SURVIVAL OF ENTERIC BACTERIA AND VIRUSES IN BIOSOLIDS

by

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As members of the Dissertation Committee, we certify that we have read the dissertation prepared by Nohelia Castro del Campo
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DEDICATION

This work is dedicated to my family Juan de Dios and Evangelina, my parents, Nohemi and Karina, my sisters, and Jesus Joel and Juanito, my adorable nephews. Their love, encouragement, and support have always kept me going on and mean everything to me.

I would also like to dedicate this dissertation to a little angel that I am waiting for with all my heart and to Edmundo, for being the special person in my life with whom I will be bringing our angel into this world. All my love to both of you.

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ABSTRACT

In the developed world most of domestic sewage is treated by the activated sludge process, which results in large volumes of sludge or biosolids being produced. This results in millions of tons of biosolids produced each year in the United States, which must either be disposed of or recycled in some manner. Land application is seen as the most economical and beneficial way of handling biosolids. Although the United States Environmental Protection Agency supports and regulates the land application of biosolids, more research is needed to ensure its safe.

The Appendix study A assessed the potential of *Salmonella* regrowth in Class A biosolids pellets and compost after land application. No *Salmonella* growth occurred in any of the soil/biosolids mixtures regardless of inoculum size or moisture content. While regrowth of *Salmonella* in biosolids may occur under saturated conditions it does not occur after Class A biosolid land application at typical agronomic rates. The Appendix B study evaluated the sensitivity of BGM and PLC/PRF/5 cell lines for detection of viruses as well as the occurrence and concentration of adenoviruses. The PLC/PRF/5 cell line was more sensitive for the detection of adenovirus and other enteroviruses in raw and Class B biosolids. The Appendix C study determined the potential of

regrowth of *Salmonella typhimurium* in vermicompost and Class A biosolids alone and after addition to soil. In summary, no regrowth of *Salmonella* was observed, and the die-off of *Salmonella* was not different in soil, biosolids or vermicompost.

INTRODUCTION

Biosolids are a product derived from the treatment of municipal wastewater by the activated sludge process. In 1993, the United States Environmental Protection Agency promulgated the 503 regulations, which established rules for the disposal of sewage sludge or biosolids. Class B biosolids must be treated by "processes which significantly reduce pathogens" (PSRP) and Class A by "processes to further reduce pathogens" (PFRP). Biosolids treated by PSRP may be land applied if certain restrictions are met with regard to crop production animal grazing, and public access to the treated site. There are no restrictions if the biosolid is treated to Class A requirements (EPA, 1999).

As an alternative to disposal by land filling or incineration, land application seeks to beneficially recycle the soil property-enhancing constituents in biosolids (Table 1), which are ultimately derived from crops grown on agricultural land, hence, biosolids are a valuable nutrient resource for agricultural land (Sidhu et al., 2001; Lemunier et al., 2005); and an excellent way of recycling both the nutrients and the organic matter contained in sludge (Sanchez-Monedero et al., 2004; Skanavis, 1994; Coker, 1983).

Land application of biosolids is an issue at the center of a national debate due in part to the possible presence of pathogens (Zaleski et al., 2005). The transfer of pathogens such as *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella enterica* ser. *typhimurium* from biosolid amended soil to fresh agricultural products has been described (Lemunier et al., 2005; Sidhu et al., 2001; Islam et al., 2004; Solomon et al., 2002; Al-Ghazail and Al-Azawi, 1990). The transmission of these pathogens might occur especially where biosolids was used as a fertilizer in those food crops eaten raw (Gerba and Smith, 2005).

Table 1. Projections of Use and Disposal of Biosolids

Year	Beneficial Use (%)				Disposal (%)			
	Land Application	Advanced Treatment	Other benefical use	**Total**	Surface disposal/ landfill	Incineration	Other	**Total**
1998	41	12	7.0	60	17	22	1	40
2000	43	12	7.5	63	14	22	1	37
2005	45	13	8 .0	66	13	20	1	34
2010	48	13	8.5	70	10	19	1	30

EPA, 2000

Biosolids Regulations

In 1993, the U.S. Environmental Protection Agency (EPA) promulgated 40 CFR Part 503 to address the Clean Water Act's (CWA) requirement that EPA develop a regulation for the use or disposal of sewage sludge.

The federal Part 503 rule (40 CFR Part 503) establishes requirements for land applying sewage sludge (including domestic septage) to ensure protection of public health and the environment when sewage sludge is used for its soil conditioning or fertilizing properties. Promulgated in 1993, Part 503 covers sewage sludge sold or given away in bulk, bags, or other containers for application to agricultural land (e.g., cropland, pastures, and rangelands), forests, reclamation sites (e.g., mine spoils, construction sites, and gravel pits), public contact sites (e.g., parks, plant nurseries), and lawns and home gardens.

The pollutant limits in the Part 503 rule were based on in-depth risk assessments that investigated the effects on human health and the environment of using or disposing sewage sludge (EPA, 2001). The pollutant limits and management practices in Part 503 protect human health and the environment, as required by the clean water act (CWA). Another key component of the rule is the operational standard that requires reduction of pathogens (i.e.,

disease-causing organisms) and of vector attraction (e.g., insects, rodents) using specified operational processes (e.g., treatment), microbiological monitoring, and physical barriers (e.g., injection or incorporation) for sewage sludge to achieve this reduction. This operational standard, in the judgment of EPA, protects public health and the environment from pathogens and vectors.

Pathogen Reduction Requirements

The Part 503 pathogen reduction requirements for sewage sludge are divided into two categories: Class A and Class B, as shown in Table 2. The implicit goal of the Class B requirements is to ensure that pathogens have been reduced to levels that are unlikely to pose a threat to public health and the environment under specific use conditions. Site restrictions on the land application of Class B sewage sludge minimize the potential for human and animal contact with the sewage sludge until environmental factors have reduced pathogens to below detectable levels. In addition, to further reduce the likelihood of human contact with pathogens, Class B sewage sludge cannot be sold or given away in a bag or other container for land application.

Table 2. Pathogen Reduction Requirements in Class B Biosolids

Pathogen	**Reduction Value**
Pathogenic bacteria	Reduced in density
Enteric viruses	Reduced in density
Fecal coliforms	<2 million MPN/total solids
Viable Helminth Ova	Not necessarily reduced

The implicit goal of the Class A requirements is to reduce the pathogens in sewage sludge (including *Salmonella* sp. bacteria, enteric viruses, and viable helminth ova) to below detectable levels (Table 3). When this goal is achieved, Class A sewage sludge can be land applied without any pathogen-related restrictions on the site.

Table 3. Pathogen Reduction Requirements in Class A Biosolids

Pathogen	**Reduction Value**
Salmonella spp	Less than 3 MPN/4 g
Enteric viruses	Less than 1 PFU/4 g
Viable Helminth Ova	Less than 1 viable helminth ova/4 g

Class A Pathogen Requirements

Sewage sludge that must meet the Class A pathogen requirements includes sewage sludge that is sold or given away in a bag or other container for application to land and bulk sewage sludge that is applied to a lawn or home garden. Part 503 Subpart D establishes six alternatives shown in Table 4 for demonstrating that sewage sludge meets Class A pathogen reduction requirements.

Table 4. Class A Pathogens Alternatives

Alternative	**Specification**
Thermally treated-sewage sludge	Use one of four time-temperature regimes
Sewage Sludge Treated in a High pH-High Temperature Process	Specifies pH, temperature, and air-drying requirements
Sewage Sludge Treated in Other Processes	Demonstrate that the process can reduce enteric viruses and viable helminth ova. Maintain operating conditions used in the demonstration
Sewage Sludge Treated in Unknown Processes	Demonstration of the process is unnecessary. Instead, test for pathogens at the time the sewage sludge is used or disposed, or when prepared to meet the requirements in 503.10(b), (c), (e), or (f)
Use of Processes to Further Reduce Pathogens (PFRP)	Sewage sludge is treated in one of the processes to further reduce pathogens (PFRP)
Use of a Process Equivalent to PFRP	Sewage sludge is treated in a process equivalent to one of the PFRPs, as determined by the permitting authority

AGRICULTURAL LAND APPLICATION OF BIOSOLIDS

Before the era of wastewater treatment, municipal wastewater was untreated and biosolids did not exist. Pathogen concentrations found in raw sludge are presented in Table 5.

Research on the value of dewatered sludge and straw-sludge composts were carried during and after the Second World War. It was found that these composts could be regarded as slow-release sources of nitrogen for crops, and as sources of phosphorus on phosphorus-deficient soil but there was little enthusiasm for the utilization of sludge at the time, and interest remained at a low level for many years afterwards (Coker, 1983).

Table 5. Concentration Levels of Organisms in Raw Sludge and Septage (Average Geometric Mean of Organisms Per Gram Dry Weight)(USEPA, 1981)

Organism	Primary	Secondary	Mixed	Septage
Total coliform bacteria	1.2×10^{8}	7.1×10^{8}	1.1×10^{9}	1.4×10^{8}
Fecal coliform bacteria	2.0×10^{7}	8.3×10^{6}	1.9×10^{5}	1.2×10^{6}
Fecal streptoccoci	8.9×10^{5}	1.7×10^{6}	3.7×10^{6}	6.6×10^{5}
Bacteriophage	1.3×10^{5}	NR[a]	NR	NR
Salmonella sp	4.1×10^{2}	8.8×10^{2}	2.9×10^{2}	5.1×10^{-1}
Shigella sp	NR	NR	ND[b]	NR
Pseudomonas aeruginosa	2.8×10^{3}	1.1×10^{4}	3.3×10^{3}	2.6×10^{1}
Parasite ova/cyst (total)	2.1×10^{2}	NR	$<5.0 \times 10^{1}$	NR
Ascaris sp	7.2×10^{8}	1.4×10^{8}	2.9×10^{2}	NR
Trichiuris trichiura	1.0×10^{1}	$<1.0 \times 10^{1}$	0	NR
Trichiuris vulpis	1.1×10^{2}	$<1.0 \times 10^{1}$	1.4×10^{2}	NR
Toxocara sp	2.4×10^{2}	2.8×10^{2}	1.3×10^{3}	NR
Hymenolepsis diminuta	$6. \times 10^{0}$	2.0×10^{1}	0	NR
Enteric viruses[c]	3.9×10^{2}	3.2×10^{2}	3.6×10^{2}[d]	NR

[a] NR= No data available
[b] ND = None detected
[c] Plaque = forming units per gram dry weight (PFU/gdw)
[d] $TCID_{50}$ = 50 percent tissue culture infectious dose

Potential disease-causing microorganisms known as pathogens, including bacteria, viruses, protozoa, and eggs of parasitic worms, are often present in municipal wastewater and raw sewage sludge. Pathogens also are present in domestic septage. Pathogens can represent a public health hazard if they are transferred to food crops grown on land to which sewage sludge or domestic septage is applied, contained in runoff to surface waters from land application sites, or transported away from the site by vectors such as insects, rodents, and birds.

Several previous studies have examined the potential for growth of *Salmonella* in class A and B biosolids during storage. Yeager and Ward, (1981) concluded that long-term storage of biosolids, especially at low moisture (less than 20%) is considered to be an effective means of inactivation of bacterial pathogens and preventing regrowth, however Sidhu et al., (2001) found that in the case of reintroduction of *Salmonella*, longer survival times could be expected in stored biosolids as compared to freshly composted biosolids.

Environmental factors affecting microbial regrowth include temperature, soil moisture, indigenous microorganisms, adsorption to soil particles and pH (Song et al., 2005;

Soares and Cardenaz, 1995; Lipson and Stotzky, 1984; Scheuerman et al., 1991; Straub et al., 1992; Ward et al., 1976).

Biosolids class A and B Land Application

Agricultural utilization of biosolids has been practiced since residual solids were first produced 160 year ago. Given the experience with the use of human excrement, sewage, and animal manure on croplands, the application of biosolids to agricultural lands was a logical development. As an early example, municipal sludge from Alliance, Ohio was used as a fertilizer as early as 1907. During the same period, Baltimore, Maryland used domestic septage in agricultural production (NRC, 1996).

Over the past 30 years, there has been an intense and concerted effort of scientific research worldwide to better understand the fate of potential toxic and pathogenic constituents in biosolids when they are applied to agricultural soils. The surge of technical information regarding agricultural application of biosolids led to the development of pollutant loading guidelines by the United States and western European countries. Since the late 1970s and early 1980s source control and industrial wastewater pre-treatment programs were initiated to limit the discharge of industrial constituents into municipal sewers. These

programs resulted in a dramatic reduction of trace elements in wastewater and biosolids. Municipal wastewater biosolids, particularly from industrialized cities, now contain significantly lower levels of trace elements than in earlier decades when much of the research on biosolids application to cropland was conducted.

Nowadays, application of a variety of wastes and biosolids to agricultural land is a widespread practice in many European countries, the UK and the USA (Gerba & Smith, 2005) and its use is regulated by the 503 regulations which are federal regulations established by the U.S (EPA, 1999). Environmental Protection Agency after years of research on the possible risks due to heavy metals, presence of pathogens and some other components of the biosolids has established that specific conditions need to be met before biosolids are land applied (Gaskin et al., 2002). Table 6 shows the pathogen concentration limits in biosolids.

The goal of Class A biosolids requirements is to reduce the pathogens to below detectable levels. Processes to further reduce pathogens (PFRP) such as heat treatment, composting, heat drying, beta ray and gamma ray irradiation, pasteurization and thermophilic aerobic digestion are most commonly used to meet Class A requirements. Class A biosolids are essentially pathogen free with no restrictions

relative to pathogens for land application and usually are sold or distributed in urban areas for gardening, landscaping or turf fertilization (Jacobs and McCreary, 2001).

