Persistence of Human Pathogens in a Crop Grown from Sewage Sludge Treated Soil

by

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I, Jacobeth Raesibe Bettina Chale-Matsau hereby declare that the work on which this thesis is based is original (except where acknowledgements indicate otherwise) and that neither the whole work or any part of it has been, is being, or is to be submitted for another degree at this or any other university

Signed:

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Summary

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Key words:
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Summary:

The advantages associated with the use of sewage sludge in agricultural land have motivated many countries to use sewage sludge for soil amendment purposes. South Africa's deteriorated agricultural soil could benefit from this nutritional and cost effective product. However, the major shortcoming of sewage sludge is the presence of various pathogenic microorganisms. This raised concern amongst researchers with regard to public safety. The focus of this study, was to investigate the prevalence of pathogens in a crop grown in soil enriched with sewage sludge and to determine risk of infection thereof and to suggest appropriate management practice for sewage sludge use.

Potato (*Solanum tuberrosum),* which is a high risk crop was used, to simulate a worst case scenario. Both the low metal sludge (LMS) and high metal sludge (HMS) were found to have associated diverse numbers of bacteria. Using culture-based technique, *E.coli* and *Salmonella* spp were found to persist in soil

throughout the experimental period. One treatment option (LMS 16 tons/ha) showed a prevalence of these microorganisms in potatoes.

Subsequent molecular studies based on amplification of 16S rRNA gene, yielded limited contamination of potatoes with enteric pathogens, however diverse types of opportunistic, pathogens (mostly environmental pathogens) were isolated from the potatoes. Enteric pathogens were isolated from the sewage treated soil in which these potatoes were grown.

This study has indicated that growing even high risk crops, may lead to limited infestation of produce with primary pathogens. However, proper treatment of sewage sludge prior to use in agriculture is recommendedl to ensure public safety.

The management requirements indicated in this study serve as recommended actions that can be implemented to ensure human safety with regard to sludge application to agricultural land.

CONTENTS

List of Abbreviations

AMP	:	ampicilin
mg	:	milligram
μg	:	microgram
μL	:	microlitre
ton	:	tonne
ha	:	hectare
ml	:	millilitre
IPTG	:	isopropyl β-D-galactopyranoside
dNTP	:	deoxyribonucleoside triphosphate
bp	:	base pairs
EtoH	:	Ethanol
HCl	:	Hydrochloric acid
NaCl	:	Sodium chloride
NaoAC		Sodium acetate
LB	:	Luria Bertani
SDS	:	Sodium dodecyl sulphate
DNA	:	Deoxyribonucleic acid
RNA	:	Ribonucleic acid
PCR	:	Polymerase Chain Reaction
X-gal	:	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Chapter 1
Introduction

1.1 Background

Sewage sludge is an inevitable end product of wastewater treatment, presented as a concentrate of waste material. As sewage sludge is rich in organic matter and nutrients, it can successfully be used in agricultural practices especially in arid countries such as South Africa. The use of human excreta for soil fertilization has been widely practised in parts of Asia for centuries and more recently sewage sludge from modern wastewater treatment plants has been used as a soil conditioner or has been spread on land as an inexpensive means of disposal (WHO, 1979). Today, even first world countries such as the United States and Canada use sewage sludge as soil amendment (NRC, 1996).

One of the problems faced by the agricultural industry in South Africa is the widespread degradation of the soils by erosion and nutrient depletion through incorrect agricultural practices. Sewage sludge serves as a suitable inexpensive alternative to fertilizers. Recycling of organic waste materials to be used for agriculture is in line with sustainable agriculture. Apart from nutrient recycling, organic matter acts as a soil conditioner by improving the soil structure and permeability, making heavy clay soils more friable and manageable (Easton, 1983). Demand for sludge for agricultural purposes appears to be on the increase as South African farmers begin to recognize the importance of using organic substances to improve soil properties (Korentajer, 1991).

Having recognized the benefits of sewage sludge and the widespread use of this product, it is important to discuss the restrictions on using sewage sludge in agricultural practice. Sewage sludge may contain toxic organic chemicals such as pesticides, heavy metals including lead, cadmium and mercury (Purves, 1990) and disease-causing pathogens (Straub *et al*., 1995).

