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Evaluation of bacteriophage and viral persistence during alkaline stabilization in sludge and biosolids intended for land application

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EVALUATION OF BACTERIOPHAGE AND VIRAL PERSISTENCE DURING ALKALINE STABILIZATION IN SLUDGE AND BIOSOLIDS INTENDED FOR LAND APPLICATION

BY

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B.S. University of New Hampshire, 1999

DISSERTATION

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in

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DEDICATION

To my parents Robert and Cheryl for always believing in me and providing me every opportunity to succeed, to my siblings Jennifer, Candice and Robert for their unwavering support and encouragement throughout this journey, and to my Nana Jacqueline for her strength and inspiration. I am so fortunate and truly grateful to have the love and support of wonderful family and friends. I could not have achieved this goal without you.

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ABSTRACT

EVALUATION OF BACTERIOPHAGE AND VIRAL PERSISTENCE DURING ALKALINE STABILIZATION IN SLUDGE AND BIOSOLIDS INTENDED FOR LAND APPLICATION

by

Jacqueline J. Brabants
University of New Hampshire, May, 2003

The use of lime to reduce or eliminate pathogen content is a cost-effective treatment method currently being employed in many Class B biosolids production plants in the United States. A bench scale model of lime stabilization was designed to evaluate pathogen persistence. The survivability of poliovirus type 1, fecal coliforms, *Salmonella*, adenovirus type 5, rotavirus Wa, and the male-specific bacteriophage MS-2 was evaluated under lime stabilization conditions in various matrices. *Salmonella* and fecal coliforms were evaluated at 28°C and poliovirus and MS-2 were evaluated at both 28°C and 4°C for survivability under lime stabilization conditions in a sludge matrix. All microorganisms evaluated were discovered to be below detectable levels following twelve hours of liming for all temperatures evaluated. Adenovirus type 5, rotavirus Wa, and MS-2 were evaluated in water, biosolids, previously limed and raw sludge and biosolids matrices at 28°C and 4°C. In all matrices evaluated, adenovirus type 5, rotavirus Wa and MS-2 were below detectable levels following a twenty-four hour incubation with lime, with inactivation rates varying depending on the sample matrix evaluated.

Evaluation of reliable and cost effective treatment processes to demonstrate sufficient inactivation of pathogens provides valuable information for implementation of treatment technologies. This research has demonstrated that lime stabilization is effective at reducing fecal coliforms and *Salmonella* in a sludge matrix at 28°C, poliovirus in a sludge matrix at 28°C and 4°C, and adenovirus type 5, rotavirus Wa and male-specific bacteriophage in sludge and biosolids matrices at 28°C and 4°C when calcium hydroxide was added to achieve a pH of 12 for 2 hours and 11.5 for 22 hours. The similar inactivation of MS-2 under lime stabilization conditions, and prevalence and ease of recovery in sludge samples, combined with the need for a representative indicator in the absence of adequate methodology for recovery and detection of enteric viruses in sludge and biosolids makes male specific bacteriophages a suitable indicator as an index for enteric viruses, to be used for the monitoring of biosolids to determine treatment effectiveness following lime stabilization.

CHAPTER ONE

AN OVERVIEW OF LEGISLATION AND PATHOGEN STANDARDS FOR THE TREATMENT AND LAND APPLICATION OF BIOSOLIDS

INTRODUCTION

In the United States, an estimated six million dry tons of sewage sludge are generated every year (NRC, 2002). This exorbitant amount is primarily due to the establishment of the Clean Water Act of 1972. The objectives of this legislation were to reduce the amount of biosolids discharged into our rivers and seas, establish minimum treatment requirements for municipal wastewater, and restore the physical, chemical and biological integrity of the nation's waterways. The implementation of the Clean Water Act resulted in enormous improvements in industrial effluent discharge and control of point source pollutants. Prior to enactment of the Clean Water Act, it was believed that dilution of waste in waterways was sufficient. This philosophy changed in the 1970's, bringing a shift in focus towards protection of the environment and ecology, and development of the modern day wastewater treatment that protects our nation's waterways. Yet, a new issue emerged as a result of the success of the Clean Water Act, that of sludge disposal (NRC, 2002).

Sewage sludge is a by-product of wastewater treatment processes and is defined as the solid, semi-solid, or liquid residue generated during the treatment of domestic

sewage. The goal of sewage sludge treatment processes is to minimize the volume and organic content of waste, in addition to reducing the presence of pathogens, thereby generating biosolids. Biosolids are the primary organic solid product from sewage sludge that has been treated to meet the regulatory requirements for land application. Biosolids are a complex mixture of organic, inorganic, and biological components obtained from the wastewaters of households, commercial businesses, and industrial facilities, combined with additional compounds that may be implemented or generated during treatment (NRC, 2002) (Straub et al., 1993).

Ideally, wastewater treatment processes will minimize pollutants while maintaining the beneficial properties of biosolids, making the material desirable for soil-amendment and land-reclamation purposes (Dumontet et al., 2001). The land application of biosolids represents a practical means of managing the large volume of sewage sludge generated at wastewater treatment plants by recycling nutrients and organic matter while avoiding the environmental and economic costs of land filling, incineration, surface site disposal and ocean dumping (Strauch, 1991) (Lasobras et al., 1999) (Wong et al., 2000). Today, approximately fifty percent of the sewage sludge generated annually in the United States is land applied, with the remaining material being disposed of by incineration, landfilling, or advanced treatment methods, to generate material that can be marketed and distributed as fertilizer (Figure 1) (Jager, 2000). The dramatic increase in the use of sewage sludge as soil amendments, fertilizer, or for land reclamation is in part due to the ban on ocean disposal of wastewater residuals in 1992 (NRC, 2002).

The land-application of biosolids for the purpose of soil amendment occurs on both agricultural and nonagricultural land, and to reclaim severely disturbed land such as

strip mines and gravel pits. Agricultural land includes sites where food crops (for human or animal consumption) and non-food crops are grown. Nonagricultural land includes forests, rangelands, and public contact sites such as public parks, golf courses and cemeteries. The Environmental Protection Agency estimates that sewage sludge is only applied to approximately 0.1% of the available agricultural land in the United States on an annual basis (Figure 1) (EPA, 1999a). Therefore, land application as a means of disposal of biosolids is an alternative that is not being exploited to its full potential (Jager, 2000).

Biosolids have been demonstrated to improve the chemical and physical properties of soils because biosolid material contains important nutrients and trace elements such as phosphorus and nitrogen, that enhance plant growth; however, hazardous pollutants potentially present in biosolids material may include inorganic contaminants, such as metals, pharmaceuticals and pesticides; organic contaminants, such as polychlorinated biphenyls (PCB's) and dioxins; as well as pathogens such as bacteria, viruses and parasites (Strauch, 1991) (Dumontet et al., 2001) (Lewis et al., 2002) (Straub et al., 1993). The presence of these pollutants creates a public health risk due to the potential for direct exposure to workers and community populations as well as the potential for runoff creating surface water contamination or movement through the soil column resulting in groundwater contamination (NRC, 2002) (Gaby, 1981) (NIOSH, 2000).

The potential hazards present in biosolids material, combined with their use as a soil amendment in areas where human contact may occur, inspired the U.S. Environmental Protection Agency to create an amendment to the Clean Water Act in

1993. The goal of this amendment was to establish regulations to protect the public health and the environment from pollutants that may be present in sewage sludge biosolids. This amendment, commonly known as the Part 503 rule, is contained within Title 40 of the Code of Federal Regulations under section 405 (d) of the Clean Water Act (EPA, 1996).

The Part 503 rule dictates the standards for the use and disposal of sewage sludge, including management practices for the land application of sewage sludge (EPA, 1996) (EPA, 1999b). This rule establishes minimum quality standards for land-applied sludge that dictate concentration limits and loading rates for chemicals, as well as providing treatment and use requirements to control and reduce pathogens and attraction of disease vectors. The pathogen standards are focused on reducing the presence of pathogens and potential exposures by treatment or a combination of treatment and use restrictions. The standards are not based on risk analysis, as are chemical standards (EPA, 1996) (EPA, 1999b). This is due to unreliable methods for the assay of pathogens combined with insufficient and inconsistent data on both the fate and transport of pathogens in the environment (Lewis et al., 2002). As a result of the lack of current scientific data on the range and persistence of pathogens in treated biosolids, the pathogen standards require the monitoring of indicator organisms. There are a large variety of pathogens potentially present in sewage sludge and it is seemingly impractical to monitor for them individually (NRC, 2002) (Baker and Hegarty, 1997).

The indicator organisms utilized in the Part 503 rules for determination of pathogen presence were chosen based on the scientific and technical information available prior to legislation. When the Part 503 rule was enacted, it was believed that the proposed treatment methods, coupled with biosolids management practices, would be

effective in preventing harmful exposure to biosolids components. It is now necessary to re-evaluate the pathogen-reduction requirements established in the part 503 rules in light of current scientific data and risk assessment methods. The effectiveness of treatment methods must be verified with regard to specific pathogens that were not cultivatable or even considered human pathogens at the time the 503 rules were enacted. Currently, there are enormous gaps in the scientific data available on reduction of pathogens during commonly employed treatment processes (Lewis et al., 2002). The lack of data makes developing accurate risk assessment models, for the purpose of evaluating the risk of land application of biosolids to the public health and the environment, extremely difficult if not impossible.

Formidable challenges exist with regard to biosolids management and legislation. The use of management practices is the primary means of controlling the exposure of human pathogens potentially found in biosolids (EPA, 1996). However, the Environmental Protection Agency's biosolids program has a low priority and as a result there is a lack of available resources to adequately monitor management practices and enforce existing legislation. There are inconsistencies in how states manage biosolids and no formal process for tracking health complaints associated with land application of biosolids. One of the most difficult challenges for the EPA's biosolids program is the burden of creating regulations that will adequately protect public health, when there is a deficit of scientific data on pathogen persistence. As a result of these shortfalls, the general public has expressed concern regarding the effects of biosolids on health, quality of life and natural resources. The perceived risks associated with land application of biosolids, including presence of heavy metals and pathogens, negative public perception,

odor complaints, and an increase in contaminants in the water supply, outweigh the economic incentives and soil improvement benefits realized by farmers (Krogmann et al., 2001). At present, the public perception of land application of biosolids is poor and this has had a significant negative effect on the successful implementation of the biosolids land application program.

The evaluation of cost-effective treatment technologies to determine the persistence of pathogens of public health importance is necessary to determine treatment effectiveness and begin to ease uncertainties associated with the land application of biosolids. Evaluation of reliable and cost-effective treatment processes to demonstrate sufficient inactivation of pathogens would provide valuable information for utilities looking to implement treatment technologies. Ultimately, the elucidation of effective treatment technology and identification of representative indicator organisms for pathogen monitoring in sewage sludge will help to ensure public health and safety, and promote the sustainable practice of biosolids land application.

Biosolids Management throughout the United States

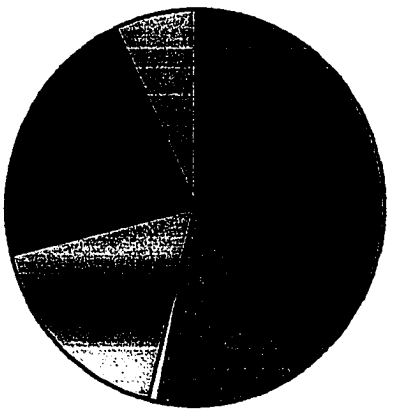

Figure 1: Biosolids Management Throughout the United States: Adapted from the Journal of the New England Water Environment Association, November 2000, Vol. 34. Approximately 60% of the biosolids generated in the United States are beneficially used with land application representing the primary method of managing biosolids in the country. In the chart legend, land application refers to the traditional practice of applying treated biosolids, including lime stabilized material, to permitted fields. Advanced treatment includes heat drying and advanced alkaline stabilization to generate a Class A product which is generally used as a fertilizer and soil amendment on lawns and gardens. Landfill refers to all forms of sludge disposal in landfills. Incineration refers to incineration of material generally in incinerators that are reserved exclusively for biosolids. Other beneficial use represents unidentified management practices and disposal methods.

Biosolids Management in New Hampshire

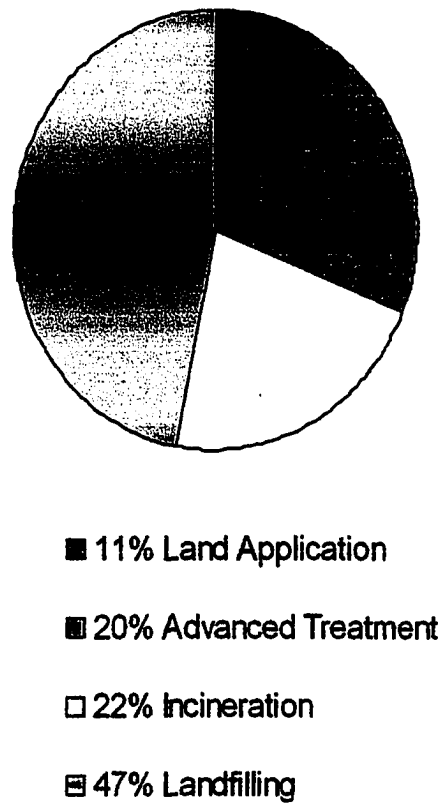

Figure 2: Biosolids Management in New Hampshire: Adapted from the Journal of the New England Water Environment Association, November 2000, Vol. 34. In the state of New Hampshire, landfilling predominates as the primary method for biosolids disposal. Beneficial use of biosolids, especially land application, is a very controversial issue in New Hampshire where many communities have adopted local ordinances severely restricting or outright banning biosolids land application. In the chart legend, land application refers to the traditional practice of applying treated biosolids, including lime stabilized material, to permitted fields. Advanced treatment includes heat drying and advanced alkaline stabilization to generate a Class A product which is generally used as a fertilizer and soil amendment on lawns and gardens. Landfill refers to all forms of sludge disposal in landfills. Incineration refers to incineration of material in the state's one incinerator which is located in Manchester, NH.

Pathogen Standards for Biosolids

Pathogens are microorganisms capable of establishing an infection and causing disease in a susceptible host (Ryan, 1994). Pathogens are able to survive in soils from hours to years depending on the specific pathogen, biosolids application methods and rates (injection, incorporation, or surface application), initial pathogen concentrations, and environmental conditions such as soil composition, and meteorological and geological conditions (Jager, 2000) (NRC, 2002) (Straub et al., 1993). The pathogen standards contained within the Part 503 regulations were developed in an effort to reduce the presence of pathogens by treatment or a combination of treatment and use restrictions; however, risk assessments were not conducted in the development of the 1993 pathogen standards due to a lack of adequate technical data (NRC, 2002) (Lewis et al., 2002) (EPA, 1996). The pathogen standards dictate treatment requirements, site restrictions, and monitoring requirements. The specific criteria for regulatory compliance with land-application guidelines is determined by the biosolid classification desired. The U.S. Environmental Protection Agency categorizes biosolids as Class A or Class B. This classification is based on several parameters including organic, metal and microbial content (EPA, 1999a) (EPA, 1996).

Class A Pathogen Requirements

Class A sludge has pathogen densities which are greatly reduced or are below detectable levels using the EPA assay for the specific pathogen. Class A sludge is

generated using a process to further reduce pathogens (PFRP). Class A certification requires demonstration of pathogen reduction by using one of several prescribed strategies combined with screening to verify reduction of bacteria, enteric viruses, *Salmonella* and viable helminth ova. Class A biosolids are treated to reduce the presence of pathogens to below detectable levels as well as meeting the high-quality pollutant concentration limits for metals. As a result, Class A products can be used without any restrictions at the application site (EPA, 1999a) (EPA, 1999b) (EPA, 1996).

To achieve Class A certification, a utility must meet treatment-process control criteria as well as pathogen reduction for either fecal coliform or *Salmonella* density. The fecal coliform density must be less than one thousand most probable number (MPN) per one gram (g) of total solids (TS) analyzed. *Salmonella* density must be less than three MPN per four grams of TS analyzed. In addition to satisfying pathogen reduction requirements for treatment control, one of a variety of treatment processes must be met in order to designate the product Class A. The goal of the available processes is to reduce pathogen densities to below specified detection limits for *Salmonella* (less than three MPN per four grams TS), enteric viruses (less than one plaque forming unit (PFU) per four grams TS), and helminths (less than one viable helminth ova per four grams TS). For biosolids to be categorized as Class A with respect to pathogen content, they must meet one of the following criteria: time and temperature requirements based on percentage of solids in the material, pH adjustment accompanied by high temperature and solids drying, monitoring of enteric viruses and helminths after a treatment process to ensure below-detectable concentrations, monitoring of enteric viruses and helminths in the biosolids at the time they are distributed or applied to land, treatment by a process for

the further reduction of pathogens (PFRP) or treatment in a process deemed equivalent to a PFRP which may include one of several processes. Processes considered to be equivalent to a PFRP include composting with minimum time and temperature conditions, heat drying with specified temperature and moisture conditions, high-temperature heat treatment, thermophilic aerobic digestion at specified time and temperature, beta irradiation at specified dosage, gamma irradiation at specified dosage or pasteurization. See Table 1 for alternative treatment processes designated to achieve Class A certification (Jager, 2000) (NRC, 2002) (EPA, 1999a) (EPA, 1999b).

Table 1: Class A Pathogen Requirements

To achieve Class A certification, treatment process control requirements and densities of either fecal coliform or *Salmonella* must be met. Density criteria for fecal coliforms are less than 1,000 MPN per gram of total solids (TS). Density criteria for *Salmonella* are less than three MPN per four grams of TS. One of the following treatment processes must be met for Class A designation, the goal of which is to reduce pathogen densities to below specified detection limits for *Salmonella* (<3 MPN per four grams TS), enteric viruses (<1 plaque forming unit (pfu) per four grams TS), and helminths (<1 viable organism per four grams TS)

Alternative Treatment Process	Description
1	**Temperature and Time Process**: Sewage-sludge must be maintained at an increased temperature for a prescribed period of time according to set guidelines.
2	**Alkaline Treatment Process**: The sewage sludge pH must be raised to greater than 12 for at least 72 hours. During this time, the sewage sludge temperature must exceed 52°C for at least twelve hours. Following the 72 hour period, the sewage sludge must be air dried to at least 50% total solids.
3	**Prior Test for Enteric Virus and Viable Helminth Ova**: The sewage sludge must be analyzed for the presence of enteric viruses and viable helminth ova. If the pathogen reduction requirements are met prior to processing, the sewage sludge is considered Class A with respect to enteric virus and viable helminth ova until the next monitoring event.
4	**Post-Test for Enteric Virus and Viable Helminth Ova**: If the sewage sludge is not analyzed before pathogen-reduction processing for enteric viruses and viable helminth ova, the density of such organisms must meet pathogen reduction criteria at the time the sewage sludge is used, disposed of, or prepared for sale.
5	**Processes to Further Reduce Pathogens**
5a	**Composting Process**: When using within-vessel or static-aerated pile composting methods, the sewage sludge temperature must be maintained at 55°C or higher for three days. If windrow composting methods are used, the sewage sludge temperature must be maintained at 55°C or higher for 15 days or longer. During this period, a minimum of five windrow turnings are required.
5b	**Heat drying Process**: The sewage sludge must be dried by direct or indirect contact with hot gases to reduce the moisture content to 10% or lower. Either the temperature of the sewage-sludge particles must exceed 90°C or the wet bulb temperature of the gas in contact with the sewage sludge leaving the dryer must exceed 80°C.
5c	**Heat Treatment Process**: Liquid sludge must be heated to a temperature of 180°C or higher for 30 minutes

5d	**Thermophilic Aerobic Digestion Process**: Sewage sludge must be agitated with air or oxygen to maintain aerobic conditions. The mean cell residence time for the sewage sludge must be 10 days at 55°C to 60°C.
5e	**Beta Ray Irradiation Process**: The sewage sludge must be irradiated with beta rays from an accelerator at a dose of at least 1.0 megarad at room temperature.
5f	**Gamma Ray Irradiation Process**: The sewage sludge must be irradiated with gamma rays from certain isotopes, such as cobalt 60 and cesium 137, at a dose of at least 1.0 megarad at room temperature.
5g	**Pasteurization Process**: The temperature of the sewage sludge must be maintained at 70°C or higher for 30 minutes or longer.
6	**Process Equivalent to Process to Further Reduce Pathogens (PFRP)**: The sewage sludge must be treated in a process that is equivalent to PFRP, as approved by the permit authority. To obtain a class A biosolid rating, the process must reduce *Salmonella* species or fecal coliforms to below Class A criteria and must operate under the specified conditions used in its application demonstration to the EPA Pathogen Equivalency Committee.
Table adapted from National Research Council: "Biosolids Applied to Land, Advancing Standards and Practices," 2002	

Class B Pathogen Requirements

Class B sludge is generated by using one or a combination of prescribed processes to significantly reduce pathogens (PSRP), or a process equivalent to a PSRP. Demonstration of pathogen reduction to achieve Class B certification requires monitoring of fecal coliform levels (EPA, 1996). Class B sludge is known to contain pathogens because it is treated to a lesser extent than Class A sludge. Class B biosolids are permitted to contain detectable concentrations of pathogens and therefore site restrictions and management practices are recommended to minimize exposure with potentially harmful constituents until environmental degradation (heat, sunlight, desiccation) has further reduced the presence of pathogens (NRC, 2002). Currently, there are no

requirements for measurement of pathogen density during on-site application. Class B sludge is less expensive and therefore most highly desired. Class B sludge is spread in great quantities on land as fertilizer and is also mixed with other material to use for gravel pit reclamation and landfill closures; however, restrictions exist with regard to public use sites. Due to the presence of pathogens contained within sludge treated to meet class B requirements, site restrictions exist with regard to land application of this material and are based on the time required to reduce the levels of pathogens to below detectable concentrations at the time of public exposure (EPA, 1996).

Fecal coliforms are the only microbiological parameter evaluated for Class B biosolids. In addition to complying with the fecal coliform limitations for Class B biosolids, which dictate that the geometric mean of seven samples must be less than two million MPN or colony forming units (CFU) per gram of total solids analyzed, treatment requirements may be satisfied by performing one of a variety of processes. Processes to significantly reduce pathogens include aerobic digestion at defined time and temperature combinations, air drying for three months with at least two months at average ambient daily temperatures above freezing, anaerobic digestion under defined time and temperature conditions, composting under defined time and temperature conditions, or lime stabilization to maintain the pH above twelve after two hours of contact. The aforementioned processes are believed to result in fecal-coliform concentrations of less than two million per gram of totals solids analyzed and reduce the concentrations of *Salmonella* and enteric virus (NRC, 2002) (Table 2).

There exists a need for data demonstrating rates of pathogen survival in soil or on crops following the land application of biosolids. It is necessary to evaluate the reliability

of the Environmental Protection Agency's prescribed treatment techniques using current pathogen detection technology, and to examine the potential for regrowth of pathogens. The indicators (fecal coliforms, enteric virus, and helminth ova) appear to be present in very low densities in biosolids and in raw sewage sludge. This is a problem because as representative pathogens, these organisms are routinely screened for and used to measure presence and treatment efficiency for Class A biosolids. In addition, it is necessary to determine whether to use indicator organisms to predict pathogen survival and recontamination, and if so which organisms are adequate and most representative. Such data will be useful for development of quantitative microbial risk-assessment models to more accurately determine the risk associated with land application of biosolids (NRC, 2002) (Jager, 2000) (EPA, 1999a) (EPA, 1999b).

Table 2: Class B Pathogen Requirements

To achieve Class B certification, management-practice requirements, including site restrictions, and pathogen control requirements for densities of fecal coliforms must be met. The geometric mean of fecal coliform densities for at least seven separate samples must be less than two million MPN (most probable number) or CFU (colony forming units) per gram of total solids (TS) analyzed.

Treatment Process	Description
1	**Aerobic Digestion:** The sewage sludge must be agitated with air or oxygen to maintain an aerobic condition for a mean cell residence time and temperature between forty days at 20°C and sixty days at 15°C.
2	**Anaerobic Digestion:** The sewage sludge must be treated in the absence of air for a specific mean cell residence time at a specific temperature. Values for the mean cell residence time and temperature must be between 15 days at 35°C to 55°C and 60 days at 20°C.
3	**Lime Stabilization:** Sufficient lime must be added to the sewage sludge to raise the pH to 12 for 2 hours of contact time.
4	**Air Drying:** The sewage sludge must be dried on sand beds or in paved or unpaved basins for a minimum of three months. During two of the three months, the ambient average daily temperature must be above 0°C.
5	**Composting:** The sewage sludge must be composted using either within-vessel, static-aerated pile, or windrow composting methods and the temperature raised to 40°C or higher for five days. For four hours at some point during each of the five days, the temperature in the compost pile must exceed 55°C.
6	**Process Equivalent to Process to Significantly Reduce Pathogens (PSRP):** Treat the sewage sludge in a process that is equivalent to a PSRP, as approved by the permit authority. (i.e. N-viro alkaline stabilization, Synox OxyOzone process)

Table adapted from National Research Council: "Biosolids Applied to Land, Advancing Standards and Practices," 2002

Biosolids Treatment Processes

Wastewater treatment generates effluent and sewage sludge. Wastewater originating from homes, businesses, and industries is combined in wastewater treatment plants where it is treated to allow effluent discharge into the surface and groundwater of the United States. The residual solid product is also treated, generally through stabilization, aerobic or anaerobic process, for specified time periods and temperatures. Sewage sludge is generated in several treatment processes including primary clarification and secondary clarification of sewage sludge. The process of treating wastewater and sewage sludge is illustrated in Figure 3.

One of the goals of biosolids treatment processes is to reduce pathogens in an effort to protect public health. There are a variety of techniques that exist to reduce pathogens. Techniques that combine physical, chemical and biological processes are employed to optimize pathogen reduction in biosolids. Physical means for pathogen reduction include heating and cavitation, which is a term for processes that impart high mechanical energy such as ultrasound and pulse power, to a fluid resulting in the creation of high temperature and pressure microenvironments (NRC, 2002) (Jager, 2000).

Chemical disinfection of biosolids is a technique that has been employed for over fifty years. The chemicals used for this purpose are classified on the basis of the mode of disinfection and stabilization. Alkaline stabilization is a method utilized extensively in the United States. Alkaline stabilization agents include quick lime (CaO), hydrated lime ($Ca(OH)_2$), cement kiln dust, or lime kiln dust, which are added to liquid biosolids or dewatered cake. Alkaline stabilization processes produce Class B biosolids (Jager, 2000).

To generate Class A biosolids, increased temperatures or addition of ammonia are necessary to achieve a higher level of treatment in an effort to inactivate highly resistant viruses, protozoan spores and helminth eggs (NRC, 2002).

Biological processing has been effective in the digesting, composting and storage of biosolids and involves mechanical or autothermal heating. To comply with current regulations for the use and disposal of sewage, treatment plants must meet a standard set of criteria for pathogen reduction and demonstration of process efficiency. This criterion differs depending on the classification of biosolids desired (NRC, 2002) (EPA, 1999a) (EPA, 1999b) (EPA, 1996).

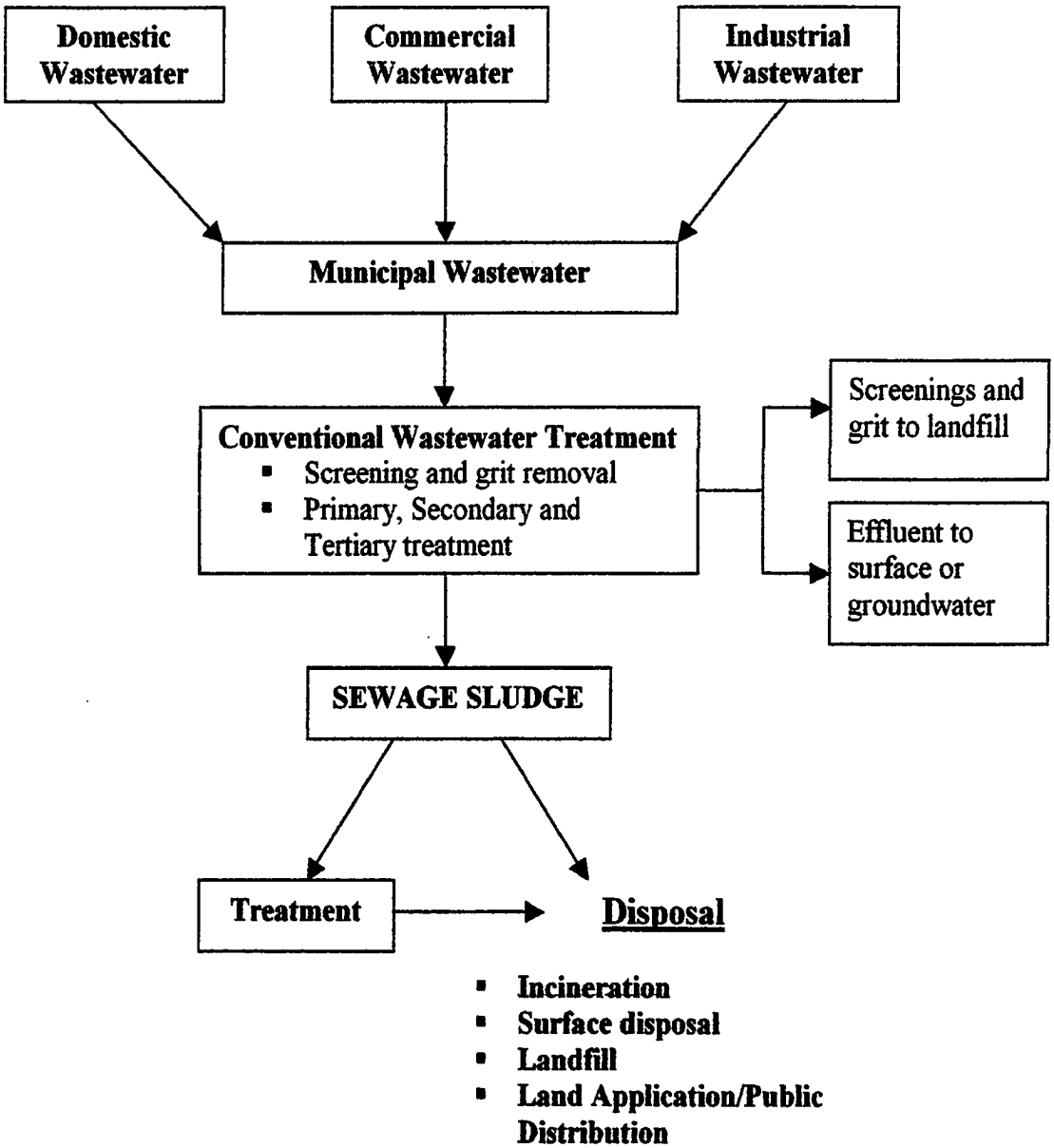

Figure 3: Schematic flow diagram of wastewater treatment and biosolids generation and disposal. Adapted from National Research Council "Biosolids Applied to Land: Advancing Standards and Practices," 2002.

Lime Stabilization

The use of lime to reduce or eliminate pathogen content in sewage sludge represents a simple and inexpensive method of treatment by which Class B sludge can be generated (Jager, 2000). Liming is a process where calcium hydroxide or calcium oxide is added and the pH elevated to 12 for 2 hours and then reduced to 11.5 for 22 hours. Lime has been in use for a number of years for the disinfection and odor suppression of solid wastes. Initial studies using lime were conducted to demonstrate the potential for phosphate removal. During these studies it was observed that the high pH achieved during addition of calcium hydroxide inactivated bacteria in addition to creating a sludge that was essentially odorless (Sattar et al., 1976). It is now known that addition of lime to raw sewage or primary effluent results in the flocculation of organic matter and the sedimentation of this flocculated material creates a relatively clear supernatant with reduced amounts of phosphates and other pollutants (Sattar et al., 1976) (NRC, 2002). Therefore, addition of calcium hydroxide (lime) to wastewaters is a way to achieve phosphate removal; thereby allowing utilities to comply with regulations that previously existed to control the discharge of chemical pollutants such as phosphates into waterways.

Early lime stabilization studies conducted for the purpose of phosphate removal and assessment of viral inactivation, involved collection of the supernatant formed during liming and its subsequent evaluation for enteric virus (Sattar et al., 1976). Results indicated a 99.99% reduction of seeded virus. However, techniques for virus recovery from sludge were not available at the time and as a result the potential viral adsorption to

particles found in the sedimented sludge was not taken into account (Sattar and Ramia, 1976). Additional research has demonstrated that maintaining a high pH over a period of time is very effective at reducing or eliminating the viral load from sludge. Studies conducted by Derbyshire and Brown in the late 1970's revealed the inactivation of porcine strains of enterovirus and adenovirus in pig slurry treated with calcium hydroxide (Derbyshire and Brown, 1979). Deng and Cliver conducted additional research to evaluate inactivation of pathogens in sludge matrices and demonstrated the inactivation of poliovirus type 1 in mixtures of septic tank effluent and dairy cattle manure slurry (Deng and Cliver, 1995). Subsequent research conducted by this team demonstrated inactivation of hepatitis A virus in a similar matrix. Such inactivation was attributed to microbial activity, more specifically to proteolytic enzymes produced by bacteria in the waste. Grabow et al., demonstrated that the reductions in the numbers of enteric viruses were higher than those of coliphages, enterococci, total plate and coliform indicators, highlighting the potential use of coliphage and bacterial counts as indicators of pathogen survival (Grabow et al., 1978).

There are many benefits associated with the process of lime stabilization including elimination of pathogenic microorganisms and odor along with reduction of heavy metal movement (Wong and Fang, 2000); however, limitations do exist and include a loss of soluble phosphate, ammonia and nitrogen content that minimizes the potential benefits for soil amendment, especially when compared to alternatives such as digested sludge, where there is no reduction of organics. Lime addition significantly increases the quantity of material for disposal and treatment results in the generation of a

high pH sludge liquor which must be treated or disposed of following dewatering of limed material (EPA, 1999a) (EPA, 1999b).

There has been very little research focusing on enteric virus survival in lime stabilized sludge since the 1970's. Currently, there is little information available on the removal and inactivation of human pathogenic viruses, such as rotavirus and adenovirus, which were not cultivatable when original studies were conducted to evaluate the effects of high-pH lime treatment of sewage.

Evaluation of the lime stabilization technique to demonstrate that it is an effective method to inactivate bacterial, viral, helminth and protozoal pathogens that could not be evaluated at the time the Part 503 rules were enacted is necessary. The availability of such data will be useful in efforts to ease the uncertainties and minimize the risks associated with the application of Class B sludge to land.

Pathogens in Biosolids

There are four major types of pathogens that may be found in biosolids (Straub et al., 1993). These pathogens are bacteria, viruses, protozoans and helminths. Since the promulgation of the Part 503 rule, many new pathogens have been recognized, and the importance of others has increased, yet it appears that research on the fate of microorganisms in biosolids ceased following the adoption of the 503 regulations. There are very little data available about the fate of emerging pathogens in biosolids treatment processes and land application programs. The pathogens considered in the current regulation are enteric viruses, helminths and *Salmonella* or fecal coliforms.

Bacteria

Bacterial pathogens of concern in sewage sludge include species of *Salmonella* and *Shigella, Yersinia* and *Vibrio cholerae, Campylobacter jejuni,* and *Escherichia coli.* Several types of *E. coli* are pathogenic to humans. Enterohaemorrhagic *E. coli* of the serotype O157:H7 has been of the greatest concern in the United States. Exposure to contaminated drinking water, recreational water, and food has resulted in numerous outbreaks of diarrhea and in some cases mortality in young children due to hemolytic uremic syndrome (NRC, 2002). *E.coli* 0157:H7 occurs in domestic wastewater and has been detected in biosolids (Lytle et al., 1999).

Campylobacter is recognized as being the most common enteric bacterial infection, yet there is relatively little research documenting its fate in biosolids treatment processes or the environment (MMWR, 1999). In addition, *Staphylococcus aureus* has been implicated as a source of illness from land-applied biosolids. It is found on the skin of a large number of people and is associated with atopic dermatitis, a superficial inflammation of the skin. For bacterial pathogens, the infectious dose will vary with the organism and can be as low as 100 bacteria to establish a *Salmonella* infection (Kowal, 1985). Bacteria are an important pathogen with regard to biosolids and land application because the potential exists for regrowth following treatment, particularly when biosolids are blended with other nutrient containing materials for the creation of soil amendment products (Yanko, 1988).

Exposure to both human and animal wastes has been associated with outbreaks of gastroenteritis although there are no scientific data to confirm this (Lewis, 2002). The

range of emerging bacterial pathogens and their potential for presence in biosolids, combined with the increasing rate at which microorganisms are developing antibiotic resistance, necessitates the demand for current data regarding pathogen persistence in land applied biosolids.

Enteric Viruses

Viruses are obligate, intracellular parasites composed of a DNA or RNA core surrounded by a protein coat. Viruses require a living host for replication and specific target cell receptors to establish an infection. Infection is accomplished by release of the viral genome into a susceptible cell. There are at least 140 types of human enteric viruses that include 72 serotypes of enteroviruses, caliciviruses, hepatitis A virus, reovirus, astroviruses, adenovirus and rotavirus (Hurst et al., 1991), all of which have the potential to be transmitted through biosolids. Examination of raw sludges in various studies in different geographical areas revealed that numbers of enteric viruses may range from 1,000 to greater than 50,000 PFU/Liter with numbers of adenovirus and rotavirus reported in even larger concentrations (Lasobras et al., 1999).

Enteric viruses are the major cause of childhood diarrhea in the United States and result in an estimated one hundred deaths per year related to gastroenteritis (EPA, 2000). Poliovirus is the most well known of the enteroviruses, and is transmitted by the fecal-oral route. Infection manifests as poliomyelitis and in some cases paralysis. The environmental fate of poliovirus has been studied extensively because it is easily grown and assayed in the laboratory.

Rotaviruses and caliciviruses are the leading causes of gastroenteritis in the United States and have been shown to be transmitted by food and water. Rotaviruses are the most important viruses causing life-threatening disease in young children and are a major cause of hospitalization of children in the United States (Gerba et al., 1996) (Estes, 2001). In a recent study by Pang et al. twenty-four percent of children under two years of age evaluated for diarrheal disease were infected with rotavirus (Pang et al., 2000). Rotaviruses have been detected in water, wastewater and more recently in biosolids, although very little data exist on their occurrence in biosolids (Chapron et al., 2000). Rotaviruses are responsible for both waterborne and foodborne outbreaks in the United States and are the only double-stranded RNA viruses currently known to be transmitted through water to humans. They are extremely resistant in the environment and present in large amounts in wastewater. Rotavirus has emerged as the most common cause of severe gastroenteritis in children and as a result has become a target for prevention and control with vaccines (NRC, 2002).

Adenoviruses are one of the most common and persistent viruses detected in wastewater, and enteric adenoviruses are the second most common cause of childhood viral diarrhea (Enriquez et al., 1995) (Gerba et al., 1996). Adenoviruses are heat resistant and have been detected in Class B biosolids (Chapron et al., 2000). Some strains cause nose, eye and respiratory infections, whereas others appear to be only enteric pathogens. In immunosuppressed cancer patients, enteric adenoviruses cause serious infections, resulting in case fatalities of up to 50% (Gerba et al., 1996). Adenoviruses have been transmitted by both recreational and drinking waters (Kukkula et al., 1997) (Papapetropoulou and Vantarakis, 1998). Adenoviruses are of particular concern because

of the ability of some strains to produce tumors in animals and transform cells *in vitro* (Russell, 2000).

There has been very little research conducted on the occurrence of enteric viruses in biosolids in recent years. The existing information provides data about the prevalence of enteroviruses, specifically poliovirus, in biosolids, and the effect of treatment; yet for many processes the amount of data on virus removal is very limited. Previous cell culture techniques were limited in their ability to detect viruses in environmental samples. With the advent of new and improved cell culture detection techniques, it is now possible to assess prevalence and persistence of pathogens, such as rotavirus and adenovirus, which were previously not cultivatable. There is a need for better estimates on the concentration of enteric viruses in biosolids, along with identification of adequate indicators. In addition, there is a need for better assessment of exposure via aerosols and groundwater transport.