Table 6. Part 503 Pathogen Density Limits Adapted from USEPA 2000

Part 503 pathogen density limits

Pathogen or indicator	Standard density limits (dry wt.)
Class A	
Salmonella	3 MPN per 4 g total solids
Fecal coliforms	<1000 MPN per g
Enteric viruses	<1 PFU per 4 g total solids
Viable helminth ova	<1 per 4 g total solids
Class B	
Fecal coliform density	<2,000,000 MPN per g total solids

Advantages of Agricultural Land Application

Sewage sludge contains several plant macronutrients, principally N and P, and in most cases, varying amounts of micronutrients such as boron (B), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), and zinc (Zn). The exact ratio of these nutrients will not be that of a well-balanced formulated fertilizer; but the nutrients in sewage sludge can be combined with nutrients from other fertilizers to provide the proper amounts of nutrients needed for crop production.

Sewage sludge can also be a valuable soil conditioner. The addition of organic materials like sewage sludge to a fine-textured clay soil can increase the amount of pore space available for root growth and the entry of water and air into the soil. In coarse-textured sandy soils, organic residues like sewage sludge can increase the water holding capacity of the soil and provide chemical sites for nutrient exchange and adsorption. In some regions of the country, the water added to the soil during sewage sludge application also is a valuable resource.

THE OCCURRENCE OF PATHOGENS IN BIOSOLIDS

Viruses

The relative abundance of adenoviruses in the primary sludge samples has been reported to be a mean of 1,320 $PFU1^{-1}$ (Williams and Hurts, 1988). Some principal pathogens of concern usually found in domestic sewage are shown in Table 7.

More than 100 different types of viruses excreted by humans (Strauch, 1991) may be absorbed on sludge organic matter and thereby protected from inactivation (Chauret, 1999). In addition to human viruses, animal viruses present from birds, dogs may reach sewage systems and then contaminate wastewater, to the detriment of human health (Dumontet et al., 2001).

The concentrations of virus in raw sewage sludge have been estimated to be 10^3 cytopathogenic units (CU) per kg (w.w.), and almost 100% of the sludge samples contained enteric viruses (Dumontet et al., 2001).

Detection of Viruses in Biosolids

The standard method for isolation of human enteric viruses from environmental samples involves the ability of viruses to produce observable cytopathogenic effects (CPE) in animal

cell cultures (Lee and Jeong, 2004). A widely used method is the total culturable virus assay (TCVA) (Fout et al., 1996).

In 1962, a continuous line from African green monkey kidney cells was cloned and designated BGM (Buffalo Green Monkey) by Almen L. Barron (Barron et al., 1970). Studies of the cell line revealed that it was far more sensitive in the isolation of enteroviruses from environmental samples than the cell lines used up until then (Dahling et al., 1974). This resulted in an increase in the use of the BGM cell line throughout the world. However, many laboratories later reported that they had not met with the same success of the cell line's sensitivity (Dahling and Wright, 1986).

The TCVA is optimized for detection and quantification of enteric viruses that can replicate in cell culture, but the assay is labor intensive and time-consuming. Also, some enteric viruses (Tiemessen and Kidd, 1994), such as the noroviruses, cannot be cultured and others, such as rotaviruses and hepatitis A virus, are difficult to cultivate in cell culture systems (Lee and Jeong, 2004).

The PLC/PRF/5 cell line has been reported to be among the most sensitive cell lines used for the detection of enteric viruses from water (Taylor et al., 1997; Grabow et al., 1999; Daemer et al., 1980; Grabow et al. 1992). No studies

have reported its use for enteric virus isolation from biosolids.

Bacteria

Competition between the indigenous microflora in the environment represents what is believed to be a major factor in controlling pathogenic bacteria survival outside of their animal host (Rogers and Smith, 2007).

Research has already shown that fecal bacteria such as *E. coli* are not well-adapted to survive in soil (Vinten et al., 2002; Mawdsley et al., 1995). However, when introduced into the soil and protected within feces, their rate of die-off may be reduced through provision of favorable micro-sites and available nutrients (Oliver et al., 2006).

The investigation of enteric bacterial die-off remains important because of the growing concern over livestock-derived pathogens in agricultural environments reaching watercourses. Using generic *E. coli* as a surrogate for pathogenic strains of these bacteria allow for a determination of a worst-case scenario of pathogenic *E. coli* persistence in the environment. This is because pathogenic strains of *E. coli* have been shown to survive less well in soil compared to nonpathogenic strains (Mubiru et al., 2000; Ogden et al., 2001).

Furthermore some other factors have been known to have an effect on the survival of pathogens such as ammonia (NH_3) which is known to be bactericidal. Therefore, bacteria die-off is enhanced at pH 8.5–9 due to an increase in the NH_3/NH_4 ratio (Venglovsky et al., 2006).

Bujoczek et al., (2001) determined the antimicrobial effects of abiotic parameters, such as pH, free ammonia (NH_3) concentration, temperature, and anoxic containment time on the survival of bacterial pathogenic and indicator organisms such as fecal coliforms, *Salmonella* sp., and bacterial spores of *Clostridium perfringens*. Lime additions at doses much lower than those required for pasteurization (t>30 min at 70°C) proved to be adequate in producing biosolids complying with the US EPA Class A requirements; Fecal coliform and *Salmonella* bacteria were not detected after 1 day and no regrowth was observed at the end of the 6-months of storage. The spores of pathogenic *Clostridium perfringens* bacteria were the least affected. A lime dose of 120 g/kg was the lowest dose effective in killing spores.

The potential of *Salmonella typhimurium* regrowth in Class A biosolid pellets and compost after land application was assessed by Castro-del Campo et al., 2006. Mixtures of sandy loam soil, sandy loam soil plus biosolids, and biosolids were inoculated with two different concentrations and

monitored during a period of 20 days. No *Salmonella* growth occurred in any of the soil/biosolid mixtures regardless of inoculum size or moisture content. No growth occurred in any of the biosolids with a moisture content of 20%. Growth of *Salmonella* did occur in all of the Class A products under saturated conditions. Under all moisture conditions indigenous microflora increased in numbers in the biosolids, soil and biosolid/soil mixtures. In conclusion, these results suggest that while regrowth of *Salmonella* in biosolids may occur under saturated conditions it does not occur after Class A biosolids land application at typical agronomic rates.

Table 7. Some Principal Pathogens of Concern Usually Found in Domestic Sewage (USEPA 1999)

Organism	Disease
Protozoa	
Entamoeba histolytica	Acute enteritis
Balantidium coli	Diarrhea and dysentery
Bacteria	
Salmonella spp.	Salmonellosis, typhoid
Shigella spp.	Bacillary dysentery
Vibrio cholera	Cholera
Escherichia coli	Gastroenteritis
Viruses	
Hepatitis A	Infectious hepatitis
Rotavirus	Acute dysentery with severe diarrhea
Helminthes	
Ascaris lumbricoides	Digestive, nutritional disturbance
Trichuris trichiura	Abdominal pain, diarrhea, anemia
Taenia saginata	Insomnia, anorexia, nervouness

PRESENT STUDY

The methods, results and conclusions of this study are presented in the papers appended to this dissertation. The following is a summary of the most important findings in these documents.

The manuscript "Assessment of *Salmonella typhimurium* Growth in Class A Biosolids and Soil/Biosolid Mixtures" in Appendix A was published in the *Journal of Residual Science and Technology* (2007) 4(2):83-88. The potential of *Salmonella typhimurium* regrowth in Class A biosolid pellets and compost after land application was assessed. Mixtures of soil, soil plus biosolids, and biosolids were inoculated with two different concentrations and monitored during a period of 20 days. No *Salmonella* growth occurred in any of the soil/biosolid mixtures regardless of inoculum size or moisture content. No growth occurred in any of the biosolids with a moisture content of 20% except the pellets from Texas when inoculated with 10,000 colony forming units/g. Growth of *Salmonella* did occur in all of the Class A products under saturated conditions. Under all moisture conditions indigenous microflora increased in numbers in the biosolids, soil and biosolid/soil mixtures. In conclusion, these results suggest that while regrowth of *Salmonella* in

biosolids may occur under saturated conditions it does not occur after Class A biosolids land application at typical agronomic rates.

The manuscript “Comparative Assessment of PLC/PRF/5 and BGM Cells for Virus Detection in Biosolids”. The use of the BGM cell line in combination with other cell lines for the surveillance of enteric viruses in water has been reported to increase the sensitivity of the Total Culturable Viral Assay (TCVA) for the detection of enteroviruses. The objective of this study was to evaluate the BGM and PLC/PRF/5 cell lines for TCVA in raw sludge and Class B biosolids. A secondary objective was to assess the occurrence and concentration of adenoviruses. In our study the PLC/PRF/5 cell line was more sensitive than the BGM cell line for the detection of culturable adenoviruses and other enteroviruses in raw and Class B biosolids, obtaining a percentage of 83% (PLC) in contrast with a 50% (BGM). In addition, CPE was observed earlier on PLC/PRF/5 cells than on BGM cells. Adenoviruses were detected in a 66% of the samples at concentrations ranging from o.95 in Class B biosolids to 381 MPN/4 g in raw sludge. Most of the viruses detected in the biosolids were adenoviruses.

The manuscript "Comparison of *Salmonella typhimurium* Survival in Agricultural Soil Amended with Vermicompost and Class A Biosolid". Vermicompost produced from cattle manure residues by the earthworm *Eisenia fetida* and biosolids from wastewater treatment are being utilized as organic fertilizers. Agricultural application of vermicomposts and/or biosolids might be contaminated with *Salmonella*. The objective of this study was to determine potential regrowth of *Salmonella typhimurium* in vermicompost and Class A biosolids alone and after addition to soil. Mixtures of soil, soil plus vermicompost or biosolids at a moisture content of 30% were inoculated with four different concentrations and monitored during a period of 20 days. In agricultural soil, and soil amended with either Class A biosolids or vermicompost *Salmonella* was able to survive for the 20 days, not presenting statistically differences. In the absence of soil, *Salmonella* declined rapidly, surviving less than 10 days in Class A biosolids and 15 in vermicompost. In summary, no regrowth of *Salmonella* was observed, and the die-off of *Salmonella* was not different in soil and soil amended with both fertilizers, but it did in biosolids or vermicompost.

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APPENDIX A:

ASSESSMENT OF *SALMONELLA TYPHIMURIUM* GROWTH IN CLASS A BIOSOLIDS AND SOIL/BIOSOLID MIXTURES[1]

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ABSTRACT

The potential of *Salmonella typhimurium* regrowth in Class A biosolid pellets and compost after land application was assessed. Mixtures of soil, soil plus biosolids, and biosolids were inoculated with two different concentrations and monitored during a period of 20 days. No *Salmonella* growth occurred in any of the soil/biosolid mixtures regardless of inoculum size or moisture content. No growth occurred in any of the biosolids with a moisture content of 20% except the pellets from Texas when inoculated with 10,000 colony forming units/g. Growth of *Salmonella* did occur in all of the Class A products under saturated conditions. Under all moisture conditions indigenous microflora increased in numbers in the biosolids, soil and biosolid/soil mixtures. In conclusion, these results suggest that while regrowth of *Salmonella* in biosolids may occur under saturated conditions it does not occur after Class A biosolids land application at typical agronomic rates.

INTRODUCTION

Biosolids are the end- product derived from the treatment of municipal wastewater. In 1993, the United States Environmental Protection Agency promulgated the Federal 503 regulations, which established rules for the disposal of specific types of sewage sludge or biosolids. Class B biosolids are produced by "processes which significantly reduce pathogens" (PSRP). In contrast, Class A biosolids only arise following "processes to further reduce pathogens" (PFRP). Biosolids treated by PSRP may be land applied if certain restrictions are met with regard to crop production, animal grazing, and public access to the treated site. There are no restrictions if Class A biosolids are land applied (Gerba et al., 2002; Gerba and Smith, 2005).

As an alternative to disposal by land filling or incineration, land application allows for the beneficial reuse of biosolids. Land application of biosolids provides valuable nutrients for crop production (Sidhu et al., 2001; Lemunier et al., 2005); and increases soil organic matter (Sanchez-Monedero et al., 2004; Skanavis, 1994; Coker, 1983).

However, Class B biosolids contain pathogens with the potential for growth or regrowth (Zaleski et al., 2005b). In addition, the transfer of pathogens such as *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella enterica* ser. typhimurium from biosolid amended soil to agricultural products has been documented (Lemunier et al., 2005; Sidhu et al., 2001; Islam et al., 2004; Solomon et al., 2002; Al-Ghazail and Al-Azawi, 1990).

Several previous studies have examined the potential for growth of *Salmonella* in Class A and B biosolids during storage. Yeager and Ward, (1981) concluded that long-term storage of raw sewage sludge, especially at low moisture (less than 20%) was an effective means of inactivation of bacterial pathogens and also prevented regrowth. However, Sidhu et al., (2001) found that following reintroduction of *Salmonella,* longer survival times occurred in stored biosolids as compared to freshly composted biosolids.

Environmental factors affecting microbial survival or regrowth include: temperature; soil moisture; indigenous microorganisms; adsorption to soil particles; and pH (Song et al., 2005; Soares and Cardenaz, 1995; Lipson and Stotzky, 1984; Scheuerman et al., 1991; Straub et al., 1992; Ward et al., 1976; Pepper et al., 2006).

The goal of this study was to assess the potential for *Salmonella typhimurium* growth or regrowth in Class A biosolids before and after addition to an agricultural soil.

MATERIAL AND METHODS

Percent solids and moisture content

Moisture content of the soils, biosolids amended soil and biosolids were determined by drying moist samples at 102°C for 24 hours. Once the moisture content was determined, sterile reverse osmosis treated water was added to adjust the moisture content to either 20% moisture content or saturated conditions.

