end-use. Once again possible end-uses include: i) agricultural product (ie. soil conditioner or fertilizer), ii) landfill cover, or iii) fill for soil remediation projects (ie. contaminated or acidic soils, mine site remediation)

7.3 Enteric Virus Indicator Study

This work demonstrated that reovirus was hardier than poliovirus in terms of temperature inactivation. This is significant in that reovirus is a safer virus to work with because it is not a human pathogen.

It would be beneficial to compare the inactivation of reovirus and poliovirus simultaneously under varying temperature conditions. It would also be beneficial to compare the inactivation of these viruses when other biosolids disinfection methods – such as alkaline disinfection – are used.

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APPENDIX A

Full-Scale ALLD Study Raw Data

Table A1: Total solids and pH readings for sampling period 1 (day12).

Treatment	Total Solids				pH
	empty crucible (g)	wet weight (g)	dry weight(g)	total solids (%)	(solids probe)
Baseline	27.4843	42.0478	31.6518	28.6161	7.87
	25.0825	40.635	29.5284	28.5864	7.62
	23.7344	39.6339	28.288	28.6399	7.68
			mean	28.6141	7.72
			st dev	0.0268	0.13
Control	25.0619	50.5854	32.2931	28.3315	8.32
	27.1219	54.0676	34.8216	28.5749	8.32
	26.6763	51.4019	33.6949	28.3860	8.19
			mean	28.4308	8.28
			st dev	0.1277	0.07
L200	23.6056	43.7643	34.3812	53.4538	12.17
	25.8604	43.8404	35.6608	54.5072	12.22
	25.7101	41.8876	34.4766	54.1895	12.24
			mean	54.0502	12.21
			st dev	0.5403	0.03
L100	26.3863	47.6264	34.9336	40.2413	12.74
	26.9715	50.3887	36.2022	39.4185	12.87
	24.7106	48.4135	34.3082	40.4912	12.32
			mean	40.0503	12.64
			st dev	0.5613	0.28
FA1500	27.485	45.6071	35.4362	43.8757	9.32
	29.0921	51.2516	39.4468	46.7280	9.14
	23.5396	42.304	31.9701	44.9282	9.14
			mean	45.1773	9.20
			st dev	1.4424	0.10
FA1000 + L100	23.6062	44.2149	34.8545	54.5803	12.46
	27.1219	44.762	36.7289	54.4611	12.47
	25.0619	44.5888	35.6495	54.2206	12.52
			mean	54.4207	12.48
			st dev	0.1833	0.03
FA1000 + L50	23.7348	44.6445	34.8685	53.2466	12.23
	25.0829	40.4044	33.2538	53.3296	12.17
	25.5404	48.099	37.7016	53.9094	11.94
			mean	53.4952	12.11
			st dev	0.3611	0.15
FA500 + L100	22.517	40.9968	32.8877	56.1191	12.25
	25.215	41.6896	34.5391	56.5968	11.99
	23.3454	39.256	32.3582	56.6465	12.27
			mean	56.4541	12.17
			st dev	0.2912	0.16
FA500 + L50	24.2743	47.7567	37.1213	54.7091	11.85
	25.0896	49.7969	38.4593	54.1123	12.01
	24.1238	45.1721	35.8257	55.5955	11.94
			mean	54.8056	11.93
			st dev	0.7463	0.08

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Table A2: Total solids and pH readings for sampling period 2 (day40).

Treatment	Total Solids				pH
	empty crucible (g)	wet weight (g)	dry weight(g)	total solids (%)	(solids probe)
Baseline (prior to experiment)	27.4843	42.0478	31.6518	28.6161	7.87
	25.0825	40.635	29.5284	28.5864	7.62
	23.7344	39.6339	28.288	28.6399	7.68
			mean	28.6141	7.72
			st dev	0.0268	0.13
Control	24.9325	48.356	31.5681	28.3288	8.49
	25.8618	46.1396	31.677	28.6777	7.74
	24.1039	44.5072	29.9428	28.6174	8.20
			mean	28.5413	8.14
			st dev	0.1865	0.38
L200	23.735	37.828	31.439	54.6654	12.24
	25.5401	38.361	32.5944	55.0219	12.27
	25.083	38.9695	32.6678	54.6200	12.29
			mean	54.7691	12.27
			st dev	0.2201	0.03
L100	25.7106	46.4431	33.7903	38.9712	12.55
	26.8019	45.645	34.0447	38.4374	12.55
	26.0771	45.5721	34.4439	42.9177	12.40
			mean	40.1088	12.50
			st dev	2.4472	0.09
FA1500	26.3868	52.1984	40.8501	56.0341	9.14
	24.7113	52.9909	40.2987	55.1189	9.03
	26.9715	45.5409	37.1598	54.8661	9.96
			mean	55.3397	9.38
			st dev	0.6145	0.51
FA1000 + L100	23.5396	44.8943	35.4186	55.6271	12.22
	29.0911	51.6825	41.6335	55.5185	12.17
	27.4849	49.6182	39.8799	56.0016	12.27
			mean	55.7157	12.22
			st dev	0.2535	0.05
FA1000 + L50	25.7842	44.6281	36.0165	54.3003	11.84
	26.1558	42.9792	34.7459	51.0604	11.81
	23.9693	43.7643	34.7236	54.3284	11.90
			mean	53.2297	11.85
			st dev	1.8787	0.05
FA500 + L100	23.5704	45.0324	33.7584	47.4699	12.12
	23.4754	44.4886	33.4261	47.3545	12.10
	22.9711	41.6502	31.6741	46.5922	12.13
			mean	47.1389	12.12
			st dev	0.4770	0.02
FA500 + L50	25.0817	47.343	35.8495	48.3700	11.64
	26.1011	54.7417	39.7698	47.7249	11.70
	24.3508	48.0556	35.6671	47.7384	11.43
			mean	47.9445	11.59
			st dev	0.3686	0.14