The subject of this study will be limited to pathogens. These pathogens originate from humans who use the sewerage systems and who suffer from acute or latent infections. Pathogens are excreted from infected individuals via faeces, urine, secretions or excretions of the nose, pharynx and skin depending on the type of infection, and reach the sewage treatment plants via sewers and sanitary installations in homes (Strauch, 1991). The spectrum and quantity of pathogens are extended by other sources connected to the system, including hospitals, abattoirs, livestock markets and related activities (Strauch, 1991).

Most of the human enteric diseases are caused by bacteria of the family *Enterobacteriaceae*, particularly *E. coli* and *Salmonella* spp. These organisms are present in high numbers in sewage.

Biological wastewater treatment processes such as lagoons, trickling filters and activated sludge treatment may substantially reduce the number of pathogens in the wastewater. However, these processes do not completely remove or inactivate pathogenic organisms as some of them are adsorbed to faecal particles (Strauch, 1991). The resulting sewage sludge still contains sufficient levels of pathogens to pose a public health and environmental concern (EPA, 1999).

1. 2 Motivation for Present Study

The South African sludge guidelines are presently being revised. The scientific premises of the current guidelines have been evaluated. This evaluation revealed that the pathogen limits used in the sludge guidelines were based on international trends and experiences. It is therefore necessary to investigate the appropriateness of the current guidelines for South African use.

However, very little information is available on the pathogen load in sludge and the human health risk associated with sludge used in agricultural practices in South Africa.

1.3 Aim and Objectives

The aim of this research is to understand the behaviour and risks associated with the agricultural use of sewage sludge in terms of pathogenic infections, so as to adequately protect humans against sludge borne pathogens associated with the agricultural application of sewage sludge.

This will be achieved by

- Evaluating the risk to human health associated with the agricultural application of inadequately disinfected sewage sludge, and
- Recommending management practices to ensure that all spheres of the population associated with the agricultural application/use of sewage sludge are adequately protected against pathogenic infections.

The aim of the study will be addressed by:

- Investigating the current microbiological quality of South African sewage sludge from various wastewater treatment plants in South Africa.
- Determining the microbial quality of sewage sludge prior to application to soil.
- Determining the persistence of microorganisms in soil following sludge application.
- Establishing the survival of pathogenic organisms using a high risk crop.
- Using the research results of the above-mentioned experiments to quantify the risk to human health associated with the agricultural application of sewage sludge that has not been adequately disinfected prior to application.
- Developing a management framework based on the literature and results from this research to adequately protect humans against sludge borne pathogens associated with the agricultural application of sewage sludge.

1.4 Approach

A countrywide survey will be performed to establish South African sludge quality using sludge collected from Wastewater Treatment Plants.

Microbiological assessment in sludge dedicated for soil amendment will be determined prior to using the sludge for planting. The crop chosen for the purpose of this study is potato (*Solanum tuberosum*) (Recke *et al*., 1997). Potato was chosen as it is a high risk crop for growing in sewage sludge treated soil (WRC, 1997; EPA, 1999). Also the season was appropriate for growing potato. Green house experiments coupled with advanced laboratory techniques will be employed to establish if any pathogens persist in soil and potato. Knowledge accrued from these experiments and from the risk assessment will then be used to recommend management approaches for adequate protection of human health.

Chapter 2
Literature Review

2.1 Introduction

The use of human excreta for fertilizer, ranging from night soil application to irrigation with sewage has been a world-wide practice for many years, especially in highly populated countries such as China and India (Rudolfs *et al*., 1950). It is especially advantageous because it recycles nutrients back to the land and can be economically attractive (Zenz *et al*., 1976).

In South Africa sludge production is increasing rapidly, and at the same time the soil condition has deteriorated markedly. As sludge contains high levels of organic matter and nutrients (Hu *et al*., 1996), use of this product in agricultural land could provide an alternative means of disposal, and also benefits the poor soil quality of most of South Africa's agricultural land. It is believed that when treated properly, and provided certain industrial contaminants are restricted from entering the sewage, the resultant sewage sludge can become a relatively innocuous organic fertilizer and soil conditioner of significant value for growing trees, grass and certain crops (WRC, 1997).