Protozoa

Protozoan pathogens of concern in municipal wastewater and sewage sludge include *Cryptosporidium parvum* and *Giardia lamblia*, *Entamoeba histolytica*, *Balantidium coli* and *Toxoplasma gondii*. *Toxoplasma gondii* is a threat to humans as a result of the severe deformations and damage caused to a developing fetus when the organism is transmitted transplacentally from an infected mother. Infection manifests differently depending on the site of growth of the organism and may include pneumonia, neurologic flu-like symptoms and retinitis. There are currently no data on the prevalence

or resistance of *Toxoplasma* oocysts in biosolids treatment and no adequate methods for screening such material (Garcia and Bruckner, 1997) (NRC, 2002).

Balantidium coli has worldwide distribution in humans and is the largest of the protozoa that parasitize humans, but is rarely recovered from clinical specimens in the United States. Infections are either asymptomatic or manifest as intermittent diarrhea (Garcia and Bruckner, 1997). In areas of the world where human infections with this parasite are common, sludges can contain large numbers of cysts. There is no information about the prevalence of *B. coli* in the United States or survival during biosolids treatment processes (NRC, 2002).

Entamoeba histolytica is transmitted from human to human by the fecal-oral route and is generally associated with poor hygiene and poor water quality. In 1984, five hundred million people worldwide were estimated to be infected with *E. histolytica* (Garcia and Bruckner, 1997). Infection manifests as severe dysentery and can be life threatening. The geographical distribution of this pathogen and prevalence in wastewater or manure is unknown and there are no routinely employed standard methods for detecting this pathogen in effluents or biosolids (NRC, 2002). However, the cyst stage has been demonstrated to be very resistant to environmental conditions and can remain viable for sixty days at 0°C in soil (Garcia and Bruckner, 1997).

Cryptosporidium parvum and *Giardia lamblia* are parasites of the small intestine that cause diarrhea. *C. parvum* oocysts and *G. lamblia* cysts have been detected in products of wastewater treatment and in biosolids and are of particular concern because of their low infectious dose, ten oocysts for *Cryptosporidium* and one cyst for *Giardia* (Kowal, 1985). *G. lamblia* is the most commonly diagnosed flagellate in the intestinal

tract. Infection manifests as giardiasis which causes nausea, diarrhea and dehydration (Faubert, 2000) (Garcia and Bruckner, 1997). Infection with the protozoan parasite *Cryptosporidium* manifests as acute gastrointestinal distress resulting in a severe diarrhea that is potentially life-threatening in immunocompromised individuals (Clark, 1999) (Current, 1988) (Leventhal and Cheadle, 1989) (Balows et al., 1991) (Bowman, 1999) (Fayer, 1997). There is currently no effective treatment for *Cryptosporidium* infection (Clark, 1999). *Cryptosporidium* has a ubiquitous geographical distribution in the United States and is commonly found in surface water. *Cryptosporidium* is a major threat to the water supply because it is resistant to chlorine, small and difficult to filter, and is present in many animals (Guerrant, 2001). Accurate and reliable methods for detecting *Giardia* and *Cryptosporidium* in water are currently being developed and such methods are not optimized for recovery of these protozoan parasites from sludge and biosolids matrices (Nieminski et al., 1995). Parasite cysts and oocysts are resistant to environmental degradation and to conventional methods of treating biosolids; and as a result have been implicated in parasitic waterborne outbreaks. For these reasons, parasitic protozoa represent potential candidates for biosolids monitoring (Olson et al., 1999) (Kuczynska and Shelton, 1999).

The effect of lime treatment on protozoan parasites is not known, nor is the potential for pathogen survival following land application. This is in part due to a lack of adequate recovery methodology for the parasites from biosolids material and a lack of rapid and sensitive viability assays. Research to determine the persistence of protozoan parasites during biosolids treatment processes is necessary to fully assess the potential use of such organisms as indicators for the presence of parasites in biosolids material.

Helminths

Helminth eggs are large and heavy and are therefore concentrated in wastewater sludge (O'Donnell et al., 1984) (Meyer et al., 1978). Helminth ova are currently utilized to indicate parasite removal, therefore demonstrating effective treatment and approval for use of biosolids for land application. Helminth worms include *Ascaris lumbricoides* and *Ascaris suum, Trichuris trichiura, Toxocara canis, Taenia saginata, Taenia solium, Necator americanus* (Hookworm), and *Hymenolepsis nana.* The clinical manifestation of a helminth infection will depend on the worm burden, the length of the infection and the age and overall health status of the host. Infection with *T. trichiura* (whipworm) is more common in warm, moist areas of the world, presenting as diarrhea or dysentery, and is often seen in conjunction with *Ascaris* infections. The potential for transmission of *T. trichiura* from animals to humans is not known but presents a potential risk especially from contaminated surface water runoff. The prevalence of this organism in sludges and the effects of treatment are not known (NRC, 2002) (Garcia and Bruckner, 1997).

Research conducted by Yanko demonstrated that the ova of *A. lumbricoides* were more resistant to inactivation by treatment processes and environmental conditions than other parasites investigated (Yanko, 1988). *A. lumbricoides* ova have been demonstrated to survive for long periods of time in sludge matrices, particularly when eggs are integrated into soil where they are afforded protection from radiation and desiccation. (Storey and Phillips, 1985) (O'Donnell et al., 1984) (Yanko, 1988). This research led to the establishment of *A. lumbricoides*, an intestinal roundworm, as the primary indicator for assessing presence of other parasites and measurement of treatment efficiency. The

use of *Ascaris* eggs as an indicator for helminths has been advocated because: 1) Ascariasis is a common and ubiquitous helminth infection 2) *Ascaris* eggs tend to settle in sludge, and 3) *Ascaris* eggs are more resistant to adverse external conditions than other enteric organisms (Meyer et al., 1978).

It is estimated that 800 million to 1.3 billion individuals worldwide suffer from Ascariasis, the infection resulting from ingestion of eggs, or ova (WHO, 1986). Infection manifests as vomiting, abdominal pain, pneumonitis and nutritional impairment, and in severe infections, small bowel obstruction (WHO, 1986). Current EPA 503 regulations require biosolids to be screened for *A. lumbricoides*; however, regional differences in parasite occurrence and difficulties in testing viability have led to suggestions for an alternative organism to be appointed to indicate the presence of parasites. Currently there is no timely method to monitor for the inactivation of *Ascaris* eggs. Inactivation of helminth ova is one of the ways of assessing whether a disinfection process produces Class A biosolids. Eggs must be recovered from biosolids and examined microscopically for viability. Original methodology was developed to detect parasite eggs in fecal samples but methods for routine analysis of sludge material, which contains fewer eggs, are not adequate (Meyer et al., 1978). Currently employed methodology is costly and labor-intensive and extremely inefficient, therefore compromising the integrity of reported results on pathogen presence in biosolids intended for land application. The low number of *Ascaris* eggs typically found in domestic sewage biosolids in the United States warrants investigation of alternative indicators for routine screening. The need exists for improved methodology to effectively recover helminth eggs from biosolids. In addition,

studies must be conducted to determine the persistence, transport and fate of helminth ova in biosolids, along with identification of representative indicators.

Pathogen Exposure from Biosolids

The major routes of potential human exposure to pathogens in biosolids are air, soil, water and transfer from vectors. Humans may be exposed to pathogens in biosolids from a variety of pathways including ingestion of contaminated food, water, or soil, dermal contact, and inhalation of bioaerosols, all of which represent primary sources of transmission. Secondary transmission may occur from exposure to pathogens shed from infected individuals either by direct contact or by routes through the environment (NRC, 2002). The hazards associated with biosolids are a function of the number and type of pathogens in the treated sludge relative to the minimum infectious dose and the exposure level (EPA, 1993). There is the potential for surface-water contamination by runoff, as well as groundwater contamination from passage of pathogens through the soil into underground aquifers, particularly during a rain event (Straub et al., 1993) (Hurst et al., 1991).

Pathogens can survive in soils from hours to years and there is very little information available about pathogen transport and survival in soils and aerosols (Straub et al., 1993). In a study conducted by NIOSH (National Institute of Occupational Safety and Health), air samples were collected and analyzed for bacteria and endotoxin, and bulk sewage sludge samples were obtained and analyzed for fecal coliform bacteria at a Class B biosolids land application processing facility. The results revealed that

potentially pathogenic bacteria were found in bulk samples and air samples, and concluded that employee gastrointestinal illness at that facility may have been caused by ingestion or inhalation of Class B biosolids (NIOSH, 2000).

Pesaro et al. demonstrated that viruses contained within manure may persist for prolonged periods of time if stored under nonaerated conditions, representing a potential source of environmental contamination at times of land application (Pesaro et al., 1995). Viruses are small and not as complex in structure as bacteria, protozoan parasites and helminths; therefore, they have the greatest potential for transport in the soil. Enteric viruses in soil are too small to be retained through size exclusion like protozoans and helminths. Rather, viruses are removed or retained by the soil through electrostatic adsorption. The specific characteristics of the soil matrix will affect viral binding. Viruses carry a negative charge in acidic soils and therefore bind to positively charged soil particles. This charge interaction is reduced during rain events creating the potential for virus survival and transport through the soil to underground aquifers.

In addition to viral transport through the soil column, groundwater may be subjected to fecal contamination from sewage treatment plant effluent, on-site septic waste treatment discharges, land runoff from urban, agricultural and natural areas, and leachates from sanitary landfills (Abbaszadegan et al., 1998) (Hurst et al., 1991). Therefore, under these circumstances, it is especially necessary to demonstrate the inactivation of pathogens in biosolids prior to land application. Demonstrating pathogen inactivation helps to ensure protection of surface and groundwater, in addition to protecting the public from exposure through ingestion, dermal contact or inhalation of aerosols.

Outbreaks

Toxic chemicals, infectious organisms, and endotoxins or cellular material may all be present in biosolids. At present, there is no documented scientific evidence that the Part 503 rule has failed to protect public health. Currently, there are no scientifically documented outbreaks or excess illnesses that have occurred from microorganisms in treated biosolids (Lewis et al., 2002). There have been anecdotal allegations of disease from exposure to biosolids. These reports attribute a range of adverse health effects from relatively mild irritations and allergic reactions to severe and chronic health outcomes such as headaches, respiratory problems and gastrointestinal illness. There have also been several allegations of human and animal deaths attributed to biosolids exposure. Odors are a common complaint from citizens living near biosolids land-application sites as well as attraction of vectors, declines in property value and damage to property from transport of biosolids (Krogmann et al., 2001). At present, a causal association between biosolids exposures and adverse health outcomes has not been documented. However, epidemiological studies have not been conducted on exposed populations, and many of the pathogens potentially present in biosolids are not reportable diseases; therefore, information on potential biosolids related outbreaks is not available. There is a considerable lack of health information on populations exposed to biosolids (Lewis et al., 2002). In order to conduct an appropriate risk analysis, scientific risk assessment data, including adequate epidemiological studies, are necessary indicating the fate of pathogens under various treatment conditions (NRC, 2002).

Indicator Organisms

The concept of indicator organisms first came about in the 1800's at a time when cholera was rampant. It was thought that such an organism could be used to indicate pollution in the water supply, particularly fecal pollution, and the definition remains the same today (Hach, 2000). There are three different types of indicator organisms. Indicator organisms can be used to control a process, such as the use of total coliforms to determine the treatment effectiveness of chlorine disinfection. They can be used to indicate a particular type of contamination, such as the evaluation of fecal coliform density following treatment as a measure of how well a process can meet Class B sewage sludge disinfection requirements. Finally, indicator organisms can be used as a model, or surrogate for other organisms, such as the use of somatic phage, F-specific phage, or phage of *Bacteroides fragilis* as an indicator of enteric virus and potentially a surrogate in experimental studies (Fewtrell and Bartram, 2001).

There are several characteristics associated with a good indicator organism. The organism should be present in high concentrations in feces, preferably human feces, to indicate human fecal contamination versus total coliforms from animals and humans. The organism should be present only when there is fecal contamination. An acceptable indicator organism should not replicate or increase in number once outside of a host. In addition, the indicator organism should be more resistant to commonly employed disinfection techniques and behave similarly to the population it is representing in its ability to be inactivated by disinfection techniques. Finally, methodology should exist to accurately and efficiently detect the indicator organisms (Hach, 2000). Ideally, in

monitoring biosolids quality, the indicator organism should provide a real-time indication of pathogen presence, but that is rarely available with biological indicator organisms aside from rapid enzymatic tests such as Colilert™ (NRC, 2002). Usually the monitoring of indicator organisms provides results 24-48 hours after analysis, at which time the sample that was taken may no longer be representative. Therefore, any action to correct contamination may be futile (Fewtrell and Bartram, 2001).

In addition to the delay in detecting and enumerating currently employed indicator organisms for biosolids testing, the methodology utilized for assay of these organisms is not sufficient to allow for adequate protection of public health (Straub et al., 1993). Fecal coliforms are evaluated routinely to indicate the potential presence of pathogens. However, it has been questioned whether fecal coliforms are truly the best indicator and if their presence or absence can be correlated with the presence of absence of pathogenic bacteria, viruses and or parasites. This is of particular concern considering that the method for recovery of fecal coliforms is cumbersome, uses a small and arguably non-representative sample, and does not generate immediate results. Studies to evaluate the reliability of traditionally employed indicators of fecal contamination in water have demonstrated that fecal coliforms do not provide adequate information about the fate and resistance of viruses to treatment (Havelaar et al., 1993).

Clostridium perfringens has received attention as a potential indicator organism in light of waterborne *Cryptosporidium* outbreaks. *Clostridium* is a spore-forming bacterium that is extremely resistant to conventional methods of water treatment potentially making it an excellent candidate for monitoring of *Cryptosporidium* in water. It is typically found at high densities in untreated biosolids; therefore, the spores may represent a surrogate for

Ascaris eggs. However, there is concern that this organism is potentially far too resistant to treatment technologies, and adequate reduction or inactivation could not be achieved and therefore it should not be included in guidelines. Bifidobacteria are prevalent in water and found in high numbers in feces; however, methods to effectively culture these types of bacteria for monitoring purposes are difficult due to their strictly anaerobic nature (Fewtrell and Bartram, 2001).

Bacteriophages have received a great deal of attention in the research of indicator organisms and surrogates due to their similarity in size and composition to the enteroviruses which are currently monitored for in drinking water (Lasobras et al., 1999). Bacteriophages are small, found in high numbers in feces and present in high numbers in wastewater, although their presence will depend on the quality of the water, pH and quantity of bacteria. Bacteriophages appear to behave similarly to enteric virus as far as their inactivation by traditionally employed methods. Some bacteriophages are more susceptible to inactivation than others. It appears that F-specific phages are more susceptible to disinfection techniques than somatic phage or phage infecting *Bacteroides fragilis*. Available information on bacteriophage as an indicator organism is scarce. The data that do exist is not uniform primarily due to variations in the host bacterium used for study, making comparisons difficult if not impossible to conclude (Lasobras et al., 1999) (Fewtrell and Bartram, 2001).

There is no one indicator that can accurately reflect the presence or inactivation of virus, bacteria, protozoa and helminth organisms under all circumstances. All of these organisms are distinct and have unique qualities that make them resistant to treatment technologies in different ways. It is not feasible or cost-effective to monitor for all of the

pathogens that are present in environmental samples (Baker and Hegarty, 1997). It may be feasible to monitor indicator organisms for process efficiency at critical control points in the treatment process. This may represent a means of providing a cost-effective monitoring regime. Potentially, bacteriophages represent a useful tool with which to monitor the fate of human enteric viruses in sludges (Lasobras et al., 1999).

Studies investigating the inactivation of virus, bacteria, protozoan and helminth will always need to be conducted as new pathogens emerge and new treatment technologies are developed. Each of these organisms is susceptible to a different treatment process and there ideally would be indicators to reflect this, i.e. *Cryptosporidia* are susceptible to ultraviolet light inactivation and ozonation but are extremely resistant to chlorination (Fewtrell and Bartram, 2001).

The selection of microorganisms for analysis in biosolids or wastewater should be based on a set of criteria that includes the following: availability of a reliable and relatively consistent assay for study of the pathogen, presence of the pathogen in wastewater and the ability to be transmitted as a result of exposure to biosolids, survivability following biosolids treatment processes, and the extent of the survival in the environment. In the Part 503 regulations, fecal coliforms are used as indicator organisms to classify biosolids, and to indicate wastewater treatment efficiency. Fecal coliforms are easy to assay and represent an appropriate indicator of treatment efficiency with regard to the potential for regrowth; however, some pathogens are more resistant to treatment processes than fecal coliforms.

Routine surveillance of enteroviruses cannot accurately reflect the likelihood of contamination by other extremely resistant viruses such as adenoviruses and rotavirus.

The focus must ultimately be on demonstrating process efficiency and taking a multiple barrier approach to adequately protect public health, along with educating the public about land application and protection of their watershed, rather than investing in costly inaccurate analysis. Research to demonstrate the effectiveness of treatment processes coupled with evaluation of bacteriophage as potential indicator organisms is required to ensure cost-effective management of biosolids land application (Fewtrell and Bartram, 2001).

Risk Assessment

The development of effective public health policy requires the integration of large collections of information that are diverse, highly variable and in most cases uncertain. Patterns of disease are caused by a complex interaction of social, biological and environmental processes. Risk assessment is a process for identifying potential adverse consequences along with their severity and likelihood (Byrd, 2000). The risk analysis process involves three main steps which are risk assessment, risk management, and risk communication. Each overlaps with the next and requires that individuals from many different disciplines join collectively to establish a program that will effectively meet the needs of the community. The objective of the risk analysis process is to quantify the risk and provide risk managers with tools to balance the level of risk against the cost of the risk reduction (Fewtrell and Bartram, 2001). The process used for conducting the Part 503 risk assessments involved four main steps. These steps were hazard identification, exposure assessment, dose-response evaluation, and risk characterization (EPA, 1995).

In order to control the exposure of the human population to environmental contaminants present in biosolids it is necessary to identify an acceptable level of exposure. This is accomplished by combining exposure assessment data with dose-response data for the specific hazard that has been identified. The resultant risk characterization is the generation of an approximate risk of developing an illness based on an exposure. An exposure assessment is necessary to determine the likelihood of an individual coming in contact with and absorbing a contaminant. An exposure assessment must take into account the region being analyzed, what it is currently used for and what it may be used for in the future. The population residing in that region needs to be characterized and any subpopulations, particularly immunocompromised populations, identified. Often risk assessment is conducted with many assumptions. In addition to the aforementioned variables, the time at which the study is conducted influences the risk assessment that will result. In addition to characterizing the region, the contaminant itself must be characterized to determine the fate of the contaminant in the environment through construction of a fate-transport model, the spatial and temporal distribution, and the cumulative effects and the degradative effects of the contaminant. In addition, pharmacokinetic studies can be performed to determine the response in the body to the pathogen, including adsorption and degradation in the body (Byrd, 2000) (Fewtrell and Bartram, 2001).

Risk assessment relies on accurate scientific and epidemiological data, neither of which currently exists as relates to biosolids quality. Epidemiological studies provide a qualitative approach to risk assessment and are often able to determine whether the effects of one exposure are greater than another. Such studies often establish cause-effect

relationships and are inherently stricken with bias in the methods and population selected for the study. There is an extremely small amount of epidemiological information available on the potential adverse health effects of biosolids and there is an absence of evidence documenting infection, and limited evidence documenting the lack of infection from biosolids. Epidemiological exposure assessment studies to assess the potential adverse health effects of biosolids are necessary to examine the exposure and potential health risks to both worker and community populations (Byrd, 2000).

A risk assessment must balance the needs of the community with the risk. Models are often employed in risk assessment. An ideal model will be an exact representation of the situation trying to be emulated; however, this is unlikely if not impossible. Therefore, models attempt to represent a situation and several types of models exist for this purpose. Physical, biological, and conceptual models are all part of risk analysis. Dose-response models, a type of biological model, are a critical part of the risk assessment process because they indicate the infectious dose for a particular pathogen in the animal being studied. Generating dose-response data is cumbersome and costly and contributes greatly to the expense of establishing an adequate risk assessment; however, it changes the risk assessment from qualitative, to quantitative, where a value can be assigned with conditional properties. The generation of a dose-response curve for a particular agent allows regulators to establish a threshold by combining the exposure data with the dose-response data to establish an acceptable risk. Development of a conceptual model for disease transmission is dynamic and population based and involves defining disease states in a population and identifying potential transmission pathways. Each model is unique to the pathogen being modeled and the various transmission pathways. The degree

of contamination and the degree of risk will depend on the contribution and interactions of all the different environmental transmission pathways (Byrd, 2000) (Fewtrell and Bartram, 2001).

The risk assessment portion of risk analysis aims to characterize a risk by determining the probability of illness from exposure to a pathogen. Once a risk has been identified and characterized, a management strategy must be implemented, and the risk effectively communicated to the public. Risk management may involve monitoring of indicator organisms to meet the standards set based on the aforementioned risk assessment, using process control organisms to monitor critical control points in the distribution system, demonstration of an effective process over a period of time, or conducting an entirely new risk assessment for the specific community (Fewtrell and Bartram, 2001).

Overall, the risk assessment process is laden with uncertainty, often from variability in the data and models employed, making it extremely difficult for regulators to establish guidelines that will adequately protect public health. Biosolids are a complex mixture of chemical and biological agents, the exact composition of which changes from time to time and place to place. It will never be possible to account for all the components of the mixture. The Part 503 rule risk assessments were carried out more than a decade ago. Due to significant changes in risk-assessment methods and policies over the last decade, there is a need to revise and update the Part 503 rule risk assessments to include new pathogens that have been recognized to be transmitted by biosolids (Gerba et al., 2002) (Lewis et al., 2002). Current scientific data measuring the fate of pathogens during biosolids treatment processes is necessary to facilitate this task. Risk assessment requires

an appreciation for the collaboration that needs to take place in order for the process to be successful. For pathogen assessment it is important to consider the cumulative effects of exposure to multiple pathogens over a period of time. Ultimately, creation of a harmonized framework that accounts for this cumulative effect to establish a combined qualitative risk of pathogen exposure in the environment will be necessary to fully predict the fate of pathogens present in biosolids and their effect on public health (Byrd, 2000) (Fewtrell and Bartram, 2001) (Gerba et al., 2002), (Straub et al., 1993).

Research Goals and Objectives

The objectives of this research were to evaluate the effectiveness of lime stabilization as a treatment technology to inactivate pathogens whose persistence under liming conditions has not previously been studied. Pathogens employed in the monitoring of biosolids treatment processes, bacteria and enteric virus, were evaluated with bacteriophage to determine their persistence in lime-stabilized sludge. Male-specific bacteriophage, fecal coliforms, *Salmonella*, poliovirus type 1, rotavirus Wa strain, and adenovirus Type 5, were evaluated during lime stabilization conditions in various matrices at room temperature (28°C) and reduced temperature (4°C). In addition, the prevalence of male-specific bacteriophage and enterovirus in raw and lime stabilized biosolids was evaluated and compared to assess the potential use of male-specific bacteriophage as an indicator of pathogen presence and inactivation during biosolids treatment. The data generated as a result of this research will be useful in the continued development of new regulations and risk analysis for the land application of biosolids and will provide insight into the magnitude of pathogen contamination in limed biosolids.

Phase 1

The goals of the first phase of this research were to evaluate the survivability of bacterial and viral pathogens in a bench-scale model of lime stabilization. Various matrices were seeded to determine the persistence of pathogens in a bench-scale model of lime stabilization. The pH of the matrix was elevated to 12 for two hours and then lowered to 11.5 for twenty-two hours. For viral pathogens, lime stabilization experiments were conducted at both room temperature (28°C) and reduced temperature (4°C), representing a temperature range found in New Hampshire. The pH of the sample was adjusted using an aqueous slurry of calcium hydroxide and samples were removed at specific time points following addition of lime. Control samples consisting of seeded organisms into water, sludge or biosolids matrix and test samples consisting of a seeded and subsequently limed water, sludge or biosolids matrix were assayed at specified time points and results of enumeration were compared to determine the amount of bacteria, phage, and virus removed or inactivated by the elevated pH.

Organisms were recovered from the limed matrix in accordance with the 503 regulations, and viability or infectivity was assessed using methods specific to the organism in question. The plaque assay technique was used to determine the survivability of male-specific bacteriophage and rotavirus. A $TCID_{50}$ technique was used for enumeration of adenovirus type 5.

The results of this research will have a significant impact on the agricultural use of biosolids for fertilizer and the regulations currently in place for the land application of biosolids. The increasing quantity of biosolids being land applied every year demands the

assessment of the survivability of pathogenic organisms in lime-stabilized sludge. Land application of biosolids is an excellent means of disposal; however, until treatment processes can demonstrate effective removal of pathogenic organisms, it should be considered a public health risk to apply sludge to land when it cannot be accurately determined whether or not pathogenic organisms are present and to what extent. This research represents initial investigation in what needs to be an on-going investigation to evaluate various methods of sludge or biosolids preparation, and the persistence of emerging pathogens during such treatment, in an effort to minimize public health threats, maximize potential benefits and move our society towards sustainability. The land application of biosolids should be done in a way such that groundwater and public health are protected. The challenge is to establish which management practices and treatment techniques (timing, application rate and stockpiling) are most important to protect groundwater quality and public health.

Phase 2

The results generated in Phase 1 of this research provided the impetus for Phase 2. The goals of the second phase of this research focused on evaluating the prevalence of and comparing the relationships between male-specific bacteriophage and human enteric virus within various raw and lime stabilized biosolids samples obtained from several sites throughout the United States. The methodology established in the Part 503 rule for recovering enteric viruses from biosolids is extremely inefficient; this is due in part to the particle association that takes place between charged viruses and soil particles and the

difficulty in separating the viruses from the solid component for assay. As a result, routine monitoring of enteric virus from biosolids samples generally fails to recover enteric virus, demonstrating that the material is in compliance with regulations and is therefore suitable for land application. The negative results for enteric virus presence in biosolids are most likely due to the inefficiency of recovering virus from the material, especially when considering that fecal material will contain measurable amounts of virus. The purpose of this study was to evaluate the presence of enteric virus and male specific bacteriophage in raw and limed biosolids in an effort to identify a potential indicator organism that may be used in conjunction with evaluation of enteric virus. The identification of such an indicator will help to ensure treatment effectiveness in the absence of adequate methodology to recover viral pathogens.

Thirty-six raw and lime stabilized match-batch samples (sampled immediately prior to and immediately following treatment) were obtained from three participating utilities in the United States which employ lime stabilization as a method of sludge treatment. Aliquots of designated samples were obtained and eluted for recovery of enteric virus and assayed by cell culture to evaluate the presence of virus cultivatable using Buffalo Green Monkey Kidney Cells (BGM) in accordance with methodology established in the Part 503 regulations. In addition, aliquots of the same samples were washed, centrifuged and assayed for male-specific bacteriophage using a double-agar overlay technique. It is unrealistic that one indicator will adequately ensure that a public health hazard does not exist; however, considering the lack of adequate methodology for recovery of enteric virus, survey of phage may provide a screening tool to be used in

conjunction with monitoring of enteric virus as they are easily recovered from wastewater solids and present in high numbers.

REFERENCES

Abbaszadegan, M., P. Stewart, M. LeChevallier, J. Rosen, and C. Gerba. 1998. Occurrence of viruses in ground water in the United States. Interim Report.

Alpert, G., L. Bell and C. Kirkpatrick. 1986. Outbreak of cryptosporidiosis in a day-care center. Pediatrics. 77:152-157.

Baker, K.H. and J.P. Hegarty. 1997. Detection and occurrence of indicator organisms and pathogens. Water Environment Research. **69**(4):403-415.

Balows, A., W. Hausler, Jr., K. Herrmann, H. Isenberg and H. Shadomy. 1991. Manual of Clinical Microbiology. 5th ed. ASM Press. Washington, D.C.

Bean, C. 1999. Public health effects of land application of municipal sludge: Is *Ascaris lumbricoides* the best indicator organism for survivability of protozoans in treated biosolids. RFP 99-HHE-3. University of New Hampshire, Durham, NH.

Biosolids Management: Assessment of Innovative Processes. 1998. Project 96-REM-1. Water Environment Research Foundation. Alexandria, Virginia.

Bowman, D. 1999. Georgis' parasitology for veterinarians. 7th ed. W.B. Saunders Company.

Bruckner, D., L. Garcia and M. Voge. 1979. Intestinal parasites in Los Angeles, California. Am J Med Technol. **45**:1020-1024.

Byrd, D. and C. R. Cothern. 2000. Introduction to risk analysis: A systematic approach to science-based decision making, Government Institutes, Rockville, MD.

Chapron, C.D., N.A. Ballester and A.B. Margolin. 2000. The detection of astrovirus in sludge biosolids using an integrated cell culture nested PCR technique. Journal of Applied Microbiology. **89**(1):11-5.

Clark, D. 1999. New insights into human cryptosporidiosis. Clinical Microbiology Reviews. **12**(4):554-563.

Cooper, R. and R. Danielson. Detection of bacterial pathogens in wastewater and sludge. Water Microbiology in Public Health.

Current, W. 1988. The biology of *Cryptosporidium*. ASM News. **54**:605-611.

Deng, M.Y. and D.O. Cliver. 1995. Persistence of inoculated hepatitis A virus in mixed human and animal wastes. Applied and Environmental Microbiology. **61**(1):87-91.

Deng, M.Y. and D.O. Cliver. 1995. Inactivation of poliovirus type 1 in mixed human and swine wastes and by bacteria from swine manures. Applied and Environmental Microbiology. **58**(6):2016-21.

Derbyshire, J.B. and E.G. Brown. 1979. The inactivation of viruses in cattle and pig slurry by aeration or treatment with calcium hydroxide. Journal of Hygiene (London). **82**(2):293-299.

Dowd, S., C. Gerba, I. Pepper and S. Pillai. 2000. Ecological risk assessment: bioaerosol transport modeling and risk assessment in relation to biosolids placement. Journal of Environmental Quality. **29**:343-348.

Dumontet, S., A. Scopa, S. Kerje and K. Krovacek. 2001. The importance of pathogenic organisms in sewage and sewage sludge. Journal of Air and Waste Management Association. **51**(6):848-60.

Enriquez, C.E., C.J. Hurst and C.P. Gerba. 1995. Survival of the enteric adenoviruses 40 and 41 in tap, sea, and wastewater. Water Resources. **29**:2548-2553.

EPA (U.S. Environmental Protection Agency). 1993. A guide to the federal EPA rule for land application of domestic septage to non-public contact sites. Office of Wastewater Enforcement and Compliance. United States Environmental Protection Agency, Washington, D.C. EPA/832-B-92-005.

EPA (U.S. Environmental Protection Agency). 1996. A plain english guide to the EPA part 503 biosolids rule. United States Environmental Protection Agency, Washington, D.C.

EPA (U.S. Environmental Protection Agency). 1999a. Biosolids generation, use and disposal in the United States. Office of Solid Waste and Emergency Response, United States Environmental Protection Agency, Washington, D.C. EPA 530-R-99-009.

EPA (U.S. Environmental Protection Agency). 1999b. Environmental regulations and technology: control of pathogens and vector attraction in sewage sludge. Office of Research and Development. United States Environmental Protection Agency, Washington, D.C. EPA/625/R-92/013.

EPA (U.S. Environmental Protection Agency). 2000. Health risks of enteric viral infections in children. United States Environmental Protection Agency. Washington, D.C. EPA/822/R/00/010.

EPA (U.S. Environmental Protection Agency). 1993. Preparing sewage sludge for land application or surface disposal: a guide for preparers of sewage sludge on the monitoring, record keeping, and reporting requirements of the federal standards for the use or disposal of sewage sludge, 40 CFR Part 503. Office of Water. United States Environmental Protection Agency, Washington, D.C.

EPA (U.S. Environmental Protection Agency). 2000. Report to Congress: EPA studies on sensitive subpopulations and drinking water contaminants. Office of Water. United States Environmental Protection Agency, Washington, D.C. EPA 815-R-00-015.

Estes, MK. 2001. Introduction: gastroenteritis viruses. *In.* Gastroenteritis Viruses. Novartis Foundation Symposium. John Wiley and Sons, LTD. Pp 1-4.

Faubert, G. 2000. Immune response to *Giardia duodenalis.* Clinical Microbiology Reviews. **13**(1):35-54.

Fayer, R. 1997. *Cryptosporidium* and cryptosporidiosis. CRC Press, Inc. New York, NY.

Fewtrell, L. and J. Bartram. 2001. Water Quality: guidelines, standards and health – assessment of risk and risk management for water-related infectious disease. World Health Organization Water Series. International Water Association Publishing, London, UK.

Gaby, W.L. 1981. Health hazards associated with solids waste disposal. Reviews of Environmental Health. **3**(3):277-91.

Garcia, L.S. and D. Bruckner. 1997. Diagnostic medical parasitology.3rd ed. American Society for Microbiology, ASM Press, Washington D.C.

Gerba, C.P., I.L. Pepper and L.F. Whitehead III. 2002. A risk assessment of emerging pathogens of concern in the land application of biosolids. Water Science and Technology. **46**(10):225-30.

Gerba, C.P., J.B. Rose and C.N. Hass. 1996. Sensitive populations: who is at the greatest risk? International Journal of Food Microbiology. **30**:113-123.

Grabow, W.O.K., I. Middendorff and N. Basson. 1978. Role of lime treatment in the removal of bacteria, enteric viruses, and coliphages in a wastewater reclamation plant. Applied and Environmental Microbiology. **35**(4):663-669.

Guerrant, R.L. 2001. Cryptosporidiosis: an emerging, highly infectious threat. Emerging Infectious Diseases. **3**(1).

Hach. 2000. The use of indicator organisms to assess public water safety. Technical Information Series. Booklet No. 13. 2nd edition.

Health, Safety and Risk Management. 1997. Water Environment Federation Digest Series. Water Environment Federation, Alexandria, Virginia.

Havelaar, A.H., M. Van Olphen and Y.C. Drost. 1993. F-Specific RNA bacteriophages are adequate model for enteric viruses in fresh water. Applied and Environmental Microbiology. **59**(9):2956-2962.

Hurst, C.J., S.A. Schaub, M.D. Sobsey, S.R. Farrah, C.P. Gerba, J.B. Rose, S.M. Goyal, E.P. Larkin, R. Sullivan, J.T. Tierney, R.T. O'Brian, R.S. Safferman, M.E. Morris, F.M. Wellings, A.L. Lewis, G. Berg, P.W. Britton and J.A. Winter. 1991. Multilaboratory evaluation of methods for detecting enteric viruses in soils. Applied and Environmental Microbiology. **57**(2):395-401.

Jager, R. 2000. Managing New England's 282,000 dry tons of biosolids. Journal of the New England Water Environment Association. **34**(2):172-199.

Koneman, E., S. Allen, W. Janda, P. Schreckenberger and W. Winn. 1994. Introduction to diagnostic microbiology. pp 411. *In* Caputo (ed.). JB Lippincott Co., Philadelphia.

Kowal, N.E. 1985. Health effects of land application of municipal sludge. Health Effects Research Laboratory, United States Environmental Protection Agency, Cincinatti, OH. EPA/600/1-85/015.

Krogmann, U., V. Gibson and C. Chess. 2001. Land application of sewage sludge: perceptions of New Jersey vegetable farmers. Waste Management Resources. **19**(2):115-25.

Kuczynska, E. and D.R. Shelton. 1999. Method for detection and enumeration of *Cryptosporidium parvum* oocysts in feces, manures and soils. Applied and Environmental Microbiology. **65**(7):2820-2826.

Kukkula, M., P. Arstila, M.L. Klossner, L. Manuela, C.H. Bonsdorff and P. Jaatinen. 1997. Waterborne outbreak of viral gastroenteritis. Scand J Infect Dis. **29**(4):415-8.

Lasobras, J., J. Dellunde, J. Jofre and F. Lucena. 1999. Occurrence and levels of phages proposed as surrogate indicators of enteric viruses in different types of sludges. Journal of Applied Microbiology. **86**:723-729.

Leventhal, R. and R. Cheadle. 1989. Medical parasitology – A self-instructional text. 3rd ed. F.A. Davis Company. Philadelphia, PA.

Lewis, D.L., D. K. Gattie, M.E. Novak, S. Sanchez and C. Pumphrey. 2002. Interactions of pathogens and irritant chemicals in land-applied sewage sludges (biosolids). BMC Public Health. **2**(11):1-8.

Lytle, D.A., E.W. Rice, C.H. Johnson and K.R. Fox. 1999. Electrophoretic mobilities of *Escherichia coli* O157:H7 and wild-type *Escherichia coli* strains. Applied and Environmental Microbiology. **65**(7):3222-5.

Meyer, K.B., K.D. Miller and E.S. Kaneshiro. 1978. Recovery of *Ascaris* eggs from sludge. Journal of Parasitology. **64**(4):380-383.

MMWR (Morbidity and Mortality Weekly Report). 1999. Incidence of foodborne illnesses: prelimlinary data from the foodborne diseases active surveillance network (Foodnet). United States. **48**(9):189-194.

National Research Council of the National Academies. 2002. Biosolids applied to land: advancing standards and practices. The National Academies Press. Washington, D.C.

Nieminski, E.C., F.W. Schaefer III and J.E. Ongerth. 1995. Comparison of two methods for detection of *Giardia* cysts and *Cryptosporidium* oocysts in water. Applied and Environmental Microbiology. **61**(5):1714-1719.

NIOSH (National Institute for Occupational Safety and Health). 2000. Workers exposed to class B biosolids during and after field application.

O'Donnell, C.J., K.B. Meyer, J.V. Jones, T. Benton, E.S. Kaneshiro, J.S. Nichols and F.W. Schaefer III. 1984. Survival of parasite eggs upon storage in sludge. Applied and Environmental Microbiology. **48**(1):618-625.

Olson, M.E., J. Goh, M. Phillips, N. Guselle and T. McAllister. 1999. *Giardia* cyst and *Cryptosporidium* oocyst survival in water, soil and cattle feces. Journal of Environmental Quality. **28**:1991-1996.

Pang, X-L, S. Honma, S. Nakata and T. Vesikari. 2000. Human caliciviruses in acute gastroenteritis of young children in the community. Journal of Infectious Disease. 181:S288-S294.

Papapetropoulou, M. and A.C. Vantarakis. 1998. Detection of adenovirus outbreak at a municipal swimming pool by nested PCR amplification. J Infect. **36**(1):101-3.

Payment, P. and M. Riley. 2002. Resolving the global burden of gastrointestinal illness: A call to action. American Academy of Microbiology, Washington, D.C.

Pesaro, F., I. Sorg and A. Metzler. 1995. In situ inactivation of animal viruses and a coliphage in nonaerated liquid and semiliquid animal wastes. Applied and Environmental Microbiology. 61(1):92-7.

Richard, M. 1989. Activated sludge microbiology. The Water Pollution Control Federation, Alexandria, Virginia.

Russell, W.C. 2000. Update on adenovirus and it vectors. Journal of General Virology. **81**:2573-2604.

Sattar, S.A. and S. Ramia. 1978. Viruses in sewage: effect of phosphate removal with calcium hydroxide (lime). Canadian Journal of Microbiology. **24**:1004-1006.

Sattar, S.A., S. Ramia and J.C.N. Westwood. 1976. Calcium hydroxide (lime) and the elimination of human pathogenic viruses from sewage: studies with experimentally-contaminated (poliovirus type 1, sabin) and pilot plant samples. Canadian Journal of Public Health. **67**:221-226.

Spellman, F. 1997. Wastewater biosolids to compost. pp 8. Tecnomic Publishing Company, Inc. Lancaster, PA.