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Table A3: Total solids and pH readings for sampling period 3 (day69).

Treatment	Total Solids				pH
	empty crucible (g)	wet weight (g)	dry weight(g)	total solids (%)	(indirect method)
Baseline (prior to experiment)	27.4843	42.0478	31.6518	28.6161	7.87
	25.0825	40.635	29.5284	28.5864	7.62
	23.7344	39.6339	28.288	28.6399	7.68
			mean	28.6141	7.72
			st dev	0.0268	0.13
Control	26.3873	47.9474	32.5595	28.6279	8.68
	24.7117	44.9806	30.4745	28.4317	8.59
	26.9724	52.0463	34.1086	28.4607	8.56
			mean	28.5068	8.61
			st dev	0.1059	0.06
L200	22.7714	39.0305	31.6486	54.5983	12.09
	25.0821	37.3275	31.6805	53.8847	12.12
	24.3515	40.5158	33.0687	53.9287	12.14
			mean	54.1373	12.12
			st dev	0.3999	0.03
L100	25.7847	52.2957	36.8092	41.5846	11.60
	23.97	49.9584	34.1201	39.0563	11.59
	23.571	48.2989	33.5058	40.1765	11.60
			mean	40.2725	11.60
			st dev	1.2669	0.01
FA1500	27.4855	48.2169	38.4353	52.8175	8.93
	23.5401	54.9946	39.8732	51.9261	8.90
	29.092	61.2972	45.4957	50.9349	8.91
			mean	51.8928	8.91
			st dev	0.9417	0.02
FA1000 + L100	25.5406	51.5409	39.7286	54.5686	11.69
	25.8617	45.5206	36.8497	55.8933	11.70
	24.1038	42.3113	34.1581	55.2207	11.68
			mean	55.2275	11.69
			st dev	0.6624	0.01
FA1000 + L50	24.9329	50.2017	38.8703	55.1566	11.40
	26.6769	45.3915	37.0271	55.3055	11.41
	25.0838	51.8523	39.7931	54.9500	11.41
			mean	55.1374	11.41
			st dev	0.1785	0.01
FA500 + L100	28.2072	55.4587	41.4046	48.4282	11.55
	28.1852	48.1717	37.7174	47.6932	11.58
	25.9124	52.6773	38.7167	47.8399	11.59
			mean	0.3890	0.02
			st dev	47.9871	11.57
FA500 + L50	27.2604	49.744	37.7687	46.7376	10.95
	25.9264	57.2525	40.4816	46.4635	10.90
	27.2073	54.5313	40.0976	47.1757	10.90
			mean	46.7923	10.92
			st dev	0.3593	0.03

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Table A4: Total solids and pH readings for sampling period 4 (day291).

	Total Solids				pH
Treatment	empty crucible (g)	wet weight (g)	dry weight(g)	total solids (%)	(indirect method)
Baseline (prior to experiment)	27.4843	42.0478	31.6518	28.6161	7.87
	25.0825	40.635	29.5284	28.5864	7.62
	23.7344	39.6339	28.288	28.6399	7.68
			mean	28.6141	7.72
			st dev	0.0268	0.13
Control	25.0889	50.1845	32.3305	28.8561	7.90
	23.5596	47.528	30.4277	28.6548	7.90
	23.5388	51.6339	31.638	28.8278	7.90
			mean	28.7796	7.90
			st dev	0.1090	0.00
L200	24.3518	42.2365	33.5513	51.4378	12.10
	26.9726	43.5643	35.4593	51.1503	12.00
	23.9705	44.9163	34.7375	51.4041	12.00
			mean	51.3307	12.03
			st dev	0.1572	0.06
L100	25.7848	47.7655	35.5399	44.3803	10.10
	23.4764	52.1737	35.9707	43.5382	10.10
	22.9721	38.1835	30.187	47.4309	10.10
			mean	45.1165	10.10
			st dev	2.0481	0.00
FA1500	24.7115	51.0813	37.7812	49.5631	8.90
	25.0822	55.999	40.8882	51.1243	9.00
	26.387	64.2151	46.7889	53.9332	9.00
			mean	51.5402	8.97
			st dev	2.2145	0.06
FA1000 + L50	27.2608	63.5773	47.4944	55.7146	11.40
	27.2076	50.8179	40.4857	56.2386	11.40
					11.40
			mean	55.9766	11.40
			st dev	0.3705	0.00
FA1000 + L100	22.9361	61.4037	44.4057	55.8122	11.90
	22.5771	54.3988	40.3103	55.7268	11.90
	24.4224	45.3391	36.1505	56.0705	11.90
			mean	55.8698	11.90
			st dev	0.1790	0.00
FA500 + L100	29.0899	56.985	42.5695	48.3225	11.50
	22.9705	52.5606	37.3007	48.4290	11.60
	25.7833	54.4296	40.1064	49.9998	11.50
			mean	0.9392	0.06
			st dev	48.9171	11.53
FA500 + L50	23.475	54.4137	38.9514	50.0228	10.50
	26.9705	64.1706	45.6309	50.1622	10.70
	23.9685	56.0953	39.4684	48.2460	10.80
			mean	49.4770	10.67
			st dev	1.0684	0.1528