Soil

A sandy loam soil (64% sand, 26% silt and 10% clay) was collected from the Campus Agricultural Center at the University of Arizona, Tucson, AZ. The soil was sieved (2-mm mesh) and after determining the moisture content it was brought to field capacity (20% moisture content) with sterile water.

Biosolids

Milorganite® Class A pellets produced in Milwaukee, WI and Kellogg® composted Class A biosolids produced in California, were obtained from commercial sources. Class A pasteurized pelleted biosolids were obtained from a wastewater treatment plant located in Houston, Texas. For studies involving soil amended with biosolids, a biosolids addition equivalent to 3 tons per acre was utilized.

Inoculum

Salmonella typhimurium (ATCC strain 23564) was grown in tryptic soy broth (TSB; Difco, Sparks, MD) overnight at 37°C on a shaker and harvested by centrifugation at 13,080 x *g* for 10 min. The pellet was washed three times in 0.1 M phosphate buffer saline (PBS). Appropriate 10-fold dilutions in PBS were made to enumerate the bacterial cell density. *Salmonella typhimurium* was inoculated into the soil/biosolids at a concentration of either one or four $\log_{10}$ cfu/g dry weight.

Regrowth potential assessment

Inoculated soil, biosolid amended soil or biosolid alone were added to 50 mL sterile centrifuge tubes. Moisture contents were adjusted by addition of sterile water, and

subsequently inoculated at room temperature (24°C) for set time periods. Duplicate tubes of each mixture were analyzed after 0, 1, 3, 5, 10, 15 and 20 days.

Salmonella assay

Salmonella typhimurium was quantified using the spread plate technique on Enteric Hektoen Agar (Difco, Sparks, MD). Appropriate dilutions of samples in Tris buffered saline (Sigma Chemical, St. Louis) were plated and incubated at 37°C for 24 hours. *Salmonella* Latex Test (Oxoid, Basingstoke, United Kingdom) was used to confirm presumptive *Salmonella* colonies.

Assay of indigenous microflora

Soil and biosolid mixtures at 20% moisture content or saturated conditions were used as controls to monitor heterotrophic plate count (HPC) bacteria, and assess any changes in the indigenous microflora during the course of experiments. HPC numbers and controls were analyzed throughout the 20-day incubations. HPC bacteria were assayed using a spread plate technique on R2A agar (Difco, Co., Detroit, MI). All soil and biosolid samples were also tested for the presence of indigenous *Salmonella* by assay on Enteric Hektoen Agar (Difco, Sparks, MD). Plates of the

appropriate dilutions were incubated at 27°C for 7-10 days before bacterial colonies were counted.

No indigenous *Salmonella* was detected in any of the soil/biosolid samples used in this study. Experimental conditions used in this study are shown in Table 1. Moisture contents were kept constant during the 20 day incubation periods at 37°C.

RESULTS

Growth of Salmonella in Milorganite®/Soil Treatments at 20% Moisture Content

No growth of *Salmonella* was detected when an inoculum of 10 CFU/g was initially added to biosolids or soil and incubated for 20 days. When a 10,000 CFU/g was added to the study mixtures, the concentration remained largely unchanged in soil or soil amended with Milorganite® during 20 days of incubation. In contrast the level of *Salmonella* decreased 3 $\log_{10}$ after one day and declined to undetectable numbers after day 20 in the Milorganite® alone (Fig 1).

Indigenous microflora in Milorganite® treatments increased in concentration by day 3 but changed little during the

remaining 20 days of incubation (Table 2). A greater increase in HPC bacteria was observed in Milorganite® followed by soil/Milorganite® and soil.

Growth of Salmonella in Class A Texas pellets at 20% moisture content

Salmonella did grow following inoculation of Houston pelleted biosolids with 10 CFU/g of *Salmonella*. The concentration of *S. typhimurium* reached 2 $\log_{10}$ over the initial inoculum on day 1 (5.6×10^3 CFU/dry g) and day 3 (5.9×10^3 CFU/dry g) and after day 5 it declined to undetectable levels and remained there for the rest of the experiment. *Salmonella* was undetected after it was inoculated in soil and soil plus pellets after one day (Fig 2). For soil alone or soil amended with the pellets, no *Salmonella* were detected at any point in the incubation.

Following an initial inoculum of 4 log/g, neither growth nor regrowth of *S. typhimurium* was observed in any of the soil, biosolids, or mixtures of soil and biosolids. The concentration of *Salmonella* decreased over time after inoculation. *Salmonella* concentrations declined more rapidly in the pellets alone followed by soil and biosolids mixtures. In soil alone, *Salmonella* concentrations declined

less rapidly (Fig 3). In this study, the numbers of indigenous HPC bacteria increased in all treatments from about 10^7 to almost 10^{10}/g of soil (Fig 4).

Milorganite® treatments under saturated conditions

S. typhimurium was observed to grow in Milorganite® alone, but not soil or soil + Milorganite® when the mixtures where saturated and when inoculated with 10 CFU/g (Fig 5). No growth occurred in soil or soil with Milorganite®. The number of *Salmonella* continued to increase in Milorganite® alone throughout the experiment reaching almost 10^7 CFU/g. Growth was also observed in Milorganite®, when inoculated with 10^4 CFU/g, reaching a peak concentration on day five (10^8 CFU/g) and declining slowly to a concentration of approximately 10^6 CFU/g by day 20 (Fig 6). In soil or soil + Milorganite® treatments, *Salmonella* numbers decreased throughout the incubation period. Indigenous microflora numbers remained relatively constant throughout this experiment (Fig 7).

Class A Compost treatments at saturated conditions

In Class A compost, *Salmonella* increased from 10 CFU/g to almost 10^4 following one day of incubation, then slowly declined to approximately 10^3/g after 15 days. In soil or soil + compost, *Salmonella* declined to undetectable levels within 5 days (Fig 8). *Salmonella* in all three mixtures decreased over time after day 0 when inoculated with $1x10^4$

CFU/g (Fig 9). Indigenous microflora levels remained relatively constant throughout this experiment (Fig 10).

DISCUSSION

Growth of *Salmonella* introduced into biosolids was only observed under saturated moisture conditions. This is consistent with other cited studies. For example Burge et al. (1987) observed that growth of *Salmonella* in Class B biosolids required moisture content greater than 20%. This also agrees with the earlier study of Thomason et al. (1975) who detected the growth of *Salmonella* within wet environments. More recently Zaleski et al., (2005a) documented the regrowth and recolonization of *Salmonella* in Class B biosolids. Specifically, Class B biosolids were allowed to desiccate in drying beds and attained Class A levels with respect to *Salmonella* after 4 weeks. However, following rainfall events, which resulted in the saturation of the biosolids, significant increases in *Salmonella* numbers were observed. Since *Salmonella* are facultative anaerobes, it is possible that they become more competitive than other biosolid-borne organisms under saturated conditions where oxygen is limiting. In the saturated drying beds, *Salmonella* increased by over four orders of magnitude. Based on these data, and this current study,

Salmonella growth appears to occur under saturated conditions and care should be taken to ensure that stored biosolids are maintained under aerobic conditions.

In contrast to biosolids, no growth was observed when biosolids were added to soil at typical agronomic rates of 3 tons per acre. Growth did not occur in this biosolid-amended soil regardless of the size of the *Salmonella* inoculant added, or the moisture content of the soil including saturated conditions. These data also agree with those of Zaleski et al. (2005a) who similarly found that *Salmonella* would not grow in Class B biosolid-amended soil, even when saturated. Zaleski et al. (2005b) suggested that growth of *Salmonella* in Class B biosolid-amended soil is inhibited by competition with indigenous soil microflora even under saturated conditions. Sidhu et al. (2001) also demonstrated the role of indigenous microflora in inhibiting the growth of *Salmonella*.

The results of this current study and earlier studies have implications with respect to the potential for growth or regrowth of *Salmonella* in biosolids. First, growth or regrowth is far more likely to occur in Class A biosolids than Class B, particularly in composted material which results in very low background levels of indigenous microflora. However, note that Milorganite® Class A pellets

appeared to be inhibitory to *Salmonella*. Secondly, growth is encouraged under saturated moisture conditions, which result in an anaerobic redox potential reducing competition for the facultative anaerobic *Salmonella*. Thirdly, growth is far less likely once biosolids are incorporated into soil, most likely due to indigenous microbial competition and lower concentration of organic matter. Based on these factors, care should be taken when biosolids, particularly Class A, are stored prior to land application. In contrast, once land applied, biosolids are unlikely to result in growth of *Salmonella*.

SUMMARY

The results of this study suggest that while growth or regrowth may occur in Class A biosolids under saturated conditions, it does not occur after Class A biosolids have been added to soil at typical agronomic rates. Data also suggest that biosolids stored prior to land application should be covered to preclude saturated moisture conditions after rainfall events, and also prevent re-inoculation via bird or animal wastes.

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Table 1. Experimental conditions

Mixture	pH	Moisture content (%)
Soil	7.5	20
Soil + Milorganite®*	7.5	20
Milorganite®	6.0	20
Soil	7.5	Saturated
Soil + Milorganite®	7.5	Saturated
Milorganite®	6.0	Saturated
Soil	7.5	20
Soil + Class A Texas	7.5	20
Class A pellets	7.5	20
Soil	7.5	Saturated
Soil + Class A	7.5	Saturated
Class A compost	7.5-8.0	Saturated

*Class A pellet

Table 2. Levels of indigenous microflora at 20% moisture content in Milorganite®/soil treatments

Mixture	Days						
	0	**1**	**3**	**5**	**10**	**15**	**20**
Soil	$6.0x10^{8}$	$6.0x10^{8}$	$7.2x10^{8}$	$7.7x10^{8}$	$4.2x10^{8}$	$4.2x10^{8}$	$3.7x10^{8}$
Soil+ Milorganite®	$2.7x10^{8}$	$6.0x10^{7}$	$1.2x10^{8}$	$9.6x10^{8}$	$7.0x10^{8}$	$7.7x10^{8}$	$6.0x10^{8}$
Milorganite®	$5.0x10^{8}$	$4.9x10^{8}$	$2.0x10^{9}$	$2.0x10^{9}$	$2.0x10^{9}$	$3.2x10^{9}$	$5.2x10^{9}$

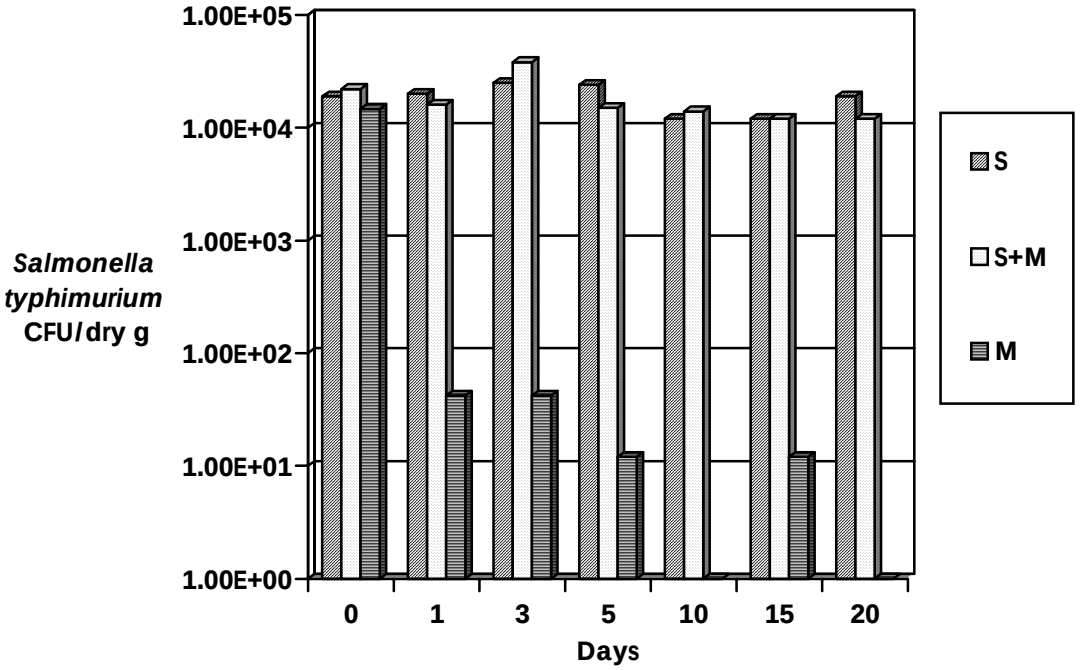

Figure 1. *Salmonella typhimurium* growth following an initial inoculum of 4 log CFU/g with 20% moisture content in Milorganite® treatments

S= Soil

S + M= Soil + Milorganite®

M= Milorganite®

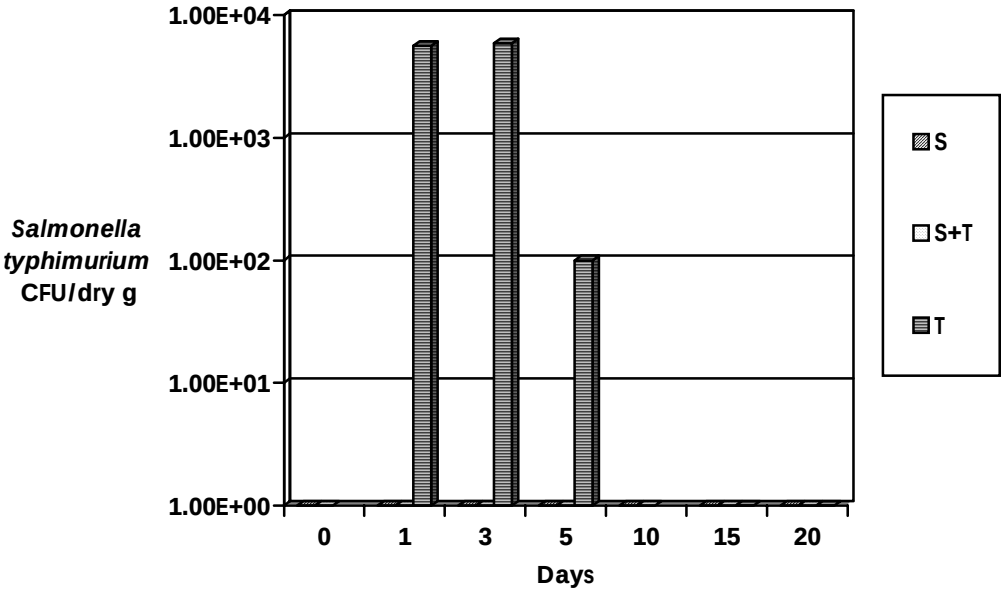

Figure 2. *Salmonella typhimurium* growth following an initial inoculum of 10 CFU/g with 20% moisture content in Texas pellets treatments.