The beneficial use of sludge for soil amendment in South Africa was also recently shown by Snyman and colleagues (1998). At present, sewage sludge is used for crop growing but limited only to fenced areas to restrict access to unauthorized persons as well as milk-, meat- and egg producing animals (WRC, 1997). Other recommendations suggest that application may only be done with planting and during the period subsequent to harvesting and prior to the next growing season. Snyman and Van der Waals (2003) reported that South African farmers, noting the increased crop production as a result of enhanced soil properties from sewage sludge use, are in favour of using sewage sludge and show adherence to the recommended dosage of 8 ton/ha as stipulated in the guidelines (WRC, 1997).

Elsewhere, application of sewage sludge to deteriorated soil, resulted in increased yields (Tester and Parr, 1983). Consequently, municipal sewage sludges are routinely utilized on agricultural lands in various parts of the world. In Canada, it is becoming a common practice such that as much as 43% of the produced sewage sludge is applied to land. By comparison, the United States and Europe apply approximately 60% and 34% respectively of their sewage sludge to agricultural land (EPA, 1999; Apedaile, 2001).

2.2 Metals and Toxic Organic Pollutants in Sludge

The composition of wastewater sludge may be highly variable depending on the quantity and quality of contributions from industrial and domestic sources. The types of constituents include among others, chlorinated hydrocarbons, polynuclear aromatic hydrocarbons and metals (Brown *et al*., 1991). Hyde (1976) pointed out that heavy metals are retained in soils following sludge application and can accumulate to the point at which they are toxic to plants. Thus, due to their uptake by crops, they may also be toxic to humans and animals. This observation was confirmed by Rost and colleagues (2001) who recently reported that heavy metals have long lasting adverse effects on biological functions in soil. The heavy metal of major concern, because of its possible phytotoxicity and danger to the human food chain, is cadmium (Cd). Other heavy metals of importance are copper (Cu), nickel (Ni) and zinc (Zn), and they are also known to be phytotoxic (Hyde, 1976; Purves, 1990).

Organic compounds such as pesticides, polychlorinated biphenyls, halogenated aliphatics, ethers and aromatic hydrocarbons are the products of industrial wastewater which could land up in wastewater sludges (Korentajer, 1991; Vorobieva *et al*., 1996; Kouloumbis *et al*., 2000). The concentration of these compounds needs to be monitored and limited by implementing source reduction.

2.3 Socio-economic Issues Regarding Sludge Use

Farmers and the food industry have expressed their concern that agricultural use of untreated sludge may affect the safety of food products and the sustainability of agricultural land, and may carry potential economic and liability risks (NRC, 1996). There is also concern that the use of contaminated sewage sludge for crop production could negatively affect the export market. For fear of foodborne illness, some countries may refuse importation of vegetables and foods produced under such agricultural practices (Sobsey, 1996; Doyle, 2000).

There has been increased public scrutiny of the potential health and environmental consequences of land spreading of sewage sludge. It appears that once fear of pathogens, odours, nuisances and possible environmental deterioration have been generated in a community, people have great difficulty in accepting the risks, even if there aren't any, of applying sewage sludge to agricultural land (Hyde, 1976; Tauxe, 1997). Thus, it is essential that aesthetic characteristics and matters affecting both long-term quality of the land and the public health must be thoroughly understood before using sewage sludge on farmland.

In spite of the increasing concerns, in their recent report to the United States Environmental Protection Agency, the National Research Council pointed out that there is no evidence that proper use of wastewater treatment sludge on land has any detrimental effect on either the people working at the site, on the population surrounding the land application site, or on people eating the crops grown in the sludge-amended soil (NRC, 2002). Vesilind (2003) is of the opinion that the aversion to sludge use emanates from the knowledge of its origin and not necessarily from diseases linked to sludge use.

Kirby (2001) pointed out that exposure to potentially lethal pathogens is linked to social factors such as class, education and income. Carneiro and his colleagues (2002) have observed that less intense *Ascaris* infection came from affluent

households with higher socio-economic profile. In many African countries including South Africa, a large percentage of the population live in poverty (Parliamentary Bulletin, 1996), thus it can be expected that these households would be intensely affected by contaminated crops. The high incidence of HIV/AIDS (Human Immunodeficiency Virus/ Acquired Immune Deficiency Syndrome) infection in the country could translate into more pathogenic infections due to their depressed immune systems, if such communities are exposed to contaminated crops. South Africa is a comparatively large country, covering 1,221,042 square kilometers and with an estimated population of about 40 million. It has been estimated that 14.2% of people in South Africa have been infected with HIV/AIDS (Dorrington *et al*., 2002). The routine surveillance conducted by the Department of Health has shown that among pregnant women attending public health clinics for antenatal care, the prevalence has increased from less than 1% in 1990 to 26.5% in 2002 (Dorrington *et al*., 2002). Overall, it is estimated that 23.3% of men and 23.5% of women are infected whereas the prevalence amongst the male and female youth is 5.8% and 21.6% respectively (Dorrington *et al*., 2002).