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Table A5: Total solids and pH readings for sampling period 5 (day356).

	Total Solids				pH
Treatment	empty crucible (g)	wet weight (g)	dry weight(g)	total solids (%)	
Baseline (prior to experiment)	27.4843	42.0478	31.6518	28.6161	7.87
	25.0825	40.635	29.5284	28.5864	7.62
	23.7344	39.6339	28.288	28.6399	7.68
			mean	28.6141	7.72
			st dev	0.0268	0.13
Control	24.2722	70.5599	37.9849	29.6249	8.40
	30.6601	64.5686	40.1835	28.0856	8.44
	25.0897	68.1167	37.2993	28.3766	8.46
			mean	28.6957	8.43
			st dev	0.8178	0.03
L200	30.1981	55.5217	43.2802	51.6597	12.40
	29.309	54.6855	42.3468	51.3775	12.50
	26.9553	40.2183	33.8198	51.7568	12.48
			mean	51.5980	12.46
			st dev	0.1970	0.05
L100	27.3919	51.6844	34.9235	31.0038	11.36
	26.3863	57.9897	35.8536	29.9566	11.02
	25.9119	62.1747	36.3793	28.8654	11.20
			mean	29.9419	11.19
			st dev	1.0693	0.17
FA1500	27.2595	77.6989	54.2042	53.4199	9.23
	27.2067	67.922	49.4518	54.6357	9.21
	25.6536	56.8537	42.3706	53.5800	9.19
			mean	53.8785	9.21
			st dev	0.6606	0.02
FA1000 + L50	23.5389	58.4888	42.6643	54.7223	11.23
	25.6574	60.9232	45.3549	55.8544	11.02
	26.9716	62.6734	47.0447	56.2243	10.79
			mean	55.6004	11.01
			st dev	0.7826	0.22
FA1000 + L100	31.0648	66.9522	51.1911	56.0818	11.98
	24.7105	54.1153	41.2098	56.1109	12.12
	25.7839	47.98	38.0675	55.3413	12.03
			mean	55.8447	12.04
			st dev	0.4362	0.07
FA500 + L100	27.4577	60.6455	43.3529	47.8947	12.19
	28.3968	60.3415	44.1294	49.2495	12.14
	29.4571	51.117	39.9459	48.4250	12.22
			mean	0.6827	0.04
			st dev	48.5231	12.18
FA500 + L50	30.7297	56.4454	42.8865	47.2738	11.15
	23.3064	65.4536	43.3422	47.5377	11.16
	28.0532	60.1979	43.4519	47.9043	11.32
			mean	47.5719	11.21
			st dev	0.3166	0.0954

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Table A6: *Clostridium perfringen* spore counts for sampling period 1 (day12).

Treatment	*Cl Perfringens* (CFU of serial dilutions)							CFU/ml	CFU/g
	-1	-2	-3	-4	-5	-6	-7		
Baseline	TNC TNC TNC	TNC TNC TNC	TNC TNC TNC	TNC TNC TNC	308 TNC TNC	66 17 41.5	1	41500000.0	145033298.1
Control	TNC	TNC	TNC TNC TNC	TNC TNC TNC	320 TNC TNC	46 44 46 45.333	6	45333333.3	159451530.6
L200	5 1	1 0 0	2 0 0	5 0 0	1 0 0	0 1 0	0	0.0	0.0
L100	TNC TNC	TNC TNC TNC	TNC TNC TNC	67 62 60 63	9 7 6	1 0 0	0 0	630000.0	1573020.0
FA1500	TNC	TNC TNC	TNC TNC TNC	TNC TNC TNC	166 136 186 162.67	25 18 19	0 3	16266666.7	36006280.3
FA1000 + L100	TNC	182	44 41 39 41.333	9 3 2	2 0 0	0 0 0	0	41333.3	75951.5
FA1000 + L50	TNC	TNC	104 118 131 117.67	14 11 13	1 2 1	0 0	0	117666.7	219957.4
FA500 + L100	TNC	TNC	95 111 123 109.67	14 10 11	1 0 2	0 0 0	0	109666.7	194257.9
FA500 + L50	TNC	TNC	88 86 106 93.333	9 19 7	1 1 2	0 0 0	0	93333.3	170298.8

* TNC = too numerous to count

Full-Scale ALLD Study Raw Data

Table A7: *Clostridium perfringen* spore counts for sampling period 2 (day40).