S= Soil

S + T= Soil + Texas pellets

T= Texas pellets

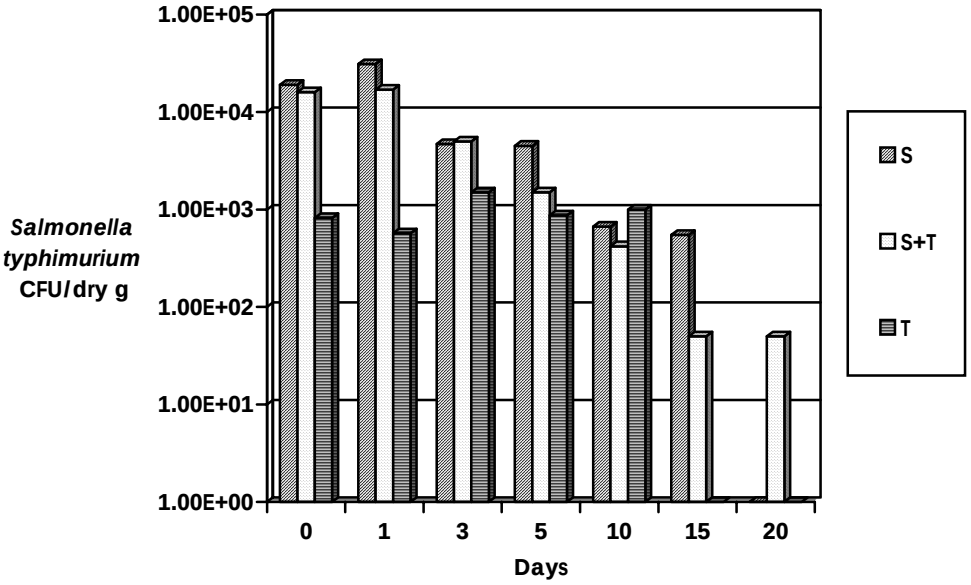

Figure 3. *Salmonella typhimurium* growth following an initial inoculum of 4 $\log_{10}$ CFU/g with 20% moisture content in soil or Texas pellets.

S= Soil

S + T= Soil + Texas pellets

T= Texas pellets

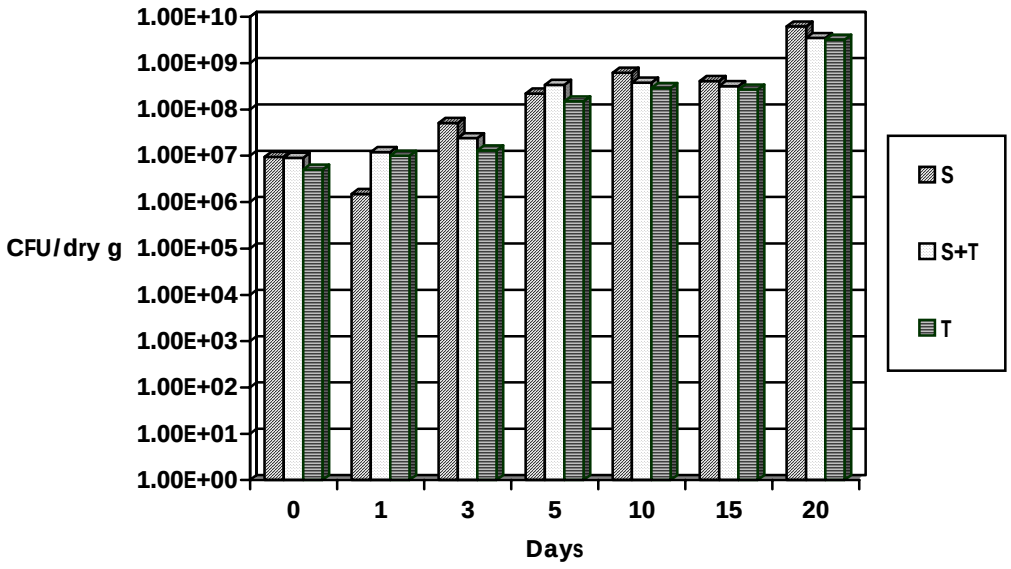

Figure 4. Indigenous microflora levels with 20% moisture content in soil or Texas pellets

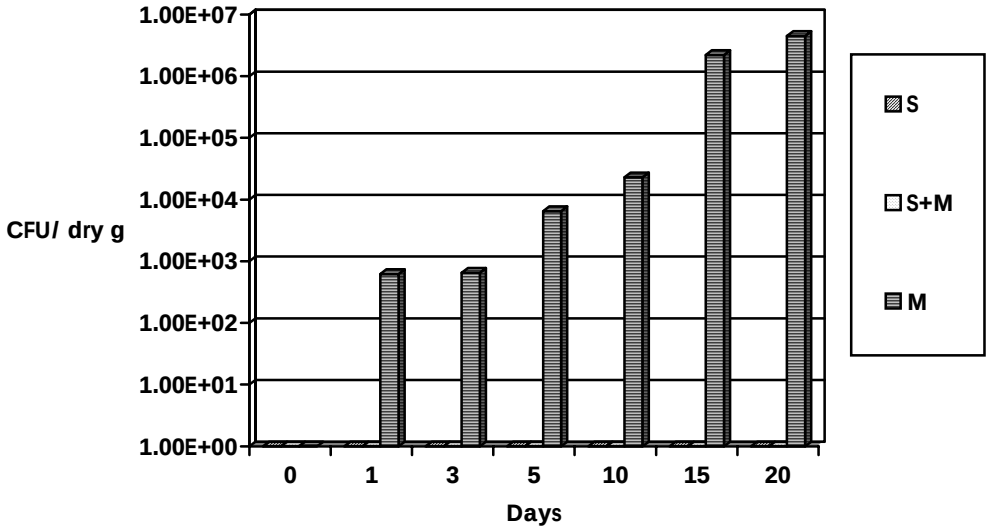

Figure 5. *Salmonella typhimurium* growth in saturated Milorganite® treatments following an initial inoculum of 10 CFU/g of *Salmonella*

S= Soil

S + M= Soil + Milorganite®

M= Milorganite®

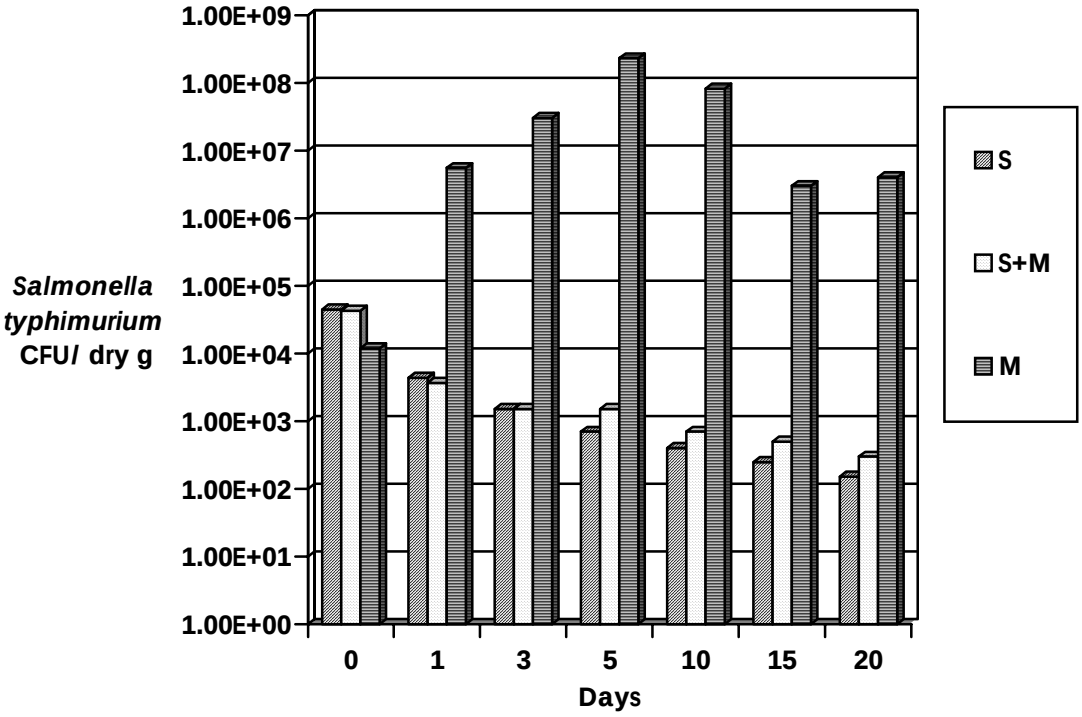

Figure 6. *Salmonella typhimurium* growth in saturated MIlorganite® treatments following an initial inoculum of 4 $\log_{10}$ CFU/g of *Salmonella*

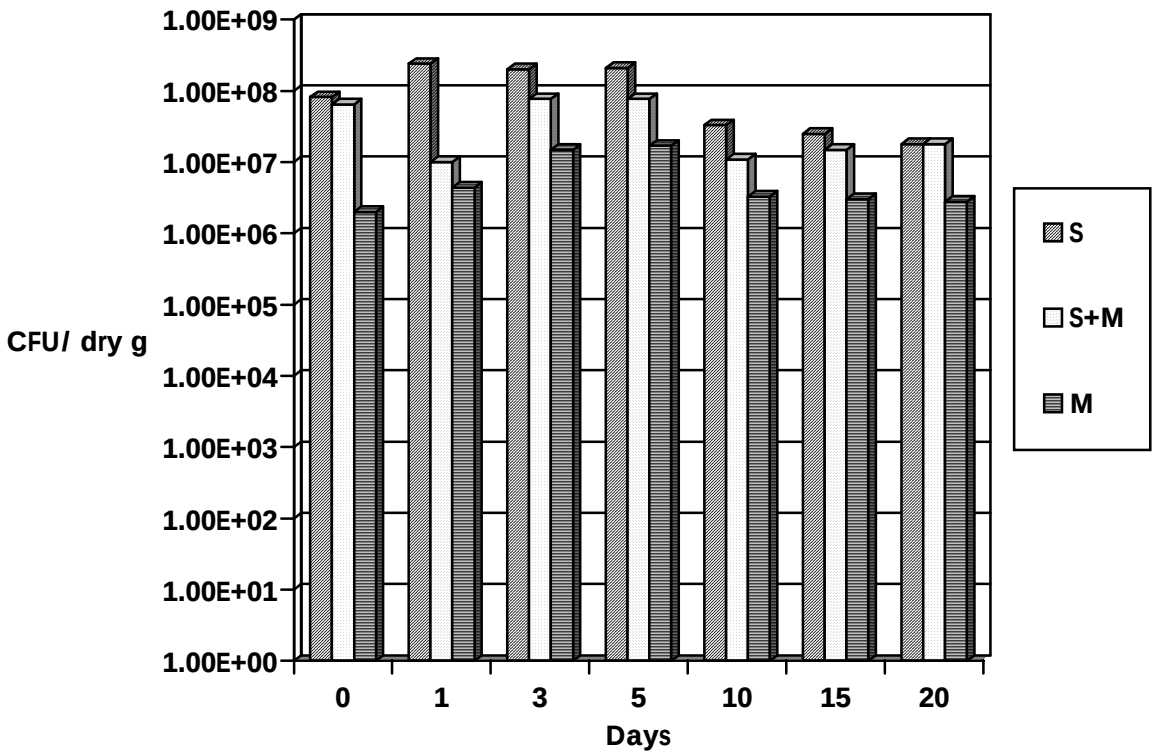

Figure 7. Indigenous microflora levels under saturated conditions in Milorganite® treatments