Storey, G.W. and R.A. Phillips. 1985. The survival of parasite eggs throughout the soil profile. Parasitology. **91**:585-590.

Straub, T.M., I.L. Pepper and C.P. Gerba. 1993. Hazards form pathogenic microorganisms in land disposed sewage sludge. Rev Environ Contam Toxicol. **132**:55-91.

Strauch, D. 1991. Survival of pathogenic microorganisms and parasites in excreted manure and sewage sludge. Rev Sci Tech. **10**(3):813-46.

Tuohy, J. 2000. CDC Sounds a warning on risks of sludge. [online] USA Today: Health. www.usatoday.com/life/dcovthu.htm. Date accessed: 7/18/00

U.S. Environmental Protection Agency. 1995. A guide to the biosolids risk assessments for the EPA Part 503 Rule, United States Environmental Protection Agency, Office of Wastewater Management, Washington, DC. EPA/832-B-93-005.

United States Environmental Protection Agency. Handbook: Septage Treatment and Disposal.

Wastewater and Sludge Treatment Processes. 1995. WRc. 149-150.

Wong, J.W.C. and M. Fang. 2000. Effects of lime addition on sewage sludge composting process. Water Resources. **34**(15):3691-3698.

World Health Organization. 1986. Parasitic diseases programme: major parasitic infections: a global view. World Health Stat Q. **39**:145-160.

Yanko, W. 1988. Occurrence of pathogens in distribution and marketing municipal sludges. EPA/600/1-87/014. USEPA, Research Triangle Park, NC.

CHAPTER TWO

EVALUATION OF POLIOVIRUS TYPE 1, FECAL COLIFORMS, *SALMONELLA*, ADENOVIRUS TYPE 5, ROTAVIRUS Wa, AND MS-2 BACTERIOPHAGE PERSISTENCE UNDER LIME STABILIZATION CONDITIONS

ABSTRACT

The use of lime to reduce or eliminate pathogen content is a cost-effective treatment method currently being employed in many Class B biosolids production plants in the United States. A bench scale model of lime stabilization was designed to evaluate pathogen persistence. The survivability of poliovirus type 1, fecal coliforms, *Salmonella* sp., adenovirus type 5, rotavirus Wa, and the male specific bacteriophage MS-2 was evaluated under lime stabilization conditions in various matrices. *Salmonella* sp. and fecal coliforms were evaluated at room temperature (28°C) and poliovirus and MS-2 were evaluated at both room temperature (28°C) and reduced temperature (4°C) for survivability under lime stabilization conditions in a sludge matrix. All microorganisms evaluated were discovered to be below detectable levels following twelve hours of liming at pH 12 for both temperatures evaluated. Adenovirus type 5 and MS-2 were initially tested in an RO water matrix and limed with an aqueous solution of calcium hydroxide for twenty-four hours at 28°C. In all water matrix trials, both adenovirus type 5 and MS-2 were below detectable levels following 0.1 hours of liming, demonstrating at least a five-log reduction and at least a seven-log reduction respectively.

Rotavirus Wa and MS-2 were also seeded into an RO water matrix and subsequently limed with an aqueous solution of calcium hydroxide for twenty-four hours at 28°C. In all water matrix trials, both rotavirus Wa and MS-2 were below detectable levels, demonstrating at least a four log reduction and at least a seven log reduction respectively. Adenovirus type 5 and rotavirus Wa, evaluated individually with MS-2, were seeded into compost, raw and previously limed matrices, representative of sludge and biosolids matrices. Each matrix was seeded and limed for twenty-four hours at 28°C and 4°C. In all matrices evaluated at 28°C and 4°C, adenovirus type 5, rotavirus Wa and MS-2 were below detectable levels following a twenty-four hour incubation with lime, demonstrating at least a four-log reduction, at least a four-log reduction, and at least a six-log reduction respectively, with inactivation rates varying depending on the sample matrix evaluated. Evaluation of reliable and cost effective treatment processes to demonstrate sufficient inactivation of pathogens provides valuable information for implementation of treatment technologies. This research has demonstrated that lime stabilization is effective at reducing fecal coliforms and *Salmonella* sp. in a sludge matrix at 28°C, poliovirus type 1 in a sludge matrix at 28°C and 4°C, and adenovirus Type 5, rotavirus Wa, and male-specific bacteriophage, in sludge and water matrices at 28°C and 4°C when calcium hydroxide is added to achieve a pH of 12 for 2 hours and 11.5 for 22 hours. In addition, the data demonstrate that male specific bacteriophages are inactivated similarly to both adenovirus type 5 and rotavirus Wa and may therefore represent a potential indicator to evaluate treatment efficiency.

INTRODUCTION

The use of lime to reduce or eliminate pathogen content in sewage sludge represents a simple and inexpensive method of treatment by which Class B sludge can be generated (Farrell et al., 1974). Liming is a process where calcium hydroxide or calcium oxide is added and the pH elevated to 12 for two hours and then reduced to 11.5 for twenty-two hours. Lime has been in use for a number of years for the disinfection and odor suppression of solid wastes. Lime treatment reduces the number of microorganisms in discharged effluent by binding to solid material, thereby facilitating flocculation in sedimentation or flotation processes while the hydroxide alkalinity of the lime has an antimicrobial effect (Grabow et al., 1978) (Wong et al., 2000). Lime is not an oxidizing chemical and the formation of hazardous compounds is unlikely. During the liming process, the splitting of complex organic molecules is likely to occur by hydrolysis. In addition, a "lime bonding" to sludge occurs where lime becomes firmly bound to sludge and cannot be dissolved off (Farrell et al., 1974). Installation of a lime stabilization treatment system is relatively rapid, with minimal capital costs, compared to alternative treatment technologies. Such a system is easy to operate and generates a relatively safe and sustainable product (Farrell et al., 1974).

The early evidence available to indicate stability of lime treated sludge was obtained primarily from qualitative observations noting the lack of odor in lime stabilized sludges (Farrell et al., 1974). Qualitative research has shown the bactericidal properties of lime by inactivating *Escherichia coli* and *Salmonella* (Farrell et al., 1974) (Wong et al.,

2001). Additional work with bacteria revealed the destruction of Gram-negative bacteria and highlighted the survival of spore-forming bacteria (Grabow et al., 1978). Previous research to evaluate the persistence of enteric virus under lime stabilization conditions has utilized poliovirus type 1 and has demonstrated that maintaining a high pH over a period of time is very effective at reducing or eliminating the viral load from sludge (Sattar et al., 1976) (Sattar and Ramia, 1976) (Derbyshire and Brown, 1979) (Deng and Cliver, 1995) (Grabow et al., 1978).

There has been very little research focusing on enteric virus survival in lime stabilized sludge since the 1970's. Currently, there is little information available on the removal and inactivation of certain human enteric pathogenic viruses, such as rotavirus and adenovirus, which were not cultivatable when original studies were conducted to evaluate the effects of high-pH lime treatment of sewage. Enteric viruses are the major cause of childhood diarrhea in the United States and result in an estimated one hundred deaths per year related to gastroenteritis (EPA, 2000). There has been very little research conducted on the occurrence of enteric viruses in biosolids in recent years. The existing information provides data about the prevalence of enteroviruses, specifically poliovirus, in biosolids, and the effect of treatment; yet for many processes the amount of data on virus removal is very limited (NRC, 2002). With the advent of new and improved cell culture detection techniques, it is now possible to assess the prevalence and persistence of pathogens such as rotavirus and adenovirus that were previously not cultivatable and identify potential indicator organisms to monitor treatment effectiveness.

Bacteriophages have received a great deal of attention in the research of indicator organisms due to their similarity in size and composition to enteroviruses. Currently,

there are no data available evaluating the inactivation of bacteriophages compared with inactivation of enteric viruses during traditionally employed sludge treatment processes. The similar morphology, structure and behavior of F-specific RNA bacteriophages, as well as other phages, to that of human enteric viruses, suggests that they could represent a better model to indicate the presence of pathogens and could potentially be utilized to demonstrate inactivation by treatment processes (Fewtrell and Bartram, 2001).

Evaluation of the lime stabilization technique to demonstrate that it is sufficient to inactivate the enteric virus, helminth and protozoan pathogens that could not be evaluated at the time the Part 503 rules were enacted is necessary. The need for such information was the impetus for conducting the following investigation.

Poliovirus

Previous research conducted to evaluate the persistence of pathogens during lime stabilization utilized poliovirus type 1. At the time such research studies were performed, methods were available for the propagation and enumeration of poliovirus in cell culture. Poliovirus type 1 was selected for experimental contamination because it is relatively harmless due to available vaccines, and relatively easy to grow and quantitate in the laboratory (Sattar et al., 1976).

Poliovirus belongs to the enterovirus family and exists as three serotypes (types 1-3). Enteroviruses are small RNA viruses that readily infect the intestinal tract and have a ubiquitous and worldwide distribution. Poliovirus infection is asymptomatic in ninety percent of cases. When disease does result it manifests in one of three ways. Abortive poliomyelitis is a nonspecific febrile illness lasting two to three days. Aseptic meningitis

includes symptoms associated with abortive poliomyelitis and is accompanied by meningeal irritation (stiff neck, pain, and stiffness in the back), with recovery in two to three days. Paralytic poliomyelitis is often preceded by a period of minor illness with signs of meningeal irritation, and asymmetric paralysis. As damaged neurons regain function, recovery will begin and may continue for a period of six months after which time remaining paralysis is permanent (Ryan, 1994).

Enteroviruses are unique in their resistance to extreme pH, high temperatures, and common disinfectants such as 70% alcohol and various detergents (Ryan, 1994). In addition, enteroviruses are normally present in sewage in large numbers with densities ranging from undetectable to ten thousand plaque forming units (PFU) per liter (Yanko et al., 2000) (Sattar et al., 1976). The aforementioned qualities make poliovirus an excellent candidate for spiking studies to evaluate persistence during lime stabilization.

Previous studies conducted by Sattar concluded that no virus could be detected in the supernate of limed sewage sludge samples following twenty minutes of liming at pH 11.5, indicating a viral removal of 99.99%; however, the group concluded that sludge associated virus in the sediment may contain infectious virus and a lack of efficient methodology to recover such virus particles may present a public health threat if the limed material is disposed of through land application (Sattar et al., 1976). It cannot be assumed that because poliovirus was inactivated under lime stabilization conditions that other enteric viruses present in sewage sludge would be inactivated in a similar manner. Therefore similar experiments were conducted using representatives of the adenovirus and reovirus groups.

Adenovirus

Adenoviruses are one of the most common and persistent viruses detected in wastewater, and enteric adenoviruses are the second most common cause of childhood viral diarrhea (Enriquez et al., 1995) (Gerba et al., 1996). Adenoviruses have been transmitted by both recreational and drinking waters (Kukkula et al., 1997) (Papapetropoulou and Vantarakis, 1998). Adenoviruses consist of almost one hundred different serotypes, forty-seven of which are known to infect humans. Adenoviruses were first isolated in 1953 and are widespread in nature, infecting birds, many mammals and man. There are two genera of adenovirus, Aviadenovirus (avian) and Mastadenovirus (mammalian). Adenoviruses are non-enveloped, sixty to ninety nanometers in diameter, icosahedral in shape and possess double-stranded DNA (Novartis, 2001). Replication and assembly of mature virus particles occurs in the nucleus of an infected cell and virions are released by cell destruction (Ryan, 1994).

Adenoviruses are characterized by their ubiquity and persistence in host tissues for periods ranging from a few days to several years. Adenoviruses have the ability to produce infection in the absence of clinical disease and this is illustrated by the frequent recovery of virus from tonsils or adenoids removed from healthy children and by "prolonged intermittent shedding of virus from the pharynx and intestinal tract" after initial infection (Ryan, 1994). Type 1 and type 2 adenoviruses are highly endemic and type 5 is the next most common. Most primary infections with these viruses occur early in life (Novartis, 2001). The spread of the virus can be either respiratory or by fecal-oral contamination. Only about 45% of adenovirus infections result in disease. Infections

caused by serotypes 1, 2, and 5 are generally most frequent during the first few years of life. All serotypes can occur during any season of the year, but are encountered most frequently during late winter or early spring (Ryan, 1994).

Adenovirus infections are very common with most being asymptomatic. Adenoviruses enter the host by inhalation of droplets or by the oral route. The virus replicates in epithelial cells, producing cell necrosis and inflammation. Viremia sometimes occurs and may result in spread to distant sites such as the kidney, bladder, liver, lymphoid tissue and occasionally the central nervous system. Following the acute phase of the illness, viruses may remain in tissues, particularly lymphoid structures such as tonsils, adenoids and intestinal Peyer's patches and become reactivated and shed without producing illness for six to eighteen months thereafter. Reactivation is enhanced by stressful events. The clinical manifestation of adenovirus infection is diverse depending on the associated serotype. Acute respiratory syndromes vary in both clinical manifestations and severity and symptoms may include fever, rhinitis, pharyngitis, cough and conjunctivitis. Acute and occasionally chronic conjunctivitis has been associated with several serotypes. Other serotypes have been recognized as significant causes of gastroenteritis (Ryan, 1994). Infection with enteric adenoviruses is universal among children early in life with infection being symptomatic. The immune response generated generally protects against subsequent severe disease (Novartis, 2001). Several adenovirus types have demonstrated oncogenic potential (Russell, 2000). There is no specific therapy for adenovirus infection (Ryan, 1994).

Rotavirus

Rotaviruses are the most important viruses causing life-threatening disease in young children and are a major cause of hospitalization of children in the United States (Gerba et al., 1996) (Estes, 2001) (Smith and Gerba, 1982). Rotaviruses have been detected in water, wastewater and more recently in biosolids, although very little data exist on their occurrence in biosolids (Chapron et al., 2000). They are extremely resistant in the environment and present in large amounts in wastewater varying in concentration from undetectable to ten thousand plaque forming units (PFU) per liter (Yanko et al., 2000). The public effects of exposure to rotavirus in drinking and recreational waters have been evaluated and the resultant dose-response data indicated that rotavirus is the most infective of all the enteric viruses (Baker and Hegarty, 1997). These qualities make rotavirus an important candidate for evaluating survivability following lime stabilization. There is a need for more data on the occurrence and potential exposure to this and other viruses (Baker and Hegarty, 1997).

Rotavirus has a worldwide presence and is believed to account for forty to sixty percent of cases of acute gastroenteritis occurring in the winter among infants and children less than two years of age. By the age of four years, more than ninety percent of individuals have humoral antibodies, suggesting a high rate of virus infection early in life (Ryan, 1994). The rotaviruses belong to the family Reoviridae. They are naked, spherical particles 65 to 75 nanometers in diameter with a segmented genome containing double-stranded RNA and a double icosahedral shell. Three serotypes have been associated with disease in humans, groups A, B, and C. Four group A serotypes (1, 2, 3 and 4) based on

type-specific antigens on the outer capsid are of major epidemiologic importance (Ryan, 1994).

Outbreaks of rotavirus induced gastroenteritis are frequently observed as a result of person to person transmission in institutional settings such as hospitals, nursing homes, day-care centers and schools (Ansari et al., 1988). Following a one to three day incubation, infection manifests as vomiting followed by diarrhea. Rotaviruses localize in the duodenum and proximal jejunum, causing destruction of villous epithelial cells. The decrease in absorptive surface in the small intestine results in severe diarrhea. Viral excretion lasts two to twelve days but can be prolonged with symptoms persisting in malnourished or immunocompromised patients. The major complications associated with infection include dehydration which can lead to death in very small or malnourished infants. There is no specific treatment for rotavirus infection. Replacement of fluids and electrolytes is recommended and is required in severe cases. Rotaviruses are highly infectious and may spread quickly in family and institutional settings (Ryan, 1994).

The use of a plaque assay is an accurate and quantitative technique that may be employed to measure virus infectivity. Plaque assays, for both simian and human strains of rotavirus, have been developed and require treatment with proteolytic enzymes as plaques will not form under an agar overlay with maintenance medium alone (Smith et al., 1978) (Wyatt et al., 1980). Rotavirus will grow and plaque in several cell lines including MA-104, LLC-MK2 and CV-1; however, Smith et al. demonstrated that the MA-104 cell line is the most sensitive with respect to virus titers obtained, plaque size and time of appearance of plaques with simian rotavirus (Smith et al., 1978) (Londrigan et al., 2000). The presence of trypsin in the maintenance medium has been shown to

greatly facilitate the growth of rotaviruses in cell culture and is essential for plaque formation (Ramia and Sattar, 1979). Trypsin added to the agar overlay used in a plaque assay enhances rotavirus infectivity due to inactivation of neutralizing antibodies in the media and cleavage of capsid proteins on the virus surface (Smith et al., 1978).

Fecal Coliforms

Fecal coliform bacteria, including *Escherichia coli*, are commonly found in the feces of humans and other warm-blooded animals and are used to indicate the potential presence of pathogens in class B biosolids (EPA, 1998). Fecal coliforms are Gram-negative, non-spore forming rods contained within the Enterobacteriaceae and are among the larger bacteria that colonize humans (Ryan, 1994) (EPA, 1998). Enterobacteriaceae are large (2-4 micrometers in length and 0.4 to 0.6 micrometers in width) Gram-negative rods that are free living in nature and part of the indigenous flora of humans and animals. Enterobacteriaceae are the most common causes of urinary tract infection and some of the species are important etiologic agents of acute diarrhea throughout the world (Ryan, 1994). *Escherichia coli* is the "most commonly encountered member of the Enterobacteriaceae in the normal intestinal flora" and is a leading cause of opportunistic infections (Ryan, 1994). The densities of fecal coliforms in sewage are estimated to be one hundred thousand colonies per one hundred milliliters (Yanko et al., 2000). The EPA Part 503 regulations require that treated sewage sludge must be monitored for fecal coliforms and that the density may not exceed one thousand most probable number (MPN) or colony forming units (CFU) per gram of total solids (dry weight basis)

analyzed if the sludge is to qualify as Class A sludge, or less than two million MPN or CFU per gram total solids analyzed if the sludge is to quality as Class B sludge (EPA, 1999). Bacteria are important pathogens with regard to biosolids and land application because the potential exists for regrowth following treatment, particularly when biosolids are blended with other nutrient containing materials for the creation of soil amendment products (Yanko, 1988).

Salmonella

Salmonella are ubiquitous pathogens and infection is most often associated with improper food handling; however, *Salmonella* is a microorganism that is of concern in sewage sludge. The densities of *Salmonella* in sewage range from less than one to one thousand most probable number (MPN) per one hundred milliliters (Yanko, 2000). The EPA Part 503 regulations require that treated sewage sludge must be monitored for fecal coliform or *Salmonella* species and have a *Salmonella* density of less than three most probable number (MPN) per four grams total solids (dry weight basis) to qualify as a Class A sludge (EPA, 2000). For bacterial pathogens, the infectious dose will vary with the organism and can be as low as 100 bacteria to establish a *Salmonella* infection (Kowal, 1985). *Salmonella* infection manifests as gastroenteritis following ingestion of contaminated food or water. Symptoms will manifest twenty-four to forty-eight hours after ingestion and consist of nausea and vomiting followed by cramping and diarrhea persisting for three to four days and resolving spontaneously (Ryan, 1994).

Bacteriophage

There are at least twelve distinct groups of bacteriophage that are diverse both structurally and genetically. Somatic coliphages, F-specific RNA phages, and *Bacteroides fragilis* phages represent types of bacteriophage that have been proposed as specific indicators of viral contamination (Fewtrell and Bartram, 2001). The best known bacteriophages are the common phages of *E. coli.* Bacteriophage may consist of single-stranded or double-stranded DNA or RNA and are further classified by size, the presence and structure of a tail, and the location of attack for infection. Phage may be male-specific, requiring a bacterial pilus for attachment or somatic, attaching directly to the outer cell membrane or cell wall.

The male-specific phage MS-2 is a single-stranded RNA bacteriophage within the family leviviridae. MS-2 is approximately twenty-six nanometers in size, 3,569 nucleotides in length, icosahedral in shape, and possesses a positive sense RNA strand; therefore, the MS-2 nucleic acid acts directly as its own messenger RNA upon entry into a susceptible cell. MS-2 attaches directly to the pilus of a susceptible cell during infection (Fewtrell and Bartram, 2001). Studies conducted by Mignotte-Cadiergues et al. concluded that inactivation of somatic coliphages, F-RNA bacteriophage and *Bacteroides fragilis* was dependent on pH and sludge matrix, with a pH of 9 being necessary for inactivation in solids matrices and a pH of 13.5 for liquid sludges (Mignotte-Cadiergues et al., 2002). The similar morphology, structure and behavior of F-specific RNA bacteriophage, as well as other phages, to that of human enteric viruses, suggests that

they could represent a model for presence of pathogens to demonstrate inactivation by treatment processes, and for routine surveillance of pathogen persistence.

MATERIALS AND METHODS

Male Specific Bacteriophage Preparation

Male specific bacteriophage levels were determined using a modified double-agar-overlay procedure previously described by Cabelli (1982). Two grams of sample to be assayed was obtained to which ten milliliters of phosphate buffered saline (pH 7.0) supplemented with magnesium chloride was added. The sample was thoroughly vortexed and subsequently centrifuged at 1,000 x g for ten minutes at room temperature (28°C). The supernate was immediately assayed for presence of male-specific bacteriophage.

Male-specific bacteriophage was propagated and enumerated using an *E. coli* host harboring a conjugative plasmid conferring both streptomycin and ampicillin resistance and pilus production (*E. coli* F. Amp HFR). The *E. coli* host was grown to log phase in Tryptic Soy broth supplemented with 1% streptomycin/ampicillin and 1% magnesium chloride at 37°C for approximately three hours. MS-2 bacteriophage was added to the log phase *E. coli* culture and incubated at 37°C for twelve to eighteen hours. Following incubation, the viral suspension was centrifuged at 10,000 rpm at 4°C for ten minutes to remove cellular debris. The supernate was removed, placed in sterile bottles and refrigerated at 4°C until use. The propagated MS-2 was enumerated by plaque assay with *E. coli* F. Amp using a double agar overlay technique. This was accomplished by growing host *E. coli* to log phase in Tryptic Soy Broth for three hours prior to assay. Serial dilutions of sample supernate to be enumerated were created using a 1X phosphate buffered saline solution supplemented with magnesium chloride. One hundred microliters

of sample and two hundred microliters of *E. coli* were inoculated into five milliliters of a sterile tempered agar overlay. The sample was gently mixed and immediately poured onto a sterile Tryptic soy agar plate and distributed evenly over the surface of the plate by swirling. Plaques were observed and counted after eighteen to twenty-four hours of incubation at 37°C. Plates containing 30-300 plaques were counted and used to calculate the final titer which was reported as the number of plaque forming units per milliliter of sample evaluated (PFU/mL). Male-specific bacteriophage densities were calculated per two grams of sample determined by the number of plaques per volume of supernate assayed times the dilution factor divided by the number of grams of sample examined.

Poliovirus Preparation

Poliovirus type 1 (LSc strain) was propagated using Buffalo Green Monkey (BGM) Kidney cells (Biowhittaker). Cells were grown to 90% confluency in closed 75 cm^2 cell culture flasks at 37°C with Eagle's Minimal Essential Media (MEM) and L-15 Medium supplemented with 8% Fetal Bovine Serum (FBS). Prior to infection, media was removed from each flask and cell monolayers were washed twice with serum-free MEM. One flask was infected with 1 mL of 10^7 PFU/mL poliovirus type 1 and the other flask was inoculated with 1 mL of serum-free MEM and maintained as a negative control. The virus was allowed to adsorb to cells for sixty minutes at 37°C with rocking of the flasks every fifteen minutes to redistribute the virus and adequately hydrate cells. Following incubation, 15 mL of Eagle's MEM supplemented with 2% FBS was added to each flask and flasks were incubated at 37°C. Flasks were observed daily for cytopathic effect. Once

a generalized cytopathic effect was achieved, virus was liberated from the cells by rapidly freeze thawing cells twice. The resultant viral suspension was transferred to a 15 mL conical tube and centrifuged at 1000 x g to separate cellular debris from virus present in the supernatant. Supernate fluid containing virus was aliquoted into cryovials for storage at -80°C.

Virus was enumerated using the plaque-forming unit (PFU) method (Dulbecco and Vogt, 1954). Buffalo Green Monkey Kidney (BGM) cells were grown to confluency with 5% CO_2 in 12-well cell culture plates with Eagle's MEM supplemented with 8% FBS. Cells were washed prior to infection with serum-free MEM. The propagated viral suspension was serially diluted in serum-free MEM. Cell culture wells were clearly labeled with the dilution of virus to be added and cells were inoculated in triplicate with 0.1 mL of the appropriate viral dilution. Plates were incubated in 5% CO_2 for sixty minutes with continual rocking to allow virus to adsorb and maintain hydration of cells. Following incubation, 2 mL of an agar overlay maintenance medium consisting of Medium 199 (Sigma) supplemented with 2% FBS, 2% flake agar (Difco), and neutral red (Sigma) was added to each well. This agar overlay provides a solid support matrix with a viability stain for plaque visualization. Following addition of overlay, the agar was permitted to solidify and the plates were returned to the 5% CO_2 incubator. Cells were examined every twenty-four hours and observed for plaques for seven days. The concentration of virus was determined by multiplying the averaged number of plaques counted in triplicate wells by the dilution factor of the wells. For a 12-well cell culture plate, dilutions containing 20-50 plaques were counted and this number was used to calculate the concentration of virus which was reported as plaque forming units per

milliliter volume evaluated (PFU/mL). Enumerated stocks of poliovirus were stored in cryovials at -80°C. Poliovirus type 1 was propagated for use in spiking studies to evaluate efficiency of inactivation by lime stabilization.

Adenovirus Type 5 Preparation

Adenovirus Type 5 was propagated using A549 cells grown to 90% confluency in closed 75 cm^2 cell culture flasks at 37°C with Eagle's Minimal Essential Media (MEM) and L-15 Medium supplemented with 10% Fetal Bovine Serum (FBS). Confluent monolayers of A549 cells were inoculated with adenovirus type 5 and incubated at 37°C for sixty minutes to permit viral attachment. Following incubation, a maintenance medium consisting of Eagle's MEM supplemented with 2% FBS was added and cells were incubated at 37°C and observed daily for cytopathic effect. Once a generalized cytopathic effect was achieved, flasks were freeze-thawed to liberate viruses trapped within cells. The resultant suspension was centrifuged at 1000 x g for ten minutes to separate cellular debris from virus present in the supernatant.

Adenovirus was enumerated using the $TCID_{50}$ method established by Reed and Muench in 1937. Serial dilutions of propagated virus were created with serum-free minimal essential media. 96-well cell culture plates containing confluent monolayers of A549 cells were inoculated according to $TCID_{50}$ protocols with 0.025 mL of the appropriate viral dilution. Adsorption of the virus was allowed to occur for 1.5 hours at 37°C. Following adsorption, a maintenance medium consisting of MEM (Sigma) supplemented with 2% FBS was added. Plates were incubated at 37°C in 5% CO_2 with

humidity and observed for cytopathic effect. Plates were observed for fourteen days post-inoculation and the number of positive wells was determined for each dilution. The number of positive wells was used to calculate a $TCID_{50}$ titer according to the formula below. Enumerated stocks of adenovirus type 5 were stored in cryovials at -80°C. Adenovirus type 5 was propagated for use in spiking studies to evaluate efficiency of inactivation by lime stabilization.

Calculation of $TCID_{50}$

Log $TCID_{50}$/mL = Log [{10 exp [X + (p-0.5)]}/Inoculum volume]

X = positive exponent from last dilution where all wells were positive
P = ratio of positive wells/total number of wells

Rotavirus Wa Preparation

Rotavirus Wa propagation and enumeration was accomplished using modified protocols established by Smith et al. (1978). Rotavirus Wa strain was propagated using Ma104 cells. Cells were grown to 90% confluency in closed seventy-five cm^2 cell culture flasks at 37°C using Eagle's MEM supplemented with 10% FBS. Prior to infection, media was removed from each flask and the cells were washed twice with serum-free MEM. One flask was infected with 1 mL of 10^6 PFU/mL rotavirus Wa and the other flask was inoculated with 1 mL of serum-free MEM and maintained as a negative control. The virus was allowed to adsorb to cells for sixty minutes at 37°C with rocking of the flasks every fifteen minutes to redistribute the virus and adequately hydrate cells. Following

incubation, 15 mL of Eagles' MEM supplemented with five micrograms per milliliter of trypsin (Gibco) was added to each flask and flasks were incubated at 37°C. Flasks were observed daily for cytopathic effect. Once a generalized cytopathic effect was achieved, virus was liberated from the cells by rapidly freeze thawing cells twice. The resultant viral suspension was transferred to a 15 mL conical tube and centrifuged at 1000 x g for ten minutes to separate cellular debris from virus present in the supernatant. Supernate fluid containing virus was aliquoted into cryovials for storage at -80°C.

Virus was enumerated using a modified plaque-forming unit (PFU) method (Smith et al., 1954). Ma104 cells were grown to confluency with five percent CO_2 in 12-well cell culture plates with Eagle's MEM supplemented with 10% FBS. Cells were thoroughly washed prior to infection with serum-free MEM. The propagated viral suspension was serially diluted in serum-free MEM. Cell culture wells were clearly labeled with the inoculum dilution to be added and cells were inoculated in triplicate with 0.1 mL of the appropriate viral dilution. Plates were incubated in 5% CO_2 for sixty minutes with continual rocking to allow virus to adsorb and maintain hydration of cells. Following adsorption, 2 mL of an agar overlay maintenance medium consisting of 2X MEM supplemented with 1 ug/mL trypsin (Gibco), and 2% agar (Sigma) was added. This agar overlay provides a solid support matrix to physically confine virus and permit plaque formation. Following addition of overlay, the agar was permitted to solidify and the plates were returned to the 5% CO_2 incubator. Plates were incubated for four days at which time 2 mL of 10% formaldehyde in normal saline solution was added to each well. Plates were returned to the CO_2 incubator for overnight incubation. Solid overlay was removed from wells by rinsing under warm tap water and 2 mL of a 0.1% crystal violet

solution was added to each well to permit visualization of plaques (Figure 1). Plaques were quantified and results from duplicate flasks averaged. A titer was determined by multiplying the average number of plaques by the dilution factor. Enumerated stocks of rotavirus Wa were stored in cryovials at -80°C. Rotavirus Wa was propagated for use in spiking studies to evaluate efficiency of inactivation by lime stabilization.

Visualization of Plaque Formation in a Rotavirus Plaque Assay

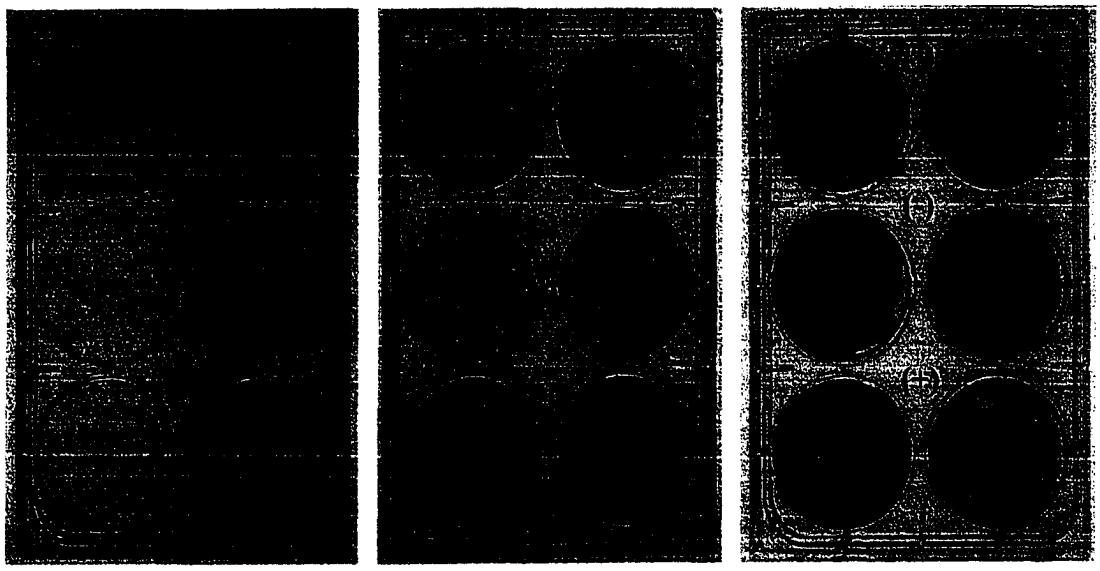

Figure 1: Visualization of plaque formation in a rotavirus plaque assay – 12 well cell culture plates were inoculated with serial dilutions of rotavirus Wa. Following a four day incubation to permit viral replication, agar overlay was removed, cells were fixed with 10% formaldehyde in normal saline, and 0.1% crystal violet solution was added to permit visualization of plaques. Crystal violet solution will stain cells purple; therefore, areas of clearing represent areas in the cell culture well where no cells are present. Plaques are easily visualized as zones of clearing in the monolayer that decrease with an increase in viral dilution. Negative controls, inoculated with one hundred microliters of serum free MEM do not contain areas of clearing and therefore are negative for plaque formation. The positive control well, inoculated with one hundred microliters of rotavirus Wa 1 x 10^3 PFU/mL displays plaque formation consistent with the concentration of the inoculum.

Elution of Viruses from Sludge Samples

Samples were evaluated for total culturable viruses, including enteroviruses, according to the procedures designated for recovery and assay of viruses from sludge in the EPA part 503 rules. The procedure for recovery of viruses from wastewater solids is an adsorption process reliant upon adsorption of viruses from the liquid phase to the sludge solids, which are subsequently eluted and concentrated by centrifugation. The supernate was discarded and viruses were desorbed from the solids by physiochemical means and further concentrated by organic flocculation. Decontamination prior to evaluation by cell culture was accomplished by incubation with antibiotics.

Liquid samples were conditioned prior to elution. A 100 mL quantity of liquid sludge was homogenized for five minutes, at which time 1 mL of aluminum chloride was added and the pH of the solution adjusted to 3.5 with 1N HCL. The sample was mixed for thirty minutes and subsequently centrifuged at 2500 x g for fifteen minutes at 4°C. The supernate was discarded and the sample eluted. Elution of conditioned liquid samples and sludge solids involved resuspension of the resultant pellet from the conditioning or measuring 100 grams of sample in the case of a solid sample and adding 100 mL of distilled water. The sample was blended gently for five minutes. Following blending, an equal volume of 20% beef extract solution was added to the sample and blended. The sample was then mixed for thirty minutes and centrifuged at 10,000 x g for thirty minutes at 4°C. The supernate fluid was decanted and the appropriate volume of distilled water added to bring the final concentration of beef extract to 3%. The resultant eluate was transferred to a clean centrifuge bottle and the pH adjusted to 3.5. The sample

was mixed for thirty minutes and centrifuged at 2500 x g for fifteen minutes. The sediment was retained, resuspended in 20 mL of sodium phosphate buffer (pH 7.0) and transferred to a conical tube where the pH was adjusted to 7.0. Samples were incubated at 37°C for three hours with 1 mL of antibiotic/antimycotic and fungizone (10,000 units/mL of penicillin and 10,000 μg/mL of streptomycin, and 25 μg/mL of amphotericin B) and 0.1 mL of gentamicin (50 mg/mL) and frozen for assay.

Fecal Coliform Isolation

Indigenous fecal coliform densities in dewatered sludge samples obtained for lime stabilization experiments were evaluated using a most probable number assay (MPN) according to method 9221 E Standard Methods for the Examination of Water and Wastewater (Greenberg et al., 1992). This method is recommended by the EPA for fecal coliform detection in biosolids by multiple tube fermentation (also referred to as the most probable number procedure) (EPA, 1999). The MPN assay is an estimation of bacterial density and employs culture specific media combined with elevated temperature to isolate and enumerate fecal coliforms. A presumptive step using lauryl tryptose broth (LTB) (Difco) as the selective enrichment medium and a completed step using *E. coli* (EC) (Difco) media, incubated at elevated temperature, permit the recovery and isolation of fecal coliforms. Thirty grams of sample was blended with 270 mL sterile phosphate buffered saline (PBS). Ten-fold serial dilutions were created with PBS and inoculated into five test tubes containing sterile LTB and glass shell vials to indicate gas production. Tubes were incubated for forty-eight hours at 35°C and observed at both 24 and 48 hours

for the presence of presumptive growth indicated by gas and/or heavy growth in the fermentation tubes. Failure to produce gas or growth in the LTB media within 48 hours was recorded as a negative presumptive test. A sterile loop was used to transfer a culture sample from each LTB presumptive positive fermentation tube to fermentation tubes containing sterile EC media and glass shell vials. EC fermentation tubes were incubated in a water bath at 44.5°C for 24 hours. Gas production in EC broth in 24 hours or less, measured by presence of a gas bubble within the glass shell vial, was considered a positive fecal coliform reaction. Failure to produce gas was a negative reaction and indicated that fecal coliform bacteria were not present. Results of the MPN procedure were reported in terms of MPN/g calculated from the number of positive EC culture tubes and total solids determination using Thomas' equation. Positive control cultures consisting of *E. coli* were included in each assay. *Pseudomonas sp.* were incorporated as a negative control in each assay.

Thomas' Equation: MPN/100mL = (# Positive Tubes x 100)/√(mL sample in negative tubes x mL sample in all tubes)

MPN/g = (10 x MPN Index/100mL)/(largest volume x % dry solids)

Salmonella Isolation

Indigenous *Salmonella* densities in dewatered sludge samples obtained for lime stabilization experiments were evaluated using a most probable number assay (MPN) according to methodology recommended by the EPA for *Salmonella* detection in biosolids by multiple tube fermentation (also referred to as the most probable number procedure) using procedures established in standard methods 9260 D (Greenberg et al., 1992). Sludges with *Salmonella* species densities below three MPN per four gram of total solids meet class A criteria.

The MPN assay is an estimation of bacterial density and employs culture specific media to isolate and enumerate *Salmonella*. Thirty grams of sample was blended with 270 mL PBS. Sample volumes were inoculated into selenite brilliant green sulfa (SBG) broth for enrichment of *Salmonella*. Following a 24 hour incubation period at 37°C, samples were streaked for isolation on xylose-lysine deoxycholate agar (XLD). Characteristic *Salmonella* colonies will appear as red or pink with a black center. Plates were incubated for 24 hours at 37°C. Positive *Salmonella* plates containing characteristic black colonies were stabbed into triple sugar iron agar (TSI) slants. Slants were incubated for 24 hours at 37°C. Positive TSI slants for *Salmonella* are characterized by a color change (black butt/red slant) with no gas production. TSI supports the growth of organisms other than *Salmonella*; therefore, a confirmation step, employing urea, serves as an additional confirmation and will result in a yellow color change if positive for *Salmonella*. Samples yielding positive urease tests are confirmed as *Salmonella* using polyvalent antisera (Difco) specific for *Salmonella*. Agglutination is measured using a

small volume from the TSI slant placed on a glass slide and observed for agglutination. Negative control samples incorporate *Proteus sp.*, which have urease capability, and generate a red color change for the urease test.

Lime Stabilization

Experimental Design Evaluation

Poliovirus type 1 was seeded into a 1-Liter volume of raw dewatered sludge and evaluated for inactivation by lime stabilization at room temperature (28°C) and reduced temperature (4°C) according to methodology established in prior studies conducted by Sattar et al. (1976). The purpose of this exercise was to determine whether or not the same inactivation rates reported in previous studies with respect to poliovirus type 1 could be achieved in addition to developing an appropriate experimental design for the evaluation of adenovirus type 5, rotavirus Wa, and MS-2 bacteriophage under lime stabilization conditions (Sattar et al., 1976). In similar trials, indigenous *Salmonella* sp. and fecal coliforms were evaluated in conjunction with seeded MS-2 to compare results obtained to the inactivation rates achieved by other investigators (Mignotte et al., 2001) (Gantzer et al., 2001). A raw dewatered sludge sample was obtained from the Durham Wastewater Treatment Plant, Durham, NH and diluted with 1.5 liters of PBS to facilitate mixing during lime treatment. A 500 mL aliquot of the sample was removed and incubated at room temperature. A background sample was removed to assay for presence of enteroviruses, fecal coliforms, *Salmonella* sp. and MS-2 bacteriophages. Enteroviruses were recovered by beef extract elution and detected by plaque assay. Fecal coliform and *Salmonella* sp. concentration was estimated using a most probable number fermentation tube test. MS-2 viability was determined by double-agar overlay plaque assay.