	Cl Perfringens (CFU of serial dilutions)							CFU/ml	CFU/g
Treatment	-1	-2	-3	-4	-5	-6	-7		
Baseline	TNC	TNC TNC TNC	TNC TNC TNC	TNC TNC TNC	308 TNC TNC	66 17 41.5	1	41500000.0	537541556.9
Control	TNC	TNC	TNC	TNC TNC TNC	327 TNC TNC	31 33 31 31.667	0	31666666.7	388866148.2
L200	0 0 0	1 2 0	0 0 0	0 0 0	0			0.0	0.0
L100	TNC	81 75 113 89.667	9 10 7	0 1 1	0			8966.7	71733.3
FA1500	TNC	TNC	TNC TNC TNC	TNC TNC TNC	94 52 92 79.333	6 8 3	2	7933333.3	84607180.9
FA1000 + L100	22?	21 15 22 19.333	2 2 0	0 0 0	0			1933.3	15821.1
FA1000 + L50	34	14 15 13 14	0 1 4	0 0 0	0			1400.0	11814.3
FA500 + L100	69	19 15 26 20	2 0 0	0 0 0	1			2000.0	16506.2
FA500 + L50	55	28 32 30	4 9 5	1 1 1	1			3000.0	25884.4

* TNC = too numerous to count

Full-Scale ALLD Study Raw Data

Table A8: *Clostridium perfringen* spore counts for sampling period 3 (day 69).

	Cl Perfringens (CFU of serial dilutions)							CFU/ml	CFU/g
Treatment	-1	-2	-3	-4	-5	-6	-7		
Baseline	TNC TNC TNC	TNC TNC TNC	TNC TNC TNC	TNC TNC TNC	308 TNC TNC	66 17 41.5	1	41500000.0	145033298.1
Control	TNC	TNC	TNC	TNC TNC TNC	327 TNC TNC	36 37 40 37.67	6	37666666.7	132132390.0
L200	11 8 12	0 0 1	0 0 0	0				0.0	0.0
L100	TNC TNC TNC	143 134 98 125	16 30 28	2				12500.0	31038.6
FA1500	TNC	TNC	TNC TNC TNC	TNC TNC TNC	120 128 97 115	9 15 16		11500000.0	22161053.0
FA1000 + L100	9 11 9 9.667	0 0 2	0 1 0	0				96.7	175.0
FA1000 + L50	14 30 10 18	9 9 8	0 1 0	0				180.0	326.5
FA500 + L100	19 20 24 21	6 3 6	0 0 1	0 0 0				210.0	437.6
FA500 + L50	21 35 48 41	58 50 64	8 20 13					413.3	883.3

* TNC = too numerous to count

Full-Scale ALLD Study Raw Data

Table A9: *Clostridium perfringen* spore counts for sampling period 4 (day291).

	Cl Perfringens (CFU of serial dilutions)							CFU/ml	CFU/g
Treatment	-1	-2	-3	-4	-5	-6	-7		
Baseline	TNC TNC TNC TNC	TNC TNC TNC	TNC TNC TNC	TNC TNC TNC	308 TNC TNC	66 17 41.5	1	41500000.0	145033298.1
Control	TNC	TNC	TNC	TNC TNC TNC	287	32 32	0	32000000.0	111190038.9
L200	0 1 1	1 0 0	0 0 0	0 0 0	0	0	0	NS	NS
L100	TNC TNC TNC	TNC TNC TNC	54 53 55 54	9 8 7	1	1	0	121000.0	268194.7
FA1500	TNC TNC TNC	TNC TNC TNC	TNC TNC TNC	TNC	144 144	15	1	14400000.0	27939350.4
FA1000 + L100	65 71 75 70.3	17 13 12	0 1 4	1	0	0	0	703.3	1256.5
FA1000 + L50	3 8 5 NS	2 4 0	0 1 0	0	0	0	0	NS	NS
FA500 + L100	0 2 1	1 0 0	0 0 1	0	0	0	0	NS	NS
FA500 + L50	NS 2 4 3 NS	0 4 3	0 1 1	0	0	0	0	NS	NS

* TNC = too numerous to count

Full-Scale ALLD Study Raw Data

Table A10: *Clostridium perfringen* spore counts for sampling period 5 (day356).