2.4 Microorganisms Encountered in Sewage Sludge

Infectious diseases are transmitted primarily through human and animal excreta, particularly faeces. If there are active cases or carriers in the community, then faecal contamination of water sources will result in the causative organisms being present in water. Pathogens in domestic sewage are primarily associated with insoluble solids. Many of these organisms become bound to solids following wastewater treatment and are transferred to wastewater sludge (Bitton, 1994). As the wastewater treatment processes concentrate these solids into sewage sludge, the sewage sludge has higher quantities of pathogens than incoming wastewater (EPA, 1999). However, the transmission of pathogens can be minimized by reducing the infectivity of sludges through effective treatment processes (Smith, 1996).

The actual species and quantity of pathogens present in sewage sludge from a particular municipality depend on the health status of the local community and may vary substantially at different times (EPA, 1999). The four major types of human pathogenic organisms, namely bacteria, viruses, protozoa and helminths may all be present in sludge. These organisms can cause infection or disease if humans or even animals are exposed to sufficient levels. The infective dose, that is, the number of pathogenic organism to which a human must be exposed to become infected, varies depending on the organism and on the health status of the exposed individual (EPA, 1999). While some pathogens may cause infections in a susceptible host by a single organism, others may require several hundreds to be present before an infection can be initiated. Symptoms may vary in severity from mild gastroenteritis to severe and sometimes fatal diarrhoea, dysentery, hepatitis or typhoid depending on the type of pathogen and pathogen load. Thus, when reclaimed water or sludge is used on fields producing food crops, it is critical to protect public health.

In the sections that follow, the major bacterial, viral and parasitic organisms found in wastewater sludge are described.

2.4.1 Bacteria

Wastewater normally contains many bacterial species, and strains (Vilanova *et al*., 2002) that may end up in the wastewater sludge. If such sludge is not adequately treated and used in agricultural land, crop contamination may be imminent. As Bubert and colleagues (1999) have pointed out, contamination of food material does not only occur during food processing, but may also begin with the production of raw food materials in the environment.

Faecal coliforms and enterococci have been used widely as faecal pollution indicators (Vilanova *et al*., 2002). Both bacterial groups include several species. For example, the genus *Enterococcus* contains 19 recognized species (Manero and Blanch, 1999). *Salmonella* spp, *Shigella* spp, *Campylobacte*r spp, *Yersinia*

spp, *Leptosporia* spp and *Escherichia coli* are bacterial pathogens of primary concern in sludge. *Escherichia coli* is particularly abundant in human and animal faeces, where numbers may reach 10^9 g^{-1} of faeces (Bitton, 1994). The major bacterial groups or species are tabulated in Table 2.1 and some of these (*) are discussed in sections i to viii. Several case studies have been cited in these sections. These case studies do not necessarily detail outbreaks due to wastewater sludge use, but are indicators of what the effects could be if the pathogens manage to survive and infect a receptor, as a worst-case scenario.

Table 2.1 Bacterial pathogens to be expected in sewage sludge (Source: EPA, 1999; Strauch, 1991)

Pathogen	Disease
Salmonella spp *	Salmonellosis (gastroenteritis)
Shigella spp *	Bacillary dysentery
Escherichia coli *	Urinary infection; diarrhoea
Yersinia enterocolitica *	Yersniosis (gastroenteritis)
Clostridium spp *	Gas gangrene
Leptospira spp	Leptospirosis
Mycobacterium spp	Tuberculosis and leprosy
Vibrio cholerae spp	Cholera
Staphylococcus spp	Osteomyelitis
Streptococcus spp	Rheumatic fever; glomerulonephritis
Klebsiella spp	Pneumonia; urinary tract infection
Enterobacter spp	Urinary tract infection
Serratia spp	Meningitis; endocarditis
Citrobacter spp	Neonatal meningitis
Proteus spp	Urinary tract infection
Providencia spp *	Urinary tract infection
Listeria monocytogenes *	Listeriosis

i *Escherichia coli*

Escherichia coli is normally found in the gastrointestinal tract of humans and other warm-blooded animals (Brooks *et al*., 1991) and is the most common cause of foodborne illness. Foods that have been implicated with *E. coli* include cheese, beef, fish, poultry, apple cider and lettuce (Reis *et al*., 1980; Kornacki and Marth, 1982; Ackers *et al*., 1998).