Enteroviruses were not detected in the background sludge sample; therefore, poliovirus type 1 was spiked to achieve a final concentration of approximately 1.0×10^4

PFU/mL. Fecal coliform concentrations and *Salmonella* sp. concentrations were determined to be 1.32×10^8 and 1.0×10^3 MPN/gram dry weight respectively, which was sufficient for experimentation and therefore, indigenous fecal coliforms and *Salmonella* sp. were evaluated without the need for additional spiking. Male specific bacteriophage was not recovered in the background sample; therefore, the 1-Liter sludge sample was spiked to achieve a final concentration of approximately 1.0×10^7 PFU/mL of MS-2. Vials of MS-2 and poliovirus used for spiking were placed at room temperature and assayed throughout the experiment to evaluate inactivation as a result of incubation at room temperature. The spiked 1-Liter sample was limed to a pH of 12 for 2 hours and incubated at pH 11.5 for 22 hours with continual mixing through the use of magnetic stir bars. Aliquots of the test sample, control sample incubated at room temperature, and MS-2/poliovirus incubated at room temperature, were removed at time 0.1, 2, 12, and 24 hours to assay for poliovirus, fecal coliform and *Salmonella* sp., and MS-2 viability.

The results of trials to evaluate experimental design were comparable to those reported by Sattar et al., demonstrating inactivation of poliovirus type 1 following lime stabilization, and those results reported by Mignotte et al., reporting an elimination of *Salmonella* sp. from a sludge sample in 24 hours at a pH of 10.0, and Gantzer et al., reporting a decrease in *E. coli* following lime stabilization (Sattar et al., 1976) (Mignotte et al., 2001) (Gantzer et al., 2001). Upon review of the experimental design it was determined that in order to achieve statistical accuracy, it would be necessary to create separate control and test beakers for individual time points. This would eliminate variability resulting from the removal of aliquots at various time points from one individual spiked sample. Therefore, in subsequent experimentation to evaluate pathogen

inactivation in RO water matrix trials and sludge and biosolids matrix trials, separate control and test beakers were employed using smaller quantities of matrix (50 mL RO water or 50 G sludge).

RO (Reverse Osmosis) Water Matrix Trials

Adenovirus type 5, rotavirus Wa, and MS-2 were initially evaluated in an RO water matrix to determine inactivation by lime stabilization. Trials were performed in an RO water matrix to provide a baseline for inactivation independent of the inhibitory components commonly found in sludge and biosolids matrices. In addition, due to poor methodology and low recovery rates for protozoa and helminth organisms in sludges, lime stabilization to evaluate these pathogens was conducted in RO water. To allow for comparisons to be made, initial trials to evaluate viral persistence during lime stabilization were also conducted in RO water matrices. RO water matrix trials were performed according to the following procedures in triplicate at room temperature (28°C). Fifty milliliter volumes of RO water in separate control and test beakers for time points 0.1, 2, 12, and 24 hours were inoculated with adenovirus type 5 and MS-2 to achieve a final concentration of 1.0×10^6 $TCID_{50}$/mL or PFU/mL respectively for each virus. The same experimental design was employed in separate trials with Rotavirus Wa and MS-2 with inoculation of 50-mL volumes of RO water to achieve a final concentration of 1.0×10^4 PFU/mL for each virus. Samples were continually mixed with magnetic stir bars and pH and temperature readings were recorded for control and test beakers hourly. The pH of the test beakers for each time point was simultaneously adjusted to 12.0 using an 8%

aqueous slurry comprised of calcium hydroxide and distilled deionized water. Approximately 0.4 - 0.5 mL of calcium hydroxide slurry was required to elevate the pH to 12.0, corresponding to a lime dose of approximately 80 g/kg total solids. The pH was maintained at 12.0 for 2 hours at which time, 0.1 N HCL was added drop by drop until a pH value of 11.5 was achieved and maintained. At time points 0.1, 2, 12 and 24 hours, test beakers were neutralized with 0.1 N HCL and aliquots were removed from designated control and test beakers for viral enumeration. Aliquots of control and neutralized test sample were diluted in phosphate buffered saline and plated immediately for male-specific bacteriophage using the previously described double agar overlay technique. Aliquots of control and neutralized test sample designated for viral enumeration were centrifuged for ten minutes at 1000 x g to remove precipitated lime, in an effort to minimize the toxic effects of the lime crystals on cells. Following centrifugation, the supernate was retained and assayed directly for adenovirus type 5 and rotavirus Wa by $TCID_{50}$ and plaque assay respectively according to methods previously described.

Sludge Matrix Trials

Sludge matrix trials were performed according to the following procedure at room temperature (28°C) and reduced temperature (4°C). Fifty grams of various sludge matrices (compost, raw and previously limed), collected from participating utilities, was placed in separate control and test beakers for removal at time points 0.1, 2, 12, and 24 hours. One hundred milliliters of RO water was added to facilitate mixing. Samples were

inoculated with adenovirus Type 5 and MS-2 to achieve a final concentration of approximately 1.0×10^5 $TCID_{50}$/mL and approximately 1.0×10^7 PFU/mL for each virus respectively. The same experimental design was employed in separate trials using rotavirus Wa and MS-2. Rotavirus Wa and MS-2 were inoculated into various sludge samples to achieve a final concentration of approximately 1.0×10^4 PFU/mL and approximately 1.0×10^7 PFU/mL for each virus respectively. The pH of the test beakers was simultaneously adjusted to 12.0 using an 8% aqueous slurry comprised of calcium hydroxide and distilled deionized water. Approximately 0.4 - 0.5 mL of calcium hydroxide slurry was required to elevate the pH to 12.0, corresponding to a lime dose of approximately 80 g/kg total solids. The pH was maintained at 12.0 for 2 hours at which time 0.1 N HCL was added drop by drop until a pH value of 11.5 was achieved and maintained. At time points 0.1, 2, 12 and 24 hours, test beakers were neutralized with 0.1 N HCL and aliquots were removed from designated control and test beakers for viral elution and enumeration. Aliquots of control and neutralized test sample were diluted in phosphate buffered saline and plated immediately for male-specific bacteriophage using the previously described double agar overlay technique. Aliquots of control and neutralized test sample designated for viral enumeration were eluted according to previously described methodology and subsequently assayed for adenovirus type 5 and rotavirus Wa by $TCID_{50}$ and plaque assay respectively.

RESULTS

MS-2, Poliovirus Type 1, Fecal Coliform and *Salmonella* Persistence in Lime Stabilized Sludge

A bench scale analysis of lime stabilized sludge to assess survivability of MS-2 bacteriophage and Poliovirus type 1 in a dewatered sludge matrix revealed the inactivation of both organisms immediately following the addition of calcium hydroxide at room temperature (28°C) (Figure 2). Evaluation at reduced temperature (4°C) revealed an increased survivability of both organisms, as compared to results obtained for experiments conducted at room temperature (28°C) (Figure 3). At 28°C, MS-2 bacteriophage was below detectable levels (< 1 PFU/mL) following the initial liming, demonstrating at least a seven-log reduction. Poliovirus type 1 was also below detectable levels (<1 PFU/mL) at the same time point, demonstrating at least a five-log reduction. At 4°C the MS-2 bacteriophage did not demonstrate any reduction following the initial liming, yet was below detectable levels following a two-hour incubation at pH 12. At the same temperature, poliovirus type 1 demonstrated at least a 1.2 log reduction following the initial liming and was below detectable levels after the two-hour lime stabilization period at pH 12. MS-2 and fecal coliforms evaluated in a dewatered sludge matrix at room temperature (28°C) were below detectable levels following 0.1 hours of lime stabilization at pH 12 demonstrating at least a seven-log and at least an eight-log reduction respectively (Figure 4). No decrease in *Salmonella* density was observed at time 0.1 hours and 2 hours

post liming. Following 12 hours of liming, *Salmonella* was below detectable levels (Figure 4).

Poliovirus Type 1 and MS-2 Persistence in a Limed Sludge Matrix at Room Temperature (28°C)

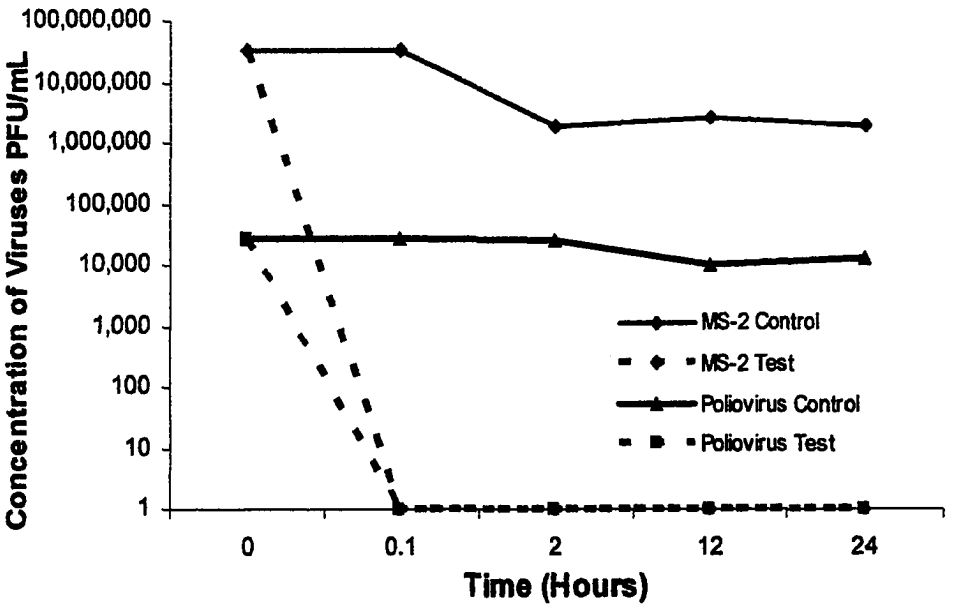

Figure 2: Evaluation of poliovirus type 1 and MS-2 persistence in a limed sludge matrix at room temperature (28°C) - Poliovirus type 1 and MS-2 seeded into a dewatered sludge matrix were below detectable levels following lime stabilization at room temperature (28°C). Poliovirus type 1 seeded into dewatered sludge to achieve a final concentration of 1 x 10^4 PFU/mL (Poliovirus Test) was below detectable levels following 0.1 hours of liming (time point 6 minutes) demonstrating at least a four log reduction. MS-2 seeded into dewatered sludge to achieve a final concentration of 1 x 10^7 PFU/mL (MS-2 Test) was also below detectable levels following 0.1 hours of liming (time point 6 minutes) demonstrating at least a seven log reduction. Control samples consisting of poliovirus type 1 (Poliovirus Control) and MS-2 (MS-2 Control) seeded into dewatered sludge maintained at room temperature did not undergo lime stabilization. Aliquots of control samples removed at time points 0.1, 2, 12 and 24 hours demonstrated no viral reduction.

Poliovirus Type 1 and MS-2 Persistence in a Limed Sludge Matrix at Reduced Temperatures (4°C)

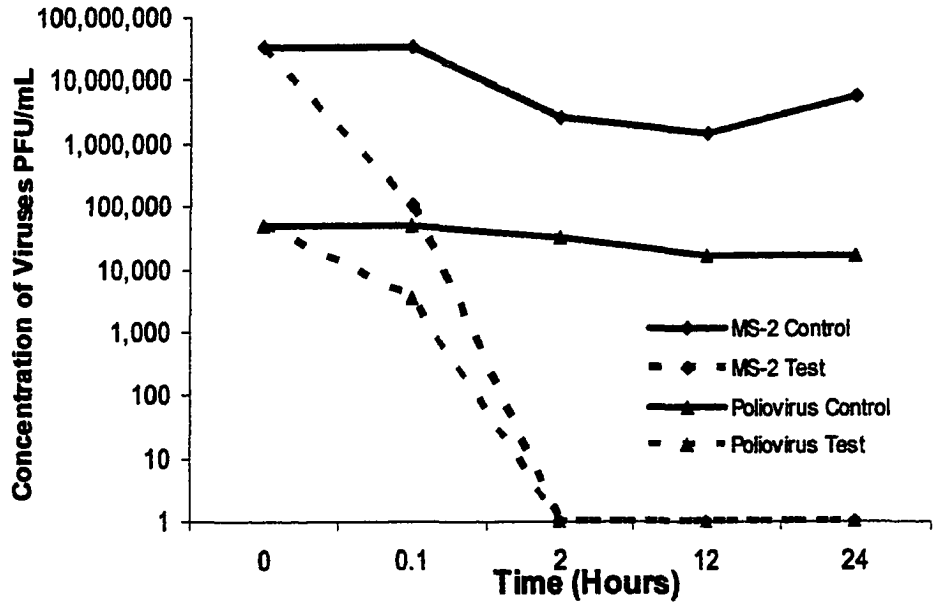

Figure 3: Evaluation of poliovirus type 1 and MS-2 persistence in a limed sludge matrix at reduced temperature (4°C) - Poliovirus type 1 and MS-2 seeded into a dewatered sludge matrix were below detectable levels following lime stabilization at 4°C. Poliovirus type 1 seeded into dewatered sludge to achieve a final concentration of 1×10^4 PFU/mL (Poliovirus Test) was below detectable levels following 2 hours of liming (time point 2) demonstrating at least a four log reduction. MS-2 seeded into dewatered sludge to achieve a final concentration of 1×10^7 PFU/mL (MS-2 Test) was also below detectable levels following 2 hours of liming (time point 2) demonstrating at least a seven log reduction. Control samples consisting of poliovirus type 1 (Poliovirus Control) and MS-2 (MS-2 Control) seeded into dewatered sludge maintained at room temperature did not undergo lime stabilization. Aliquots of control samples, removed at time points 0.1, 2, 12 and 24 hours, demonstrated no viral reduction.

MS-2, Poliovirus Type 1, *Salmonella*, & Fecal Coliform Persistence in a Limed Sludge Matrix at Room Temperature (28°C)

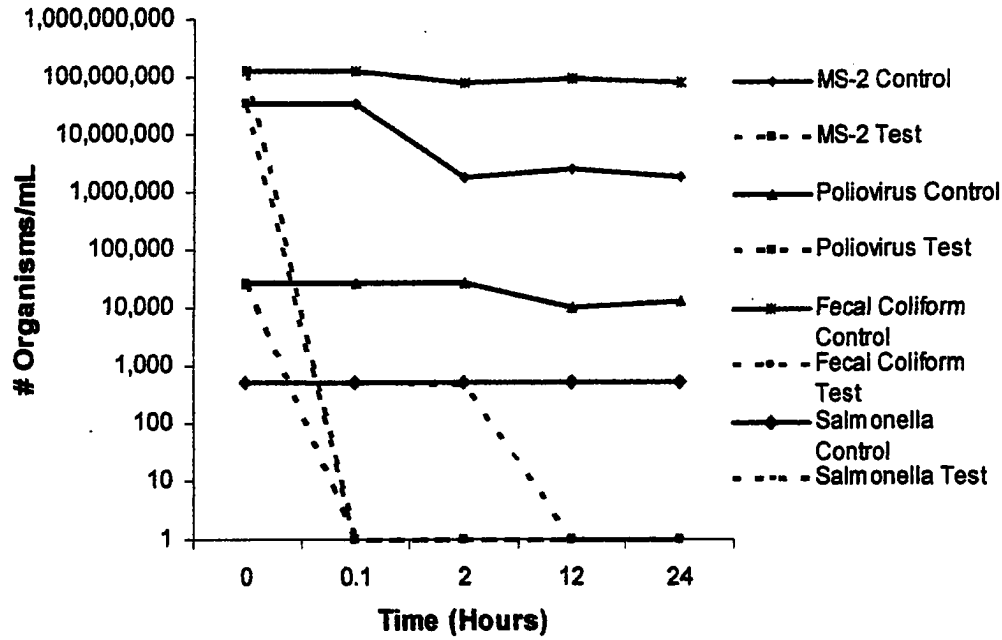

Figure 4: Evaluation of MS-2, poliovirus type 1, *Salmonella*, & fecal coliform persistence in a limed sludge matrix at room temperature (28°C) – MS-2, poliovirus type 1, *Salmonella*, and fecal coliforms evaluated in a dewatered sludge matrix were below detectable levels following lime stabilization at room temperature (28°C). Poliovirus type 1 seeded into dewatered sludge to achieve a final concentration of 5×10^4 PFU/mL (Poliovirus Test) was below detectable levels following 0.1 hours of liming (time point 6 minutes) demonstrating at least a four and a half log reduction. MS-2 seeded into dewatered sludge to achieve a final concentration of 5×10^7 PFU/mL (MS-2 Test) was also completely inactivated following 0.1 hours of liming (time point 6 minutes) demonstrating at least a seven log reduction. Indigenous fecal coliform and *Salmonella* sp. at concentrations of 1×10^8 (Fecal Coliform Test) and 1×10^3 MPN (*Salmonella* Test) per gram total solids respectively, were below detectable levels following 0.1 (time point 6 minutes) and 12 hours (time point 12) of liming respectively. Control samples consisting of seeded MS-2 (MS-2 Control) and poliovirus type 1 (Poliovirus Control), and indigenous fecal coliforms (Fecal Coliform Control) and *Salmonella* sp. (*Salmonella* Control) in dewatered sludge maintained at room temperature did not undergo lime stabilization. Aliquots of control samples removed at time points 0.1, 2, 12 and 24 hours demonstrated no reduction for organisms evaluated.

Adenovirus Type 5 and MS-2 Lime Stabilization Evaluated in a Water Matrix at Room Temperature (28°C)

Adenovirus Type 5 and male specific bacteriophage MS-2 were spiked into a water matrix and limed for twenty-four hours with removal of aliquots at time points 0.1 hours (immediately following addition of lime), 2, 12, and 24 hours post lime addition. Following removal of aliquots at designated time points and immediate neutralization, virus was enumerated using previously described methods. The results of three trials conducted with adenovirus type 5 and MS-2 spiked into a limed water matrix at room temperature are presented in figures 5, 6, 7, and 8. In all trials, adenovirus was below detectable levels by $TCID_{50}$ ($< 10^{0.5}$ $TCID_{50}$/mL) indicating inactivation following 0.1 hours of liming (time point 6 minutes). In all trials, MS-2 was below detectable levels (< 1 PFU/mL) following 0.1 hours of liming (time point zero). In all RO water trials, Adenovirus was spiked into RO water to achieve a final concentration of approximately 10^4 $TCID_{50}$/mL and MS-2 was spiked into RO water to achieve a similar final concentration of approximately 10^4 PFU/mL. In all RO water trials both Adenovirus and MS-2 were below detectable levels following 0.1 hours of liming, exhibiting at least a four log reduction for both viruses.

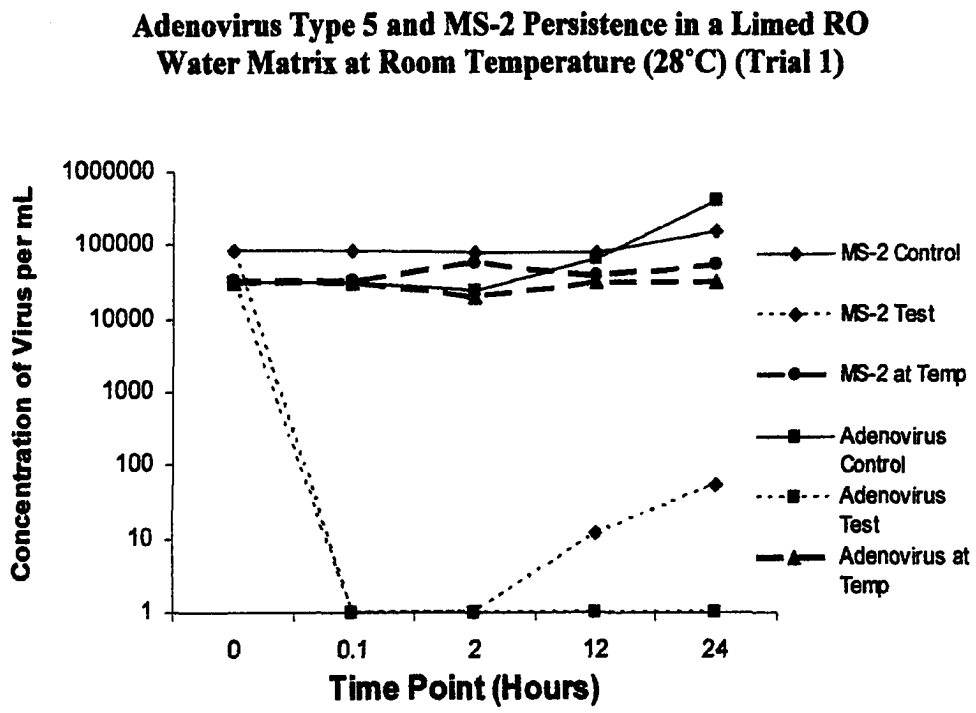

Figure 5: Evaluation of adenovirus type 5 and MS-2 persistence in a limed RO water matrix at room temperature (28°C) (trial 1) - Adenovirus type 5 and MS-2 seeded into an RO water matrix were below detectable levels following lime stabilization at 28°C. Adenovirus type 5 seeded into RO water to achieve a final concentration of 1 x 10^4 $TCID_{50}$/mL (Adenovirus Control) was below detectable levels following an initial liming (time point 6 minutes) demonstrating at least a four log reduction. MS-2 seeded into RO water to achieve a final concentration of 1 x 10^4 PFU/mL (MS-2 Control) was below detectable levels following initial liming (time point 6 minutes) demonstrating at least a four log reduction. Control samples consisting of adenovirus type 5 (Adenovirus Control) and MS-2 (MS-2 Control) seeded into an RO water matrix maintained at room temperature did not undergo lime stabilization. In addition, aliquots of seeded virus (MS-2 at Temp and Adenovirus at Temp) were incubated at room temperature and enumerated at each time point. Aliquots of control samples removed at time points 0.1, 2, 12 and 24 hours demonstrated no viral reduction.

Adenovirus Type 5 and MS-2 Persistence in a Limed RO Water Matrix at Room Temperature (28°C) (Trial 2)

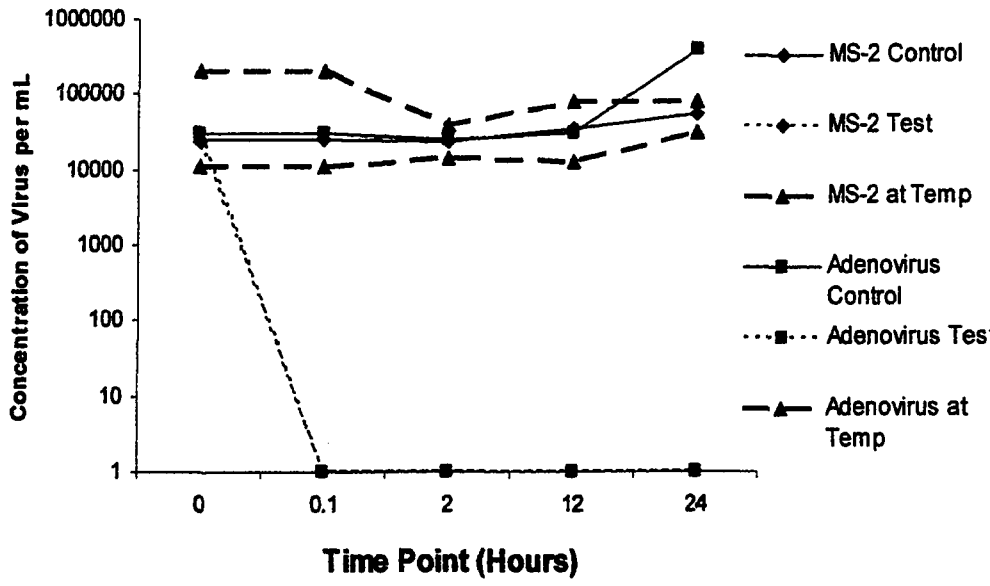

Figure 6: Evaluation of adenovirus type 5 and MS-2 persistence in a limed RO water matrix at room temperature (28°C)(trial 2) - Adenovirus type 5 and MS-2 seeded into an RO water matrix were below detectable levels following lime stabilization at 28°C. Adenovirus type 5 seeded into RO water to achieve a final concentration of 1 x 10^4 $TCID_{50}$/mL (Adenovirus Control) was below detectable levels following an initial liming (time point 6 minutes) demonstrating at least a four log reduction. MS-2 seeded into RO water to achieve a final concentration of 1 x 10^4 PFU/mL (MS-2 Test) was below detectable levels following initial liming (time point 6 minutes) demonstrating at least a four log reduction. Control samples consisting of adenovirus type 5 (Adenovirus Control) and MS-2 (MS-2 Control) seeded into an RO water matrix maintained at room temperature did not undergo lime stabilization. In addition, aliquots of seeded virus (MS-2 at Temp and Adenovirus at Temp) were incubated at room temperature and enumerated at each time point. Aliquots removed at time points 0.1, 2, 12 and 24 hours demonstrated no viral inactivation.

Adenovirus Type 5 and MS-2 Persistence in a Limed RO Water Matrix at Room Temperature (28°C) (Trial 3)

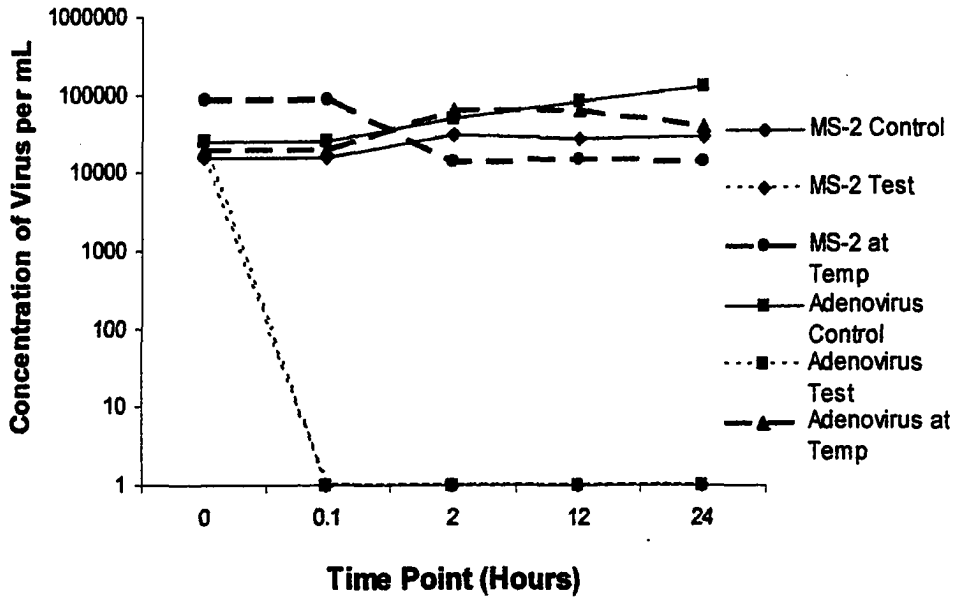

Figure 7: Evaluation of adenovirus type 5 and MS-2 persistence in a limed RO water matrix at room temperature (28°C) (trial 3) - Adenovirus type 5 and MS-2 seeded into an RO water matrix were below detectable levels following lime stabilization at 28°C. Adenovirus type 5 seeded into RO water to achieve a final concentration of 1 x 10^4 $TCID_{50}$/mL (Adenovirus Control) was below detectable levels following an initial liming (time point 6 minutes) demonstrating at least a four log reduction. MS-2 seeded into RO water to achieve a final concentration of 1 x 10^4 PFU/mL (MS-2 Control) was below detectable levels following initial liming (time point 0) demonstrating at least a four log reduction. Control samples consisting of adenovirus type 5 (Adenovirus Control) and MS-2 (MS-2 Control) seeded into an RO water matrix maintained at room temperature did not undergo lime stabilization. In addition, aliquots of seeded virus (MS-2 at Temp and Adenovirus at Temp) were incubated at room temperature and enumerated at each time point. Aliquots removed at time points 0.1, 2, 12 and 24 hours demonstrated no inactivation of organisms.

Adenovirus Type 5 and MS-2 Inactivation for Three Trials Conducted in a Limed RO Water Matrix at Room Temperature (28°C)

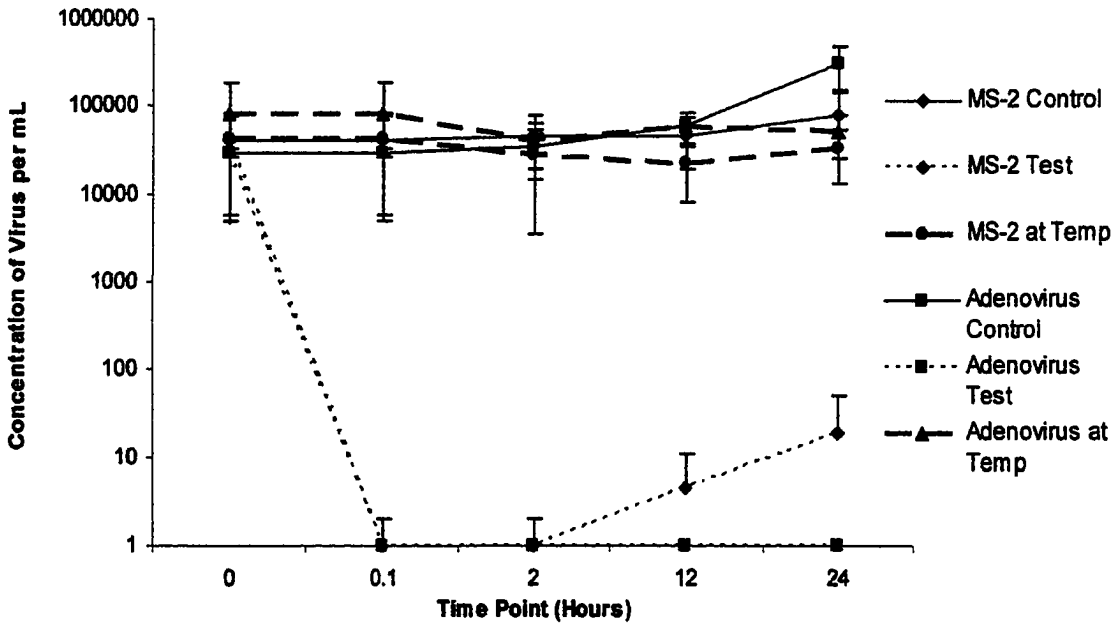

Figure 8: Adenovirus Type 5 and MS-2 Persistence for Three Trials Conducted in a Test Lime and Control RO Water Matrix at Room Temperature (28°C) - Adenovirus type 5 and MS-2 seeded into an RO water matrix at room temperature were below detectable levels following lime stabilization for 0.1 hours in all three trials conducted. Adenovirus type 5 seeded into RO water to achieve a final concentration of 1×10^4 $TCID_{50}$/mL (Adenovirus Test) was below detectable levels following an initial liming (0.1 hours) demonstrating at least a four log reduction. MS-2 seeded into RO water to achieve a final concentration of 1×10^4 PFU/mL (MS-2 Test) was below detectable levels following initial liming (0.1 hours) demonstrating at least a four log reduction. Control samples consisting of adenovirus type 5 (Adenovirus Control) and MS-2 (MS-2 Control) seeded into an RO water matrix maintained at room temperature did not undergo lime stabilization. In addition, aliquots of seeded virus (MS-2 at Temp and Adenovirus at Temp) were incubated at room temperature and enumerated at each time point. Aliquots removed at time points 0.1, 2, 12 and 24 hours demonstrated no viral inactivation.

Statistical Analysis of Three Trials of Adenovirus Type 5 and MS-2 Lime Stabilization evaluated in a RO Water Matrix at 28°C

Statistical analysis of three trials of Adenovirus type 5 and MS-2 lime stabilization evaluated in a water matrix at room temperature were conducted using the SPSS statistical software package. A general linear model was constructed to evaluate the following null hypotheses 1) lime stabilization does not have a statistically significant effect on the inactivation of adenovirus type 5 and MS-2 bacteriophage 2) the effect of liming is not statistically different between adenovirus type 5 and MS-2 bacteriophage when the effect of liming over a period of time is considered for each organism individually 3) there is no statistical difference between the inactivation of adenovirus type 5 and MS-2 bacteriophage under lime stabilization conditions.

Analysis of differences in treatment effectiveness for inactivation of adenovirus type 5 and MS-2 indicates that there is no significant difference between the effect of lime treatment on either organism (p value = 0.119). Therefore, the hypothesis that both viruses behave the same with regard to treatment by lime stabilization cannot be rejected. Overall treatment effect on adenovirus type 5 and MS-2 was significant (p value = 0.000) and both viruses were below detectable levels following 0.1 hours of liming. In addition, there appears to be no significant effect of liming over time for adenovirus type 5, therefore, treating for longer periods of time will generate no greater inactivation effect because adenovirus is inactivated immediately. In addition, the effect of time does not appear to differ between adenovirus type 5 and MS-2 (p value 0.001); therefore, treating for a period of time beyond 0.1 hours in RO water provides no added advantage with regard to inactivation of viruses during lime stabilization.

Adenovirus Type 5 and MS-2 Lime Stabilization evaluated in Various Sludge Matrices at Room Temperature (28°C) and Reduced Temperature (4°C)

Adenovirus type 5 and male-specific bacteriophage MS-2 were spiked into various sludge matrices and limed for twenty-four hours with removal of aliquots at time points 0.1 (immediately following addition of lime), 2, 12 and 24 hours post lime addition. Following removal of aliquots at designated time points and immediate neutralization, virus was enumerated using previously described methods. The results of three trials conducted with adenovirus type 5 and MS-2 spiked into a various sludge matrices at room temperature (28°C) are presented in figures 9, 10, 11 and 12. The results of similar trials conducted at reduced temperature (4°C) are presented in figures 13, 14, 15, and 16. In all sludge trials conducted at room temperature (28°C) and reduced temperature (4°C), adenovirus was below detectable levels by $TCID_{50}$ ($< 10^{0.5}$ $TCID_{50}$/mL) indicating inactivation following 24 hours of liming (time point 24). Inactivation rates varied with different matrices. In trials conducted at 28°C, adenovirus was inactivated at 2 hours, 0.1 hours and 0.1 hours for composted, previously limed and raw samples respectively. In these same trials, MS-2 was inactivated at 2 hours, 12 hours, and 24 hours post-lime addition for composted, previously limed and raw samples respectively. In trials conducted at 4°C, adenovirus was determined to be below detectable levels at 2 hours, 0.1 hours, and 0.1 hours for composted, previously limed and raw samples respectively. In these trials, MS-2 was below detectable levels (< 1 PFU/mL) at 24 hours, 12 hours and 12 hours for composted, previously limed and raw samples respectively. In all sludge trials, adenovirus type 5 was spiked into the sludge

matrix to achieve a final concentration of approximately 10^5 $TCID_{50}$/mL and MS-2 was spiked to achieve a final concentration of approximately 10^7 PFU/mL.

Adenovirus Type 5 and MS-2 Persistence under Liming Conditions in a Composted Biosolids Matrix at Room Temperature (28°C)

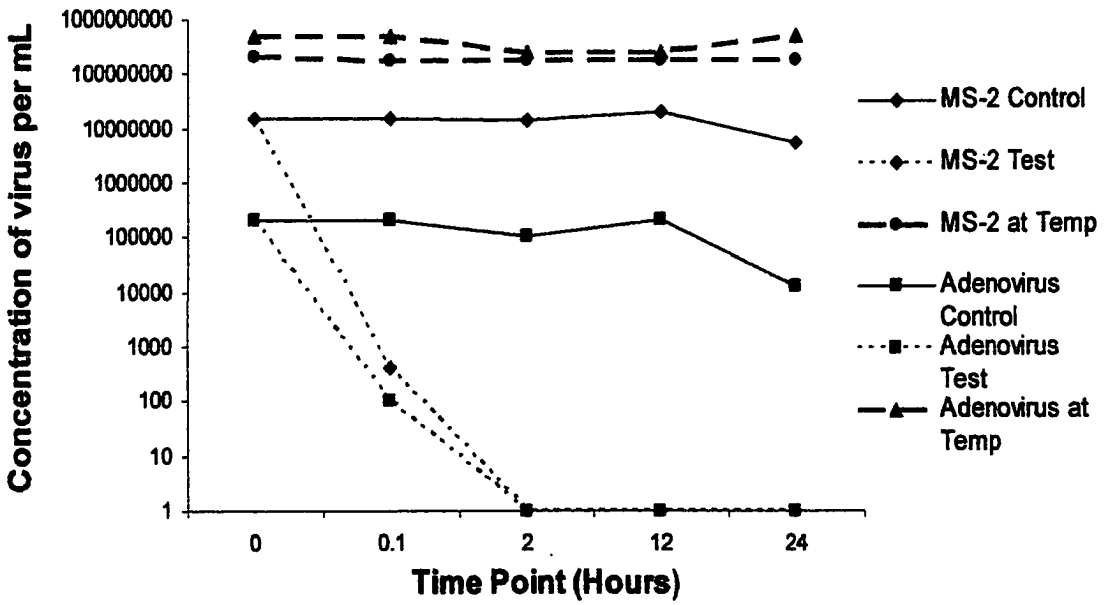

Figure 9: Adenovirus type 5 and MS-2 persistence in a test lime and control composted biosolids matrix at room temperature (28°C) - Adenovirus type 5 and MS-2 seeded into a composted biosolids matrix at room temperature were below detectable levels following lime stabilization for 2 hours. Adenovirus type 5 seeded into a composted biosolids matrix to achieve a final concentration of 1×10^5 $TCID_{50}$/mL (Adenovirus Test) was below detectable levels following 2 hours of liming (time point 2) demonstrating at least a five log reduction. MS-2 seeded into the same matrix to achieve a final concentration of 1×10^7 pfu/mL (MS-2 Test) was below detectable levels following 2 hours of liming (time point 2) demonstrating at least a seven log reduction. Control samples consisting of adenovirus type 5 (Adenovirus Control) and MS-2 (MS-2 Control) seeded into composted biosolids maintained at room temperature did not undergo lime stabilization. In addition, aliquots of seeded virus (MS-2 at Temp and Adenovirus at Temp) were incubated at room temperature and enumerated at each time point. Aliquots of control samples removed at time points 0.1, 2, 12 and 24 hours demonstrated no viral reduction.