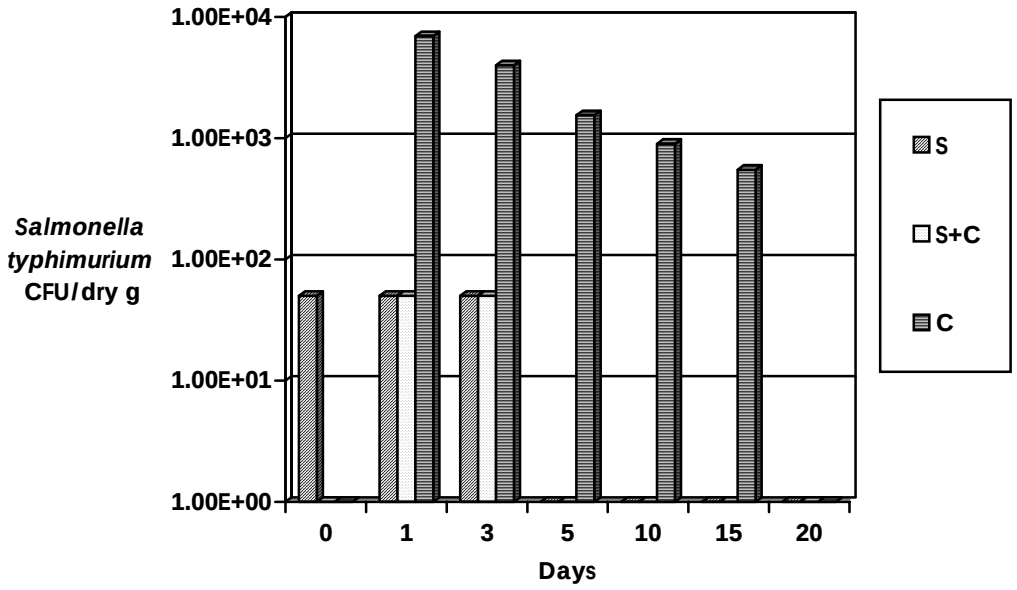

Figure 8. *Salmonella typhimurium* growth in saturated Class A compost treatments following an initial inoculum of 10 CFU/g of *Salmonella*

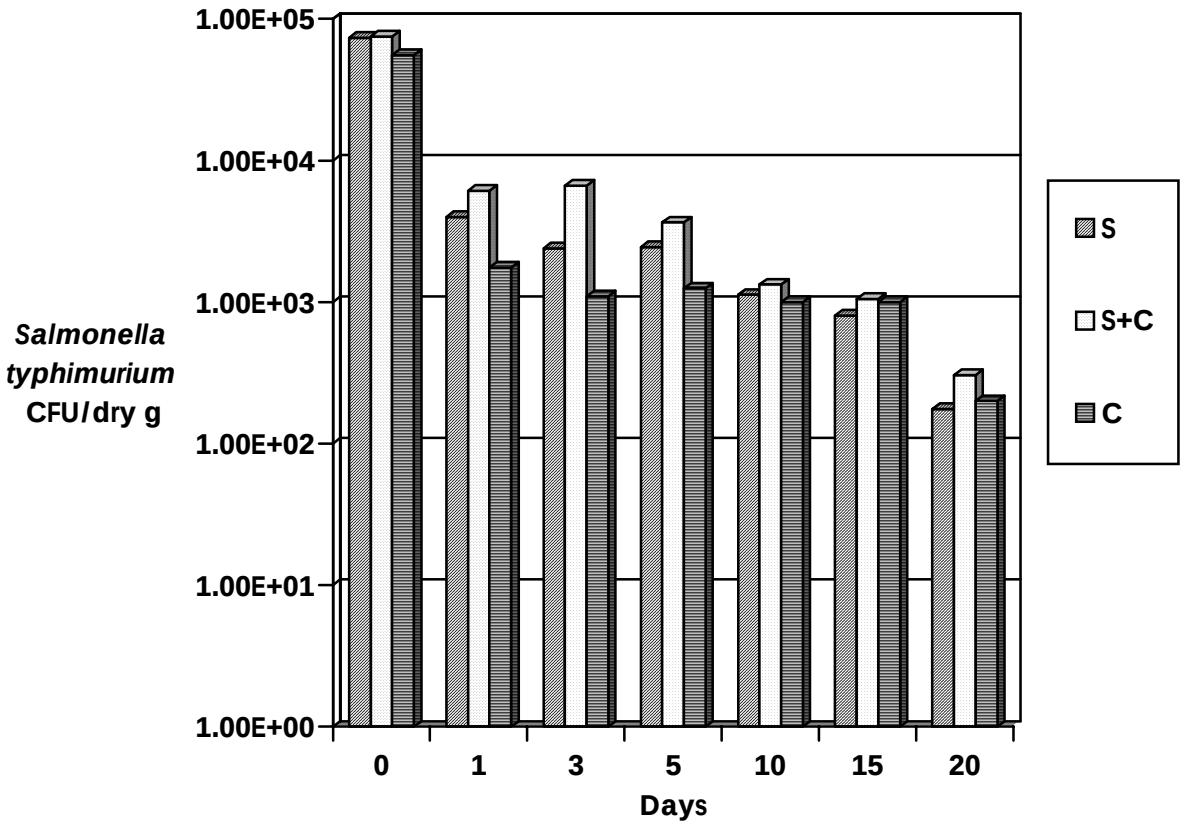

Figure 9. *Salmonella typhimurium* growth in saturated Class A compost treatments following an initial inoculum of 4 $\log_{10}$ CFU/g of *Salmonella*

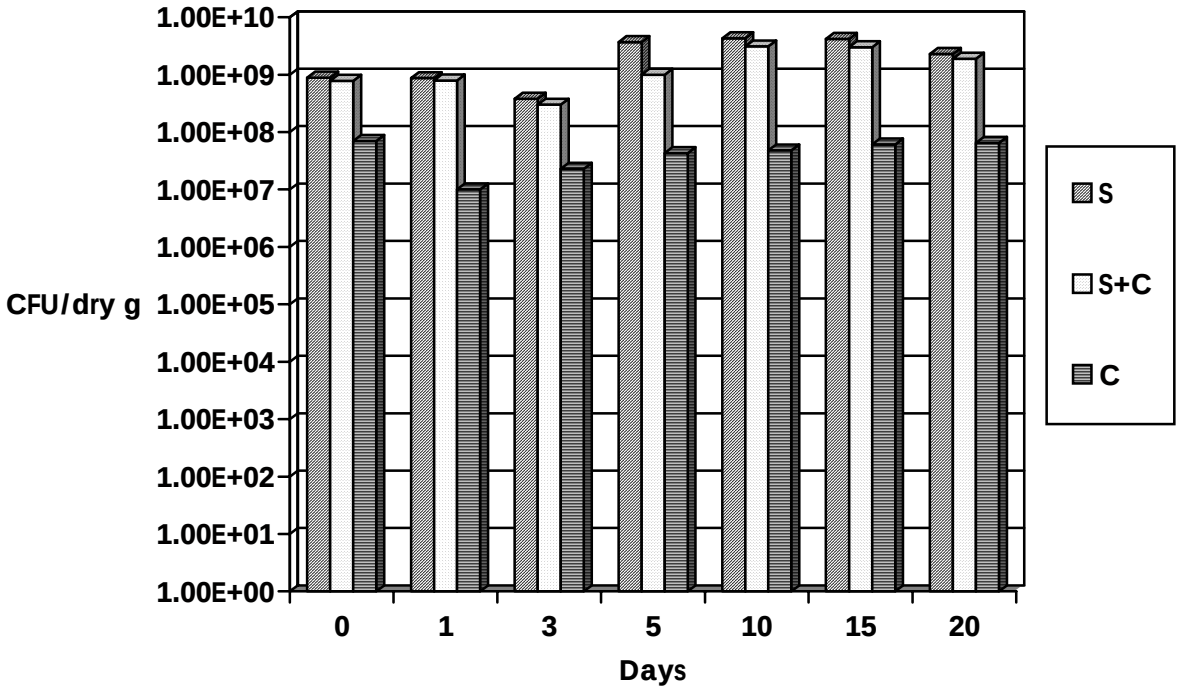

Figure 10. Indigenous microflora levels under saturated conditions in Class A compost treatments

APPENDIX B:

COMPARATIVE ASSESSMENT OF PLC/PRC/5 AND BGM CELL LINES FOR VIRUS DETECTION IN BIOSOLIDS

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Key Words: Adenovirus, PLC/PRF/5 cell line, BGM cell line.

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ABSTRACT

The use of the BGM cell line in combination with other cell lines for the surveillance of enteric viruses in water has been reported to increase the sensitivity of the Total Culturable Viral Assay (TCVA) for the detection of enteric viruses. The objective of this study was to evaluate the BGM and PLC/PRF/5 cell lines for TCVA in raw sludge and Class B biosolids. A secondary objective was to assess the occurrence and concentration of adenoviruses in these samples. In our study, the PLC/PRF/5 cell line detected greater numbers of culturable adenoviruses and other enteroviruses than the BGM cell line in raw and Class B biosolids. Viruses were detected in 75% of the samples using PLC/PRF/5 cells and 50% using BGM cells. In addition, CPE was observed earlier with the PLC/PRF/5 cells than with the BGM cells. Adenoviruses were detected in 66% of the samples with means ranging from 0.95 MPN/4 g in Class B biosolids to 381 MPN/4 g in raw sludge. Most of the viruses detected in biosolids were adenoviruses.

INTRODUCTION

In the United States, biosolids are classified as either Class A or B depending on the concentration of enteric pathogens (EPA, 1999). Class B biosolids potentially contain detectable levels of enteric viruses, while Class A biosolids must contain less than one plaque forming unit/4 grams of total culturable virus using the BGM cell line (EPA, 2001). The most common method to obtain Class B biosolids is mesophilic anaerobic digestion, a process of treating biosolids in the absence of air for at least 15 days at 35˚C.

Human enteric viruses are normally present in Class B biosolids at concentrations less than 0.25 most probable number (MPN) per 4 g (Chetochine et al., 2006). Detection of infectious enteroviruses currently requires the use of animal cell culture. For greater than 20 years, the BGM cell line has been the recommended cell line for the assay of enteric viruses in water and biosolids (EPA, 2001). Use of this cell line allows for the detection of enteroviruses and is especially sensitive for the Coxsackie B viruses (Grabow et al., 1999). However, in recent years, other cell lines including CaCo-2 and PLC/PRF/5 have been shown to isolate greater numbers of enteric viruses from water (Chapron et

al., 2000; Lee et al., 2004; Sedmak et al., 2005; Vivier et al., 2004). The PLC/PRF/5 cell line in particular is useful for the detection of enteric adenoviruses which have been identified as a cause of recreational and drinking water outbreaks (Shenk, 1996; van Heerden et al., 2005a; van Heerden et al., 2005b; Vivier et al., 2004).

The objective of this study was to assess the occurrence of adenoviruses in biosolids and to compare the BGM and PLC/PRF/5 cell lines for total culturable virus assay (TCVA) of raw sludge and Class B biosolids.

MATERIALS AND METHODS

Sample Processing

The type, treatment method and sources of biosolids used in this study are shown in Table 1. Twelve samples (7 Class B biosolids, 5 raw sludge) were concentrated by organic flocculation as described previously (EPA, 1984) to a final volume of 30 mL. The efficiency of the organic flocculation was determined using poliovirus type 1 (LSc-2ab)(courtesy of the Department of Virology and Epidemiology, Baylor College of Medicine, Houston, TX) and averaged 2%.

The concentrate of each sample was divided in a different number of replicates according to the dilutions needed for each and then split into sub-samples of 2.5 mL and inoculated in 75 cm^2 flasks containing cell monolayers; sub-samples of one mL and 0.1 mL were inoculated in 25 cm^2 flasks. The PLC/PRF/5 cell line (American Type Culture Collection, Manassas, VA; ATCC #CRL-8024) and BGM cell line (USEPA, Cincinnati, OH) used in this study were passage 59 to 85. The cells were 3 to 5 days old with a confluent monolayer at the time of infection. Inoculated flasks were incubated at 37°C with slow agitation using a platform shaker (Gyratory Shaker-Model G2, New Brunswick Scientific Co. Inc. Edison, N.J. USA) for two hours. The concentrate was then removed, the flasks covered with Eagles maintenance media (Gibco™ Invitrogen Corporation, Grand Island, NY) containing 2% fetal bovine serum (Hyclone, Logan, UT) and incubated for 14 days at 37°C. The flasks were checked every day for viral cytopathogenic effects (CPE). The media was changed every four days. Flasks showing signs of CPE were frozen at -20°C and thawed twice in order to destroy the cells and release the viruses. This solution was then filtered through a 0.22 μm pore size membrane filter and inoculated onto flasks containing fresh cell monolayers (3-5 days old) to confirm viral CPE. After 14 days of incubation, negative flasks were frozen and thawed twice and

one mL of the supernatant was assayed on monolayers (3-5 days old) in 25 cm^2 flasks. This second passage was monitored for 14 days for CPE. Samples were passed up to three times. All CPE positive flasks were confirmed as described previously. The viral MPN/L was determined using the MPN General Purpose Program by Hurley and Roscoe (1983).

Identification of Adenoviruses by Polymerase Chain Reaction (PCR)

Adenovirus was detected by PCR (Van Heerden et al. 2003) using the primers for adenovirus developed by Avellón et al. (2001) (Table 2). The PCR mixture consisted of the following: 1X PCR buffer, 2.5 mM $MgCl_2$, 200 μM of each dNTP, 1 μM of each primer, 1 unit GOLD TAQ polymerase, 10 μL sample, water (total volume of 50 μL). The conditions for the PCR and the nested PCR were: 5 minutes at 94°C, then 35 cycles of 30 s at 94°C, 30 s at 50°C, 30 s at 72°C, followed by a final extension of 10 min at 72°C.

Quality control assurance

The cell culture facility and incubator are physically separated from the PCR facility. Two separate PCR workstations with no airflow and UV light were used for the

reagent preparation and one biological type II hood was used for processing the samples. Reagents and samples were stored in separate rooms to prevent cross contamination (PCR QA/QC protocol). The workstations were cleaned with a 10% bleach solution and exposed to UV light for at least 20 min. Equipment was designated for sample vs. reagent processing (i.e. pipets, tips and lab coats were used only in one specific room). The PCR thermocyclers are in another separate room outside of the work area. The tubes containing the PCR product were only opened in the workstation designated for samples and in the room for electrophoresis.