Escherichia coli, depending on the infective strain, can cause a variety of illnesses that include infantile diarrhoea, traveler's diarrhoea, hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) and thrombocytopenic purpura (TP) (Pell, 1997; Penner, 1998). Hemorrhagic colitis is a severe illness and is characterized by bloody diarrhoea and severe abdominal cramps while HUS is characterized by bloody diarrhoea followed by renal failure. Thrombocytopenic purpura yield symptoms similar to those of HUS but the central nervous system is also affected. Death often occurs in patients with HUS and TP (Pell, 1997). Hemolytic uremic syndrome can be a serious complication in children and is a leading cause of acute kidney failure (Penner, 1998).

ii *Salmonella* spp

Salmonellosis was normally associated with contamination of food of animal origin, but in recent years, it has been indicated that *Salmonella* spp contamination may also occur in foods of plant origin. For instance *Salmonella* spp outbreaks have been associated with consumption of celery, watercress, watermelon, lettuce, cabbage, tomatoes, potatoes and carrots (Wells and Butterfield, 1997; Guo *et al*., 2000). These organisms have also been implicated in infections due to wastewater spreading (Melloul and Hassani, 1999). *Salmonella* spp are capable of surviving and multiplying in fruits and vegetables. Asplund and Nurmi (1991) have demonstrated that tomatoes can provide a favourable environment for growth of *S. enteitidis*, *S. infantis* and *S. typhimurium* in spite of their low pH value, showing that the high acidity is not necessarily

effective enough to inhibit *Salmonella* spp growth. *Salmonella* spp can grow and multiply at temperatures of 22^{o}C (Asplund and Nurmi, 1991) suggesting that once produce has been contaminated, microorganisms may continue to grow on the shelf in retail stores increasing the risk of infection.

Enteric fever is caused by the microorganism *S. typhosa*, in which the organism, ingested along with food finds its way into the bloodstream. Another organism, *S. cholera-suis* causes septicemia resulting in blood poisoning. The *S. typhimurium* and *S. enteritidis* cause gastroenteritis, an infection very commonly associated with contaminated food. Symptoms of Salmonellosis include nausea, vomiting, headache, chills, diarrhoea, fever and can even lead to reactive arthritis. In most cases the disease is short-lived, but salmonellosis can be fatal (Penner, 1998). Infants, once infected, frequently become long-term carriers and cause family outbreaks (Burge and Marsh, 1978).

iii *Listeria* spp

Listeriosis is rare in non-pregnant healthy adults, however, adults with conditions such as type 1 diabetes, cardiovascular disease, renal transplant, neoplasm, alcoholism and AIDS are more susceptible (Penner, 1998). Due to its ability to survive for long periods and its capability to grow at refrigerator temperature (Penner, 1998) this organism poses a serious threat in regard to foodborne illness. Healthy animals can be asymptomatic carriers of *L. monocytogenes* (Pell, 1997).

Listeria monocytogenes is a human and animal pathogen capable of causing nausea, vomiting, headache, fever, and severe infections like septicemia, encephalitis and meningitis, especially in immunocompromised individuals, newborns and pregnant women where it can result in stillbirths. About 100 cells of *L. monocytogenes* are sufficient to cause illness (Brooks *et al*., 1991). In the USA this organism has a fatality rate of 20 – 40% (Penner, 1998).

Several outbreaks have been associated with contaminated commercial foodstuffs, such as vegetables, milk and meat products on which these bacteria can multiply even at low temperatures (Bubert *et al*., 1999). Both *L. monocytogenes* and *L. innocua* have been isolated from various environmental samples such as soil, vegetation and human and animal faeces (Bubert *et al*., 1999).

iv *Yersinia enterocolitica*

Yersinia enterocolitica causes yersiniosis and is found in a variety of animals, particularly pigs. It will grow at refrigerator temperatures, but grows best at room temperature. Infection with this organism yields symptoms that range from a mild gastroenteritis to sever conditions of polyarthritis and meningitis (Prescott *et al*., 2002).