Adenovirus Type 5 and MS-2 Persistence under Liming Conditions in a Previously Limed Biosolids Matrix at Room Temperature (28°C)

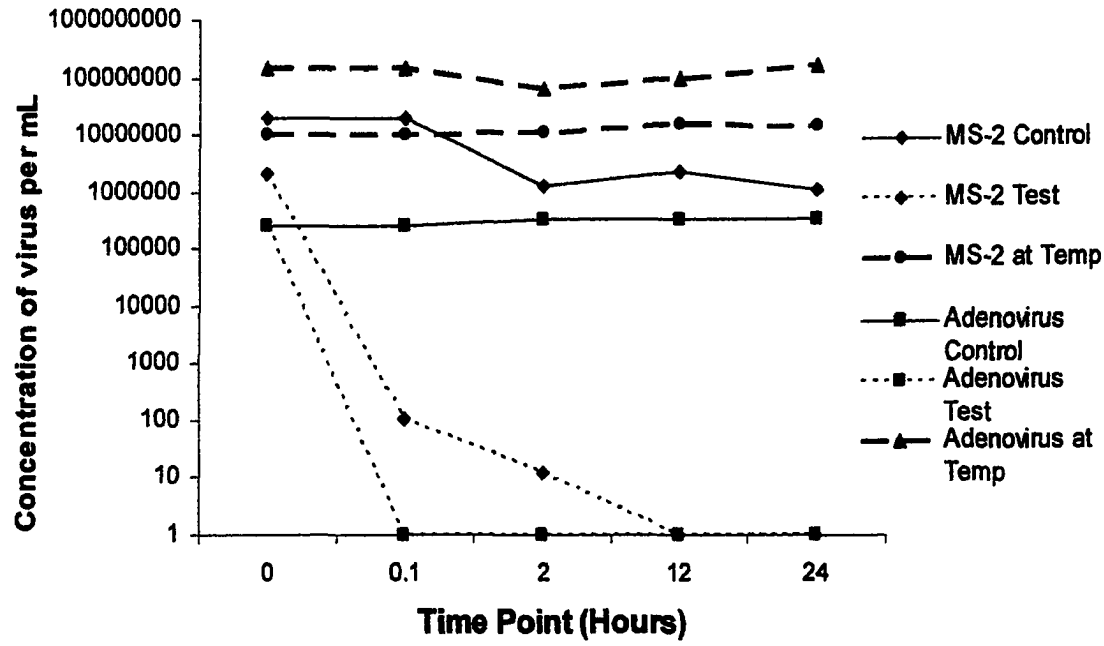

Figure 10: Adenovirus type 5 and MS-2 persistence in a test lime and control previously limed biosolids matrix at room temperature (28°C) - Adenovirus type 5 and MS-2 seeded into a previously limed biosolids matrix at room temperature were below detectable levels following lime stabilization for 0.1 and 12 hours respectively. Adenovirus type 5 seeded into previously limed biosolids to achieve a final concentration of 1 x 10^6 $TCID_{50}$/mL (Adenovirus Test) was below detectable levels following an initial liming (0.1 hours) demonstrating at least a six log reduction. MS-2 seeded into previously limed biosolids to achieve a final concentration of 1 x 10^7 PFU/mL (MS-2 Test) was detectable following 0.1 and 2 hours of liming and was below detectable levels following 12 hours of liming (time point 12) demonstrating at least a seven log reduction. Control samples consisting of adenovirus type 5 (Adenovirus Control) and MS-2 (MS-2 Control) seeded into previously limed biosolids maintained at room temperature did not undergo lime stabilization. In addition, aliquots of seeded virus (MS-2 at Temp and Adenovirus at Temp) were incubated at room temperature and enumerated at each time point. Aliquots of control samples removed at time points 0.1, 2, 12 and 24 hours demonstrated no viral reduction.

Adenovirus Type 5 and MS-2 Persistence under Liming Conditions in a Raw Sludge Matrix at Room Temperature (28°C)

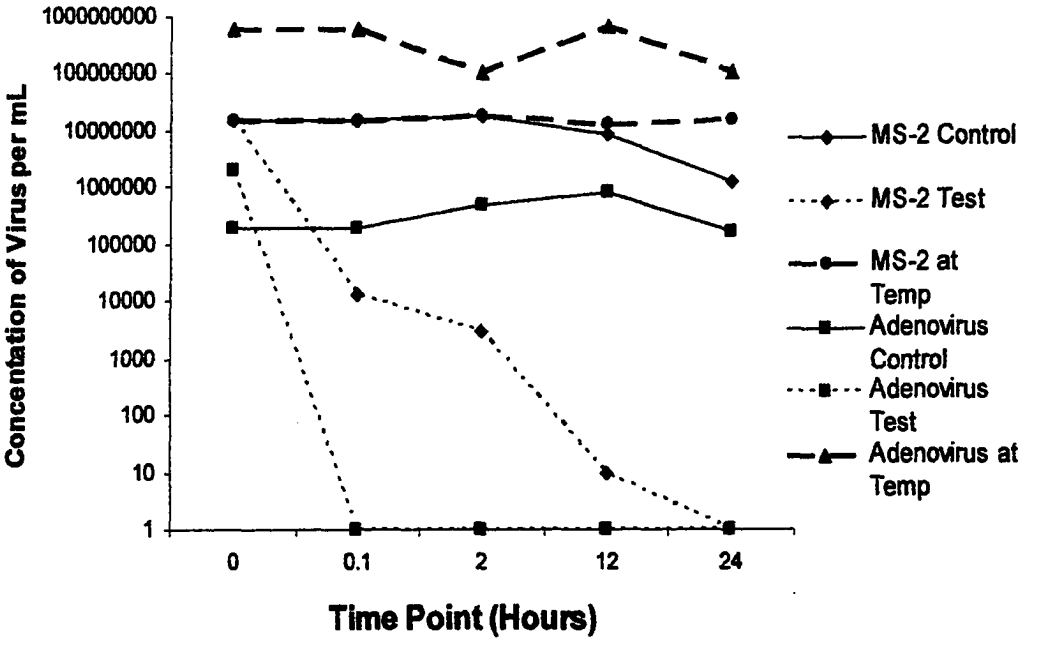

Figure 11: Adenovirus type 5 and MS-2 persistence in a test lime and control raw sludge matrix at room temperature (28°C) - Adenovirus type 5 and MS-2 seeded into a raw sludge matrix at room temperature were below detectable levels following lime stabilization for 0.1 hours and 24 hours respectively. Adenovirus type 5 seeded into raw sludge to achieve a final concentration of 1 x 10^5 $TCID_{50}$/mL (Adenovirus Test) was below detectable levels following an initial liming (0.1 hours) demonstrating at least a five log reduction. MS-2 seeded into the same raw sludge to achieve a final concentration of 1 x 10^7 PFU/mL (MS-2 Test) was below detectable levels following 24 hours of liming demonstrating at least a seven log reduction. Control samples consisting of adenovirus type 5 (Adenovirus Control) and MS-2 (MS-2 Control) seeded into an RO water matrix maintained at room temperature did not undergo lime stabilization. In addition, aliquots of seeded virus (MS-2 at Temp and Adenovirus at Temp) were incubated at room temperature and enumerated at each time point. Aliquots of control samples removed at time points 0.1, 2, 12 and 24 hours demonstrated no viral reduction.

Overall Adenovirus Type 5 and MS-2 Inactivation for Three Trials Conducted in Various Sludge Matrices Lime Stabilized at Room Temperature (28°C)

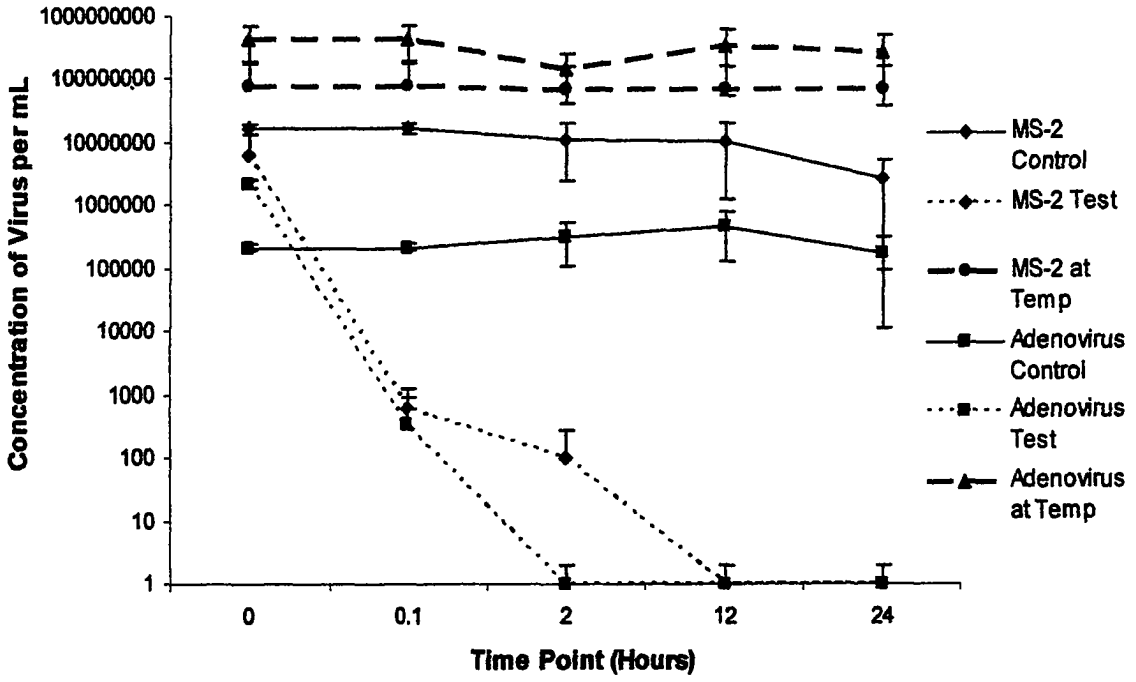

Figure 12: Overall adenovirus type 5 and MS-2 inactivation for three trials conducted in various sludge matrices lime stabilized at room temperature (28°C) – Adenovirus type 5 and MS-2 seeded into varying sludge matrices were below detectable levels following 24 hours of lime stabilization at 28°C in all trials conducted, with inactivation times varying depending on the matrix evaluated. The results presented represent the average concentrations of adenovirus type 5 (Adenovirus Control, Adenovirus Test) and MS-2 (MS-2 Control, MS-2 Test) in spiked control and test samples for the three lime stabilization trials conducted in the various matrices evaluated (compost, raw and previously limed) at room temperature. Average enumerations of aliquots of seeded virus (MS-2 at Temp and Adenovirus at Temp), incubated at room temperature and enumerated at each time point, are also presented.

Adenovirus Type 5 and MS-2 Persistence under Liming Conditions in a Composted Biosolids Matrix at Reduced Temperature (4°C)

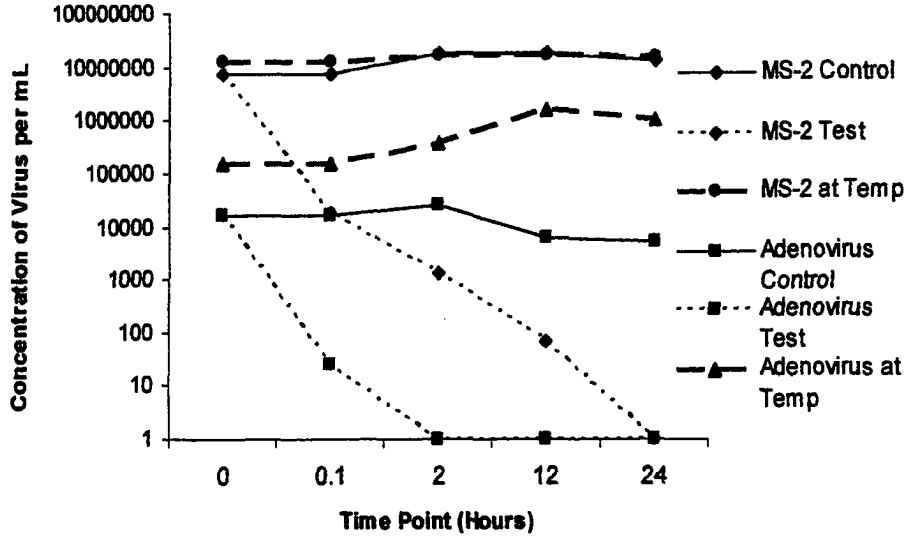

Figure 13: Adenovirus type 5 and MS-2 persistence in a test lime and control composted biosolids matrix at reduced temperature (4°C) - Adenovirus type 5 and MS-2 seeded into a composted biosolids matrix at 4°C were below detectable levels following lime stabilization for 2 hours and 24 hours respectively. Adenovirus type 5 seeded into a composted biosolids matrix to achieve a final concentration of 1×10^5 $TCID_{50}$/mL (Adenovirus Test) was below detectable levels following 2 hours of liming (time point 2) demonstrating at least a five log reduction. MS-2 seeded into the same matrix to achieve a final concentration of 1×10^7 PFU/mL (MS-2 Test) was below detectable levels following 24 hours of liming (time point 24) demonstrating at least a seven log reduction. Control samples consisting of adenovirus type 5 (Adenovirus Control) and MS-2 (MS-2 Control) seeded into composted biosolids maintained at room temperature did not undergo lime stabilization. In addition, aliquots of seeded virus (MS-2 at Temp and Adenovirus at Temp) were incubated at 4°C and enumerated at each time point. Control sample aliquots removed at time points 0.1, 2, 12 and 24 hours demonstrated no viral reduction.

Adenovirus Type 5 and MS-2 Persistence under Liming Conditions in a Previously Limed Biosolids Matrix at Reduced Temperature (4°C)

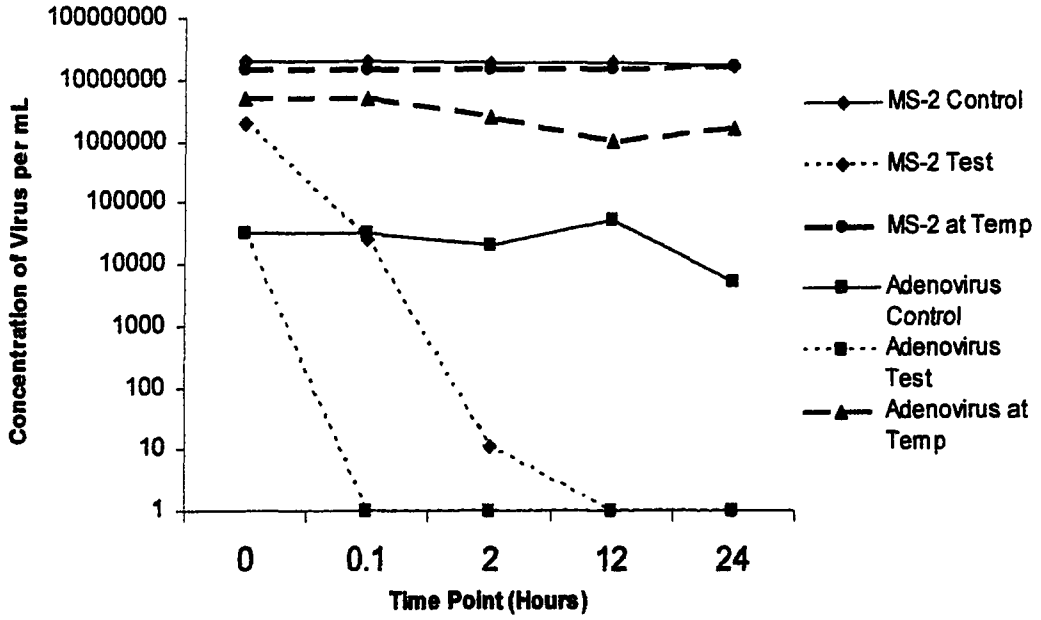

Figure 14: Adenovirus type 5 and MS-2 persistence in a test lime and control previously limed biosolids matrix at reduced temperature (4°C) - Adenovirus type 5 and MS-2 seeded into a previously limed biosolids matrix at 4°C were below detectable levels following lime stabilization for 0.1 and 12 hours respectively. Adenovirus type 5 seeded into previously limed biosolids to achieve a final concentration of 1×10^5 $TCID_{50}$/mL (Adenovirus Test) was below detectable levels following an initial liming (0.1 hours) demonstrating at least a five log reduction. MS-2 seeded into previously limed biosolids to achieve a final concentration of 1×10^7 PFU/mL (MS-2 Test) was detectable following 0.1 and 2 hours of liming and was below detectable levels following 12 hours of liming (time point 12) demonstrating at least a seven log reduction. Control samples consisting of adenovirus type 5 (Adenovirus Control) and MS-2 (MS-2 Control) seeded into previously limed biosolids maintained at 4°C did not undergo lime stabilization. In addition, aliquots of seeded virus (MS-2 at Temp and Adenovirus at Temp) were incubated at 4°C and enumerated at each time point. Aliquots removed at time points 0.1, 2, 12 and 24 hours demonstrated no viral reduction.

Adenovirus Type 5 and MS-2 Persistence under Liming Conditions in a Raw Sludge Matrix at Reduced Temperature (4°C)

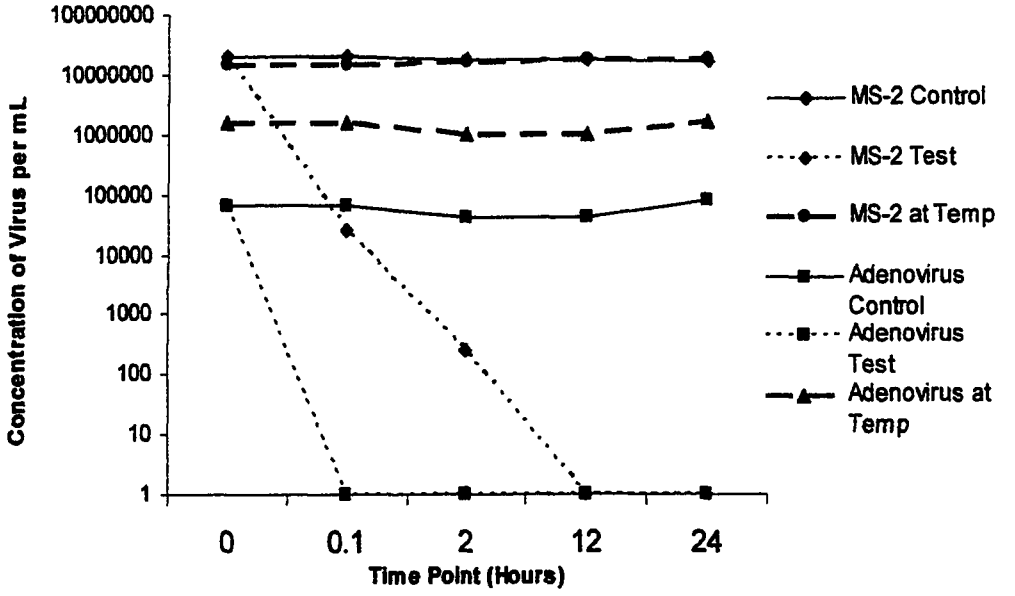

Figure 15: Adenovirus type 5 and MS-2 persistence in a test lime and control raw sludge matrix at reduced temperature (4°C) - Adenovirus type 5 and MS-2 seeded into a raw sludge matrix at 4°C were below detectable levels following lime stabilization for 0.1 hours and 12 hours respectively. Adenovirus type 5 seeded into raw sludge to achieve a final concentration of 1 x 10^5 $TCID_{50}$/mL (Adenovirus Test) was below detectable levels following an initial liming (0.1 hours) demonstrating at least a five log reduction. MS-2 seeded into the same raw sludge to achieve a final concentration of 1 x 10^7 PFU/mL (MS-2 Test) was below detectable levels following 12 hours of liming demonstrating at least a seven log reduction. Control samples consisting of adenovirus type 5 (Adenovirus Control) and MS-2 (MS-2 Control) seeded into raw sludge matrix maintained at 4°C did not undergo lime stabilization. In addition, aliquots of seeded virus (MS-2 at Temp and Adenovirus at Temp) were incubated at 4°C and enumerated at each time point. Aliquots removed at time points 0.1, 2, 12 and 24 hours demonstrated no viral reduction.

Overall Adenovirus Type 5 and MS-2 Inactivation for Three Trials Conducted in Various Sludge Matrices Lime Stabilized at Reduced Temperature (4°C)

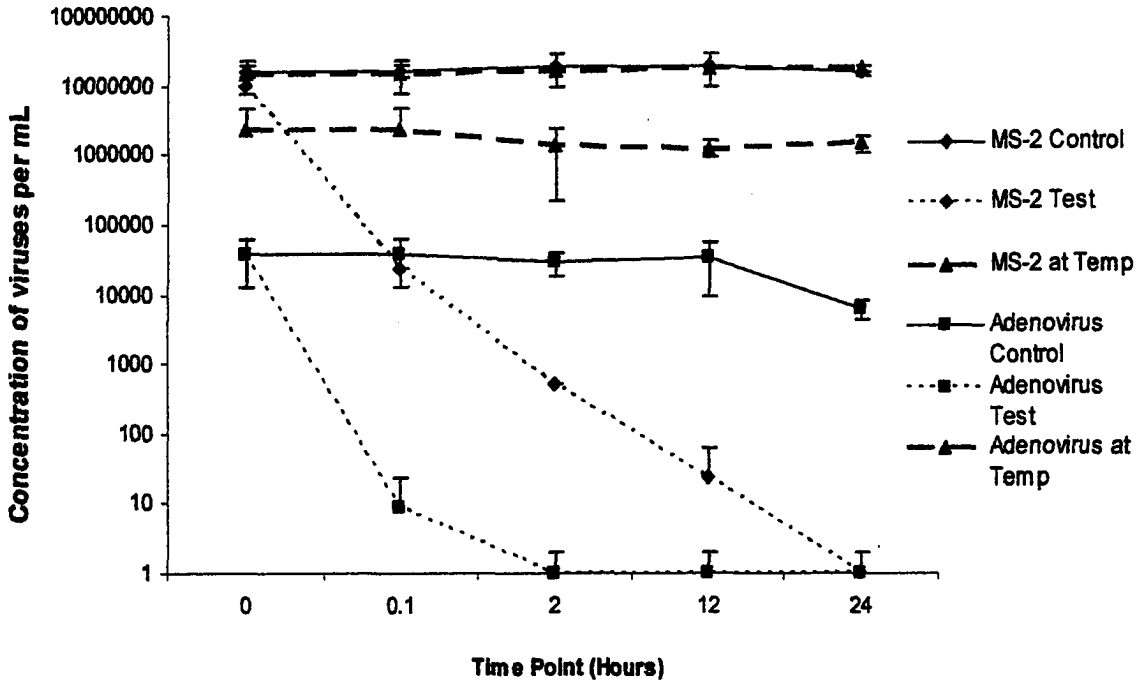

Figure 16: Overall adenovirus type 5 and MS-2 inactivation for three trials conducted in various sludge matrices lime stabilized at reduced temperature (4°C) – Adenovirus type 5 and MS-2 seeded into varying sludge matrices were below detectable levels following 24 hours of lime stabilization at 4°C in all trials conducted, with inactivation times varying depending on the matrix evaluated. The results presented represent the average concentrations of adenovirus type 5 (Adenovirus Control, Adenovirus Test) and MS-2 (MS-2 Control, MS-2 Test) in spiked control and test samples for the three lime stabilization trials conducted in the various matrices evaluated (compost, raw and previously limed) at 4°C. Average enumerations of aliquots of seeded virus (MS-2 at Temp and Adenovirus at Temp), incubated at 4°C and enumerated at each time point, are also presented.

Statistical Analysis of Three Trials of Adenovirus Type 5 and MS-2 Lime Stabilization evaluated in Various Sludge Matrices (compost, previously limed, raw) at 28°C and 4°C

Statistical analysis of three trials of adenovirus type 5 and MS-2 lime stabilization evaluated in various sludge matrices (compost, previously limed, raw) at room temperature (28°C) and reduced temperature (4°C) were conducted using the SPSS statistical software package. A general linear model was constructed to evaluate the following null hypotheses 1) lime stabilization does not have a statistically significant effect on the inactivation of adenovirus type 5 and MS-2 bacteriophage 2) the effect of liming is not statistically different between adenovirus type 5 and MS-2 bacteriophage when the effect of liming over a period of time is considered for each organism individually 3) there is no statistical difference between the inactivation of adenovirus type 5 and MS-2 bacteriophage under lime stabilization conditions.

Analysis of lime treatment effectiveness for all trials in all matrices evaluated at both 28°C and 4°C revealed a statistically significant effect of treatment on both adenovirus type 5 and MS-2 generating a p value of 0.000. Therefore, the hypothesis that both viruses behave the same with regard to treatment by lime stabilization cannot be rejected. In addition, it was determined that there is no significant difference between the effect of liming on adenovirus type 5 and MS-2 at both temperatures generating a p value of 0.315 at 28°C and 0.072 at 4°C. A p value of 0.072 is on the border of what would be considered statistically significant. This can be explained by the larger effect size for adenovirus, with greater than 99.99% inactivation at almost all time points except for the 0.1 hour time point in trial #1. MS-2 was not inactivated until two hours of liming;

therefore, the overall effect size (control data minus test data) used for statistical calculation was smaller for MS-2 in earlier time points. The percent inactivation for virus and bacteriophage was calculated for each time point in each trial. The percent recovery was used in statistical analysis to draw comparisons between organisms due to a difference in starting concentrations. If the actual effect size value were used it would provide a false impression because the effect size for MS-2 would be extremely high due to a higher starting concentration; therefore, a statistically significant difference in effect of treatment on organisms would be a function of the starting concentration.

For trials conducted at 28°C, there is no statistically significant effect of time on the inactivation of virus or bacteriophage by lime stabilization (p value = 0.108). Therefore the hypothesis that time has no effect on treatment cannot be rejected. In addition, there is no statistically significant difference between the effect of time on adenovirus or MS-2 during stabilization, both organisms are affected the same over time (p value = 0.824). In trials conducted at 4°C, there appears to be a significant effect of liming over time; therefore, the hypothesis that time does not have an effect and that treating for longer periods of time will generate no greater effect can be rejected (p value = 0.000). The significant effect of time is most likely a result of MS-2 inactivation. In order to achieve 99.99% inactivation of MS-2, a lime exposure of at least two hours was necessary, therefore demonstrating the potential for enhanced survivability at 4°C. Liming in this instance can be demonstrated to be time dependent, whereas at 28°C it was not evident because inactivation was achieved at 0.1 hours and time points below 0.1 hours were not evaluated. Even though there is a statistically significant effect of time seen at 4°C, the effect of time does not differ significantly between organisms. Therefore

there is no statistically significant difference between the effect of time on adenovirus or MS-2 during lime stabilization; however, the p value is close to 0.05, therefore, on the border of what would be considered significant. This can be explained by the fact that MS-2 is inactivated after two hours of liming whereas adenovirus is inactivated at 0.1 hours post-lime addition. There is no statistical difference yet MS-2 was detectable in all two hour time points when adenovirus was not detected. The variation between trials was not evaluated statistically because trials were performed in different matrices. The rationale behind performing the experiments this way is that no two sludge samples or aliquots of a single sludge matrix will be the same. Therefore, in an effort to obtain information about the effect of lime stabilization it was most practical to evaluate liming in several different matrices rather than different aliquots of the same matrix that can never be considered consistent.

Rotavirus Wa and MS-2 Lime Stabilization evaluated in a RO Water Matrix at Room Temperature (28°C)

Rotavirus Wa and male specific bacteriophage MS-2 were spiked into a water matrix and limed for twenty-four hours with removal of aliquots at time points 0.1 hours, (immediately following addition of lime), 2, 12, and 24 hours post lime addition. Following removal of aliquots at designated time points and immediate neutralization, virus was enumerated using previously described methods. The results of three trials conducted with rotavirus Wa and MS-2 spiked into a limed water matrix at room temperature are presented in figures 17, 18, 19 and 20. In all trials, rotavirus was below detectable levels (< 1 PFU/mL) by plaque assay indicating inactivation following 0.1 hours of liming (time point zero). In all trials, MS-2 was below detectable levels (< 1 PFU/mL) following 2 hours of liming (time point two hours). In all RO water matrix trials, rotavirus Wa was spiked into RO water to achieve a final concentration of approximately 10^4 PFU/mL and MS-2 was spiked into RO water to achieve a similar final concentration of approximately 10^4 PFU/mL. In all RO water trials both rotavirus and MS-2 were below detectable levels following 0.1 and 2 hours of liming respectively, exhibiting at least a four log reduction for both viruses.

Rotavirus Wa & MS-2 Persistence in a Limed RO Water Matrix at Room Temperature (28°C) (Trial 1)

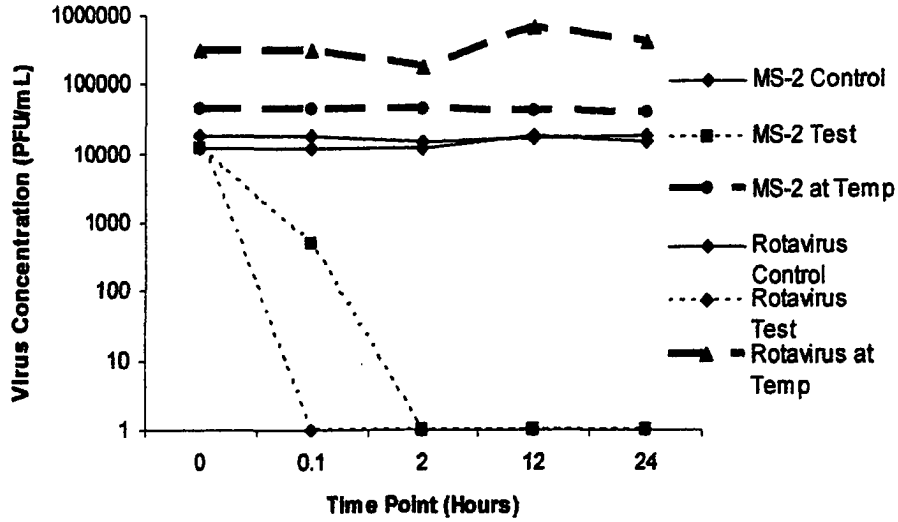

Figure 17: Evaluation of rotavirus Wa & MS-2 persistence in a limed RO water matrix at room temperature (28°C) (Trial 1) – Rotavirus Wa and MS-2 seeded into an RO water matrix were below detectable levels following lime stabilization at 28°C. Rotavirus Wa seeded into RO water to achieve a final concentration of 1 x 10^4 PFU/mL (Rotavirus Test) was below detectable levels following an initial liming (time point 0.1 hours) demonstrating at least a four log reduction. MS-2 seeded into RO water to achieve a final concentration of 1 x 10^4 PFU/mL (MS-2 Test) was below detectable levels following 2 hours of liming (time point 2 hours) demonstrating at least a four log reduction. Control samples consisting of rotavirus Wa (Rotavirus Control) and MS-2 (MS-2 Control) seeded into an RO water matrix maintained at room temperature did not undergo lime stabilization. In addition, aliquots of seeded virus (MS-2 at Temp and Adenovirus at Temp) were incubated at 28°C and enumerated at each time point. Aliquots removed at time points 0.1, 2, 12 and 24 hours demonstrated no viral reduction.

Rotavirus Wa & MS-2 Persistence in a Limed RO Water Matrix at Room Temperature (28°C) (Trial 2)

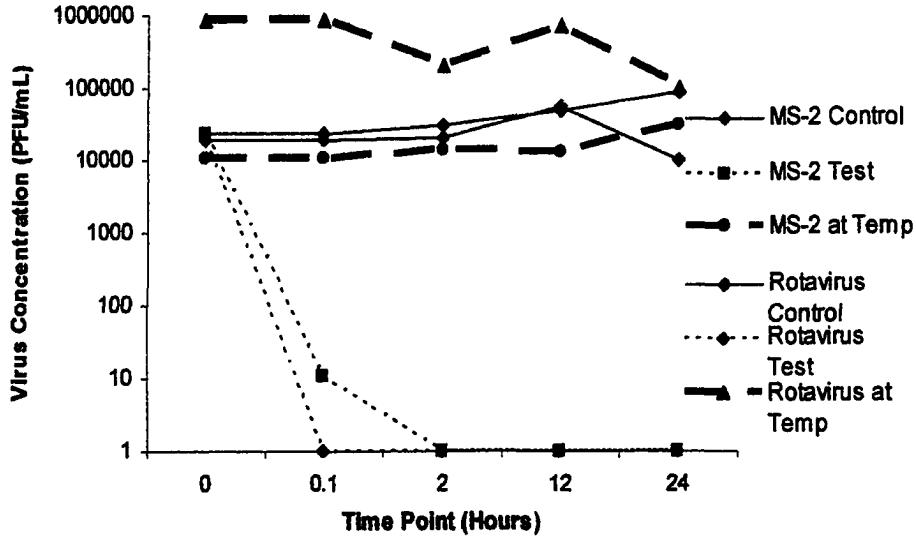

Figure 18: Evaluation of rotavirus Wa and MS-2 persistence in a limed RO water matrix at room temperature (28°C) (Trial 2) - Rotavirus Wa and MS-2 seeded into an RO water matrix were below detectable levels following lime stabilization at 28°C. Rotavirus Wa seeded into RO water to achieve a final concentration of 1 x 10^4 PFU/mL (Rotavirus Test) was below detectable levels following an initial liming (time point 0.1 hours) demonstrating at least a four log reduction. MS-2 seeded into RO water to achieve a final concentration of 1 x 10^4 PFU/mL (MS-2 Test) was below detectable levels following 2 hours of liming (time point 2 hours) demonstrating at least a four log reduction. Control samples consisting of Rotavirus Wa (Rotavirus Control) and MS-2 (MS-2 Control) seeded into an RO water matrix maintained at room temperature did not undergo lime stabilization. In addition, aliquots of seeded virus (MS-2 at Temp and Adenovirus at Temp) were incubated at 28°C and enumerated at each time point. Aliquots removed at time points 0.1, 2, 12 and 24 hours demonstrated no viral inactivation.

Rotavirus Wa & MS-2 Persistence in a Limed RO Water Matrix at Room Temperature (28°C) (Trial 3)

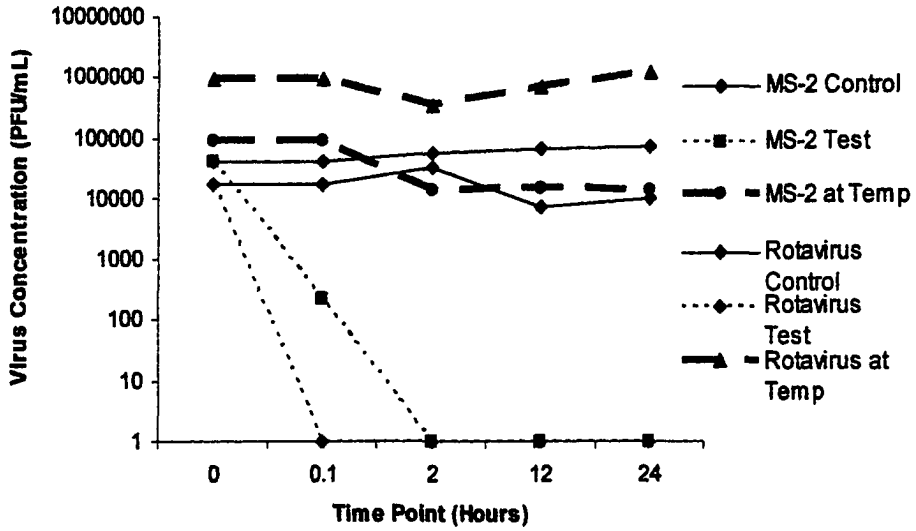

Figure 19: Evaluation of rotavirus Wa and MS-2 persistence in a limed RO water matrix at room temperature (28°C) (Trial 3) - Rotavirus Wa and MS-2 seeded into an RO water matrix were below detectable levels following lime stabilization at 28°C. Rotavirus Wa seeded into RO water to achieve a final concentration of 1 x 10^4 PFU/mL (Rotavirus Test) was below detectable levels following an initial liming (time point 0.1 hours) demonstrating at least a four log reduction. MS-2 seeded into RO water to achieve a final concentration of 1 x 10^4 PFU/mL (MS-2 Test) was below detectable levels following 2 hours of liming (time point 2 hours) demonstrating at least a four log reduction. Control samples consisting of rotavirus Wa and MS-2 seeded into an RO water matrix maintained at room temperature did not undergo lime stabilization. In addition, aliquots of seeded virus (MS-2 at Temp and Adenovirus at Temp) were incubated at 28°C and enumerated at each time point. Aliquots removed at time points 0.1, 2, 12 and 24 hours demonstrated no viral reduction.

Overall Rotavirus Wa and MS-2 Inactivation for Three Trials Conducted in a Limed RO Water Matrix at Room Temperature (28°C)

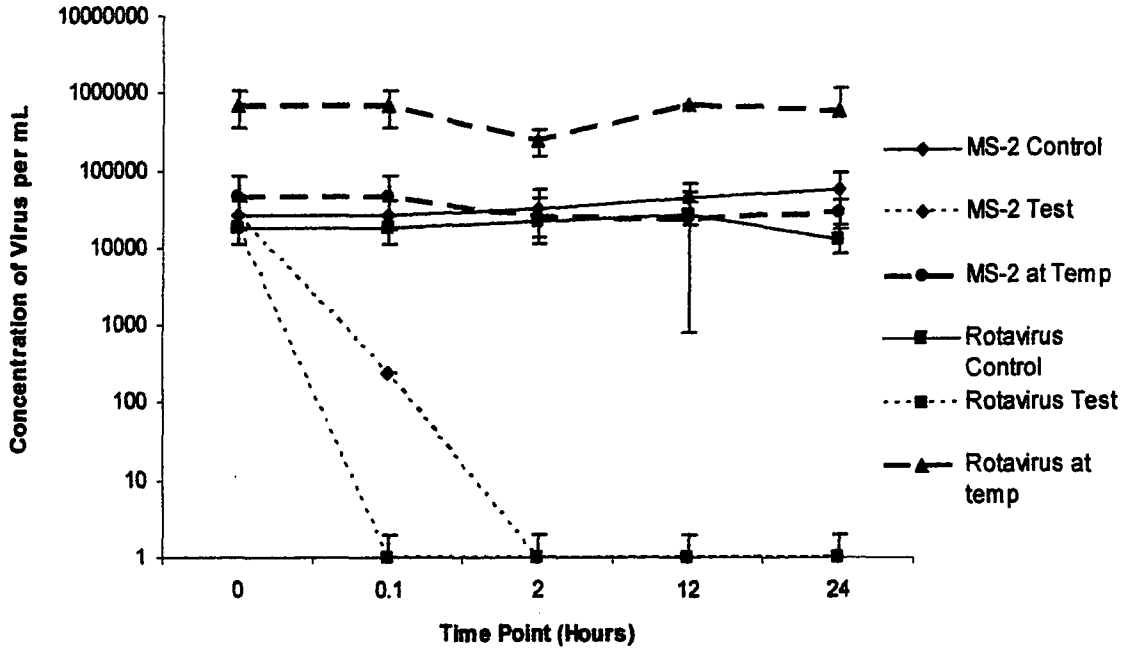

Figure 20: Overall rotavirus Wa and MS-2 persistence for three trials conducted in a test lime and control RO water matrix at room temperature (28°C) - Rotavirus Wa and MS-2 seeded into an RO water matrix were below detectable levels following lime stabilization at 28°C in all three trials conducted. Rotavirus Wa seeded into RO water to achieve a final concentration of 1 x 10^4 PFU/mL (Rotavirus Test) was below detectable levels following an initial liming (0.1 hours) demonstrating at least a four log reduction. MS-2 seeded into RO water to achieve a final concentration of 1 x 10^4 PFU/mL (MS-2 Test) was below detectable levels following two hours of liming (time point 2 hours) demonstrating at least a four log reduction. Control samples consisting of rotavirus Wa (Rotavirus Control) and MS-2 (MS-2 Control) seeded into an RO water matrix maintained at room temperature did not undergo lime stabilization. In addition, aliquots of seeded virus (MS-2 at Temp and Adenovirus at Temp) were incubated at 28°C and enumerated at each time point. Aliquots removed at time points 0.1, 2, 12 and 24 hours demonstrated no viral reduction.

Statistical Analysis of Rotavirus Wa and MS-2 Lime Stabilization for Three Trials Evaluated in an RO Water Matrix at Room Temperature (28°C)

Statistical analysis of three trials of rotavirus Wa and MS-2 lime stabilization evaluated in a water matrix at room temperature were conducted using the SPSS statistical software package. A general linear model was constructed to evaluate the following null hypotheses 1) lime stabilization does not have a statistically significant effect on the inactivation of rotavirus Wa and MS-2 bacteriophage 2) the effect of liming is not statistically different between rotavirus Wa and MS-2 bacteriophage when the effect of liming over a period of time is considered for each organism individually 3) there is no statistical difference between the inactivation of rotavirus Wa and MS-2 bacteriophage under lime stabilization conditions.