RESULTS

Nine of the 12 samples (75%) demonstrated positive CPE for viruses on the primary liver carcinoma (PLC/PRF/5) cells, whereas only six out of 12 samples (50%) were positive on the BGM cell (Table 3). Even though the difference in the detection of viruses between these two cell lines was evident, there was no statistical difference between the numbers of viruses detected between the two cell lines (P = 0.06). Testing of a greater number of samples may be necessary to demonstrate a statistical difference between these two cell lines.

Viruses were detected in all five raw sludge samples on the BGM cells and in 80% of the samples on the PLC/PRF/5 cells. Of the Class B biosolid samples, 71% were positive on PLC/PRF/5 and 14% on BGM. Statistical analysis revealed that when comparing raw sludge MPN values to those of Class B biosolids, there was a statistical significance (P=0.03). The greatest numbers of viruses were detected in raw sludge using the PLC/PRF/5 cells (381 MPN/4 g). The lowest numbers of viruses detected were in the lime treated biosolids (<1 MPN/4 g). The remainder of the class B biosolids had been treated anaerobically and averaged 14 MPN/4g.

Four of the nine Class B biosolid samples were positive on PLC/PRF/5 cells and negative on BGM cells. In contrast, only one raw sludge sample that was positive on the BGM cells was not positive on the PLC/PRF/5 cells. CPE was also observed earlier with the PLC/PRF/5 cells than with the BGM cells, averaging 3 and 5.8 days, respectively.

Since PLC/PRF/5 cells are known to support the growth of adenoviruses, all of the flasks, including monolayers without CPE, were tested for the presence of adenoviruses using PCR. In the Class B biosolids, adenoviruses were detected in 43% of the samples. Other enteric viruses were detected in only 12.5% of the samples. A non-CPE producing

adenovirus (1.78%) was also detected on PLC/PRF/5 cells. Adenoviruses were confirmed in 32% of the raw sludge samples; 39% of the CPE were apparently due to other enteric viruses.

DISCUSSION

Adenoviruses cause a wide range of illnesses including respiratory tract, eye and throat infections and gastroenteritis. They have also been associated with obesity in animals and humans. The importance of human adenoviruses, as well as the potential health risks caused by human adenoviruses in the environment, has been widely recognized (Enriquez et al., 1995; Puig et al., 1994). They are currently included on the Candidate Contaminant List (CCL) of the U. S. Environmental Protection Agency (EPA, 2005). The CCL is a list of contaminants regarded as highest priority for research on the development of detection technology and inclusion in water-quality treatment standards.

In this study, the PLC/PRF/5 cell line was able to detect culturable viruses in more raw sludge and Class B biosolid samples than the BGM cell line. Grabow et al. (1999)

reported similar results in a study comparing the sensitivity of PLC/PRF/5, BGM, PVK and L20B cell lines in which adenovirus in river water was only detected by PLC/PRF/5 cells. Grabow et al. (1992) showed that the PLC/PRF/5 cell line was superior to 293 and Chang conjunctival cells for the propagation and enumeration of typical laboratory strains of adenovirus 40 and adenovirus 41 as well as two stool isolates of adenovirus 41. The greater susceptibility of PLC/PRF/S cells to adenovirus was reflected by the earlier appearance of CPE. These results agree with our data in which PLC/PRF/5 also demonstrated earlier CPE than BGM cells, therefore reducing the time required for virus detection.

Chapron et al. (2000) found that the BGM cell line is effective for propagating enteroviruses in surface water; nevertheless, they also reported the isolation of enteroviruses in CaCo-2 cells from samples that were negative on BGM cells. This can be explained by the fact that not all enteroviruses can infect and replicate in BGM cells. This also supports the idea that no one cell line is effective for the detection of all enteric viruses (Lee and Jeong, 2004). Therefore, it may be difficult to observe CPE if samples contain very low numbers of culturable viruses or certain viruses replicate poorly on BGM cells.

Enteric viruses were detected in raw sludge samples as well as in Class B biosolids. The concentrations were greater in the biosolids. This corresponds with the observations of Nielsen and Lydholm (1980) and is explained by pathogen reduction following different treatments such as heat drying, composting, lime stabilization, etc. in the wastewater treatment plant.

This is the first report of the utilization of PLC/PRF/5 cells for the detection of adenoviruses in raw sludge and biosolids and one of few studies on the occurrence of human adenoviruses in biosolids (Bofill-Mas et al., 2006; Williams and Hurst, 1988; Lyndholm and Nielsen, 1983). Their presence in biosolids suggests that they are capable of surviving anaerobic mesophilic digestion. Adenoviruses usually occur in greater numbers than enteroviruses in effluents activated sludge with means of 1,950 infectious units/L and 1,400 infectious units/L, respectively (Irving and Smith, 1981). They may also survive for longer periods of time in water than enteroviruses (Enriquez et al., 1995).

Even though non-CPE adenoviruses can be present in environmental samples and could therefore result in an underestimation of the incidence of adenoviruses, the use of procedures like PCR may limit the reporting of false negatives in cell culture. The percentage of non-CPE

producing adenoviruses in this study was 1.78%. Van Heerden et al. (2003) found adenoviruses in raw and treated wastewater using PLC/PRF/5 and CaCo-2 cells. Non-CPE producing adenoviruses were also detected by nested polymerase chain reaction (nested-PCR) in both cell lines.

Based on our results, the occurrence of adenoviruses in sewage sludge is greater than that of enteroviruses, which corresponds to the results of previous studies (Komninou et al., 2004; Williams and Hurst, 1988; Krikelis et al., 1985). Adenoviruses display greater resistance than either enteric viruses or spore-forming bacteria to UV light disinfection (Roessler and Severin, 1996) because they contain double-stranded DNA and are able to utilize host cell enzymes to repair UV damage to their DNA. Enriquez et al. (1995) found that adenovirus 40 and 41 were somewhat more stable in primary and secondary wastewater than poliovirus 1 and significantly more stable than either poliovirus 1 or hepatitis A virus in tap and sea water. Studies evaluating the survival of different serotypes of viruses in the environment have shown adenoviruses to be more resistant to temperature and humidity fluctuations than some enteroviruses (Mahl and Sadler, 1975; Irving and Smith, 1981).

CONCLUSIONS

This is the first study establishing the use of the PLC/PRF/5 cell line for the detection of adenoviruses in raw sludge and biosolids.

Our results suggest that the use of PLC/PRF/5 cells is superior for the detection of enteric viruses in biosolids in comparison to BGM cells and will provide a better estimation of the risks from enteric viruses. In addition, the susceptibility of the PLC/PRF/5 cell line allows for more rapid virus detection. The reason for the exceptional susceptibility of PLC/PRF/5 to adenoviruses is not clear. PLC/PRF/S cells do carry a genetically integrated part of the hepatitis B virus genome; however, they are not known to carry any genetic information which may complement the replication of enteric adenoviruses.

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Table 1. Description of samples processed.

Type/Sample	Location	Treatment	Total Solids (%)
[1]Raw-15	Tucson, AZ	Raw	3.7
Raw-77	Tucson, AZ	Raw	4.8
Raw-79	Tucson, AZ	Raw	3.9
Raw-81	Tucson, AZ	Raw	4.3
Raw-83	Tucson, AZ	Raw	2.3
[2]Class B-10	Wyoming, MI	Lime stabilized – 2h at pH 12, 24h at pH 11.5	8.3
Class B-18	Grand Haven, MI	Lime stabilized – 2h at pH 12.0.	7.5
Class B-7	Duluth, MN	Anaerobic digestion – mesophilic digestion 21d thermophilic digestion 7d	2.9
Class B-11	Mankato, MN	Anaerobic digestion – mesophilic 26d	20.0
Class B-12	Chippewa Falls, WI	Anaerobic digestion – Mesophilic	17.6
Class B-14	Gillete, WY	Anaerobic digestion – mesophilic 60d	3.7
Class B-16	Cleansboro, OR	Anaerobic digestion - mesophilic 30d	22.6

[1]Raw Sludge
[2]Class B biosolids

Table 2. Nested PCR primers used to detect human adenovirus in raw sludge and Class B biosolids

Primer	Sequence (5’- 3’)	Location	Product (bp)
ADHEX1F	AACACCTAYGASTACATGAAC	20 380–20 400	473
ADHEX2R	KATGGGGTARAGCATGTT	20 836–20	854
ADHEX2F	CCCMTTYAACCACCACCG	20 485–20 503	168
ADHEX1R	ACATCCTTBCKGAAGTTCCA	20 632–20	652

B= G+T+C, K= G+T

M= A+C, R= A+G

S= G+C, Y= C+T

Table 3. MPN results for the total culturable virus assays in Biosolids

Sample	Cell culture detection MPN/4 g		PCR detection of adenovirus MPN/4 g
	PLC/PRF/5	BGM	
Class B-7	45	<1	18.7
Class B-10	<1	<1	<1
Class B-11	3.3	<1	0.95
Class B-12	15.7	<1	15.7
Class B-14	5.4	<1	5.4
Class B-16	3.1	0.88	3.1
Class B-18	<1	<1	<1
Raw-15	370	3	7.1
Raw-77	381	28	381
Raw-79	31	10	5.3
Raw-81	49	100	<1
Raw-83	<1	33	<1

APPENDIX C:

COMPARISON OF *SALMONELLA TYPHIMURIUM* SURVIVAL IN AGRICULTURAL SOIL AMENDED WITH VERMICOMPOST AND CLASS A BIOSOLID

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ABSTRACT

Vermicompost produced by the earthworm *Eisenia fetida* and Class A biosolids are utilized as organic fertilizers. Their nutrient content and the low concentration or absence of microbial pathogens makes them adequate for agriculture. However, their use has been questioned because of the potential for *Salmonella* regrowth. The aim of the present study was to determine the potential for *Salmonella typhimurium* regrowth in, vermicompost, Class A biosolids, agricultural soil, and agricultural soil amended with vermicompost. *Salmonella* inoculums of 10^1, 10^4 and 10^6 CFU/g were added to the mixtures and adjusted to 30% moisture content and incubated at 28°C for 20 days. *Salmonella* concentration declined to undetectable levels in the biosolids, followed by vermicompost, and the slowest in soil and soil amended with both organic fertilizers. No *Salmonella* regrowth was observed in any of the treatments tested at 30% moisture content. *Salmonella* was shown to survive in agricultural land and agricultural land amended with Class A biosolids and vermicompost for at least 20 days. In contrast, the pathogen survived for less than 5-15 days in Class A biosolids and vermicompost, depending on the initial inoculum.

INTRODUCTION

Organic waste generation has increased in recent years. México generates an estimated 16½ million tons/year (SEMARNAT, 2002). Organic wastes are derived from plant or animal residues such as manure, food, crops and organic residues such as biosolids (FDA, 2002). Agricultural re-use of organic waste is considered an important approach for reducing inadequate waste disposal (Jurado *et al.*, 2004).

Vermicompost produced from cattle manure residues by the earthworm, *Eisenia fetida*, along with biosolids from wastewater treatment are being utilized as organic fertilizers. These are rich in organic matter and nutrients and have improved texture and water holding capacity that make them adequate for crop production (Cooker, 1988).

Vermicompost and biosolids might contain microbial pathogens including *Salmonella*, a pathogenic bacteria commonly present in wastewater and organic wastes. Biosolids generally contain concentrations of 10^2 to 10^3 *Salmonella* colony forming units (CFU)/g of dry weight (Sidhu *et al*, 2001). In England, animal manure has been reported to contain concentrations ranging from 10^2 to 10^7 *Salmonella* CFU/g (Pell, 1997).

Composting, alkaline stabilization, heat drying, solar drying beds and vermicomposting are treatments commonly used to reduce the levels of pathogens in vermicompost and biosolids before they are applied to land. Nevertheless studies have documented the survival of *Salmonella* at low initial concentrations during the composting process. *Salmonella* has also been demonstrated to regrow at favorable environmental conditions (Russ & Yanko 1981; Burge *et al,* 1987). In addition, other studies have shown that recontamination of the final product might occur by external sources (birds and other animals)(Zaleski *et al.,* 2005). *Salmonella* survival and regrowth are determined by factors such as temperature, soil type, pH, organic matter, moisture content, nutrients and the antagonistic effects of indigenous microflora such as bacteria and fungi (Estrada *et al,* 2004).

The objective of the present study was to determine the potential of *Salmonella typhimurium* to survive in agricultural soil, vermicompost, and Class A biosolids.

MATERIAL AND METHODS

Soil and organic fertilizers

Soil was collected from the Instituto Nacional de Investigaciones Forestales, Agricolas y Pecuarias (INIFAP) located at Culiacán-Eldorado km 17.5. Vermicompost and biosolids were purchased from the Humi-Bac Company (Culiacan, Sin.) and Milorganite® (Class A pellets, Milwaukee, WI), respectively. Both vermicompost and Class A biosolids were tested for the presence of *Salmonella* before proceeding with the experiments. Tables 1-3 list the vermicompost, biosolid and agricultural soil physical characteristics. The rate of application recommended by fertilizer companies for vermicompost and biosolid is equivalent to 2 and 7.4 t/ha, respectively.

Preparation of inoculum

Salmonella typhimurium (ATCC 23564) was obtained from the American Type Culture Collection (Manassas, VA). The inoculum was prepared by adding a loop of *Salmonella* in 75 mL of Trypticase Soy Broth (TSB)(Bioxon, México). After overnight incubation at 37°C in a shaking water bath, the culture of *Salmonella* was centrifuged for 10 min at 13,800 x *g* at 4°C. The pellet was then washed two times and re-

suspended in 100 mL of sterile 0.1 M monophosphate buffer. The concentration of the inoculum was determined by spread plate assay onto Hektoen agar (Difco Co., Detroit, MI).