v *Shigella* spp

Shigellosis is caused by bacteria of the genus *Shigella* (Brooks *et al*., 1991). This disease is characterized by diarrhoea, abdominal pain, vomiting and fever. As few as 10 to 100 microorganisms are sufficient to cause an illness (Penner, 1998). *Shigella* are readily killed by heat and do not survive well in acidic environments (Prescott *et al*., 2002).

vi *Clostridium* spp

Clostridium botulinum produces a neurotoxin that cause botulism. After the toxin is absorbed, it binds to nerve endings and causes vomiting and diarrhoea, fatigue, dizziness and headache. Later there may follow constipation, double vision, difficulty speaking and swallowing, involuntary muscles may become paralyzed leading to cardiac and respiratory failure and eventually death. (Penner, 1998). The *C. botulinum* spores are heat resistant (Brooks *et al*., 1991).

Clostridium perfringens produces toxins that cause diarrhoea and severe abdominal pain. However, death is uncommon. Although spores of this organism are common in raw foods and they are heat resistant, large numbers of vegetative cells of *C. perfringes* are necessary for an illness to occur (Penner, 1998).

vii *Campylobacter jejuni*

Campylobacter jejuni causes camphylobacteriosis characterized by cramps, nausea, diarrhoea, headache and fever. Onset of the disease following consumption of contaminated food is within two to five days. Prolonged illness may lead to complications such as meningitis, urinary tract infection and reactive arthritis, but death occurs rarely (Penner, 1998). The high incidence of *C. jejuni* infections in persons infected with the human immunodeficiency virus points to the widespread transmission of low levels of this organism (Blaser, 1996). *Campylobacter* cells survive for several weeks at temperatures even as low as 4 ^{o}C (Waage *et al*., 1999). The infective dose of *C. jejuni* is very small, it has been estimated that about 500 cells of this organism can cause human illness (Black *et al*., 1988). Also, *Campylobacter* cells may enter a viable but non-culturable state due to starvation and physical stress, making them even more difficult to detect (Brooks *et al*., 1991).

viii *Providencia* spp

Providencia spp are members of the normal intestinal flora. They cause urinary tract infection and are often resistant to antimicrobial therapy (Brooks *et al*., 1991).

2.4.2 Persistence of Bacteria in Soil

The survival of microorganisms added to soil is influenced by a number of factors that include, water-holding capacity of soil, temperature, rainfall, sunlight, organic material in soil and the hydrogen ion (Rudolfs *et al*., 1950; deRopp, 1999).

Faecal coliforms can survive for several years under optimum conditions, and the *Salmonella* spp may survive for a year in rich, moist organic soil (deRopp, 1999). The survival period of *Salmonella* spp has been reported to be as long as between 15 – 117 weeks in contaminated soil (Rudolfs *et al*., 1950; Jones,1980; Strauch, 1991; Sidhu *et al*., 1999; Baloda *et al*., 2001). The *L. monocytogenes* grows well in sewage and survives for long periods in soil (Strauch, 1991). Other bacteria such as *Streptococcus jaecelis*, *Clostridium botulinium*, *Clostridium tetani*, *Clostridium perfringes* and butyl-butyric *Clostridia spp* were found in small numbers 7 months after sludge application (Hyde, 1976).

Campylobacters spp are not capable of proliferating in the environment, but may remain dormant and survive in the environment for several weeks at low temperatures (Waage *et al*., 1999). However, the infective dose is very small which increases the risk of infection (Black *et al*., 1988).

One of the most important factors influencing the survival of pathogenic bacteria in soil is competition with the existing soil microflora. In soils with low microbial activity, the newly added microorganisms may persist for much longer (Bitton, 1994). Thus the application of large quantities of sludge to soil with low existing microbial activity will increase the ability of the pathogens to persist in soil environment and hence increase the potential risk for transfer of pathogens to crops grown in the soil. On the other hand, in biologically active soils, microorganism numbers are rapidly reduced due to competition (Penner, 1998). The soils in South Africa are typically biologically active, which could be advantageous due to the fact that introduced microorganisms are rapidly out

competed. However, as a result of high microbial activity, the organic material in agricultural soil is low (Korentajer, 1991).