Analysis of differences in treatment effectiveness for inactivation of rotavirus Wa and MS-2 indicates that there is no significant difference between the effect of lime treatment on either organism (p value = 0.431). Therefore, the hypothesis that both viruses behave the same with regard to treatment by lime stabilization cannot be rejected. Overall treatment effect on Rotavirus Wa and MS-2 was significant (p value = 0.000) and both viruses were below detectable levels following 0.1 hours of liming. Statistical analysis revealed that there is no statistically significant effect of liming over time, as both rotavirus Wa and MS-2 were below detectable levels following 0.1 hours of liming (p value = 0.660).

Rotavirus Wa and MS-2 Lime Stabilization evaluated in Various Sludge Matrices at Room Temperature (28°C) and Reduced Temperature (4°C)

Rotavirus Wa and male-specific bacteriophage MS-2 were spiked into various sludge matrices and limed for twenty-four hours with removal of aliquots at time points 0.1 (immediately following addition of lime), 2, 12 and 24 hours post lime addition. Following removal of aliquots at designated time points and immediate neutralization, virus was enumerated using previously described methods. The results of three trials conducted with rotavirus Wa and MS-2 spiked into a various sludge matrices at room temperature (28°C) are presented in figures 21, 22, 23 and 24. The results of similar trials conducted at reduced temperature (4°C) are presented in figures 25, 26, 27, and 28. In all sludge trials conducted at room temperature (28°C) and reduced temperature (4°C), rotavirus was below detectable levels by plaque assay (<1 PFU/mL) indicating inactivation following 0.1 hours of liming (time point 0). Inactivation rates varied with different matrices. In trials conducted at 28°C, rotavirus was inactivated at 0.1 hours following the addition of lime for composted, previously limed and raw samples respectively. In these same trials, MS-2 was below detectable levels (<1 PFU/mL) at 2 hours, 2 hours, and 12 hours post-lime addition for composted, previously limed and raw samples respectively. In trials conducted at 4°C, rotavirus was below detectable levels (<1 PFU/mL) at 0.1 hours following the addition of lime for composted, previously limed and raw samples respectively. In these trials, MS-2 was below detectable levels (<1 PFU/mL) at 12 hours, 12 hours and 24 hours for composted, previously limed and raw samples respectively. In all sludge trials, rotavirus Wa was spiked into the sludge matrix

to achieve a final concentration of approximately 10^4 PFU/mL and MS-2 was spiked to achieve a final concentration of approximately 10^4 PFU/mL.

Rotavirus Wa and MS-2 Persistence under Liming Conditions in a Composted Biosolids Matrix at Room Temperature (28°C)

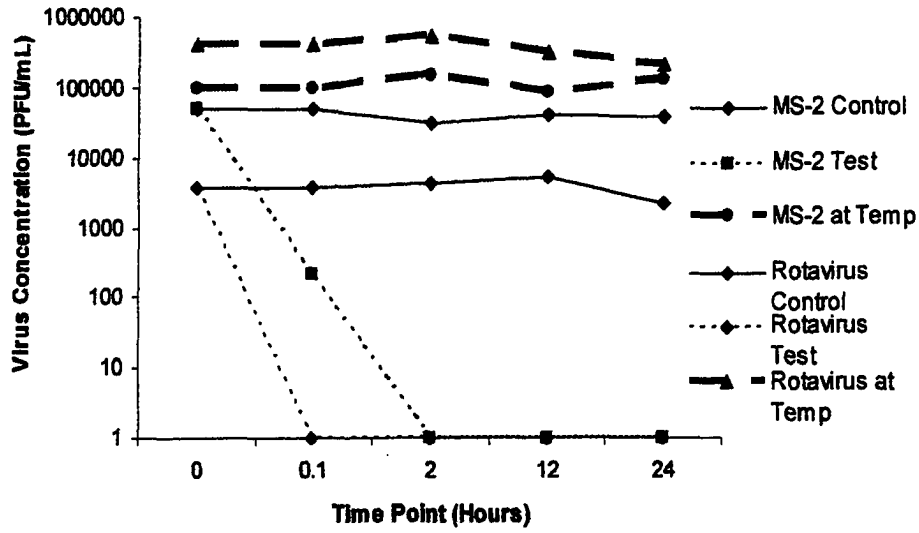

Figure 21: Evaluation of rotavirus Wa and MS-2 persistence in a test lime and control composted biosolids matrix at room temperature (28°C) – Rotavirus Wa and MS-2 seeded into a composted biosolids matrix at room temperature were below detectable levels following lime stabilization for 0.1 and 2 hours respectively. Rotavirus Wa seeded into a composted biosolids matrix to achieve a final concentration of 1×10^4 PFU/mL (Rotavirus Test) was below detectable levels following initial liming (time point 0.1 hours) demonstrating at least a four log reduction. MS-2 seeded into the same matrix to achieve a final concentration of 1×10^4 PFU/mL (MS-2 Test) was below detectable levels following 2 hours of liming (time point 2) demonstrating at least a four log reduction. Control samples consisting of rotavirus (Rotavirus Control) and MS-2 (MS-2 Control) seeded into composted biosolids maintained at room temperature did not undergo lime stabilization. In addition, aliquots of seeded virus (MS-2 at Temp and Rotavirus at Temp) were incubated at 28°C and enumerated at each time point. Control sample aliquots removed at time points 0.1, 2, 12 and 24 hours demonstrated no viral reduction.

Rotavirus Wa and MS-2 Persistence under Liming Conditions in a Raw Sludge Matrix at Room Temperature (28°C)

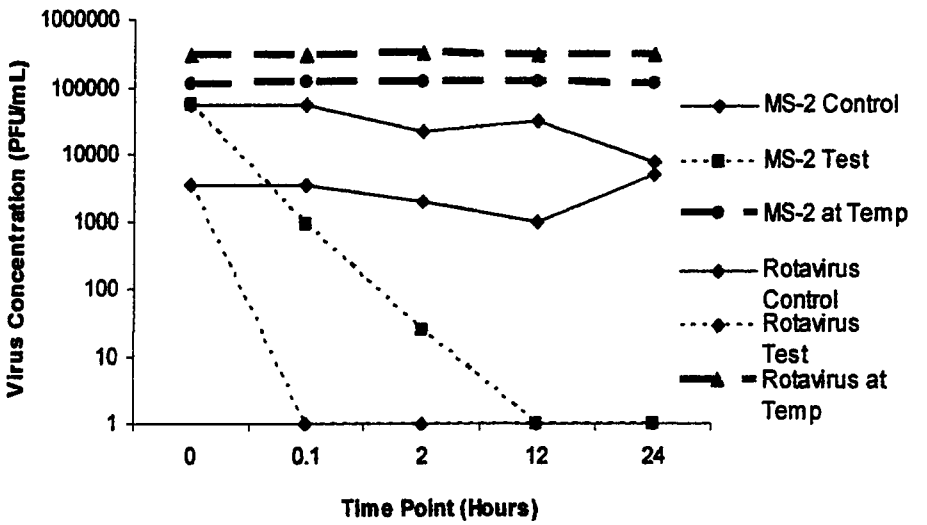

Figure 22: Evaluation of rotavirus Wa and MS-2 persistence in a test lime and control raw biosolids matrix at room temperature (28°C) – Rotavirus Wa and MS-2 seeded into a raw biosolids matrix at room temperature were below detectable levels following lime stabilization for 0.1 and 12 hours respectively. Rotavirus Wa seeded into a composted biosolids matrix to achieve a final concentration of 1 x 10^4 PFU/mL (Rotavirus Test) was below detectable levels following an initial liming (time point 0.1 hours) demonstrating at least a four log reduction. MS-2 seeded into the same matrix to achieve a final concentration of 1 x 10^4 PFU/mL (MS-2 Test) was below detectable levels following 12 hours of liming (time point 12) demonstrating at least a four log reduction. Control samples consisting of rotavirus Wa (Rotavirus Control) and MS-2 (MS-2 Control) seeded into composted biosolids maintained at room temperature did not undergo lime stabilization. In addition, aliquots of seeded virus (MS-2 at Temp and Rotavirus at Temp) were incubated at 28°C and enumerated at each time point. Control sample aliquots removed at time points 0.1, 2, 12 and 24 hours demonstrated no viral reduction.

Rotavirus Wa and MS-2 Persistence under Liming Conditions in a Previously Limed Biosolids Matrix at Room Temperature (28°C)

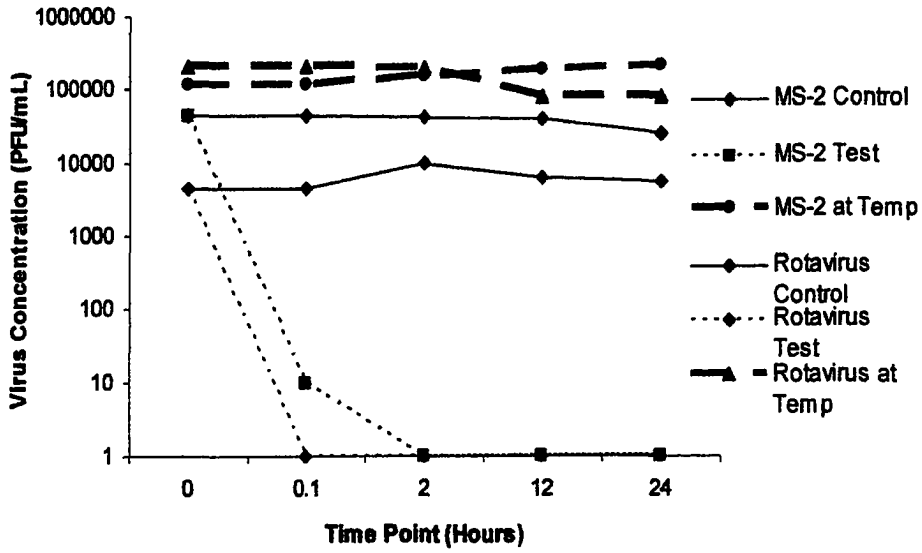

Figure 23: Evaluation of rotavirus Wa and MS-2 persistence in a test lime and control previously limed biosolids matrix at room temperature (28°C) – Rotavirus Wa and MS-2 seeded into a previously limed biosolids matrix at room temperature were below detectable levels following lime stabilization for 0.1 and 2 hours respectively. Rotavirus Wa seeded into a previously limed biosolids matrix to achieve a final concentration of 1 x 10^4 PFU/mL (Rotavirus Test) was below detectable levels following an initial liming (time point 0.1 hours) demonstrating at least a four log reduction. MS-2 seeded into the same matrix to achieve a final concentration of 1 x 10^4 PFU/mL (MS-2 Test) was below detectable levels following 2 hours of liming (time point 2) demonstrating at least a four log reduction. Control samples consisting of rotavirus Wa (Rotavirus Control) and MS-2 (MS-2 Control) seeded into a previously limed biosolids matrix maintained at room temperature did not undergo lime stabilization. In addition, aliquots of seeded virus (MS-2 at Temp and Rotavirus at Temp) were incubated at 28°C and enumerated at each time point. Control sample aliquots removed at time points 0.1, 2, 12 and 24 hours demonstrated no viral reduction.

Overall Rotavirus Wa and MS-2 Inactivation for Three Trials Conducted in Various Sludge Matrices Lime Stabilized at Room Temperature (28°C)

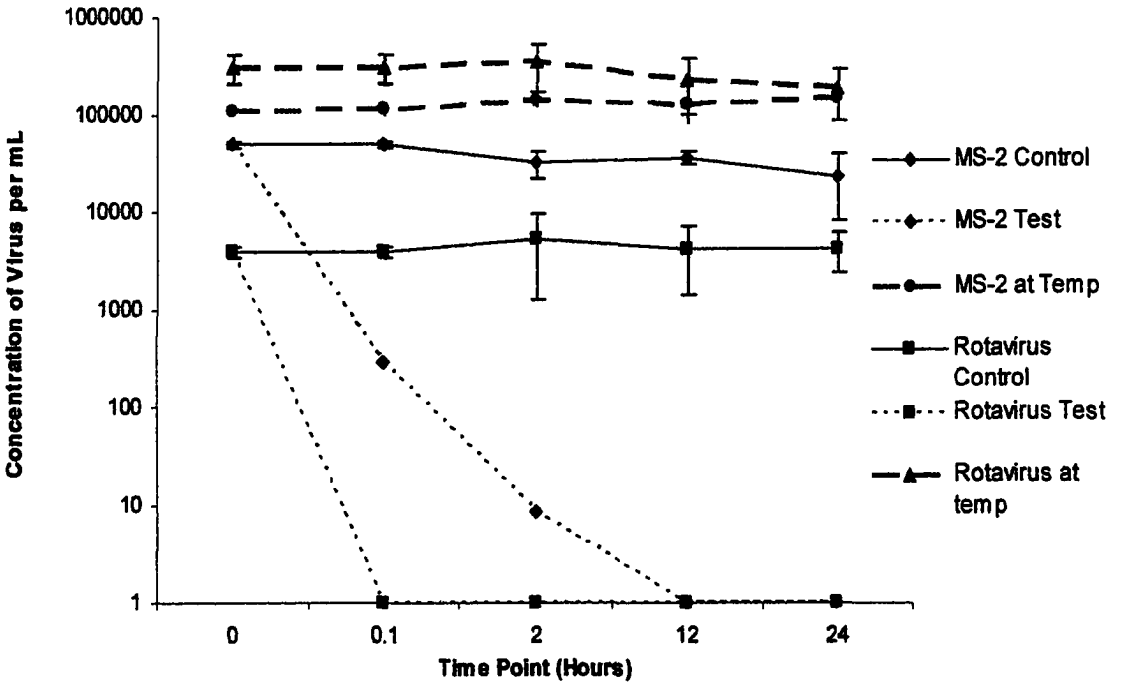

Figure 24: Overall rotavirus Wa and MS-2 inactivation for three trials conducted in various sludge matrices lime stabilized at room temperature (28°C) – Rotavirus Wa and MS-2 seeded into varying sludge matrices were below detectable levels following 12 hours of lime stabilization at 28°C in all trials conducted, with inactivation times varying depending on the matrix evaluated. The results presented represent the average reduction of rotavirus Wa and MS-2 in spiked control and test samples for the three lime stabilization trials conducted in the various matrices evaluated (compost, previously limed and raw) at room temperature. In addition, aliquots of seeded virus (MS-2 at Temp and Rotavirus at Temp) were incubated at 28°C and enumerated at each time point.

Rotavirus Wa and MS-2 Persistence under Liming Conditions in a Composted Biosolids Matrix at Reduced Temperature (4°C)

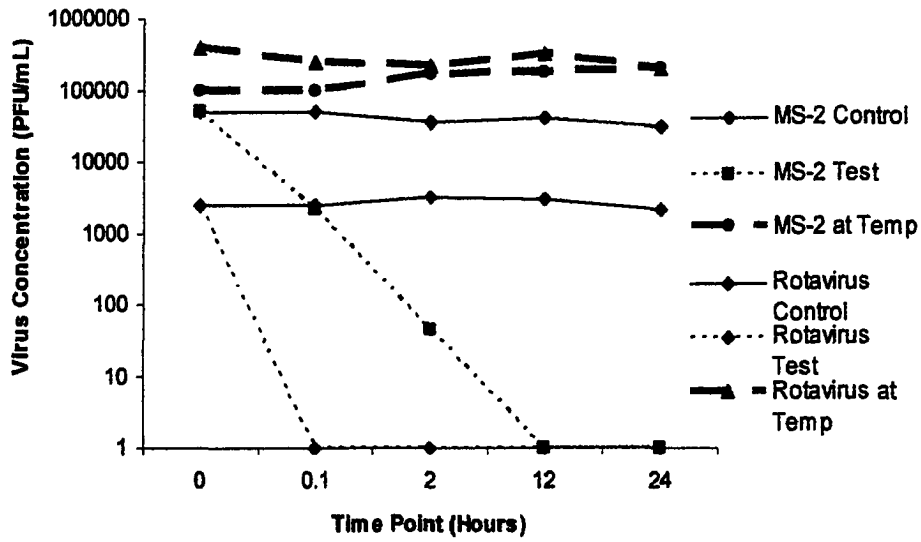

Figure 25: Evaluation of rotavirus Wa and MS-2 persistence in a test lime and control composted biosolids matrix at reduced temperature (4°C) – Rotavirus Wa and MS-2 seeded into a composted biosolids matrix at reduced temperature were below detectable levels following lime stabilization for 0.1 and 12 hours respectively. Rotavirus Wa seeded into a composted biosolids matrix to achieve a final concentration of 1×10^4 PFU/mL (Rotavirus Test) was below detectable levels following an initial liming (time point 0.1 hours) demonstrating at least a four log reduction. MS-2 seeded into the same matrix to achieve a final concentration of 1×10^4 PFU/mL (MS-2 Test) was below detectable levels following 12 hours of liming (time point 12) demonstrating at least a four log reduction. Control samples consisting of rotavirus Wa (Rotavirus Control) and MS-2 (MS-2 Control) seeded into composted biosolids maintained at reduced temperature did not undergo lime stabilization. In addition, aliquots of seeded virus (MS-2 at Temp and Rotavirus at Temp) were incubated at 4°C and enumerated at each time point. Control sample aliquots removed at time points 0.1, 2, 12 and 24 hours demonstrated no virus reduction.

Rotavirus Wa and MS-2 Persistence under Liming Conditions in a Raw Sludge Matrix at Reduced Temperature (4°C)

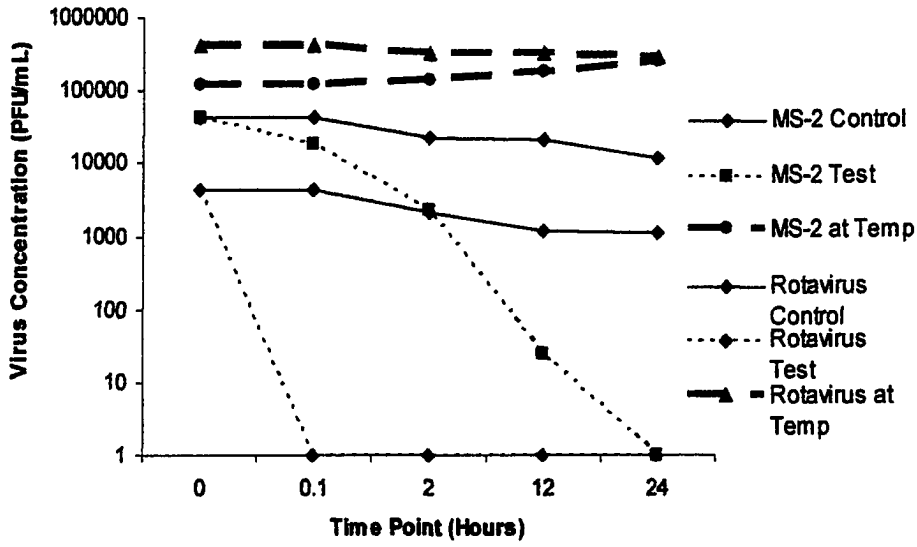

Figure 26: Evaluation of rotavirus Wa and MS-2 persistence in a test lime and control raw sludge matrix at reduced temperature (4°C) - Rotavirus Wa and MS-2 seeded into a raw sludge matrix at reduced temperature were below detectable levels following lime stabilization for 0.1 and 24 hours respectively. Rotavirus Wa seeded into a raw sludge matrix to achieve a final concentration of 1×10^4 PFU/mL (Rotavirus Test) was below detectable levels following an initial liming (time point 0.1 hours) demonstrating at least a four log reduction. MS-2 seeded into the same matrix to achieve a final concentration of 1×10^4 PFU/mL (MS-2 Test) was below detectable levels following 24 hours of liming (time point 24) demonstrating at least a four log reduction. Control samples consisting of rotavirus Wa (Rotavirus Control) and MS-2 (MS-2 Control) seeded into a raw sludge matrix maintained at reduced temperature did not undergo lime stabilization. In addition, aliquots of seeded virus (MS-2 at Temp and Rotavirus at Temp) were incubated at 4°C and enumerated at each time point. Control sample aliquots removed at time points 0.1, 2, 12 and 24 hours demonstrated no viral reduction.

Rotavirus Wa and MS-2 Persistence under Liming Conditions in a Previously Limed Biosolids Matrix at Reduced Temperature (4°C)

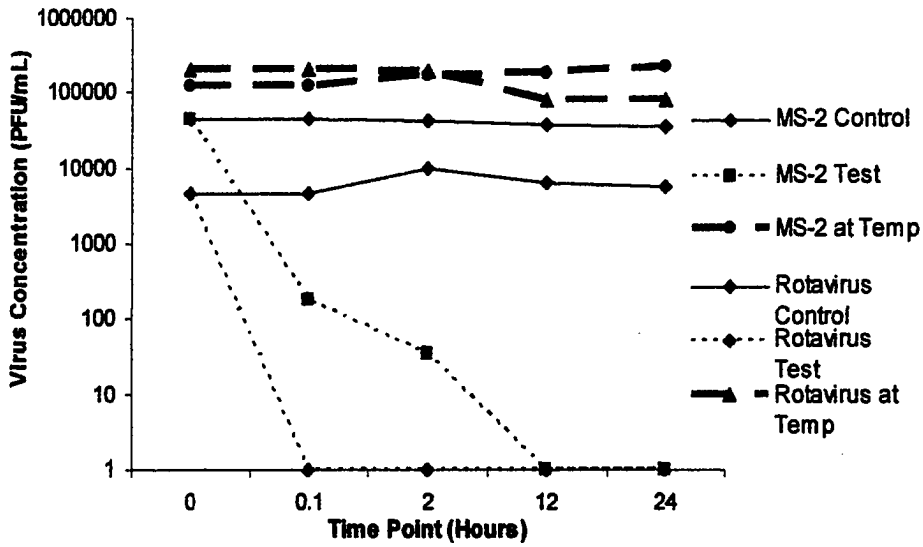

Figure 27: Evaluation of rotavirus Wa and MS-2 persistence in a test lime and control previously limed biosolids matrix at reduced temperature (4°C) - Rotavirus Wa and MS-2 seeded into a previously limed biosolids matrix at reduced temperature were below detectable levels following lime stabilization for 0.1 and 12 hours respectively. Rotavirus Wa seeded into a previously limed biosolids matrix to achieve a final concentration of 1 x 10^4 PFU/mL (Rotavirus Test) was below detectable levels following an initial liming (time point 0.1 hours) demonstrating at least a four log reduction. MS-2 seeded into the same matrix to achieve a final concentration of 1 x 10^4 PFU/mL (MS-2 Test) was below detectable levels following 12 hours of liming (time point 12) demonstrating at least a four log reduction. Control samples consisting of rotavirus Wa (Rotavirus Control) and MS-2 (MS-2 Control) seeded into previously limed biosolids maintained at reduced temperature did not undergo lime stabilization. In addition, aliquots of seeded virus (MS-2 at Temp and Rotavirus at Temp) were incubated at 4°C and enumerated at each time point. Control sample aliquots removed at time points 0.1, 2, 12 and 24 hours demonstrated no viral reduction.

Overall Rotavirus Wa and MS-2 Inactivation for Three Trials Conducted in Various Sludge Matrices Lime Stabilized at Reduced Temperature (4°C)

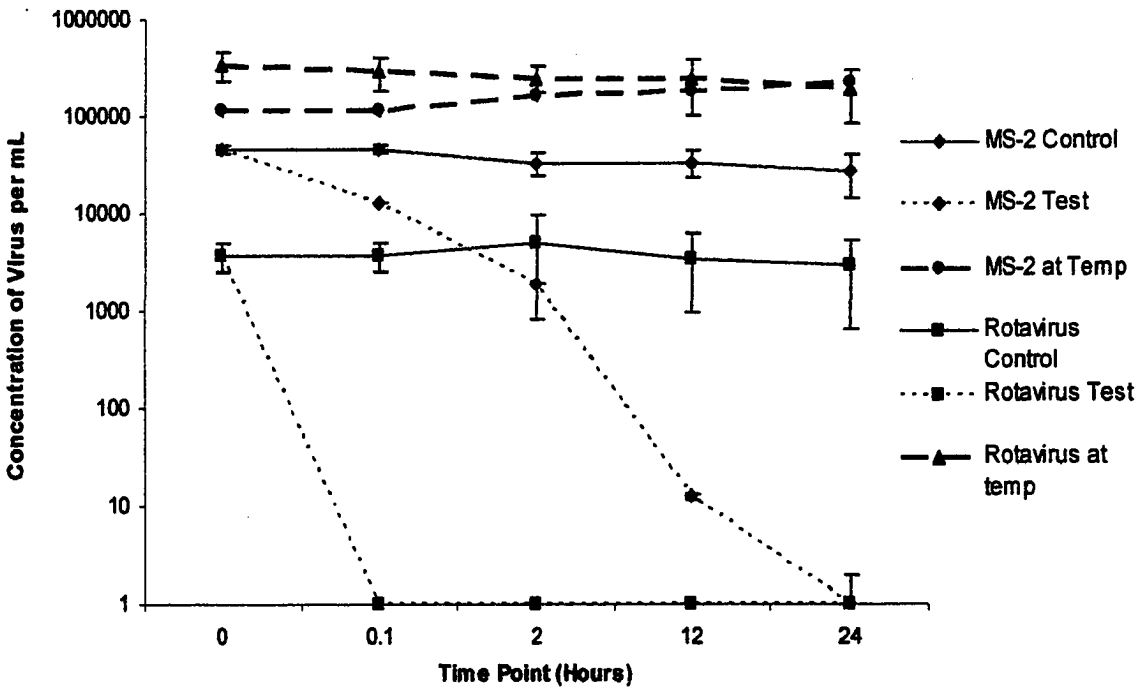

Figure 28: Overall rotavirus Wa and MS-2 inactivation for three trials conducted in various sludge matrices lime stabilized at reduced temperature (4°C) – Rotavirus Wa and MS-2 seeded into varying sludge matrices were below detectable levels following 24 hours of lime stabilization at 4°C in all trials conducted, with inactivation times varying depending on the matrix evaluated. The results presented represent the average inactivation of rotavirus Wa and MS-2 in spiked control (Rotavirus Control, MS-2 Control) and test (Rotavirus Test, MS-2 Test) samples for the three lime stabilization trials conducted in the various matrices evaluated (compost, previously limed and raw) at reduced temperature. In addition, aliquots of seeded virus (MS-2 at Temp and Rotavirus at Temp) were incubated at 28°C and enumerated at each time point.

Statistical Analysis of Three Trials of Rotavirus Wa and MS-2 Lime Stabilization evaluated in Various Sludge Matrices (compost, previously limed, raw) at 28°C and 4°C

Statistical analysis of three trials of rotavirus Wa and MS-2 lime stabilization evaluated in various sludge matrices (compost, previously limed, raw) at room temperature (28°C) and reduced temperature (4°C) were conducted using the SPSS statistical software package. A general linear model was constructed to evaluate the following null hypotheses 1) lime stabilization does not have a statistically significant effect on the inactivation of rotavirus Wa and MS-2 bacteriophage 2) the effect of liming is not statistically different between rotavirus Wa and MS-2 bacteriophage when the effect of liming over a period of time is considered for each organism individually 3) there is no statistical difference between the inactivation of rotavirus Wa and MS-2 bacteriophage under lime stabilization conditions.

Analysis of lime treatment effectiveness for all trials in all matrices evaluated at both 28°C and 4°C revealed a statistically significant effect of treatment on both rotavirus Wa and MS-2 generating a p value of 0.000, where a p value of < 0.05 was used as the parameter for statistical significance. In trials conducted at 28°C it was determined that there was no significant difference between the treatment effect of liming on either virus or bacteriophage (p value = 0.315). The same was true for trials conducted at 4°C where the p value was 0.184. Statistical analysis was performed with calculated percent inactivation values for rotavirus and MS-2 in the same way as was done for adenovirus and MS-2 data. For all trials conducted at 28°C and 4°C, there was no statistically significant effect of time on treatment, and no statistically significant difference between

the effect of time on either rotavirus or MS-2 inactivation, with p values of 0.155 and 0.352, indicating that both organisms behaved similarly over time when exposed to lime regardless of matrix.

Statistical Analysis of Adenovirus Type 5, Rotavirus Wa, and MS-2 evaluated in Various Sludge Matrices (compost, previously limed, raw) at 28°C and 4°C

Percent reduction values generated for adenovirus type 5, rotavirus Wa, and MS-2 were analyzed to make comparisons between trials evaluated at different temperatures in an effort to determine if liming conducted at 4°C reduced inactivation of virus and bacteriophage. Bacteriophage data from all trials conducted at 28°C and 4°C were compiled and analyzed. The results of such analysis revealed a significant effect of treatment regardless of temperature (p value = 0.000), and no significant difference in treatment effectiveness for inactivation of MS-2 at either temperature (p value = 0.201). The same result was true for adenovirus type 5 where the effect of treatment with lime was significant at both temperatures (p value = 0.000) and there was no significant difference in treatment effectiveness at different temperatures (p value = 0.856). Rotavirus was not detected in any of the test samples analyzed in any of the matrices at both temperatures evaluated; there was no difference between samples or temperatures.

DISCUSSION

Lime stabilization is a method employed by many sludge generators to inactivate pathogens, immobilize heavy metals by precipitating and locking heavy metals into stable compounds, modify acidic soils, which is of particular importance in new England, to produce higher yields, and to reduce vector attraction. The use of lime as a treatment method is inexpensive with regard to cost of implementation and operation. Lime can be purchased at $20 a ton, is easy to use, reduces odor which is the primary reason for the lack of public acceptance of land application of biosolids, and results in an end product that is beneficial and acceptable for agricultural use. Very little data are currently available indicating the persistence of pathogens under lime stabilization conditions. The goal of this study was to evaluate the persistence of virus and bacteriophage at various stages of the lime treatment process in water, sludge and biosolids matrices at room temperature, 28°C and reduced temperature, 4°C.

Organisms were initially evaluated in an RO water matrix. This was to evaluate the inactivation of pathogens as a result of lime addition independent of the many inhibitory substances that may be present in sludge and biosolids matrices. In addition, pathogens such as *Cryptosporidium* and viable helminth ova have poor recovery efficiencies using currently available techniques for recovery. Therefore, in order to draw comparisons against pathogens evaluated in a water matrix, as a result of problems with recovery efficiency from solid matrices, it was necessary to have a baseline inactivation for viral pathogens in a water matrix.

The results generated indicate that lime stabilization effectively inactivated adenovirus type 5, rotavirus Wa and male-specific bacteriophage MS-2 in a water matrix at room temperature (28°C). The data also demonstrate that male specific bacteriophages are inactivated similarly to both adenovirus type 5 and rotavirus Wa. The methodology established in the Part 503 rule for recovering enteric viruses from biosolids is extremely inefficient; this is due in part to the particle association that takes place between charged viruses and soil particles and the difficulty in separating the viruses from the solid component for assay as discussed in Chapter 3. Ideally, indigenous viruses would be present in high enough concentrations and easily recovered from sludge and biosolids samples so that spiking would not be necessary; however, until such methods are available to assess and evaluate the nature of the association of viruses to sludge particulates and effectively extract such viruses, spiking of virus is necessary to evaluate treatment effectiveness. Although it is important to keep in mind when interpreting the results the potential difference between the interactions of lime, which requires surface contact on viruses that are tightly bound to sludge particulates, and potentially protected, versus viruses which are freely suspended in a sludge that is homogenously limed. The data presented elucidate a new area of research to evaluate the difference in treatment effectiveness and rates of inactivation on seeded versus indigenous organisms in sludge and biosolids.

This highlights another point, which is the requirement that in order to achieve inactivation rates demonstrated in this investigation it was necessary that the matrix be adequately mixed and the lime added to achieve a homogeneous distribution in the sample. If the sludge were not adequately mixed it would be possible for the creation of

microenvironments resulting in a non-homogeneous mixture which would not achieve the high pH levels necessary for viral inactivation. Therefore, effective liming is contingent upon the lime coming in contact with the sludge particulates in a homogenous nature thereby ensuring that small areas that do not achieve the high pH do not exist. Such areas, subsequently land applied, may contain virus that could later be mobilized in a rain event and move through the soil column into underground aquifers. The most effective way to ensure adequate contact of lime is to utilize large tanks for mixing and addition of lime to a sludge that is not dewatered rather than dewatering and subsequently liming. There are advantages and disadvantages to incorporating this type of system into a treatment facility. The dewatering step removes a large component of the sludge material to be treated, therefore eliminating a great deal of the bulk, creating a smaller quantity of sludge for treatment and disposal. The dewatering process, which is most often accomplished using large centrifuged, or belt and filter presses, generates a liquid component which can be re-circulated back into the treatment processes, then chlorinated and finally discharged as effluent. Yet, once sludge is dewatered it becomes increasingly difficult to adequately homogenize the sample for liming to ensure that pathogens potentially present in the material have an equal opportunity of coming in contact with the alkaline slurry to be inactivated. Sludge that is not dewatered and can be incorporated with the lime slurry in large tanks and held for a period of time prior to dewatering is more easily homogenized and therefore more likely to inactivate pathogens. However, a drawback associated with this technique of liming is that sludge must be dewatered following lime stabilization to reduce the bulk of material that must ultimately be land applied. Dewatering of the limed sludge generates a high pH liquid that must be disposed.

This alkaline liquid cannot be easily re-circulated back into the wastewater treatment process unless there is sufficient influent to dilute the effects of the high pH so that the alkaline pH does not disturb the normal aeration process that takes place as part of the wastewater treatment process. The high pH liquid re-circulated could potentially kill the microorganisms necessary for clarification of the wastewater in the early stages of the wastewater treatment process.

Ultimately, the decision as to which method of liming is most effective should be determined on an individual plant basis. Plants that have small influent volumes cannot effectively dilute the high pH liquid that results from dewatering after liming and therefore in order to prevent the disruption of the wastewater treatment process it may be necessary to dewater prior to liming. In this instance it is imperative that the material be adequately homogenized with lime and held for sufficient time to inactivate virus present in the material prior to land application.

Additional experiments to evaluate the effectiveness of lime stabilization to inactivate *Cryptosporidium parvum* and *Ascaris lumbricoides* were conducted jointly with Christine Bean (Graduate Student, Department of Microbiology). Such experiments were conducted in water due to the ease with which the organisms could be recovered. *A. lumbricoides* remained viable following 72 hours of liming. Similarly, *C. parvum* strain MD, evaluated in a limed water matrix due to low recovery efficiencies in biosolids matrices, remained infectious in neonatal mice following 72 hours of liming. Dose-response analysis of *C. parvum* infectivity demonstrated that lime stabilization for 24 hours did not inactivate the oocysts. To the contrary, the treatment increased the

infectivity of the oocysts by more than 4-fold. The same effect was observed after a 2 hour lime exposure resulting in over 7-fold reduction in ID_{50} for treated oocysts.

Evaluation of reliable and cost-effective treatment processes to demonstrate sufficient inactivation of pathogens provides valuable information for implementation of treatment technologies. This research has demonstrated that lime stabilization is effective at reducing fecal coliforms, *Salmonella*, adenovirus type 5, rotavirus Wa, and male-specific bacteriophage in a water matrix when calcium hydroxide is added in sufficient quantity to raise the pH to 12 for 2 hours and 11.5 for 22 hours. In addition, the data demonstrate that male specific bacteriophages are inactivated similarly to both adenovirus type 5 and rotavirus Wa and may therefore represent a potential indicator to evaluate treatment efficiency. Currently fecal coliforms are the only microbiological indicator evaluated in Class B sludge. Preliminary research has demonstrated that *C. parvum* and *A. lumbricoides* persist long after fecal coliforms have been inactivated. Further investigation is required to optimize recovery efficiencies to evaluate inactivation of pathogens in a sludge matrix in an effort to accurately characterize the risks associated with land application of Class B biosolids. Fecal coliforms were inactivated immediately upon exposure to lime. This data suggests that fecal coliforms are not a reliable indicator for the inactivation of *Cryptosporidium* by lime stabilization.

Liming studies conducted to assess survivability of *Cryptosporidium* in water evaluated by DAPI/PI, Excystation, and animal infectivity reveal persistence of *Cryptosporidium* beyond the twenty-four hour time point. Incorporation of additional time points to represent 24 hours, 48 hours and 72 hours revealed 80% inactivation of *Cryptosporidium* oocysts with inactivation varying depending on the viability/infectivity

assay used to make the determination. When assessed by vital stains, liming has a statistically significant effect on *Cryptosporidium* viability. When assessed by excystation, liming has no statistically significant effect on *Cryptosporidium* viability. When assessed by mouse infectivity liming has an effect immediately at time point 0 and this effect does not increase over time. The results demonstrate not only the persistence of *Cryptosporidium* following lime stabilization but the variation in results depending on the assay used for measurement. Vital dyes and Excystation are methods used to determine the viability of *Cryptosporidium* oocysts, but viability is not synonymous with infectivity and infectivity is measured by inoculating oocysts into animals.

The *Cryptosporidium* liming trials were performed in an RO water matrix due to the ease with which the organisms could be recovered and the lack of appropriate or efficient methodology for recovery of such organisms from sludge and biosolids matrices. Ultimately the effects of liming on *Cryptosporidium* and *Ascaris lumbricoides* in a sludge or biosolids matrix must be evaluated. In order to accomplish this recovery methods must be optimized. In addition, with regard to *Cryptosporidium*, it is not known what the long term effects of holding the organism after treatment and neutralization are or if reactivation is possible over time. In conclusion, fecal coliforms are not a reliable indicator of *Cryptosporidium* or *Ascaris lumbricoides* presence or viability following treatment by lime stabilization. For class B biosolids, fecal coliforms are the only microbiological parameter evaluated and yet the data show that fecal coliforms are inactivated almost immediately upon the addition of lime whereas *Cryptosporidium* oocysts and *Ascaris lumbricoides* ova persist for long periods of time, up to 72 hours following the addition of lime, which is the time requirement for Class A certification.

Therefore sludges that are tested for pathogens to achieve Class B or Class A certification, and fall within the acceptable levels for fecal coliform density, are not necessarily free of *Cryptosporidium* and viable helminth ova. This is especially important when considering that in a survey conducted it was determined that of thirty-eight samples evaluated, 20% were positive for the presence of *Cryptosporidium* whereas only 15% were positive for *Ascaris* (Chapter 3). For Class B biosolids, reduced pathogen levels are acceptable for due to the incorporation of management practices. However, the results demonstrate pathogen persistence following 72 hours of liming which is the time requirement for alkaline stabilization to achieve Class A biosolids certification, a certification which deems the material "virtually pathogen free," or containing pathogens below detectable levels.

The land application of biosolids is a sustainable means of recycling the nutrient and organic matter present in waste and can be done in such a way that groundwater and the public health are protected. The true challenge is to establish which management practices and treatment techniques are the most relevant and important to protecting groundwater quality and public health.

The data herein demonstrate that bacterial indicators and enteric viruses were inactivated more rapidly than bacteriophages. This is promising information illustrating the potential for bacteriophage use to indicate the fate of enteric virus in treatment processes (Chapter 3). Future studies to determine the variation in sensitivity of bacteriophages to high pH to determine if MS-2 is adequate would be valuable to choose which, if any, bacteriophages are optimal. In conclusion, based on this research, it appears that bacteriophages are a potential model organism for determining the fate of

human viruses in different types of sludges as they are inactivated at a similar rate, are easily recovered, are present in high concentrations in raw sludge and rapid, reliable and cost-effective methods exist for their recovery.