	Cl Perfringens (CFU of serial dilutions)							CFU/ml	CFU/g
Treatment	-1	-2	-3	-4	-5	-6	-7		
Baseline	TNC	TNC	TNC	TNC	308	66	1		
		TNC	TNC	TNC	TNC	17			
		TNC	TNC	TNC	TNC				
						41.5		41500000.0	145033298.1
Control	TNC	TNC	TNC	TNC	125	12			
				TNC	122	20			
				TNC	129	9		12533333.3	43676686.4
					125.3				
L200	0	1	0	0	0	0			
	1	0	0	0					
	1	0	0	0					
	NS							NS	NS
L100	TNC	TNC	TNC	28	0	0			
	TNC	TNC	TNC						
	TNC	TNC	TNC						
				28				280000.0	935143.5
FA1500	TNC	TNC	TNC	TNC	28	3			
				TNC	37	3			
				TNC	29	4			
					144			14400000.0	26726780.7
FA1000 + L100	0	0	1						
	2	0	3						
	2	0	0						
	NS							NS	NS
FA1000 + L50	0	2	0	0					
	0	0	0						
	0	0	0					NS	NS
	NS								
FA500 + L100	2	1	0	0					
	2	0	0						
	2	0	0						
								NS	NS
	NS								
FA500 + L50	2	0	0	0					
	0	0	0						
	0	0	0						
	NS							NS	NS

* TNC = too numerous to count

Full-Scale ALLD Study Raw Data

Table A11: Fecal coliform bacteria MPN results for sampling period 1 (day12).

	Fecal Coliform Densities (Bounded by 95% Confidence Limits)					
	MPN index	Dilution	MPN/100ml	Lower Bound	Upper Bound	MPN/g
Baseline	2.5	1.0E-08	2.5E+09	5.3E+08	1.2E+10	8.7E+07
Control	2.5	1.0E-08	2.5E+09	5.3E+08	1.2E+10	8.8E+07
L200	0	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
L100	0	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
FA1500	2.5	1.0E-03	2.5E+04	5.3E+03	1.2E+05	5.5E+02
FA1000+L100	0	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
FA1000+L50	0	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
FA500+L100	0	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
FA500+L50	0	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00

Table A12: Fecal coliform bacteria MPN results for sampling period 2 (day40).

	Fecal Coliform Densities (Bounded by 95% Confidence Limits)					
	MPN index	Dilution	MPN/100ml	Lower Bound	Upper Bound	MPN/g
Baseline	2.5	1.0E-08	2.5E+09	5.3E+08	1.2E+10	8.7E+07
Control	9.5	1.0E-04	9.5E+05	2.0E+05	4.4E+06	3.3E+04
L200	0	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
L100	0	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
FA1500	0.3	1.0E-01	3.0E+01	6.4E+00	1.4E+02	6.6E-01
FA1000+L100	0	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
FA1000+L50	0	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
FA500+L100	0	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
FA500+L50	0	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00

Full-Scale ALLD Study Raw Data

Table A13: Fecal coliform bacteria MPN results for sampling period 3 (day69).

	Fecal Coliform Densities (Bounded by 95% Confidence Limits)					
	MPN index	Dilution	MPN/100ml	Lower Bound	Upper Bound	MPN/g
Baseline	2.5	1.0E-08	2.5E+09	5.3E+08	1.2E+10	8.7E+07
Control	4.5	1.0E-02	4.5E+03	9.6E+02	2.1E+04	1.6E+02
L200	0	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
L100	0	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
FA1500	0	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
FA1000+L100	0	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
FA1000+L50	0	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
FA500+L100	0	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
FA500+L50	0	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00

Table A14: Fecal coliform bacteria MPN results for sampling period 4 (day291).

	Fecal Coliform Densities (Bounded by 95% Confidence Limits)					
	MPN index	Dilution	MPN/100ml	Lower Bound	Upper Bound	MPN/g
Baseline	2.5	1.0E-08	2.5E+09	5.3E+08	1.2E+10	8.7E+07
Control	4.5	1.0E-02	4.5E+03	9.6E+02	2.1E+04	1.6E+02
L200	0	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
L100	0	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
FA1500	0	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
FA1000+L100	0	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
FA1000+L50	0	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
FA500+L100	0	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
FA500+L50	0	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00

Full-Scale ALLD Study Raw Data

Table A15: Fecal coliform bacteria MPN results for sampling period 5 (day356).

	Fecal Coliform Densities (Bounded by 95% Confidence Limits)					
	MPN index	Dilution	MPN/100ml	Lower Bound	Upper Bound	MPN/g
Baseline	2.5	1.0E-08	2.5E+09	5.3E+08	1.2E+10	8.7E+07
Control	4.5	1.0E-02	4.5E+03	9.6E+02	2.1E+04	1.6E+02
L200	0	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
L100	0	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
FA1500	0	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
FA1000+L100	0	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
FA1000+L50	0	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
FA500+L100	0	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
FA500+L50	0	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00

Table A16: Reovirus PFU/g over the entire duration of the study.