Inoculation of Salmonella typhimurium

Vermicompost, biosolid, soil and amended soil were placed in sterile plastic containers and separately inoculated by spraying with suspensions of 10^1, 10^4, or 10^6 CFU/mL. The final moisture content was 30%. Next, 25 g of each inoculated substrate was placed in sterile 50 mL polypropylene conical centrifuge tubes with screw caps (two replicates per day), held at 28°C, and assayed at intervals of 0, 1, 3, 5, 10, 15 and 20 days.

Salmonella typhimurium assay

Salmonella concentrations were determined by removing 10 g of sample from each conical tube and placing in a flask containing 95 mL of sodium novobiocin (40μg/mL) buffered peptone water (Difco Co., Detroit, MI) and placed on a shaker for 15 min. Serial dilutions were made in sterile monophosphate buffer and 0.1 mL was spread onto Hektoen agar (Difco Co., Detroit, MI). Plates were incubated at 37°C for 24 h and then *Salmonella* colonies were enumerated (Yeager and Ward, 1981). Randomly selected typical colonies of *Salmonella* from the hektoen plates were confirmed by

polymerase chain reaction (PCR) using the kit PCR Core System I (Promega, Madison, WI).

Heterotrophic plate count

Samples of 10 g from each treatment were collected and placed into 95 mL of Trypticase Soy Broth (TSB) and agitated for 15 min. Heterotrophic plate count bacteria (HPC) were assayed on R2A agar (Difco Co., Detroit, MI) and incubated at 28°C for 5 days (Zaleski *et al,* 2005).

Experimental Design

The experimental design corresponded to a design of three variables, totally random. The data was analyzed using MINITAB 14 with a significance level of $P = 0.05$.

RESULTS

Analysis of variance (ANOVA) showed that all treatments were significantly different in terms of die-off of *Salmonella* ($P = 0.00$)(Table 4).

Salmonella inoculated at a concentration of $1x10^1$ CFU/g was not detected after 1 day in soil or any other mixture (Fig 1a). *Salmonella* inoculated at a concentration of $1x10^4$ CFU/g declined rapidly in the biosolids, followed by vermicompost

(Fig. 1b) and was not detectable after 5 and 10 days, respectively. In soil and soil amended with both organic fertilizers, *Salmonella* survived over the entire 20 days, declining by only one log. A similar trend was observed at the inoculated concentration of $1x10^6$ CFU/g (Fig. 1c), where *Salmonella* declined to non-detectable levels in the biosolids within 10 days and vermicompost within 15 days, but not in amended soil.

No significant differences between treatments (in terms of die-off) was observed at the initial inoculated concentration of $1x10^1$ CFU/g. *Salmonella* inoculated at $1x10^4$ CFU/g in soil amended with biosolid survived longer, only being reduced by one log after 20 days. In the agricultural soil and soil amended with vermicompost, *Salmonella* declined only at day 10, whereas in vermicompost, a 3-log reduction occurred by day 5 and *Salmonella* was not detected after 10 days. *Salmonella* decreased by 2 logs at day 3 and did not survive for more than 5 days in the Class A biosolid. *Salmonella* inoculated at a concentration of $1x10^6$ CFU/g survived for 20 days in soil, but only 10 and 15 days when inoculated in biosolid and vermicompost, respectively. *Salmonella* was reduced at a similar rate regardless of initial concentration.

Salmonella added to agricultural soil and soil amended with biosolid and vermicompost behaved similarly. In contrast, both organic fertilizers alone were statistically different (Fig 2 and 3). In agricultural soil, *Salmonella* was able to survive during the 20 days of the experiment and the addition of organic fertilizers reduced the rate of *Salmonella* decline; however, there were no statistical differences in *Salmonella* survival. Without organic fertilizers, *Salmonella* decreased rapidly in soil and was not detected after 10 to 15 days.

High concentrations of HPC (>9 log/dry g) were detected in both organic fertilizers, in soil amended with both fertilizers, and in the soil alone. The concentrations of HPC remained stable throughout the experiment (Fig. 4).

DISCUSSION

The rate of *Salmonella* decline was greater in vermicompost and Class A biosolid, followed by soil, and then the mixtures of soil + fertilizer. It was observed that *Salmonella* was not detectable after 10 days in the Class A biosolid and vermicompost, compared to soil and the mixtures of soil + vermicompost or biosolid in which *Salmonella* decreased an average of 2.0 $\log_{10}$ by day 20. Nevertheless,

there was no significant difference between these reductions. It has been observed that *Salmonella* survives longer in clay-type soils than in sand-type soils (Nicholson et al., 2005). Bacterial pathogens such as *Salmonella, Escherichia coli* O157 and *Listeria* survive longer in manure amended with clay loam grassland soil than in sandy arable soil. Comparing our results to those obtained previously (Castro-del Campo et al., 2007) using a sandy loam soil, we can confirm that clay loam provided a better environment for *Salmonella*, enabling it to survive longer. Soil particles are believed to function as micro-ecological niches rich in nutrients in which species of *Salmonella* can survive, replicate, and be protected from predation (Winfield and Groisman, 2003). Pepper et al. (1993) observed that soil with fine texture provided greater protection to enteric bacteria and thus their longer persistence in the environment.

Salmonella were detected for longer periods in mixtures containing fertilizers and soil. This corresponds with the results obtained by Paluszac et al. (2005) in which the survival of *E. coli, Streptococcus* and *Salmonella* in soil increased with the addition of animal manure. This effect is due to the higher availability of nutrients following the addition of the fertilizer. A field study by Zaleski et al. (2005) was performed to determine the regrowth of *Salmonella*

in aerobically and anaerobically digested biosolids following rainfall. This study concluded that *Salmonella* regrowth occurred only after rainfall episodes and the reintroduction of *Salmonella* from external sources.

In the current study, we observed that neither Class A biosolids nor vermicompost by themselves support *Salmonella* regrowth and that the initial *Salmonella* concentration decreased following inoculation. These results are similar to those of a previous study (Castro-del Campo et al., 2007) in which *Salmonella* concentrations declined at a higher rate in biosolids after inoculation at 20% moisture content. Hussong et al. (1985) evaluated *Salmonella* growth in 30 composted samples inoculated with *Salmonella* at a concentration of $1x10^{7}$ CFU/g. *Salmonella* did not survive more than 7 days in 50% of the samples. Several authors have reported the antagonistic effect of indigenous microflora on the survival of pathogenic microorganisms in biosolids and vermicompost (Russ and Yanko, 1981; Hussong et al., 1985; Szczech, 1999). In the present study, it was observed that the *Salmonella* decrease was greater in Class A biosolid or vermicompost; one possible reason for this was the high concentrations of heterotrophic plate count found in both. Millner et al. (1987) showed that *Salmonella* suppression is not due to a specific group of microorganisms; however, they reported that the presence of Gram negative bacteria, high

levels of actinomycetes, and mesophilic bacteria play an important role in *Salmonella* inhibition. This correlates with our results in which we found the presence of *Bacillus spp.*, actinomycetes, and *Pseudomonas fluorescens* in high concentrations in the various substrates influencing *Salmonella* behavior (Data not shown). Although *Salmonella* reduction depends on the type and concentration of microorganisms, it does not depend on a single microbial species or group of organisms.

Pietronave *et al.* (2004) studied the role of indigenous microflora in the suppression of *Salmonella* in sterile and non-sterile compost under different moisture contents. They demonstrated that *Salmonella* grows rapidly in sterile compost in comparison to non-sterile compost, where the inactivation rate of the bacterium was greater. Szczech and Smolinska (2001) reported that vermicompost microflora produced from animal manure significantly reduced populations of *Phytophthora nicotianae*, preventing infection in plants. Szczech (1999) observed that *Fusarium oxysporum* is similarly eliminated and that the inhibitory effect against this fungus increases as the vermicompost application increases.

CONCLUSIONS

No *Salmonella typhimurium* regrowth was observed in Class A biosolids or vermicompost individually or after addition to agricultural soil at 30% moisture content at typical agronomic rates.

Salmonella was shown to survive in agricultural soil and agricultural land amended with Class A biosolids and vermicompost for at least 20 days. In contrast, the pathogen survived for less than 5 to 15 days in Class A biosolids and vermicompost, depending on the initial inoculum.

It is very important to monitor agricultural land, especially land amended with organic fertilizers or where food crops that are eaten raw are harvested to diminish the risk of external sources of contamination with pathogenic microorganisms due to the ability of some pathogens to survive for long periods of time. The greater the survival of *Salmonella* land applied organic fertilizers, the greater the risk of exposure to this pathogen.

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Table 1. General characteristics of vermicompost

Characteristics	
N	0.84 ± 0.05 %
P_2O_5	1.33 ± 1.05 %
Ca	4.98 ± 4.86 %
Fe	2975.9 ± 214.3 %
Zn	87.76 ± 30.35 %
Cu	9.54 ± 6.39 %
Organic matter	13.65 ± 3.39 %
pH	7.4 ± 0.59

Table 2. General characteristics of Milorganite®

Characteristics	
N	6 %
P_2O_5	2 %
Ca	1.2 %
Fe	4 %
Cl	1 %
Zn	500 ppm
Cu	240 ppm

Table 3. General characteristics of agricultural soil

Characteristics	
Sand 25%, silt 18% and clay 57%	
Texture : clay	
N	43.2 ppm
P_2O_5	16 ppm
Ca	2050 ppm
Fe	3.3 ppm
Zn	0.9 ppm
Cu	3.2 ppm
Organic matter	1.33 %
pH	7.6

Table 4. ANOVA of *Salmonella* and the different treatments

Source	DF	Seq SS	Adj MS	F	P
Mixture	4	100	25	482	**0.000**
Concentration	2	568	284	5460	**0.000**
Mixture & Concentration	8	36	4	88	**0.000**
Time	6	164	27	525	**0.000**
Mixture & Time	24	36	1	29	**0.000**
Concentration & Time	12	66	5	106	**0.000**
Mixture & Concentration & Time	48	43	0.908	17	**0.000**
Error	90	4	0.052		

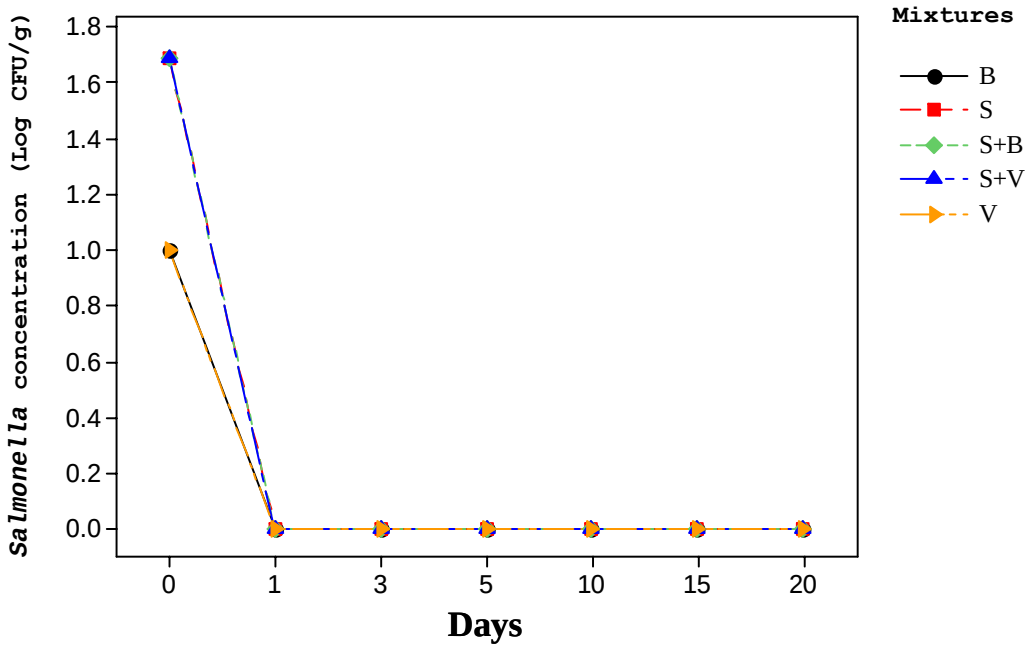


FIGURE 1a. *Salmonella* survival in the different mixtures when inoculated at $1x10^{1}$ CFU/g

[B]Biosolid
[S]Agricultural soil
[S+B]Agricultural soil amended with biosolid
[S+V]Agricultural soil amended with vermicompost

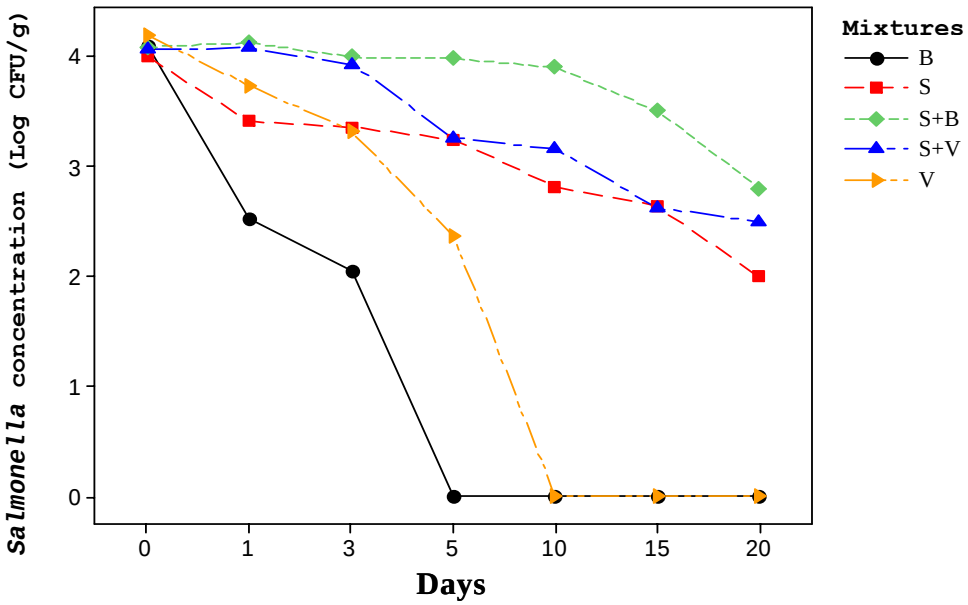


Figure 1b. *Salmonella* survival in the different mixtures when inoculated at $1x10^4$ CFU/g