The data provided in this research are useful for developing recommendations for implementing successful treatment technologies along with preventing risks to workers and community populations in settings where exposure to biosolids may occur. This research represents initial study in what needs to be an on-going investigation to evaluate methods of sludge or biosolids preparation in an effort to minimize public health threats associated with land application of biosolids, maximize potential benefits and move our society towards sustainability.

REFERENCES

Alhajjar, B.J., S.L. Stramer, D.O. Cliver and J.M. Harkin. 1988. Transport modeling of biological tracers from septic systems. Water Resources. **22**(7):907-915.

Ansari, S.A., S.A. Sattar, V.S. Springthorpe, G.A. Wells and W. Tostowaryk. 1988. Rotavirus survival on human hands and transfer of infectious virus to animate and nonporous inanimate surfaces. Journal of Clinical Microbiology. **26**(8):1513-1518.

Baker, K.H. and J.P. Hegarty. 1997. Detection and occurrence of indicator organisms and pathogens. Water Environment Research. **69**(4):403-415.

Cabelli, V.J. 1982. Microbial indicator systems for assessing water quality. Antonie van Leeuwenhoek. **48**:613-618.

Chapron, C.D., N.A. Ballester and A.B. Margolin. 2000. The detection of astrovirus in sludge biosolids using an integrated cell culture nested PCR technique. Journal of Applied Microbiology. **89**(1):11-5.

Deng, M.Y. and D.O. Cliver. Inactivation of poliovirus type 1 in mixed human and swine wastes and by bacteria from swine manures. Applied and Environmental Microbiology. **58**(6):2016-21.

Derbyshire, J.B. and E.G. Brown. 1979. The inactivation of viruses in cattle and pig slurry by aeration or treatment with calcium hydroxide. Journal of Hygiene (London). **82**(2):293-299.

Dulbecco, R. and M. Vogt. 1954. Plaque formation and isolation of pure lines with poliomyelitis viruses. J Exp Med. **99**:167-182.

Enriquez, C.E., C.J. Hurst and C.P. Gerba. 1995. Survival of the enteric adenoviruses 40 and 41 in tap, sea and wastewater. Water Resources. **29**:2548-2553.

EPA (U.S. Environmental Protection Agency). 1999. Environmental regulations and technology: control of pathogens and vector attraction in sewage sludge. United States Environmental Protection Agency. Office of Research and Development, Washington, D.C. EPA/625/R-92/013.

EPA (U.S. Environmental Protection Agency). 1998. Method 1680: Fecal coliform in biosolids by multiple tube fermentation and membrane filter procedures. United States Environmental Protection Agency. Office of Water, Washington, D.C. EPA-821-R-98-003.

EPA (U.S. Environmental Protection Agency). 1999. Sample preparation for fecal coliform tests and *Salmonella* sp. Analysis. Appendix F. United States Environmental Protection Agency. Office of Water and Wastewater, Washington, D.C.

EPA (U.S. Environmental Protection Agency). 2000. Health risks of enteric viral infections in children. EPA/822/R/00/010. United States Environmental Protection Agency. Washington, D.C.

Estes, M.K. 2001. Introduction: gastroenteritis viruses. *In.* Gastroenteritis Viruses. Novartis Foundation Symposium. John Wiley and Sons, LTD. 1-4.

Farrell, J.B., J.E. Smith Jr., S.W. Hathaway and R.B. Dean. 1974. Lime stabilization of primary sludges. Journal WPCF. **46**(1):113-121.

Fewtrell, L. and J. Bartram. 2001. Water quality: guidelines, standards and health – assessment of risk and risk management for water-related infectious disease. World Health Organization Water Series. International Water Association Publishing, London, UK.

Gantzer, C., P. Gaspard, L. Galvez, A. Huyard, N. Dumouthier and J. Schwartzbrod. 2001. Monitoring of bacterial and parasitological contamination during various treatment of sludge. Water Resources. **35**(16):3763-70.

Gerba, C.P., J.B. Rose and C.N. Haas. 1996. Sensitive populations: who is at the greatest risk? International Journal of Food Microbiology. **30**(4):663-669.

Ghiglietti, R., R. Rossi, M. Ramsan and A. Colombi. 1995. Viability of *Ascaris suum*, *Ascaris lumbricoides* and *Trichuris muris* eggs to alkaline pH and different temperatures. Parassitologia. **37**(2-3): 229-32.

Grabow, W.O.K, I. G. Middendorff and N. Basson. 1978. Role of lime treatment in the removal of bacteria, enteric viruses, and coliphages in a wastewater reclamation plant. Applied and Environmental Microbiology. **35**(4):663-669.

Greenberg, A. E., L.S. Clesceri and A.D. Eaton. 1992. Standard methods for the examination of water and wastewater. 18th ed. American Public Health Association, American Water Works Association, Water Environment Federation, Washington, D.C.

Jenkins, M.B., D.D. Bowman and W. Ghiorse. 1998. Inactivation of *Cryptosporidium parvum* oocysts by ammonia. Applied and Environmental Microbiology. **54**(2):784-788.

Jenkins, M.B., M.J. Walker, D.D. Bowman, L.C. Anthony and W.C. Ghiorse. 1999. Use of a sentinel system for field measurements of *Cryptosporidium parvum* oocyst inactivation in soil and animal waste. Applied and Environmental Microbiology. **65**(5):1998-2005.

Kowal, N.E. 1985. Health effects of land application of municipal sludge. Health Effects Research Laboratory. United States Environmental Protection Agency, Cincinatti, OH. EPA/600/1-85/015.

Kukkula, M., P. Arstila, M.L. Klossner, L. Manuela, C.H. Bonsdorff and P. Jaatinen. 1997. Waterborne outbreak of viral gastroenteritis. Scand J Infect Dis. **29**(4):415-8.

Londrigan, S.L., M.J. Hewish, M.J. Thompson, G.M. Sanders, H.Mustafa and B.S. Coulson. 2000. Growth of rotaviruses in continuous human and monkey cell lines that vary in their expression of integrins. **81**:2203-2213.

Mawdsley, J.L., A.E. Brooks and R.J. Merry. 1996. Movement of the protozoan pathogen *Cryptosporidium parvum* through three contrasting soil types. Biol Fertil Soils. **21**:30-36.

Mendex, J.M., B.E. Jimenez and J.A. Barrios. 2002. Improved alkaline stabilization of municipal wastewater sludge. Water Science and Technology. **46**(10):139-46.

Mignotte-Cadiergues, B.C., A. Maul, A. Huyard, S. Capizzi and L. Schwartzbrod. 2001. The effect of liming on the microbiological quality of urban sludge. Water Science and Technology. **43**(12):195-200.

Mignotte-Cadiergues, B.C., Gantzer and L. Schwartzbrod. 2002. Evaluation of bacteriophages during the treatment of sludge. Water Science and Technology. **46**(10):189-94.

National Research Council of the National Academies. 2002. Biosolids applied to land: advancing standards and practices. The National Academies Press. Washington, D.C.

(Novartis) Novartis Foundation. 2001. Gastroenteritis Viruses: Novartis Foundation Symposium 238. John Wiley and Sons, LTD.

Papapetropoulou, M. and A.C. Vantarakis. 1998. Detection of adenovirus outbreak at a municipal swimming pool by nested PCR amplification. J Infect. **36**(1):101-3.

Ramia, S. and S. Sattar. 1979. Simian rotavirus SA-11 plaque formation in the presence of trypsin. Journal of Clinical Microbiology. **10**(5):609-614.

Reed, L.J. and H. Muench. 1937. A simple method of estimating fifty percent endpoints. The American Journal of Hygiene. **27**(3):493-497.

Russell, W.C. 2000. Update on adenovirus and its vectors. Journal of General Virology. **81**:2573-2604.

Ryan, K. 1994. Sherris Medical Microbiology: An introduction to infectious diseases. 3rd Ed. Appleton and Lange, Norwalk, CT.

Sattar, S., S. Ramia and J.C.N. Westwood. 1976. Calcium hydroxide (lime) and the elimination of human pathogenic viruses from sewage: studies with experimentally-contaminated (poliovirus type 1, Sabin) and pilot plant samples. Canadian Journal of Public Health. **67**:221-226.

Smith, E.M., M.K. Estes, D.Y. Graham and C.P. Gerba. 1978. A plaque assay for the simian rotavirus. 513-518.

Smith, E.M. and C.P. Gerba. 1982. Development of a method for detection of human rotavirus in water and sewage. Applied and Environmental Microbiology. **43**(6):1440-1450.

Wong, J.W.C. and M. Fang. 2000. Effects of lime addition on sewage sludge composting process. Water Resources. **34**(15):3691-3698.

Wong, J.W.C., M. Fang and R. Jiang. 2001. Persistency of bacterial indicators in biosolids stabilization with coal fly ash and lime. Water Environment Research. 73(5):607-11.

Wyatt, R., W. James, E. Bohl, K. Theil, L. Saif, A. Kalica, H. Greenberg, A. Kapikian and R. Chanock. 1980. Human rotavirus type 2: cultivation in vitro. Science. **207**:189-191.

Yanko, W., R.C. Cooper, R.E. Danielson, A. Garcia, J. Pulz, M. Meckes and W. Telliard. 2000. Comparison of methods for detection of *Salmonella* in biosolids: initial validation of USEPA method 1692. United States Environmental Protection Agency. Washington, D.C.

Yanko, W. 1988. Occurrence of pathogens in distribution and marketing municipal sludges. United States Environmental Protection Agency, Research Triangle Park, NC. EPA/600/1-87/014.

CHAPTER THREE

BIOSOLIDS SURVEY FOR MALE SPECIFIC BACTERIOPHAGE AS A POTENTIAL MICROBIAL INDICATOR TO EVALUATE BIOSOLIDS TREATMENT EFFECTIVENESS

ABSTRACT

Biosolids intended for land application must be screened for pathogens, including enteric viruses and viable helminth ova, to comply with regulatory requirements. The effectiveness of existing methodology to recover enteric viruses and viable helminth ova from biosolids is questionable. Enteric viruses are recovered from biosolids using an elution technique and are subsequently detected by cell culture. Viable helminth ova are recovered from biosolids samples by flotation. The methods employed to evaluate both classes of pathogens are labor intensive, time consuming and subject to interference by environmental inhibitors present in samples. There exists a need to develop rapid and sensitive assays to recover and detect human enteric viruses and viable helminth ova in biosolids samples and ensure public health safety when such material is land applied. Bacteriophages have received a great deal of attention in the research of indicator organisms due to their similarity in size and composition to enteroviruses. In the absence of adequate methodology for recovery and detection of enteric viruses from biosolids, an alternative approach of surveying for bacteriophage was evaluated.

The primary objective of this study was to survey and compare results obtained from raw and lime stabilized biosolids intended for land application when evaluated for the presence of male specific bacteriophage and enteric virus. In addition, the samples were surveyed to evaluate the prevalence of viable helminth ova. This was accomplished by analyzing thirty-six raw and lime stabilized sludge samples for the presence of the male specific bacteriophage MS-2, enteric virus, and viable helminth ova. Samples were obtained from six wastewater treatment utilities employing lime stabilization as a treatment method, between May 2002 and February 2003.

Of the eighteen raw samples evaluated, all were positive for the presence of male specific bacteriophage in varying concentrations, and all were negative for the presence of enteric virus. Male specific bacteriophage and enteric virus were not recovered from any of the eighteen lime stabilized biosolids samples evaluated. Viable helminth ova were not recovered from any of the raw or lime stabilized samples evaluated. The data demonstrates that, using EPA approved methodology for biosolids testing, male specific bacteriophages are easily and consistently recovered from raw sludge samples when enteric viruses are not. Based on these results, coupled with data demonstrating that MS-2 is inactivated similarly to enteric virus under lime stabilization conditions, bacteriophages represent a suitable indicator for the monitoring of biosolids to determine treatment effectiveness following lime stabilization. For regulatory purposes, male-specific bacteriophage may represent an adequate indicator organism that can be used as an index of enteric virus presence and inactivation following treatment by lime stabilization.

INTRODUCTION

Land application of biosolids represents an effective means of recycling nutrients and organic matter but public health risks do exist with respect to human pathogens potentially contained within treated biosolids. Human exposure to land applied biosolids may result from direct contact, inhalation or ingestion of water from contaminated aquifers (NRC, 2002). Current regulations require the monitoring of four classes of organisms for Class A certification. These include surveillance of *Salmonella,* fecal coliforms, enteric virus, and viable helminth ova. Class B certification of biosolids requires screening for fecal coliforms (EPA, 1999). The microbiological analysis required for certification is labor intensive, costly, and employs a small sample size, thereby raising concern about the validity of results. In addition, methods for biosolids analysis are currently being developed and optimized. Available methodology for recovering pathogens from biosolids is inadequate, making it difficult to adequately assess biosolids for the presence of pathogens and to accurately determine whether or not pathogens have been inactivated following treatment processes (Brashear, 1982). The identification of an indicator organism that is both easily recovered and assayed for, and is representative of other pathogens potentially present in biosolids is necessary.

It is impractical to screen for all pathogens posing a threat to public health individually. Therefore, there is a need to be able to assess the efficacy of indicators. Currently, there is no one organism that is considered to be the ideal indicator. A study aiming to correlate the inactivation of the most resistant human pathogens to

bacteriophage inactivation would be valuable in determining the efficacy of phage as a viable indicator. This is of particular importance considering the inefficiency of existing methodology for recovery of pathogens in biosolids samples. Methods to evaluate the presence of human viruses in sludge have been developed. These methods involve elution of viruses from sludges by incorporating a proteinaceous material such as beef extract to compete with viruses for binding sites; thereby separating viruses from sludge particulates. Following elution, viruses are concentrated to reduce the sample size to a manageable volume, and subsequently detected by cell culture or molecular techniques such as PCR. Recovery of viruses using commonly employed elution methodology is challenging due to the tight association of viruses to solid particulates, which are then lost during centrifugation (Brashear, 1982). In addition, detection of viruses in a sludge matrix is problematic due to the presence of toxic substances in the eluate that interfere with cell culture and inhibit PCR. The use of phage as an indicator of enteric virus presence and inactivation represents an alternative approach (Fewtrell, 2001).

Bacteriophages have received a great deal of attention in the research of indicator organisms and surrogates due to their similarity in size and composition to the enteroviruses, a microbiological parameter evaluated for class A certification. Bacteriophages, like enteroviruses, are small in size, found in high numbers in feces and present in high numbers in wastewater, although their presence will depend on the quality of the water, pH and quantity of bacteria. Bacteriophage must be evaluated to determine whether or not they fit all of the criteria which must exist for an indicator organism to be considered reliable in predicting a health risk. These criteria are: 1) the organism must be exclusively of fecal origin and consistently present in fecal waste, 2) the indicator must

occur in greater numbers than the associated pathogen, 3) the indicator must be more resistant to environmental stresses and persist for a greater length of time than the pathogen, 4) the indicator must not proliferate to any great extent in the environment, 5) simple, reliable and inexpensive methods should exist for the detection, enumeration and identification of the indicator organism (Fewtrell and Bartram, 2001).

Bacteriophages appear to be inactivated in the same way as enteric viruses when exposed to traditionally employed methods of treatment. Research has shown variations in phage susceptibility to treatment. F-specific phages appear to be more susceptible to disinfection techniques than somatic phage or phage infecting *Bacteroides fragilis* (Fewtrell, 2001). However, there is a need for updated research to evaluate the persistence of bacteriophage during conventional treatment processes and assess its potential as an indicator organism.

There is a substantial lack of available scientific information indicating the prevalence of phage in biosolids and their persistence during treatment. The existing data are not uniform because of variations in the host bacterium used for study; however such data indicates that bacteriophage may be a useful tool with which to model the fate of human enteric viruses in sludges. Available data on the occurrence of phage in raw and treated wastewater indicate that bacteriophages are adequate indicators of enterovirus contamination (Gantzer, 1998). Research conducted by Lasobras et al. concluded that bacteriophages will accumulate in primary sludge in the same way as bacteria and viruses. This promising discovery prompted the group to evaluate the recovery and detection of phages from sludges to determine that the numbers of bacteriophage found in

various sludge types are high enough to be considered as model microorganisms (Lasobras et al., 1999).

The majority of research to determine the relevance of phage as an indicator has been conducted in the area of water quality. The microbiological quality of water is evaluated by the use of indicators of fecal contamination (fecal coliforms, *Escherichia coli* and fecal streptococci). Early research conducted by Metcalf et al. revealed that bacterial indicators are inactivated more successfully than bacteriophages (Metcalf, 1978). Later studies conducted by Havelaar et al in 1993, corroborated earlier findings by demonstrating that bacterial indicators do not provide adequate information about the fate and resistance of viruses to treatment (Havelaar et al., 1993). Additional research conducted by Havelaar et al. demonstrated that F-specific RNA bacteriophages were adequate model organisms for enteric viruses in fresh water and concluded that enteric virus concentrations can be predicted from F-RNA phage data (Havelaar et al., 1993). Recent research conducted by Jiang et al. comparing the prevalence of human adenoviruses and coliphages in coastal waters affected by urban runoff concluded that the presence of human adenovirus was not significantly correlated with the concentration of coliphage but was significantly correlated with the concentration of F-specific coliphage (Jiang et al., 2001). These findings illustrate the promise of bacteriophage as a useful indicator for monitoring purposes. The implementation of such an indicator would be highly rewarding with regard to cost, turn around time for results and feasibility of assay. The alternative to assessing the currently approved bacterial indicators for water quality is to evaluate samples for the presence of enteroviruses by cell culture or molecular biology techniques. The isolation of enteroviruses by cell culture permits determination

of the infectious nature of the virus isolated; however, this process is time-consuming, difficult to perform and not all viral serotypes can be detected. Molecular biology techniques such as reverse transcription-polymerase chain reaction (RT-PCR) can be used for sensitive, specific and rapid detection of the enterovirus genome and represent a valuable alternative to cell culturing; however, the detection of the viral genome does not dictate infectivity.

Currently, there are no data available evaluating the inactivation of bacteriophages compared with inactivation of enteric viruses during traditionally employed sludge treatment processes. The objective of this study was to determine the levels of and compare the relationships between male-specific bacteriophage and enteric virus found in raw and lime stabilized samples obtained from six utilities in three states that employ lime stabilization as a sludge treatment method.

Bacteriophage

Bacteriophages were first described from the intestinal tract of man in the early 1900's. In the 1930's phage was used as a model for indicating the likely presence of pathogenic enteric bacteria, at which time, a direct correlation was established between the presence of certain bacteriophage and the intensity of fecal contamination. There are at least twelve distinct groups of bacteriophage which are diverse both structurally and genetically. Somatic coliphages, F-specific RNA phages, and *Bacteroides fragilis* phages represent types of bacteriophage that have been proposed as specific indicators of viral contamination (Lasobras et al., 1999) (Fewtrell and Bartram, 2001) (Gantzer et al., 1998).

The best known bacteriophages are the common phages of *E. coli.* Bacteriophage may consist of single-stranded or double-stranded DNA or RNA and are further classified by size, the presence and structure of a tail, and the location of attack for infection. Phage may be male-specific, requiring a bacterial pilus for attachment or somatic, attaching directly to the outer cell membrane or cell wall. The male-specific phage MS-2 is a single-stranded RNA bacteriophage within the family leviviridae. MS-2 is approximately twenty-six nanometers in size, and is 3,569 nucleotides in length, icosahedral in shape, with a positive sense RNA strand. Therefore, the MS-2 nucleic acid acts directly as its own messenger RNA upon entry into a susceptible cell. MS-2 attaches directly to the F+ pilus of a susceptible cell (Fewtrell and Bartram, 2001).

There are many variables that affect the incidence, survival and behavior of phages in different environments, including the densities of both host bacteria and phage along with temperature and pH. Bacteriophages have been shown to persist for long periods of time, particularly at low temperatures as do enteric viruses (Lasobras et al., 1999). The only microbiological indicator evaluated for Class B biosolids certification is fecal coliforms and studies have shown that bacterial indicators are inactivated more successfully than other pathogens potentially present in biosolids (Lasobras et al., 1999). This is not surprising as the inadequacy of fecal coliforms for predicting the virological quality of water is well documented even in marine environments where human enteric viruses have been shown to accumulate in water, crabs, shellfish and bottom sediments, in the absence of fecal indicator bacteria (Goyal et al., 1984) (Dore et al., 2000). Concentrations of F-specific RNA bacteriophages have been significantly correlated with enterovirus concentrations in water environments and with adenovirus concentrations in

recreational water (Havelaar et al., 1993) (Jiang et al., 2001). The use of alternative indicators in conjunction with existing microbiological indicators offers a new way to distinguish sources of fecal contamination. The similar morphology, structure and behavior of F-specific RNA bacteriophages, as well as other phages, to that of human enteric viruses, suggests that they could represent a better model for presence of pathogens. Bacteriophage could therefore potentially be used to demonstrate inactivation of enteric virus by treatment processes, and for routine surveillance of pathogen persistence.

Bacteriophages represent a potential indicator; however, limitations do exist. Many of these limitations have been noted with regard to the use of bacteriophage as an indicator for water quality, yet many of the same issues will apply when proposing to use bacteriophage as an indicator in sludge matrices. Phages are excreted by a percentage of humans and animals all the time while viruses are excreted only by infected individuals for a short period of time. The excretion of viruses is dependent upon epidemiology, outbreaks and administration of vaccine. There is no direct correlation between the numbers of phages and viruses excreted by humans. The methods for detecting coliphages recover a wide range of phages with different properties. Some coliphages have been shown to replicate in water environments, and therefore may also replicate in a sludge environment where host bacteria exist. Enteric viruses have been detected in water environments in the absence of coliphages. Human enteric viruses associated with waterborne diseases are excreted almost exclusively by humans whereas phages used as models in water quality assessment are excreted by humans and animals. The feces of animals generally contain high densities of phages compared to humans. The percentage

of animals excreting phage is higher than for humans. The microbiota of the gut, diet and physiological state of animals seems to affect the number of coliphages in feces. The composition and numbers of phages excreted by humans is variable. The differences between phage and enteric viruses are also reflected by differences in the efficiency of adsorption elution techniques for their recovery. The differences are due to differences in adsorption properties which have major implications for behavior in water environments and some treatment processes (Fewtrell and Bartram, 2001).

Detection of bacteriophage is contingent upon the use of an appropriate host bacterium. In this survey, male specific bacteriophages were targeted for recovery. The bacterial host used was *E. coli* harboring a conjugative plasmid conferring both streptomycin and ampicillin resistance and pilus production (*E. coli* F. Amp HFR). This host was chosen for its antibiotic resistance, making it extremely useful for studies involving sludge and wastewater, where the potential for contamination is extremely high. MS-2 was evaluated as a potential indicator for monitoring the fate of enteric viruses in sludge and biosolids samples and was employed as an internal biocontrol in lime treatment studies.

MATERIALS AND METHODS

Sample Collection and Handling

Raw and lime stabilized sludge samples intended for land application were obtained from six wastewater treatment plant sites in three states within the United States. The sites chosen each employ lime stabilization as a method of treatment for wastewater solids. Samples were collected by treatment plant workers at participating utilities, placed in clean, sterile screw cap bottles and shipped overnight on ice to the Virology and Waterborne disease laboratory at the University of New Hampshire. Samples were stored at 4°C until analysis was performed. The samples were mixed and aliquoted into four portions in preparation for sample analysis, one fifty gram or fifty milliliter portion was obtained and evaluated to determine percent total solids, one one-hundred gram or one-hundred milliliter portion was obtained and eluted for enteric virus assay, one ten gram or ten milliliter portion was obtained and eluted for male-specific bacteriophage assay, and one fifty gram or four hundred and fifty milliliter portion was obtained and processed for viable helminth ova recovery.

Total Solids

Total solids are material residue that is retained following evaporation of a sample and its subsequent drying in an oven at a defined temperature. Results obtained for pathogen analysis of biosolids samples are reported as the concentration of organism

detected with regard to the percentage of total solids in the sample. The determination of total solids concentration involved drying an aliquot of sample at 103° to 105°C to remove water. The mass of total solids in the sample was then determined by comparing the mass of the sample before and after each drying step. Total solids concentrations were calculated for each sample obtained for this study. Sample aliquots of fifty grams were placed in a weighed porcelain evaporating dishes previously stored in a desiccator. A combined weight was obtained to include the weight of the sample and the evaporating dish prior to drying. Liquid samples were dried at 103° to 105°C for one hour at which time the sample was cooled in a desiccator and weighed. The sample was repeatedly heated, cooled and weighed until the weight change was less than fifty milligrams. Solid samples were dried at 103° to 105°C overnight. Following overnight drying, solid samples were cooled in a desiccator and weighed. The drying process was repeated until the weight change of the sample was less than fifty milligrams. The percentage total solids in the sample was calculated by subtracting the weight of the dish (B) from the weight of the dried residue and the dish combined (A), multiplying by one hundred and dividing the resultant figure by the weight of the wet sample and the dish (C) minus the weight of the dish (B).

Percent Total Solids = [(A)-(B)] x 100 / [(C)-(B)]

Elution of Bacteriophages from Sludge Samples

Two methods were initially evaluated for the isolation of bacteriophages from raw and lime stabilized biosolids using six raw sludge samples, and compared to the results obtained form evaluating the sample without any elution manipulation. The method employed for elution of viruses from wastewater solids as dictated in the EPA part 503 rules was evaluated for efficiency at recovering bacteriophages. This method is described in detail below and is outlined in Figure 1. The second method involved measuring two grams or two milliliters of sample and placing it into a 15-mL conical tube. Ten milliliters of a 1% phosphate buffered saline solution supplemented with magnesium chloride was added and the pH adjusted to 7.0. The sample was vortexed for thirty seconds and centrifuged at 1000 x g for 10 minutes. Following centrifugation, the supernate was immediately assayed using a double-agar overlay plaque assay technique with an F. amp *E. coli* host used for the isolation of male-specific bacteriophage.

Elution of Viruses from Sludge Samples

Samples were evaluated for total culturable viruses, including enteroviruses, according to the procedures designated for recovery and assay of viruses from sludge in the EPA part 503 rules. The procedure for recovery of viruses from wastewater solids is an adsorption process reliant upon adsorption of viruses from the liquid phase to the sludge solids, which are subsequently eluted and concentrated by centrifugation. The supernate is discarded. Viruses are desorbed from the solids by physiochemical means

and further concentrated by organic flocculation. Decontamination prior to evaluation by cell culture is accomplished by incubation with antibiotics.

Liquid samples were conditioned prior to elution as follows. A one hundred milliliter quantity of liquid sludge was homogenized for five minutes, at which time one milliliter of aluminum chloride was added and the pH of the solution adjusted to 3.5 with 1N HCL. The sample was mixed for thirty minutes and subsequently centrifuged at 2500 x g for fifteen minutes at 4°C. The supernate was discarded and the sample eluted. Elution of conditioned liquid samples and sludge solids involved resuspension of the resultant pellet from the conditioning or measuring one hundred grams of sample in the case of a solid sample and adding one hundred milliliters of distilled water. The sample was blended gently for five minutes. Following blending, an equal volume of 20% beef extract solution was added to the sample and blended. The sample was then mixed for thirty minutes and centrifuged at 10,000 x g for thirty minutes at 4°C. The supernate fluid was decanted and the appropriate volume of distilled water added to bring the final concentration of beef extract to 3%. The resultant eluate was transferred to a clean centrifuge bottle and the pH adjusted to 3.5. The sample was mixed for thirty minutes and centrifuged at 2500 x g for fifteen minutes. The sediment was retained, resuspended in twenty milliliters of sodium phosphate buffer and transferred to a conical tube where the pH was adjusted to 7.0. Samples were incubated at 37°C for three hours with one milliliter of antibiotic/antimycotic (100x) and one hundred microliters of gentamicin and frozen for assay. A flow chart of the sludge processing procedure for viral analysis is presented in Figure 1.

Viral Elution Methodology

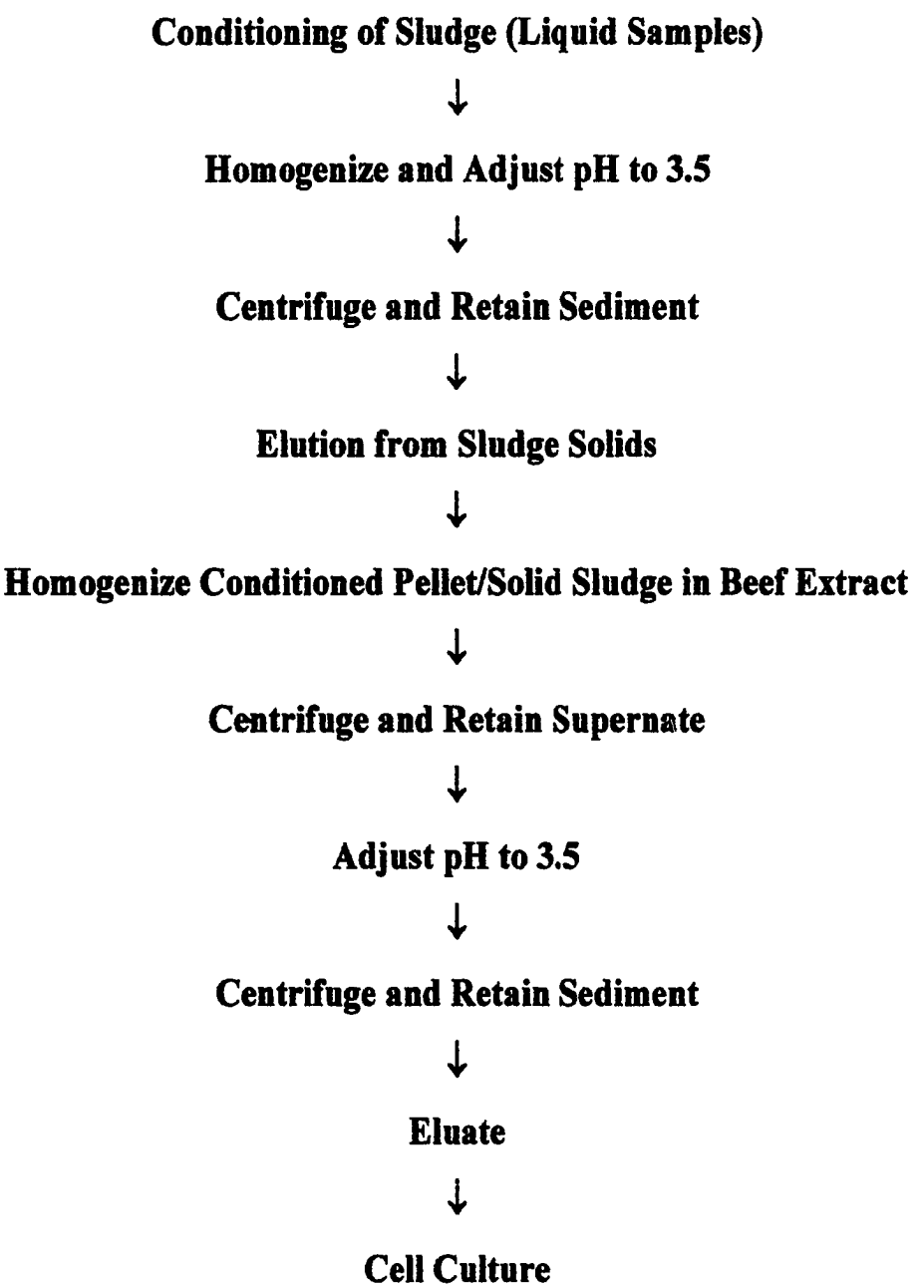

Figure 1. Sludge preparation for cell culture

Cell Culture Analysis

The procedure for detection of viruses in sludge by plaque assay was performed according to protocols established in the EPA part 503 regulations (EPA, 1999). This procedure involves the use of an agar medium to localize virus growth following attachment of infectious virus particles to cells contained within a confluent cell monolayer. Localized lesions of dead cells will develop several days following infection and visualization of such plaques is enhanced by the incorporation of a neutral red stain in the agar overlay. The neutral red viability stain will stain live cells only. The number of unstained plaques are counted and reported as plaque forming units, whose number is proportional to the amount of infectious virus particles inoculated.

The viral assay was performed using a continuous cell line of Buffalo Green Monkey Kidney (BGMK) cells. Cells were grown in minimal essential media (MEM) (Sigma) and L-15 Medium supplemented with 8% fetal calf serum, 100 U/ml penicillin, 100 ug/ml streptomycin, and 50 ug/ml kanamycin.

Prior to each viral assay cells were prepared by passaging previously prepared cell culture flasks containing confluent monolayers of BGMK cells. The cells were passaged by: 1) decanting and discarding the growth medium, 2) washing the cell monolayer with pre-warmed serum-free minimal essential media, 3) adding 1X trypsin (Sigma) solution to the monolayer and placing flasks in a 37°C incubator for five minutes or sufficient time to allow cells to lift from the bottom of the flask. Once the cells were dissociated, fresh medium was added and the cell suspension centrifuged at 1000 x g for ten minutes to remove residual trypsin. Cells were re-seeded into new flasks at a 1:4 dilution. Once

the cells reached confluency they were used to assay infectivity for enterovirus by means of a plaque assay technique.

T-25 cell culture flasks were used for plaque assay. Samples were diluted to 10^{-3} with phosphate buffered saline and 0.1 ml of the appropriate dilution inoculated in triplicate onto confluent monolayers of BGMK cells. Adsorption of virus was allowed to proceed for 1.5 hours at 37°C with rocking every fifteen minutes. Following adsorption, cells were washed with phosphate buffered saline and an agar overlay maintenance medium containing 2% fetal bovine serum added. Flasks were inverted and incubated at 37°C and examined daily for the presence of plaques up to seven days following inoculation.

Male Specific Bacteriophage

Male specific bacteriophage levels were determined using a modified double-agar-overlay procedure previously described by Cabelli (1982). Two grams of sample to be assayed was obtained to which ten milliliters of phosphate buffered saline with magnesium chloride was added. The sample was thoroughly vortexed and subsequently centrifuged at 1,000 x g for ten minutes at room temperature. The supernate was immediately assayed for presence of male-specific bacteriophage.

Male specific bacteriophage for positive control was propagated and enumerated using an *E. coli* host harboring a conjugative plasmid conferring both streptomycin and ampicillin resistance and pilus production (*E. coli* F. Amp HFR). The host was grown to log phase in Tryptic Soy broth supplemented with 1% (100x) Streptomycin/Ampicillin

and 1% Magnesium chloride at 37°C for approximately three hours. MS-2 bacteriophage was added to the log phase *E. coli* culture and incubated at 37°C for twelve to eighteen hours. Following incubation, the viral suspension was centrifuged at 10,000 rpm (4°C) for ten minutes to remove cellular debris. The supernate was removed, placed in sterile bottled and refrigerated at 4°C until use. The propagated MS-2 was enumerated by plaque assay with *E. coli* F. Amp using a double agar overlay technique. Plates were incubated at 37°C and observed twelve to eighteen hours later for plaques. For assay of sample supernate, host *E.coli* was grown to log phase in Tryptic Soy Broth for three hours prior to assay. Serial dilutions of sample supernate were created using a 1X phosphate buffered saline solution supplemented with magnesium chloride. One hundred microliters of sample and two hundred microliters of *E.coli* were inoculated into five milliliters of sterile tempered agar overlay. The sample was gently mixed and immediately poured onto a sterile Tryptic soy agar plate and distributed evenly over the surface of the plate by swirling. Plaques were observed and counted after eighteen to twenty-four hours of incubation at 37°C. Male-specific bacteriophage densities were calculated per two grams of sample determined by the number of plaques per volume of supernate assayed times the dilution factor divided by the number of grams of sample examined.

Recovery of Viable Helminth Ova from Sludge Samples

Viable helminth ova were recovered from sewage sludge samples using modified guidelines established by the U.S. Environmental Protection Agency (EPA) for the detection of *Ascaris* ova in water, wastewater, solids, and compost (EPA, 1999). The

magnesium sulfate flotation procedure is the current EPA approved method for detection of viable helminth ova. This procedure has been evaluated and modified to improve recovery efficiencies. Modifications to the approved methodology include addition of an initial step of soaking dry or thick samples overnight to liquefy, the incorporation of an additional blending and settling step to ensure liquification, and the addition of a four hundred mesh sieve to capture the *Ascaris* ova. Recovery studies performed by spiking viable Ascaris lumbricoides ova into various sample matrices (liquid, semi-solid, and solid) revealed higher recovery efficiencies for the modified EPA protocol when compared to recovery rates obtained from the approved protocol without modifications. In the thirty-six samples evaluated, the modified EPA protocol was employed for recovery of viable helminth ova (Bean and Brabants, 2000).

Flotation methods take advantage of liquids, such as magnesium sulfate, that have a higher specific gravity than that of eggs or cysts, causing parasites to float to the surface where they are retrieved for microscopic survey. Fifty grams or four hundred and fifty milliliters of sample was processed by blending with phosphate buffered saline and surfactant. The sample was allowed to settle overnight and the supernatant was aspirated and discarded. This blending and settling was repeated and sediments were subsequently screened to remove large particles. The resulting sediment was centrifuged, the supernatant removed and discarded, and the pellet resuspended in magnesium sulfate solution (specific gravity 1.2) to create a layer of ova. The specific gravity of the solution was adjusted with RO water and the sample centrifuged. Ethyl acetate was added to the supernate to remove excess fatty materials and the pellet was immediately examined microscopically for the presence of viable helminth ova using a Sedgwick rafter counting

chamber and a light microscope at 10X magnification. If helminth ova were present, the ova were observed for movement of internal larval forms. If movement could not be confirmed, samples were incubated for three to four weeks at which time, the material was observed for larval forms.

RESULTS

Efficiency of Methods Assayed for Bacteriophage Elution

Six raw sludge samples were obtained from five different locations and evaluated for efficiency of bacteriophage recovery using the two previously described elution methods. The use of these two methods was then compared to the efficiency of bacteriophage recovery with no elution. Two of the six raw samples were spiked with the male-specific bacteriophage MS-2 to achieve a final concentration of 10^4 PFU/mL, to evaluate the presence of inhibitors and potential viral loss from elution. The results of this study are presented in Figure 2. In all but one of the samples evaluated, phage was recovered in higher concentrations when a washing and centrifugation step was incorporated prior to plaque assay. In all samples evaluated, phage was recovered in low concentrations or not recovered at all when the method for viral recovery specified in the EPA Part 503 regulation for recovery of virus from wastewater solids was employed.

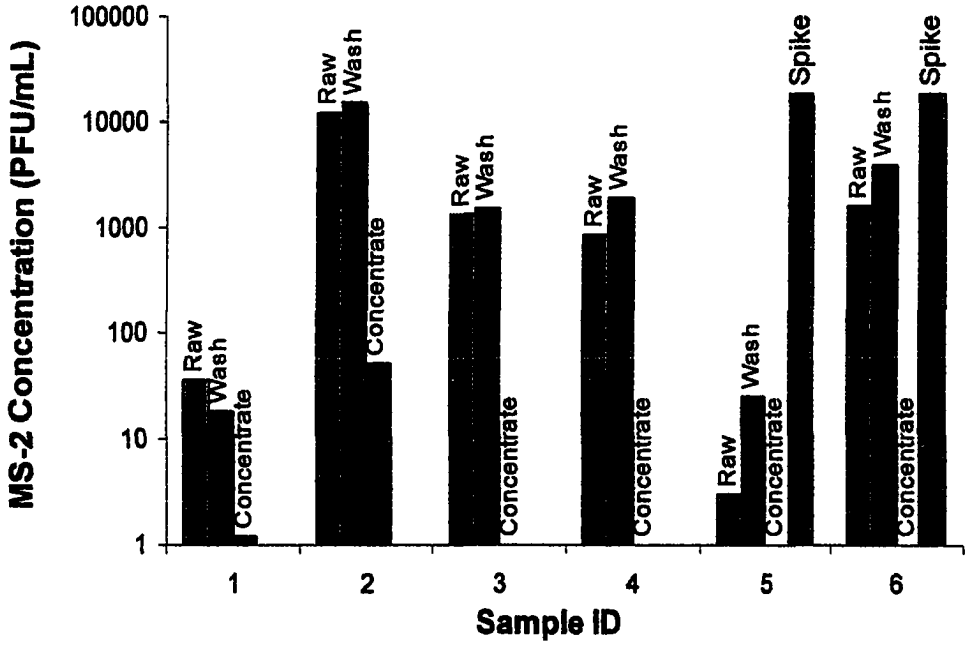

Figure 2: Evaluation of two methods for male-specific bacteriophage recovery – Six samples were evaluated for the presence of phage using the elution method recommended for enteric virus recovery according to the EPA Part 503 Regulations (Concentrate), and a washing and centrifugation procedure (Wash). The concentrations of male specific bacteriophage recovered using the two elution methods are compared to the assay of the raw sample without elution (Raw). Two of the six samples evaluated were spiked with MS-2 to achieve a 1 x 10^4 pfu/mL concentration to evaluate sample inhibition to elution. In five of the six samples evaluated, incorporation of a washing and centrifugation step prior to assay yielded a higher recovery of male-specific bacteriophage when compared to recoveries generated from assay of the raw sample directly and the concentrated sample.