Treatment	Reovirus Viability (PFU/g)				
	12	40	69	291	356
Control	4.05E+07	4.50E+07	4.22E+07	2.09E+07	2.99E+07
L 200	0	0	0	0	0
L 100	0	0	0	0	0
FA 1500	1.84E+07	1.48E+06	1.39E+06	1.73E+04	7.75E+02
L50 + FA500	0	0	0	0	0
L100 + FA500	0	0	0	0	0
FA1000 + L50	0	0	0	0	0
FA1000 + L100	0	0	0	0	0

Full-Scale ALLD Study Raw Data

Table A17: Free ammonia results for sampling period 1 (day 12).

Treatment	Reading	Conversion	Dilution Factor	Conc. (1:10)	TS (%)	Conc. Final	pH	Frac. Of total	Free NH3 (mg/L)
CNTL	54	58.7	5.0	293.7	28.43	3986.6	8.28	7.93	316.035
L200	9	7.4	5.0	37.1	54.05	764.1	12.21	99.86	763.03
L100	24	24.5	5.0	122.6	40.05	1963.7	12.64	99.95	1962.68
FA1500	33.5	35.4	5.0	176.8	45.17	3078.8	9.2	41.73	1284.77
FA1000 + L100	17	16.5	5.0	82.7	54.42	1716.2	12.48	99.93	1714.92
FA1000 + L50	17	16.5	5.0	82.7	53.5	1683.9	12.11	99.83	1680.97
FA500 + L100	17	16.5	5.0	82.7	56.45	1792.3	12.17	99.85	1789.64
FA500 + L50	18	17.7	5.0	88.4	54.81	1849.5	11.93	99.74	1844.72

Table A18: Free ammonia results for sampling period 2 (day 40).

Treatment	Reading	Conversion	Dilution Factor	Conc. (1:10)	TS (%)	Conc. Final	pH	Frac. Of total	Free NH3 (mg/L)
CNTL	72	82.2	2.5	205.4	28.54	2792.7	8.14	5.87	163.97
L200	28	29.8	2.5	74.6	54.77	1558.5	12.3	99.88	1556.6
L100	40	44.1	2.5	110.3	40.11	1767.2	12.5	99.93	1766
FA1500	55	61.9	2.5	154.9	55.34	3275.9	9.38	52.01	1703.9
FA1000 + L100	25	26.3	2.5	65.7	55.72	1400.0	12.2	99.87	1398.2
FA1000 + L50	28.5	30.4	2.5	76.1	53.233	1539.8	11.9	99.69	1535
FA500 + L100	29	31.0	2.5	77.5	47.14	1397.9	12.1	99.83	1395.5
FA500 + L50	34	37.0	2.5	92.4	47.95	1690.4	11.6	99.43	1680.9

Table A19: Free ammonia results for sampling period 3 (day 69).

Treatment	Reading	Conversion	Dilution Factor	Conc. (1:10)	TS (%)	Conc. Final	pH	Frac. Of total	Free NH3 (mg/L)
CNTL	38	28.8	5.0	144.0	28.61	1958.7	8.61	15.55	304.5
L200	18	10.3	5.0	51.3	54.14	1058.4	12.1	99.83	1056.6
L100	20	12.1	5.0	60.6	40.27	973.4	11.6	99.45	968
FA1500	30	21.4	5.0	106.9	51.89	2106.6	8.91	26.86	565.89
FA1000 + L100	18	10.3	5.0	51.3	55.23	1082.9	11.7	99.55	1078
FA1000 + L50	21	13.0	5.0	65.2	55.14	1373.5	11.4	99.15	1361.8
FA500 + L100	24	15.8	5.0	79.1	47.99	1448.0	11.6	99.41	1439.4
FA500 + L50	25	16.7	5.0	83.7	46.79	1500.1	10.9	97.41	1461.2

Full-Scale ALLD Study Raw Data

Table A20: Free ammonia results for sampling period 4 (day 291).

Treatment	Reading	Conversion	Dilution Factor	Conc. (1:10)	TS (%)	Conc. Final	pH	Frac. Of total	Free NH3 (mg/L)
CNTL	51	75.4	2.5	188.4	28.78	2569.6	7.9	3.46	89.032
L200	9	8.2	2.5	20.6	51.33	400.8	12	99.79	400.02
L100	20	25.8	2.5	64.5	45.12	1122.8	10.1	85.05	954.9
FA1500	38	54.6	2.5	136.5	51.54	2671.0	8.97	29.66	792.26
FA1000 + L100	19	24.2	2.5	60.5	55.87	1295.0	11.9	99.72	1291.4
FA1000 + L50	16	19.4	2.5	48.5	50.51	931.3	11.4	99.13	923.17
FA500 + L100	22	29.0	2.5	72.5	48.92	1350.3	11.5	99.35	1341.6
FA500 + L50	18	22.6	2.5	56.5	49.48	1063.7	10.7	95.48	1015.6

Table A21: Free ammonia results for sampling period 51 (day 356).