Microorganisms may move through the contaminated soil as a result of rainfall or irrigation (Gerba *et al*., 1975). Gagliardi and Karns (2000) have indicated that if soil pores do not become clogged, *E. coli* can travel below the top layers of soil for more than two months. They also indicated that *E.coli* from manure applied to soil could survive, replicate and move vertically in the soil (Gagliardi and Karns 2000). While soil contaminated with sewage sludge could lead to crop contamination, it has been indicated that water bodies such as groundwater, storm-water and rivers could be contaminated following rainfall or irrigation as a result of runoff from contaminated agricultural land (Lee and Jones-Lee, 1993; Bilgrami and Kumar, 1998).

2.4.3 Viruses

Sludge from wastewater treatment may contain demonstrable numbers of viruses even after anaerobic digestion (Damgaard-Larsen *et al*., 1977). Some of the viruses that can be expected in sewage sludge are tabulated in Table 2.2.

Human enteric viruses are excreted in faeces, and can be shed in high numbers (10^8 to 10^{10} particles per gram of faeces) by infected individuals (Abbaszadegan *et al*., 1999). The persistence of enteroviruses in sludge and sludge-amended soil was demonstrated by Damgaard-Larsen *et al*. (1977) and by Straub *et al*. (1994). The virus of greatest potential concern appears to be Hepatitis A, a disease with potential for long-term liver damage (Pahren *et al*., 1979).

Table 2.2 Viruses that can be expected in sewage sludge (Sources: EPA, 1999; Strauch, 1991; Bofill-Mas *et al*., 2000)

Pathogen	Disease
Enteroviruses	
Coxsackievirus A	Acute hemorrhagic conjunctivitis
Coxsackievirus B	Meningoencephalitis
Echovirus	Acute hemorrhagic conjunctivitis
Poliovirus	Poliomyelitis
Adenovirus	Respiratory and systemic infections
Reovirus	Acute respiratory infections
Hepatitis A virus	Infectious hepatitis
Rotavirus	Acute gastroenteritis
Astrovirus	Gastroenteritis
Calicivirus	Acute gastroenteritis
Coronavirus	Gastroenteritis
BK virus	Uretal stenosis and hemorrahgic colitis
JC virus	Multifocal leukoencephalopathy
Norwalk and Norwalk-like viruses	Acute gastroenteritis

Human polyomaviruses JC virus and BK virus were also indicated as being present in urban sewage obtained from widely divergent geographical areas in Europe and Africa (Bofill-Mas *et al*., 2000). The JC virus is aetiologically associated with a fatal demyelinating disease known as progressive multifocal leukoencephalopathy, which has emerged as a frequent complication of AIDS in HIV infected individuals. Infection with BK virus has been associated with diseases of the urinary tract including hemorrhagic cystitis and ureteral stenosis (Bofill-Mas *et al*., 2000).

Virus inactivation under natural conditions is a slow process (Damgaard-Larsen *et al*., 1977). Viruses may become eluted and travel through the soil (Damgaard-Larsen *et al*., 1977) which includes both vertical and lateral migration (Straub,

1995). For instance, other enteroviruses such as the coxsackie B3 virus have been isolated 18m below the soil surface after wastewater recharge (Straub *et al*., 1995). Rainfall and irrigation events may contribute to viral transport (Straub *et al*., 1995). Viruses readily adsorp to soil particles, and this has been reported to prolong their survival (WHO, 1979). However these viruses remain as infectious to humans as free viruses.

Viruses can survive for up to six months in cold weather and for three months in warm weather. Enteric viruses can survive up to 170 days in loamy and sandy soil. Poliomyelitis virus has been detected in soil irrigated with infected sewage sludge and effluent after 96 days in winter and 11 days in summer in the UK, and on the surface of mature vegetables 23 days after irrigation had ceased (Tierney *et al*., 1977; WHO, 1979). Viral survival on crops may be shorter than in the soil if viruses on crops surfaces are directly exposed to detrimental environmental factors such as sunlight and desiccation (Pahren *et al*., 1979; WHO, 1979). The warm climate in some regions of South Africa may reduce the survival of these viruses. However, more prolonged survival can be expected in the moist or more protected parts of plants, such as within the folds of leafy vegetables, in deep stem areas and on rough cracked surfaces of edible roots. It is also likely that viruses can penetrate damaged roots and under certain conditions enter the stem and leafy parts of edible plants (Pahren *et al*., 1979).