[B]Biosolid
[S]Agricultural soil
[S+B]Agricultural soil amended with biosolid
[S+V]Agricultural soil amended with vermicompost

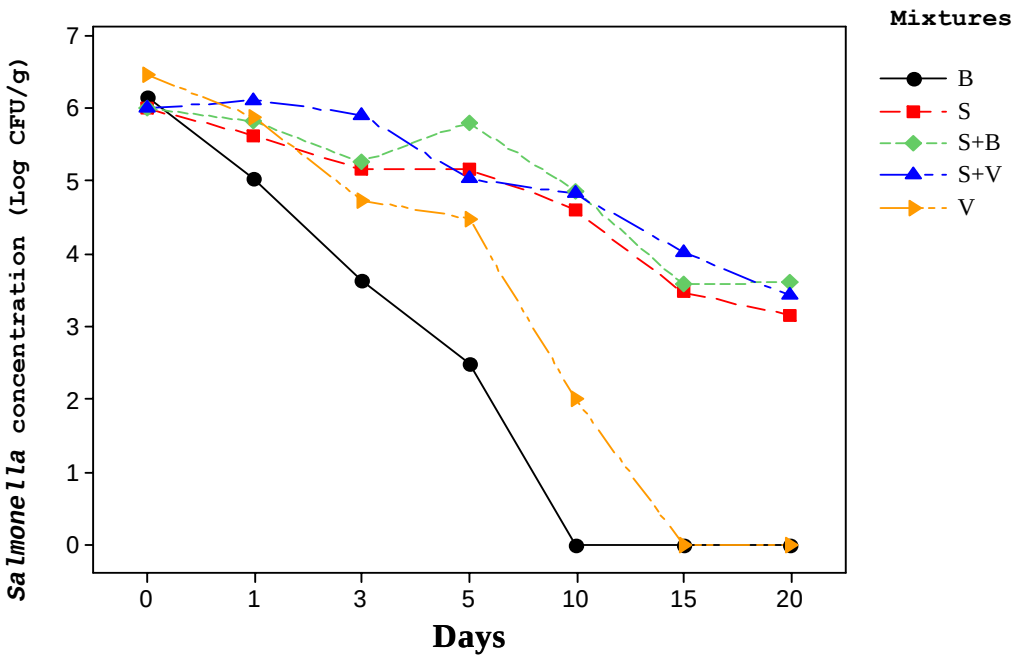
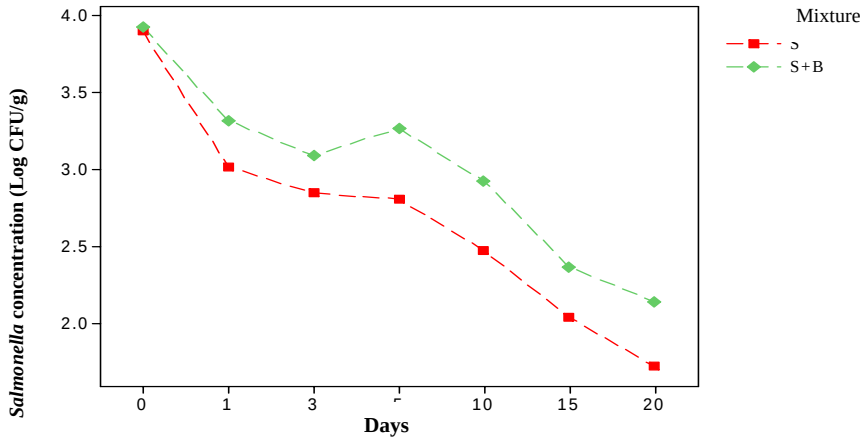
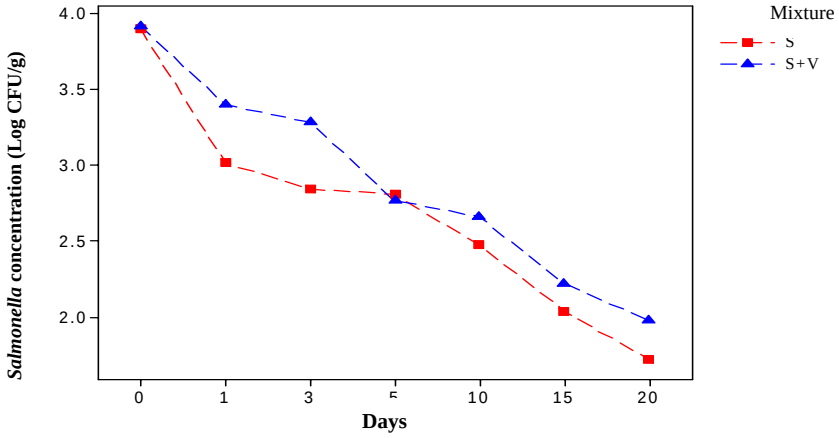


Figure 1c. *Salmonella* survival in the different mixtures when inoculated at $1x10^6$ CFU/g

[B]Biosolid
[S]Agricultural soil
[S+B]Agricultural soil amended with biosolid
[S+V]Agricultural soil amended with vermicompost
[V]Vermicompost



(a) ^{S}Soil
$^{S+B}$Soil+ class A biosolids



(b) ^{S}Soil
$^{S+V}$Soil+ vermicompost

Figure 2. Decrease in *Salmonella* concentration and comparison of its behavior between soil and soil amended with both fertilizers.

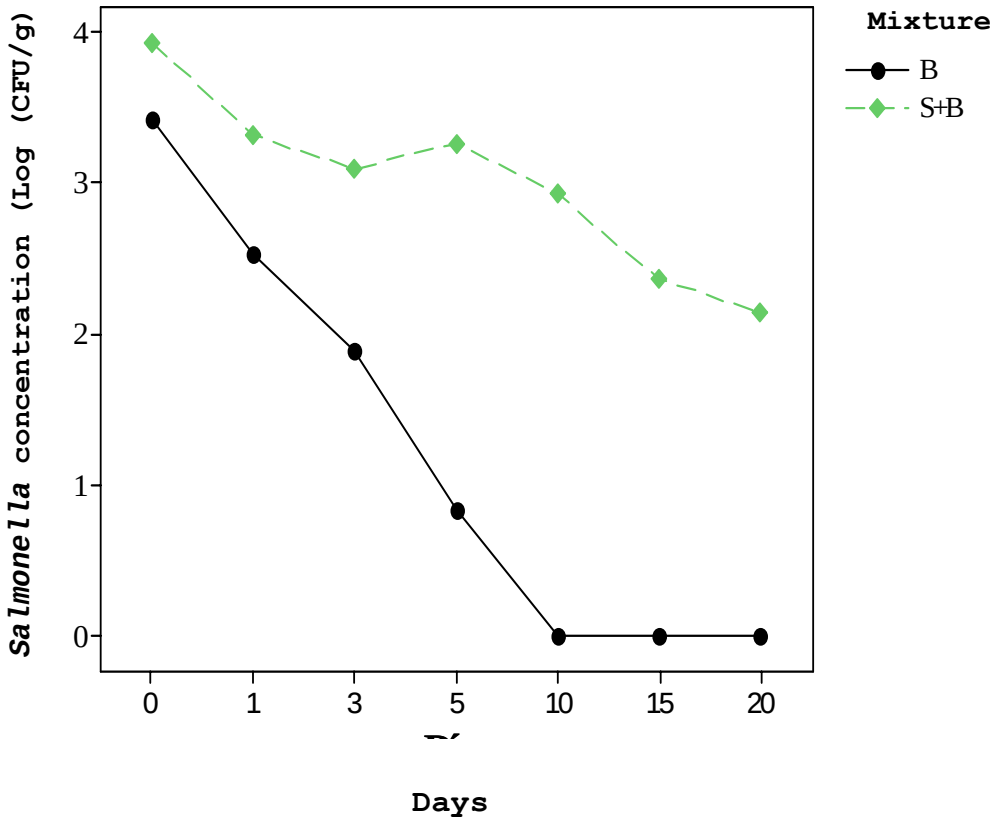


Figure 3a. *Salmonella typhimurium* survival in class A biosolids and soil amended with Class A biosolid

[B]Class A biosolid
[S+B]Soil + Class A biosolids

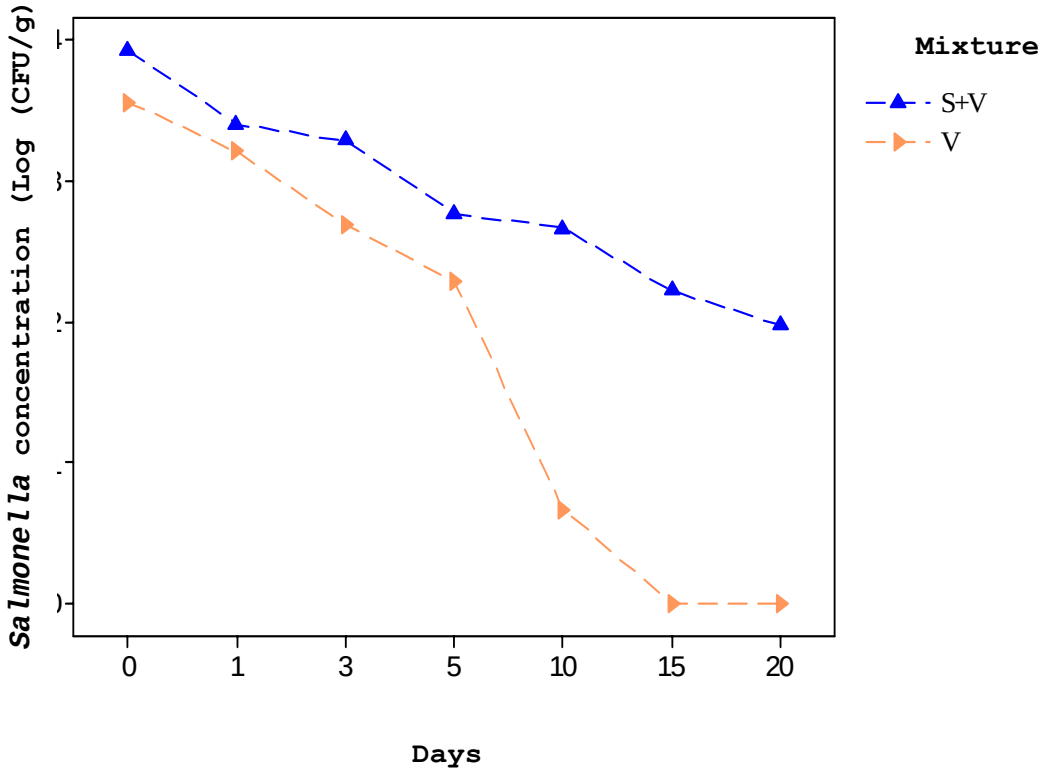


Figure 3b. *Salmonella typhimurium* survival in vermicompost and soil amended vermicompost

[B]Class A biosolids
[S+B]Soil + Class A biosolid

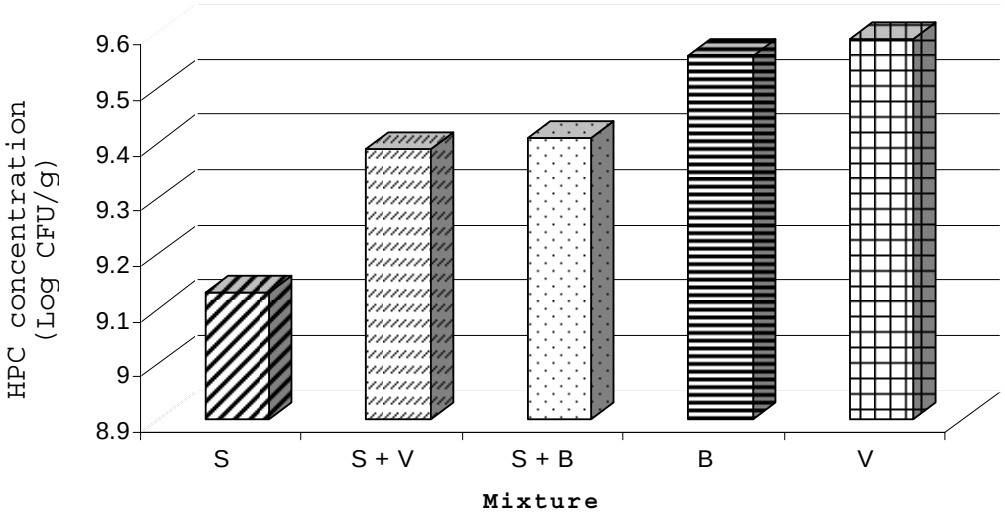


Figure 4. HPC average during the 20 day-experiment for each one of the different mixtures.

[B]Class A biosolids

[S]Soil

[S+B]Soil + Class A biosolids

[S+V]Soil + Vermicompost

[V]Vermicompost