Treatment	Reading	Conversion	Dilution Factor	Conc. (1:10)	TS (%)	Conc. Final	pH	Frac. Of total	Free NH3 (mg/L)
CNTL	50	57.4	2.5	143.5	28.7	1955.2	8.43	10.84	212.01
L200	5	0.4	2.5	1.1	51.6	21.2	12.5	99.92	21.19
L100	35	38.4	2.5	96.0	0	960.4	11.2	98.59	946.91
FA1500	33	35.9	2.5	89.7	53.88	1840.4	9.21	42.29	778.32
FA1000 + L100	10	6.8	2.5	16.9	55.6	359.7	11	97.88	352.05
FA1000 + L50	15	13.1	2.5	32.7	55.84	699.9	12	99.80	698.51
FA500 + L100	28	29.6	2.5	73.9	48.52	1365.6	12.2	99.85	1363.6
FA500 + L50	34	37.2	2.5	92.9	47.57	1687.2	11.2	98.65	1664.5

APPENDIX B

Bench-Scale PAM Study Raw Data

Table B1: Time-pH results for the control treatments and the lime only class B trials. Each pH result is an average of three readings

DU Control		DD Control		DD L80		DU L115		DU L80		DU L90	
Time (hrs)	pH	Time (hrs)	pH	Time (hrs)	pH	Time (hrs)	pH	Time (hrs)	pH	Time (hrs)	pH
0	5.8	0	7.8	0	12.3	0	12.3	0	12.1	0	12
1	5.8	1	7.9	0.5	12.1	0.5	12.1	0.5	11.3	0.5	11.8
3	5.8	3	7.7	1	12.2	1	12.1				
5	5.8	5	7.4	1.5	12.1	1.5	12.1				
10	5.7	10	7.3	2	12.1	2	12.1				
22	5.6	24	7.4								
27	5.6	32	7.3								
41	5.6	47	7.3								
49	5.6	71	7.3								
64	5.7	95	7.3								
88	5.7	119	7.4								
112	5.8	143	7.5								
136	5.8	172	7.5								
160	5.8										
189	5.9										

Table B2: Time-pH results for the PAM alone and PAM/lime combination treatments for the class B trials. Each pH result is an average of three readings

DD PAM 800		DU PAM 1500		DD L40+PAM600		DU L60+PAM1200		DD PAM 2000	
time (hrs)	pH	time (hrs)	pH	time (hrs)	pH	time (hrs)	pH	time (hrs)	pH
0	9.9	0	10	0	11.4	0	11.6	0	10.4
1	10.1	1	10.1	0.5	11.2	0.5	11.5	0.5	10.6
3	10.3	3	10.1	1	10.8	2	11.2	1	10.6
10	10.3	10	10.1	2.5	10.6	5	10.9	2.5	10.5
25	10.4	23	10.1	5	10.6	23	10.6	5	10.5
32	10.3	47	10.1	24	10.5	27	10.5	9	10.6
47	10.3	70.5	10.2	27	10.5	74	10.2	21	10.6
71	10.5	122	10.3	30	10.4	96	10.2	23	10.6
92.5	10.5	143.5	10.2	48	10.2	122	10.1	26	10.6
144	10.4	169	10.2	100	10.2	145.5	10.1	49	10.6
165.5	10.4			121	10.2			76	10.6
				146.5	10.2			97.5	10.6
				170.5	10.1			121.5	10.7
								145.5	10.6
								196	10.7
								247	10.7
								264	10.6
								289	10.7
								302	10.6

Bench-Scale PAM Study Raw Data

Table B3: Time-pH results from the lime only treatments for the class A trials with 5 min mixing and room temperature storage.

DD L300		DD L500		DU L500	
time (hrs)	pH	time (hrs)	pH	time (hrs)	pH
0	12.5	0	12.2	0	12.3
1	12.3	1	12.4	1	12.2
3	12.3	23	12.2	23	12.2
23	12.3	48	12.3	48	12.2
48	12.3	72	12.3	72	12.3
72	12.2				

Table B4: Time-pH results from the lime only treatments for the class A trials with 1 min mixing and room temperature storage.

DD L250		DU L250		DU L350		DD L350		DU L500		DD L500	
time (hrs)	pH	time (hrs)	pH	time (hrs)	pH	time (hrs)	pH	time (hrs)	pH	time (hrs)	pH
3	12.3	0	12.3	0	12.3	0	12.3	0	12.3	0	12.6
48	12.7	23	12.2	22	12.4	22	12.3	24	12.2	24	12.2
72	12.6	48	12.1	48	12.4	48	12.5	48	12.1	48	12.3
90	12.6	72	12.2	72	12.3	72	12.5	72	12.2	72	12.2

Table B5: Time-pH results from the lime only treatments for the class A trials with 1 min mixing and control chamber storage (35°C).