Survey of Bacteriophage, Enteric Virus and Viable Helminth Ova in Raw and Lime Stabilized Biosolids

Samples of raw and limed match-batch sludge and biosolids samples (collected immediately prior to and following lime stabilization) were obtained from six sites in three states during the period of May 2002 to February 2003. The microbiological quality of the sludges evaluated is presented in Table 1. The male-specific bacteriophage MS-2 was detected in all raw samples evaluated for all sites evaluated. Levels of bacteriophage recovered varied among wastewater treatment sites. Sixteen of the raw and lime stabilized samples evaluated were obtained from a wastewater treatment plant in the state of Texas. The highest levels of bacteriophage were detected in raw sludge samples obtained from the Texas wastewater treatment plant. Ten of the raw and lime stabilized samples evaluated were obtained from a wastewater treatment plant in the state of Arkansas and ten of the samples evaluated were obtained from Pennsylvania. Bacteriophage was not detected in any of the lime stabilized biosolids samples evaluated. Enteric virus and viable helminth ova were not detected in any of the raw or lime stabilized biosolids samples evaluated.

Bacteriophage, Enteric Virus and Helminth Surveillance in Raw and Lime Stabilized Biosolids						
Date Collected	Treatment	Location (United States)	Total Solids (%)	Enteric Virus (PFU/4 grams TS)	Viable Helminth Ova (Ova/4 grams TS)	Male Specific Bacteriophage MS-2 (PFU/mL)
14-May-02	Raw	Texas	4.22	<1	<1	1.9×10^3
14-May-02	Lime Stabilized	Texas	30.2	<1	<1	0
25-Jun-02	Raw	Arkansas	2.19	<1	<1	1.8×10^1
25-Jun-02	Lime Stabilized	Arkansas	32.4	<1	<1	0
15-Oct-02	Raw	Texas	2.5	<1	<1	1.4×10^3
15-Oct-02	Lime Stabilized	Texas	32.4	<1	<1	0
16-Oct-02	Raw	Pennsylvania	4.29	<1	<1	5.5×10^2
16-Oct-02	Lime Stabilized	Pennsylvania	18.2	<1	<1	0
2-Nov-02	Raw	Arkansas	2.2	<1	<1	6.0×10^2
2-Nov-02	Lime Stabilized	Arkansas	40.7	<1	<1	0
6-Nov-02	Raw	Arkansas	2.04	<1	<1	1.2×10^3
6-Nov-02	Lime Stabilized	Arkansas	43.19	<1	<1	0
7-Nov-02	Raw	Pennsylvania	2.71	<1	<1	1.0×10^3
7-Nov-02	Lime Stabilized	Pennsylvania	26.12	<1	<1	0
14-Nov-02	Raw	Texas	2.12	<1	<1	1.6×10^3
14-Nov-02	Lime Stabilized	Texas	30.35	<1	<1	0
15-Nov-02	Raw Dewatered	Pennsylvania	18.06	<1	<1	2.0×10^2
15-Nov-02	Lime Stabilized	Pennsylvania	31.66	<1	<1	0
3-Dec-02	Raw	Texas	2.5	<1	<1	1.4×10^3
3-Dec-02	Lime Stabilized	Texas	31.6	<1	<1	0
17-Dec-02	Raw	Arkansas	5.05	<1	<1	1.7×10^2
17-Dec-02	Lime Stabilized	Arkansas	44.13	<1	<1	0
17-Dec-02	Raw	Arkansas	2.29	<1	<1	1.4×10^3
17-Dec-02	Lime Stabilized	Arkansas	39.98	<1	<1	0
10-Jan-03	Raw	Pennsylvania	3.63	<1	<1	1.0×10^2
10-Jan-03	Lime Stabilized	Pennsylvania	23.56	<1	<1	0
14-Jan-03	Raw	Texas	3.46	<1	<1	2.0×10^3
14-Jan-03	Lime Stabilized	Texas	27.72	<1	<1	0
14-Jan-03	Raw	Texas	3.46	<1	<1	2.0×10^3
14-Jan-03	Lime Stabilized	Texas	29.37	<1	<1	0
11-Feb-03	Raw	Texas	4.13	<1	<1	1.0×10^3
11-Feb-03	Lime Stabilized	Texas	29.55	<1	<1	0
11-Feb-03	Raw	Texas	4.01	<1	<1	1.0×10^3
11-Feb-03	Lime Stabilized	Texas	31.25	<1	<1	0
14-Feb-03	Raw Dewatered	Pennsylvania	16.64	<1	<1	1.1×10^2
14-Feb-03	Lime Stabilized	Pennsylvania	29.06	<1	<1	0

Table 1: Bacteriophage, enteric virus, and helminth surveillance in raw and lime stabilized biosolids- Raw and lime stabilized samples collected from three states from May 2002 to February 2003, were evaluated for bacteriophage, enteric virus and viable

helminth ova. Data presented in the table are organized by sample date and are presented by pairing the raw sample data with the corresponding treated sample data following treatment by lime stabilization. Male-specific bacteriophage was recovered in all raw samples evaluated with concentrations varying depending on sample location. Male specific bacteriophage was not detected in any of the corresponding lime stabilized samples. Enteric virus was not detected in any of the raw samples or any of the corresponding lime stabilized samples evaluated. Viable helminth ova were not recovered from any of the raw samples or any of the corresponding lime stabilized samples evaluated.

Levels of Bacteriophages Detected in Raw and Lime Treated Biosolids

Male specific bacteriophage was recovered from all raw samples evaluated. The average concentration of bacteriophage recovered was 1 x 10^3 PFU/mL. Phage concentrations varied depending on location surveyed as seen in Figure 3, but did not appear to vary based on date collected, although the sample set for individual locations is limited as the primary goal of the survey was to assess overall detection of enteric virus as compared to bacteriophage detection. To fully assess variation of phage recovery for locations and seasons the sample set should be increased.

Male Specific Bacteriophage Detection in Raw Sludges Obtained from Three States

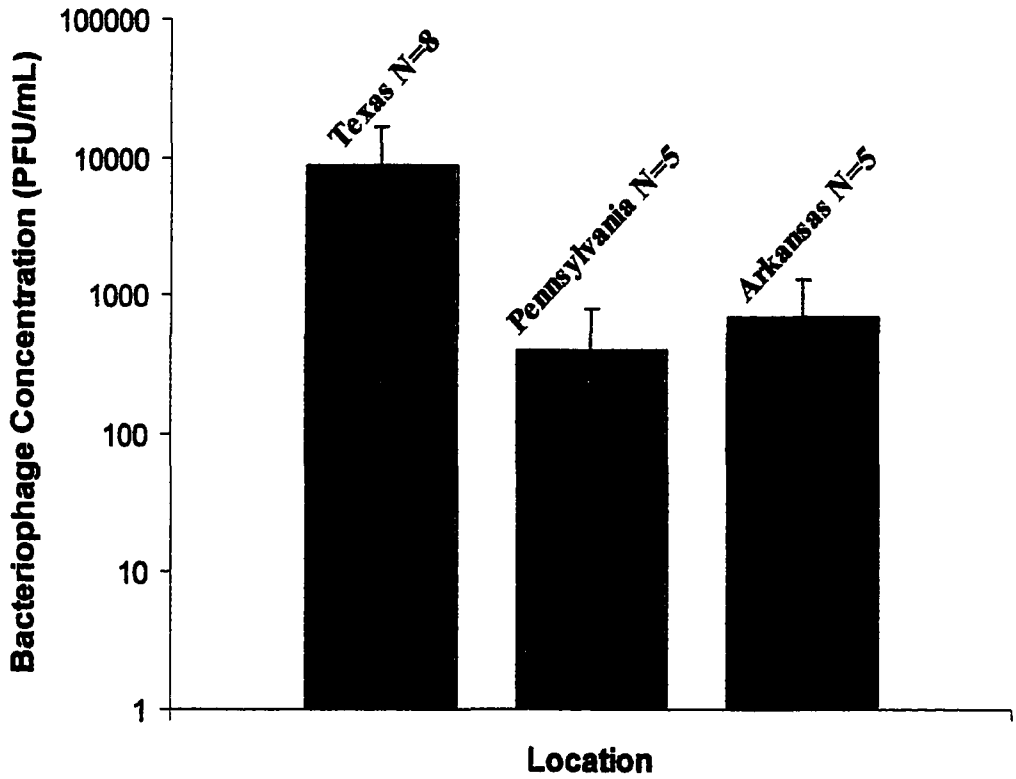

Figure 3: Male specific bacteriophage detection in raw sludges obtained from three states – The concentration of male specific bacteriophage recovered from raw sludges varied depending on the sample site. The average concentration of bacteriophage recovered from samples obtained from the Texas wastewater treatment plant site was 8.6×10^3 PFU/mL. The error bar represents +/- one standard deviation with a standard deviation of 778.61. The average concentration of bacteriophage recovered from samples obtained from the Pennsylvania wastewater treatment plant site was 4.0×10^2 PFU/mL. The error bar represents +/- one standard deviation with a standard deviation of 386.1. The average concentration of bacteriophage recovered from samples obtained from the Arkansas wastewater treatment plant site was 6.8×10^2 PFU/mL. The error bar represents +/- one standard deviation with a standard deviation of 611.1.

Levels of Enteric Virus and Viable Helminth Ova Detected in Raw and Lime Treated Biosolids

Enteric virus was not detected in any of the eighteen raw sludge samples evaluated. In addition, enteric virus was not detected in any of the eighteen corresponding lime stabilized biosolids samples evaluated. Viable helminth ova were not recovered in any of the thirty-six raw or lime stabilized samples surveyed.

DISCUSSION

Biosolids intended for land application are evaluated according to several microbiological parameters depending on the classification of biosolid desired. The microbiological parameter evaluated for Class B certification is fecal coliforms. The microbiological parameters evaluated for Class A biosolids certification, the most highly desired classification, may include screening for enteric virus and viable helminth ova in addition to fecal coliforms and *Salmonella*. Previously described treatment studies to evaluate the persistence of virus and male-specific bacteriophage under lime stabilization conditions demonstrated that rotavirus Wa and adenovirus type 5 were more susceptible to the inactivation effects of lime than male specific bacteriophage. As a result of this interesting observation, male specific bacteriophage was considered a potential indicator for the inactivation of virus during treatment by lime stabilization. Additional study to evaluate the prevalence and recovery of indigenous male specific bacteriophage from sludge samples was conducted to assess the efficacy of using bacteriophage as a potential indicator. This study evaluated thirty-six sludge samples, representing both raw and lime stabilized sludges, for the presence of enteric virus and also viable helminth ova and compared this to the presence of bacteriophage. Bacteriophage was evaluated because of its ease of detection, its reported high prevalence in sewage sludge, and its resistance during lime stabilization trials.

Biosolids samples cannot be effectively evaluated for the presence of bacteriophage or enteric virus without the incorporation of an initial elution step. In this

study, indigenous enteric virus was assayed using protocols established by the EPA Part 503 Rule for the recovery of virus from wastewater solids, as this is the protocol used to evaluate sludge samples for compliance with land application regulations. There is no such established protocol for the recovery of bacteriophage from sewage sludges. Therefore, two methods were evaluated and compared to determine the efficiency of recovering bacteriophage directly from sludges without the incorporation of an elution step. Evaluation of the EPA Part 503 Rule methodology for recovery of virus from wastewater solids to recover bacteriophage proved inefficient in comparison to the use of a simple washing and centrifugation step. The incorporation of a washing and centrifugation step resulted in higher recoveries of bacteriophage when compared to direct assay of the raw sample. This may be due to the removal of inhibitory substances through washing and centrifugation allowing for enhanced formation and visualization of plaques. Therefore, when conducting the survey of wastewater sludges for the presence of enteric virus and bacteriophage, a washing and centrifugation step was employed for elution of bacteriophage and the supernate collected was evaluated for presence of male-specific bacteriophage.

In all raw and lime stabilized sludge samples evaluated, enteric virus was not detected, and results were reported as less than one plaque forming unit per four grams total solids. Such a result indicates compliance with the established EPA Part 503 Rule regulation for enteric virus limits for land application. Therefore, all raw and lime stabilized sludge samples evaluated according to the established EPA protocol did not exceed the enteric virus limits for land application, and represent a potential threat to the public health. This is most likely due to the inefficiency of the viral elution method for

recovery of virus from the sludge samples rather than the reported absence of virus in such samples. Earlier studies have documented the ineffectiveness of the enteric virus elution and recovery procedure established for screening biosolids samples, and the need for improved methodology and identification of adequate indicator organisms. There is a need for optimization of the current methodology to enable recovery of enteric virus from sludge samples.

The samples evaluated in the survey represent a composite collection of raw samples prior to lime treatment and a composite collection of the corresponding limed biosolids following treatment. The exact nature of the association of viruses with particles in sludge is not entirely understood. There may be differences between the associations of virus particles to sludge particulates in raw sludge compared to lime stabilized sludge and the viruses once associated to limed sludge may be irreversibly bound. Such sludge bound viruses when applied to land represent a potential threat to public health. Without being able to efficiently extract the virus particles from sludge material it is difficult to successfully evaluate treatment technologies. This is why it is so important to have information about virus survivability in treated biosolids intended for land application.

Enteric viruses do not behave the same as other pathogens such as bacteria and helminth in soil matrices. Viruses are too small to be retained through size exclusion alone and are either removed or retained by the soil through electrostatic adsorption. Viruses will bind to the soil based on charges in the viral capsid and the soil properties. The characteristics of the soil will affect viral binding such as the soil type, organic load, moisture content and rainfall. The cation binding of a virus to a soil particle is reduced

during rain events. This is because part of the binding process of a virus to a soil particle is dependent on the large multivalent cations which are diluted during a rain event resulting in de-adsorption of the virus from the particle and ultimate movement of the virus through the soil. It is known that viruses can potentially survive for weeks, months or even years under the appropriate moist, cool conditions. This survival rate combined with the likelihood of transport through the soil presents a threat to underground aquifers, particularly in New England, where the groundwater table may be only a few feet from the surface of the soil in some areas, and where the soil composition may be brittle and contain cracks making it easy for virus to move into underground aquifers.

As a result of method inefficiencies, routine monitoring of enteric virus from biosolids samples generally fails to recover enteric virus, demonstrating that the material is in compliance with regulations and is therefore suitable for land application. Virus that is spiked into sludge and biosolids matrices is easily recovered from the matrix using the currently approved method for recovery of viruses from such matrices. However, it is difficult to mimic in the laboratory the exact association of virus particles to soil particulates and as a result spiking is not the ideal means of evaluating the persistence of virus during treatment process. This is illustrated by the fact that enteric virus was not recovered in any of the raw sludge samples surveyed. This is most likely because of the inefficiency of the beef extract elution method to extract the particle associated viruses from the solid component rather than the possibility of all the raw sludge samples evaluated being negative for virus. Historically, a variety of methods have been evaluated to develop elution and concentration techniques for the separation of viruses from sludge particulates and the reduction of sample size to a manageable volume. The elution

method most often used is to chemically separate viruses from sludge particulates through mixing and subsequent centrifugation. Such methods have incorporated solvents such as Freon, phenol and chloroform (Brashear et al., 1982) (Hurst et al., 1991) (Monpoeho et al., 2001). Currently, human enteroviruses are most commonly eluted from matrices and solids using aqueous solutions of beef extract. The beef extract elution technique must be optimized and in the absence of such technology an alternate indicator identified.

Currently employed methods for viral recovery from sludge were developed by conducting seeding and recovery experiments with sludges. Therefore, indigenous virus was not used in development of methodology for viral recovery. Seeded viruses and indigenous viruses clearly have different associations with sludge particulates as indigenous viruses are embedded in sludge particulates whereas the seeded viruses are merely surface-associated. In these instances, viral recoveries from seeded samples may not have significant relevance when compared to recoveries seen with indigenous viruses. Poor recovery efficiency coupled with the fact that different cell lines used to evaluate for viral infectivity will have different plaquing efficiencies with varying virus type's results in the creation of a methodology that is cost-limiting, time-consuming, inefficient and unreliable for detection of viral pathogens in sludge and biosolids matrices.

Until such time comes where adequate methodology exists for the rapid recovery and detection of human enteric virus in biosolids samples, a multifaceted approach should be taken, one in which there are multiple barriers to ensure protection of public health. The incorporation of a rapid, inexpensive and reliable bacteriophage test as an additional monitoring tool to ensure treatment effectiveness represents a novel approach

to regulating the management of biosolids intended for land application. This study demonstrates that bacteriophages are easily recovered from sludge matrices with no interfering problems associated with toxicity to the host bacteria used for the detection of bacteriophages. This is an advantage over the use of cell culture techniques required for the detection of infectious human viruses that are extremely sensitive to toxins found in sludge.

Indigenous male specific bacteriophage was easily recovered in high numbers from the raw sewage sludge samples evaluated where enteric virus was not, even though the two viruses are similar in size and composition. The reason for this difference in recovery efficiency is most likely due to variations in charge and the likely higher concentration of bacteriophage in the environment. Even though only a small percentage of individuals carry male specific phages, phages are present in high concentrations in wastewater and sludge possibly because they are capable of replicating in a biosolids matrix where a bacterial host for infection is present in high concentrations (LeClerc et al., 2000) (Havelaar et al., 1990). This is not the case for enteric viruses, which are specific to humans (poliovirus type 1 and rotavirus Wa for example) and are only released from an infected host. Such viruses are not capable of replicating within the biosolids matrix where there is no host present.

The binding efficiencies of virus to soil and sludge particulates are related to the charges of each. Soil and sludge particles, particularly in New England, are acidic and therefore carry a positive charge. Poliovirus has an isoelectric point of 7.0 and therefore will be negatively charged when the pH of the environment is below 7.0. When the virus is negatively charged it will tightly bind to the positively charged soil or sludge particles.

This interaction has been demonstrated by Taylor et al. who revealed that poliovirus was not adsorbed in soil samples with a pH above 9.0. In addition, Taylor et al. concluded that when both the virus and the substrate are highly negatively charged, repulsive electrostatic effects will inhibit adsorption (Taylor et al., 1981). The isoelectric point of MS-2 is 5.24; therefore, MS-2 will carry a negative charge below a pH of 5.24. This means that MS-2 will tightly bind in acidic soils below a pH of 5.24.

In addition to varying concentrations of phage and enteric viruses in sludge, the charge differences of phage and enteric virus may explain the higher recoveries from raw sludges. The MS-2, which may not be as tightly bound to soil particles, will be more easily eluted when compared to enteric virus, which will carry a negative charge and therefore bind tightly to positively charged soil particles. The individual and characteristic isoelectric point of viruses not only affects the binding to soil and sludge particulates but also their sensitivity to disinfection. Treatment by lime may affect the ability of virus to adsorb to cells or engage in the normal replication process by adsorbing, penetrating and uncoating. Treatment may potentially causes alterations in the antigenic and other structural features of virus as well. These are some of the reasons why the rotavirus and adenoviruses evaluated during lime stabilization treatment may have be more susceptible to inactivation by lime stabilization than phage.

In addition to surveying for enteric virus and bacteriophage in this study, viable helminth ova were evaluated using current EPA approved methodology for the recovery of viable helminth ova from sewage sludge and biosolids. The results show that in all raw and lime stabilized sludge samples evaluated, viable helminth ova were not detected, and results were reported as less than one viable helminth ova per four grams total solids. As

seen with enteric virus survey results, this indicates compliance with the established EPA Part 503 Rule regulation for viable helminth ova limits for land application. Therefore, all raw and lime stabilized sludge samples evaluated according to the established EPA protocol did not exceed the viable helminth ova limits for land application. This may be due to a combination of factors including the inefficiency of currently employed methodology for recovery of viable helminth ova from the sludges in addition to variations in the regional distribution of viable helminth ova.

In a recent survey of thirty-eight samples analyzed for the presence of viable helminth ova, it was determined that of all samples evaluated, 15.8% were positive for viable helminth ova, 23.7% were positive for *Cryptosporidium* sp. and 15.8% were positive for *Giardia* sp. It was concluded that there was no correlation between the presence of viable helminth ova (*Ascaris* sp.) and the presence of *Cryptosporidium* oocysts and *Giardia* cysts, and that percent recoveries varied with different methodologies, demonstrating the need for more adequate indicator organisms and more efficient recovery methods (Bean and Brabants, 2001). The results of the previous study were of particular importance not only in providing data documenting the potential for use of protozoan parasites as an indicator but also in light of studies that have shown the potential for leaching of oocysts and cysts through the soil profile into groundwater (Mawdsley et al., 1996). It is generally assumed that larger parasites will be retained in the soil column through size exclusion; however, Mawdsley et al. demonstrated that purified oocysts will leach through the soil profile with the extent of movement being affected by soil type. This study also concluded that organic matter and clay will play a major role in the adsorption of microbes into soil, due to their large surface area and

negative charge and studies have demonstrated increased adsorption in soils with an increase in organic matter or clay content (Mawdsley et al., 1996).

The current study demonstrates that in all of the raw samples evaluated, viable helminth ova were not recovered. This is most likely a function of regional occurrence combined with method inefficiency. The sites evaluated in the current survey differed from the sites evaluated in the previous survey; therefore, regional distribution may have been a factor in obtaining positive viable helminth ova samples. In addition, with methods of recovery being only 55% efficient for recovery of viable helminth ova (Bean and Brabants, 2000), it is likely that even if viable helminth ova were present in the samples they were lost in the recovery process. This is especially disconcerting considering the cost and labor-intensity of the methodology for processing viable helminth ova samples. The identification of an alternative indicator, such as the use of protozoan parasites, or the development and optimization of new and improved methodology for recovery of parasites from sewage sludge and biosolids is absolutely necessary. The current methodology and indicator organisms are not adequately protective of public health as is evidenced in the negative results for all samples evaluated for viable helminth ova and enteric virus.

The data obtained from the present survey demonstrate that male-specific bacteriophages are rapidly and easily recovered in relatively high concentrations in raw sludge samples where enteric virus and viable helminth ova were not recovered. Evaluating the efficacy of biosolids management strategies depends on the availability of accurate methods to determine concentrations of pathogens of concern in sludge and biosolids. Without the availability of efficient methodology to recover enteric virus from

sewage sludge samples, an alternative indicator must be chosen. The survey of bacteriophage as an indicator for the treatment inactivation of enteric virus represents a cost-effective alternative to monitoring for enteric virus directly in biosolids intended for land application. Survey of bacteriophage can be easily incorporated into the testing requirements for Class A and Class B biosolids certification. Samples collected before and after treatment are routinely tested for fecal coliforms in order to meet Class B requirements and fecal coliforms and *Salmonella* in order to satisfy the Class A requirement. The addition of bacteriophage to the testing requirements will add confidence in the reported results that treatment has been effective. It is likely that enteric virus is present in raw samples but is not easily recovered, but this research has demonstrated that not only is bacteriophage present and easily recovered in raw samples, it is also less susceptible to inactivation by lime stabilization than the viruses evaluated. The use of male specific bacteriophage as an indicator of enteric virus reduction during treatment processes should be implemented because methods are currently available and are rapid, generating results in less than 24 hours, and a large sample size is not required. Survey of raw sludge samples is likely to result in bacteriophage recovery, as demonstrated by the survey results contained within.

A monitoring regime that incorporates testing for male-specific bacteriophage would involve sampling enough solids to provide at least two grams (total solids) of composite material for bacteriophage assay prior to and following treatment (Figure 4). To prepare a sludge sample for analysis, a utility would generate a composite representative sample by collecting 5-10 samples prior to treatment and 5-10 samples following treatment. Two grams (or sufficient solids material to provide one gram total

solids, as calculated by the total solids equation) of the composite raw and lime stabilized sample, as was tested in the survey conducted, can be easily obtained from a utility that will normally be collecting samples for pathogen analysis and subsequently evaluated for presence of bacteriophage. Samples would be collected and shipped overnight to the testing laboratory on ice. Once received, two grams of sample would be resuspended in phosphate buffered saline supplemented with magnesium chloride, vortexed and centrifuged according to the bacteriophage recovery assay described previously. Sample supernate would be assayed using a double agar overlay technique as previously described. Results obtained 12-16 hours after assay would be reported as the total number of PFU/g total solids analyzed, similar to the manner in which enteric virus results are currently reported. Treated samples should contain no more than one plaque per gram total solids sample analyzed and results should be reported as less than one PFU/g total solids or below detectable levels, equivalent to at least a four log reduction, using the double agar overlay for assay of male specific bacteriophage. Such a result would indicate compliance and treatment effectiveness for inactivation of male specific bacteriophage and consequently enteric virus, and approval for land application of the treated material. Less than one PFU/g total solids has been chosen as a limit based on the results of these liming studies which revealed that following 24 hours of liming at a pH of 12 for 2 hours and a pH of 11.5 for 24 hours, male-specific bacteriophage was reduced to below detectable levels, or <1 PFU/mL; therefore, if the lime stabilization process is performed properly such an inactivation of male specific bacteriophage should be expected. A positive sample, generating a result of greater than one PFU/g total solids would require a repeat sampling. Upon re-sampling if a negative result of less than one

PFU/g total solids is generated than the plant would be in compliance and the normal monthly sampling monitoring regime should be followed. In the event that the sample is greater than one PFU/g total solids, this indicates a process control problem and would require corrective action with regard to the lime stabilization treatment process. Following corrective action, compliance and treatment efficiency must be demonstrated by calculating the geometric mean of atleast seven separate samples. The geometric mean must be less than one PFU/g total solids. This standard is based on the fecal coliform monitoring requirement that currently exists for pathogen screening; therefore, implementation of an additional assay would not require additional sampling. Once process efficiency is established through the addition of lime to the sewage sludge to raise the pH to 12 for atleast 2 hours of contact and maintain the pH at 11.5 for 22 hours of contact, monitoring should proceed monthly to ensure process efficiency and compliance.

For Class B requirements, materials containing more than 2 million MPN per gram of total solids are not acceptable. For Class A requirements, materials containing more than 1000 MPN fecal coliforms per gram total solids or 3 MPN *Salmonella* per four grams of total solids are not acceptable as Type A materials. This research has demonstrated the persistence of MS-2 during lime stabilization after which time fecal coliforms are inactivated.

The data presented here in indicate that naturally occurring male-specific bacteriophages are a useful tool for evaluating the effect of lime stabilization of sludges on rotavirus Wa and adenovirus type 5, in the absence of adequate methodology to recover such organisms. Future studies should evaluate the inactivation of male specific

bacteriophage using different treatment strategies in addition to evaluating the resistance of different types of bacteriophage to treatment in an effort to identify the optimal bacteriophage to be used as an indicator for enteric virus. Ultimately, research to assess the rates of pathogen survival in soil or on crops following the land application of biosolids must be conducted.

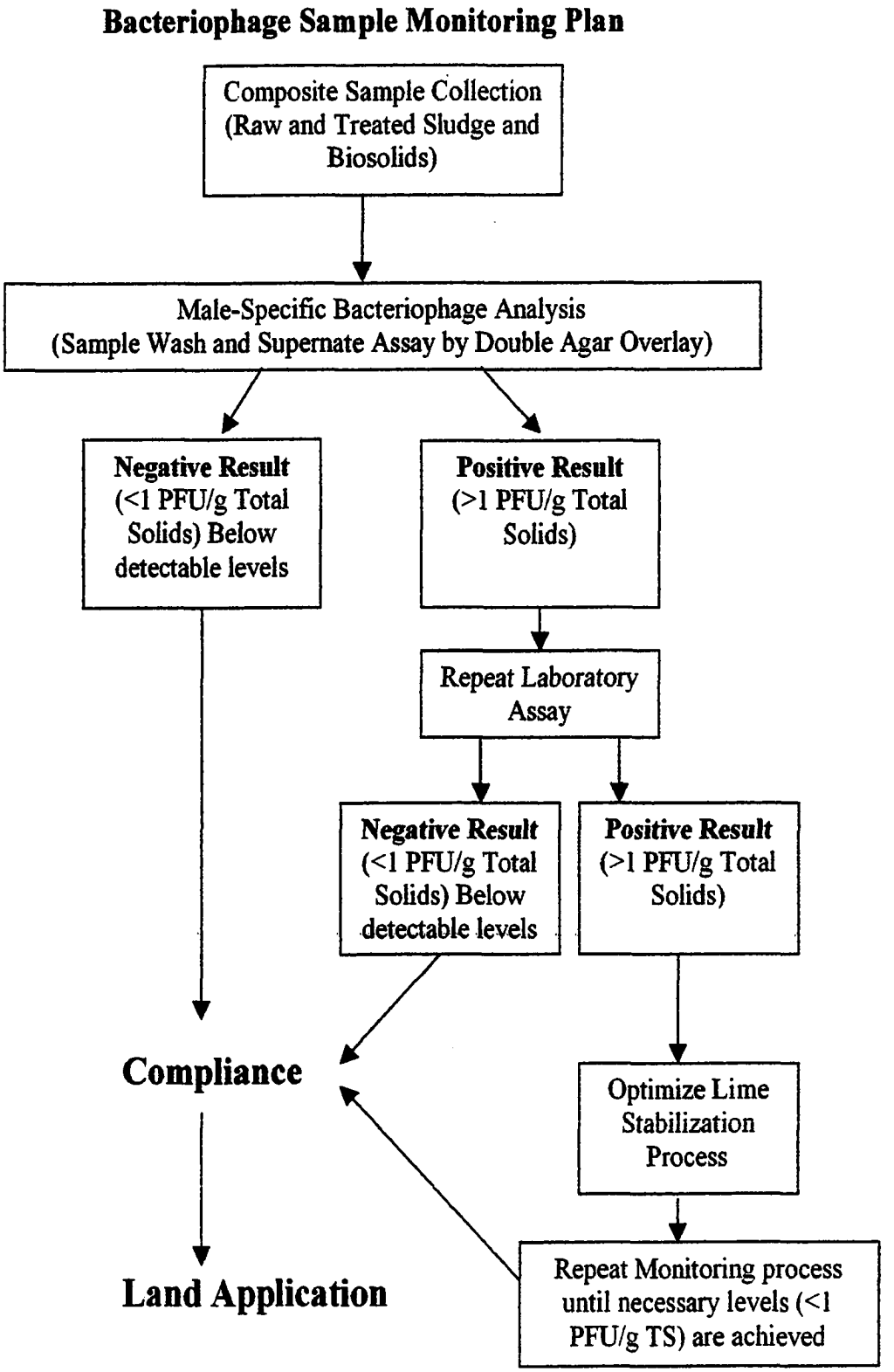

Figure 4: Bacteriophage Sample Monitoring Plan

REFERENCES

Bean, C.L. and J.J. Brabants. 2000. Successful implementation of EPA's protocol for determining the viability of *Ascaris* ova in wastewater solids. Water Environment Laboratory Solutions. 7(6):14-15.

Bean, C.L and J.J. Brabants. 2001. American Society for Microbiology 101st general meeting. May 23, Poster Title: Survey of wastewater solids to assess the prevalence of *Cryptosporidium* and *Giardia* Species and *Ascaris lumbricoides*: Evaluation of risks associated with land application of biosolids. Orlando, FL.

Bean, C.L. and J.J. Brabants. 2000. Lab analyzes *Ascaris* ova recovery rates using revised protocol. Biosolids Technical Bulletin. 7(1):12-14.

Brashear, D.A. and R.L. Ward. 1982. Comparison of methods for recovering indigenous viruses from raw wastewater sludge. Applied and Environmental Microbiology. **43**(6):1413-1418.

Cabelli, V.J. 1982. Microbial indicator systems for assessing water quality. Antonie van Leeuwenhoek. **48**:613-618.

Dore, W.J., K. Henshilwood and D.N. Lees. 2000. Evaluation of f-specific RNA bacteriophage as a candidate human enteric virus indicator for bivalve molluscan shellfish. Applied and Environmental Microbiology. **66**(4):1280-5.

Gantzer, C., A. Maul, J. M. Audic and L. Schwartzbrod. 1998. Detection of infectious enteroviruses, enterovirus genomes, somatic coliphages, and *Bacteroides fragilis* phages in treated wastewater. Applied and Environmental Microbiology. **64**(11):4307-4312.

Goyal, S.M., W.N. Adams, M.L. O'Malley and D.W. Lear. 1984. Human pathogenic viruses as sewage sludge disposal sites in the middle atlantic region. Applied and Environmental Microbiology. **48**(4):758-63.

EPA (United States Environmental Protection Agency). 1999. Environmental regulations and technology: control of pathogens and vector attraction in sewage sludge. Office of Research and Development, Washington, D.C. EPA/625/R-92/013.

Fewtrell, L. and J. Bartram. 2001. Water quality: guidelines, standards and health, assessment of risk and risk management for water related infectious disease. World Health Organization Water Series. International Water Association publishing, London, UK.

Greenberg, A.E., L.S. Clesceri and A.D. Eaton. 1992. Standard methods for the examination of water and wastewater. 18th ed. American Public Health Association, American Waterworks Association and Water Environment Federation. Washington, D.C.

Havelaar, A.H., M. Van Olphen and Y.C. Drost. 1993. F-Specific RNA bacteriophages are adequate model for enteric viruses in fresh water. Applied and Environmental Microbiology. **59**(9):2956-2962.

Havelaar, A.H., W.M. Pot-Hogeboom, K. Furuse, R. Pot and M.P. Hormann. 1990. F-specific RNA bacteriophages and sensitive host srains in feces and wastewater of human and animal origin. Journal of Applied Bacteriology. **69**(1):30-7.

Hurst, C., S. Schaub, M. Sobsey, S. Farrah, C. Gerba, J. Rose, S. Goyal, E. Larkin, R. Sullivan, J. Tierney, R. O'Brian, R. Saffferman, M. Morris, F. Wellings, A. Lewis, G. Berg, P. Britton, and J. Winter. 1991. Multilaboratory evaluation of methods for detecting enteric viruses in soils. Applied and Environmental Microbiology. **57**(2):395-401.

Jenkins, M.B., D.D. Bowman and W. Ghiorse. 1998. Inactivation of *Cryptosporidium parvum* oocysts by ammonia. Applied and Environmental Microbiology. **54**(2):784-788.

Jiang, S., R. Noble and W. Chu. 2001. Human adenoviruses and coliphages in Urban runoff-impacted coastal waters of southern California. Applied and Environmental Microbiology. **67**(1):179-184.

Lasobras, J., J. Dellunde, J. Jofre and F. Lucena.1999. Occurrence and levels of phages proposed as surrogate indicators of enteric viruses in different types of sludges. Journal of Applied Microbiology. **86**:723-729.

LeClerc, H., S. Edberg, V. Pierzo and J.M. Delattre. 2000. Bacteriophages as indicators of enteric viruses and public health risk in groundwaters. Journal of Applied Microbiology. **88**(1):5-21.

Mawdsley, J.L., A.E. Brooks and R.J. Merry. 1996. Movement of the protozoan pathogen *Cryptosporidium parvum* through three contrasting soil types. Biol Fertil Soils. **21**:30-36.

Metcalf, T.G. 1978. Indicators for viruses in natural water. *In* Water Pollution Microbiology. Vol. 2. ed. R. Michel. pp. 124-129. New York: Wiley and Sons.

Monpoeho, S., A. Maul, B. Mignotte-Cadiergues, L. Schwartzbrod, S. Billaudel and V. Ferre. 2001. Best viral elution method available for quantification of enteroviruses in sludge by both cell culture and reverse transcription-PCR. Applied and Environmental Microbiology. **67**(6):2484-8.

APPENDIX A

BUFFERS AND REAGENTS

Aluminum Chloride Solution ($AlCl_3 \cdot 6H_2O$)

Dissolve 12.07 g of aluminum chloride in 500 ml of water and dilute to 1000 ml. Autoclave solution at 121°C, 15 psi for 15 minutes. Store at room temperature.

Buffered Beef Extract Solution

Dissolve 10 g of beef extract powder, 1.34 g of Disodium hydrogen phosphate and 0.12 g of citric acid in 100 ml of water by stirring on a magnetic stirrer. Autoclave at 121°C, 15 psi for 15 minutes. Store at 4°C.

Disodium Hydrogen Phosphate Solution ($Na_2HPO_4 \cdot 7H_2O$)

Dissolve 4 g of disodium hydrogen phosphate in 100 ml of water and autoclave at 121°C for 15 minutes. Store at room temperature.

Magnesium Chloride

Dissolve 1 g magnesium chloride in 99 mL distilled water. Filter sterilize and store at room temperature.

Phosphate Buffered Saline (1X PBS)

Dissolve 9.785 g of phosphate buffered saline powder (Sigma) into 1 Liter with RO water. pH to 7.0 and autoclave for 15 minutes at 121°C, 15 psi. Store at 4°C.

Sodium Hydroxide Solution

Dissolve 4.0 g of dry sodium hydroxide (NaOH) in water and dilute to 100 mL.

Streptomycin/Ampicillin Antibiotic Solution

Dissolve 0.15 g streptomycin and 0.15 g ampicillin in 100 mL distilled water. Filter sterilize and store at 4°C.

APPENDIX B

MEDIA

1X Agar Overlay

Dissolve 15 g tryptic soy broth, 2.5 g sodium chloride, 5 g yeast extract, 0.075 g calcium chloride, and 7.5 g bacto agar in 1 liter of distilled water. Boil to facilitate dissolution. Dispense 5 mL per test tube. Cap and autoclave tubes at 125°C, 15 psi for 20 minutes. Store at room temperature. To melt when needed, alutoclave at 121C, 15 psi for 2 minutes.

Flake Agar (2%)

Dissolve 2 g of flake agar (Difco) in 98 mL RO water. Autoclave for 15 minutes at 121°C, 15 psi and store at 56°C immediately prior to use.

Medium 199 (2X)

1.96 g Medium 199 (Sigma)
0.13 g $NaHCO_3$ (Sigma)
0.95 g hepes (Sigma)
0.06 g L-glutamine (Sigma)
2 mL antibiotic/antimycotic (Gibco)1.2 mL neutral red solution (Sigma)
1 mL 1% $MgCl_2$
Dissolve components in 100 mL of RO water. Filter sterilize and store at 4°C.

Minimal Essential Media/L-15 Growth Medium

4.7 g Eagles Minimal Essential Medium (Sigma)
7.4 g Leibowitz (L-15) Medium (Sigma)
4.22 g hepes (Sigma)
0.292 g L-glutamine (Sigma)
0.75 sodium bicarbonate (Sigma)
10 mL Non-essential amino acids (Gibco)
Dissolve components completely in 1 Liter of RO water and pH solution to 7.2-7.4. Filter sterilize and store at 4°C.

Tryptic Soy Broth

Dissolve 40 g tryptic soy broth in 1 liter of distilled water, stir to dissolve. Autoclave at 121C, 15 psi for 15 minutes and store at room temperature.