DD L500		DU L500		DD L600		DU L600		DD L500+PAM1000		DU L500+PAM1000	
time (hrs)	pH	time (hrs)	pH	time (hrs)	pH	time (hrs)	pH	time (hrs)	pH	time (hrs)	pH
0	12	0	12	0	12.1	0	12.5	0	12.3	0	12.2
24	12.3	24	12.5	24	12.5	24	12.7	24	12.6	24	12.4
48	12.9	48	12.8	48	12.8	48	12.6	48	12.6	48	12.3
72	12.6	72	23.5	72	12.5	72	12.4	72	12.5	72	12.4

Table B6: Initial time-pH results for the PAM

Time (hrs)	PAM 200	PAM 400	PAM 600	1/10 PAM/H2O
0	8.2	8.8	9.2	12.2
1	2.6	9.3	9.6	12.5
6	8.7	9.3	9.7	12.3
21	8.9	9.5	9.8	12.3
33	8.8	9.3	9.8	12.3
46	8.8	9.3	9.8	12.3

APPENDIX C

Enteric Virus Indicator Study Raw Data

Table C1: Reovirus (T1L) percent survival at thermophillic temperatures

°C	Time (min)	Titre	% Survival
50	0	3.10E+09	100.00
	5	5.50E+09	177.42
	15	3.60E+09	116.13
	30	4.50E+09	145.16
52.5	0	1.00E+09	100.00
	5	3.50E+08	35.00
	15	1.00E+06	0.10
	30	X	X
55	0	1.00E+09	100.00
	2	2.20E+07	2.20
	5	1.30E+06	0.13
	15	8.00E+04	0.00308
	30	3.00E+04	0.00115

Table C2: Reovirus (T1L) percent survival at mesophillic temperatures °C

Temp (°C)	Percent Survival of Reovirus (T1L) Time (Days)										
	0	1	2	3	4	7	10	14	17	21	28
4	100	100	97.5	100	100	100	100	100	100	98.9	100
37	100	100	100	85	100	100	100	100	100	81.4	58.3
39.5	100	98.6	98.6	95.6	100	100	100	48.6	100	73.9	
42	100	100	88.3	78.9	100	61.3		28.8		3.9	0.005
45	100	100	75.5	59.2	100	13.7	0.46	0.04	0.045	9E-04	2E-04
50	100	49.1	18.3	0.004	0.02	1E-04	0	0	0	0	0

Enteric Virus Indicator Study Raw Data

Table C1: Reovirus (T1L) percent survival at varying low lime doses.

Day	Treatment	Titre (PFU/ml)	% survival
2	control	590000.00	100
	L10	134000.00	22.7118644
	L20	400000.00	67.7966102
	L40	27000.00	4.57627119
	L80	0.00	0
	L100	0.00	0
4	control	600000.00	101.694915
	L10	765000.00	129.661017
	L20	445000.00	75.4237288
	L40	11200.00	1.89830508
	L80	0.00	0
	L100	0.00	0
8	control	770000.00	130.508475
	L10	0.00	0
	L20	0.00	0
	L40	3900.00	0.66101695
	L80	182000.00	30.8474576
	L100	0.00	0
11	control	47000.00	7.96610169
	L10	1740000.00	294.915254
	L20	320000.00	54.2372881
	L40	1200.00	0.20338983
	L80	0.00	0
	L100	0.00	0
24	control	320000.00	54.2372881
	L10	460000.00	77.9661017
	L20	230000.00	38.9830508
	L40	3200.00	0.54237288
	L80	0.00	0
	L100	0.00	0

APPENDIX D

STANDARD OPERATING PROCEDURE (SOP)
FOR ENUMERATION OF BACTERIAL SPORES
OF *Clostridium perfringens* IN WASTEWATER
AND WASTEWATER SOLIDS
University of Manitoba, Department of Civil Engineering
Environmental Engineering Laboratory

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 <u>not</u> 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. <u>Do not heat</u>. 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 °C and check for gelatin liquefaction. If medium solidifies, re-incubate additional 24 hours at 35 °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 made anaerobic by using a vacuum-purge manifold or 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.

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STANDARD OPERATING PROCEDURE (SOP)
FOR ENUMERATION OF HELMINTH EGGS
(Including Genus *Ascaris*)
IN WASTEWATER AND WASTEWATER SOLIDS
(based on Tulane University procedure and modified)
University of Manitoba, Department of Civil and Geological Engineering
Environmental Engineering Laboratory

Modified August 2001, Jamie Brewster

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.

materials, reagents and apparatus

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.

Reagents

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.

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.

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).

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.

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.

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.

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.

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.

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.

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.

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.

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.

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).

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 (using a needle and syringe).
- 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 discard supernatant to the Erlenmeyer flask (using a needle and syringe) . 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.

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 discard supernatant to the Erlenmeyer flask (using a needle and syringe).
- 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 (using a needle and syringe).
- Add 10-15 mL of culture fluid, mix, and centrifuge again. Discard supernatant (using a needle and syringe).
- 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).

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).

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.