Once crops are harvested, enteric viruses can survive for prolonged periods during commercial and household storage at low temperature. The risk of human infection associated with virus-contaminated crops is greatest in the case of fruits and vegetables consumed raw (WHO, 1979).

2.4.4 Parasites

Parasites are a group of foodborne pathogens that have received relatively little attention. Parasites that are usually encountered in sludge are indicated in Table 2.3, and some of these (*) are briefly discussed.

Table 2.3 Parasites that can be expected in sewage sludge (EPA, 1999; Strauch, 1991)

Pathogen	Disease
Entamoeba histolytica	Amebiasis
Giardia lamblia *	Giardiasis
Toxoplasma gondii	Toxoplasmosis
Sarcocystis spp	Intestinal infection
Taenia spp *	Taeniasis
Diphyllobothrium latum	Pernicious anaemia
Echinococcus granulosus	Echinococcosis
Ascaris spp *	Ascariasis
Toxocara spp	Pneumonic symptoms
Trichuris trichiura	Trichuriasis
Toxoplasma gondii	Toxoplasmosis
Cryptosporidium *	Cryptosporidiosis

Some of the common types of parasites that have been detected in fresh fruits and vegetables include *Giardia lamblia*, *Entamoeba histolytica* and *Ascaris* spp. (Brackett, 1987). As little as 10 or fewer *Giardia* cysts are sufficient to cause illness (Brooks *et al*., 1991). Ayres and colleagues (1992) recovered viable *Ascaris* eggs from lettuce irrigated with raw sewage, while Gaspard and Scwartzbrod (1993) recovered viable *Ascari*s from both tomatoes and lettuce following raw sewage irrigation. It has also been demonstrated that farm workers may be infected with enteric parasites as a result of occupational exposure (Clark *et al*.,1984). It should be noted that these incidents were associated with the irrigation of raw sewage and not wastewater sludge. However, it does give an indication of potential risk.

The parasites most often found in sludge are *Ascaris* species such as *A. lumbricoides* (human intestinal roundworm) and *A. suum* (pig's roundworm) as

well as some *Toxocara* and *Trichuris* species (Bitton, 1994; Gaspard *et al*., 1995).

Ascaris eggs and certain larval stages of trichostrongylids can survive for over a year in soil that has been irrigated with sewage sludge (Strauch, 1991), and the eggs of *Cryptosporidium parvum* and *Taenia saginata* are known to survive in sewage for more than 12 months (NRC, 1996). *Cryptosporidium* species and *Giardia* species pose a serious threat to human health as these organisms are difficult to inactivate with disinfectants and their infective doses in humans are very low (Finch and Belosevic, 2001).

Protozoan parasites, such as *Giardia* spp have been found in sludge in Western Australia where they remain the most common cause of enteric disease (Hu *et al*., 1996). The most noxious are the *Ascaris* eggs and coccidial oocysts as they have high resistance (Pahren *et al*., 1979; Gaspard and Schwartzbrod, 1993). Helminths larvae are usually killed by composting, but often remain viable in slurry during storage (Shuval *et al*., 1984).

Also encountered in sludge are the organisms of the genus *Cryptosporidium* (Kuckzynska and Shelton, 1999; EPA, 1999). Of the *Cryptosporidium* species, *C. parvum* is the agent of clinical cryptosporidiosis in humans and livestock. The *C.parvum* oocysts are shed by infected mammals and are known to be resistant to standard disinfectants (Champliaud *et al*., 1998). Waterborne *C. parvum* oocysts may remain viable for several months (Kuczynska and Shelton, 1999).

Table 2.4 indicates the concentrations of pathogens as indicated by other countries.

Table 2.4 Concentrations of pathogens in sludge from other countries (Jimenez *et al*., 2002)

Pathogen	Concentration	Country
Fecal coliforms (MPN/gTS)	$3.6 \times 10^4 - 1.4 \times 10^6$	United Kingdom
	$2.3 \times 10^7 - 9.3 \times 10^{10}$	Mexico
	2.0×10^7	United States
E.coli (PFU/gTS)	$1.0 \times 10^6 - 1.9 \times 10^6$	Mexico
	1.3×10^5	United States
Ascaris/gTS	2.40 – 8.98	United Kingdom
	66 – 136	Mexico
	1.4 – 9.7	United States
	0.60 – 2.4	France

2.5 Disinfecting Treatment Processes

Previous sections provided detailed discussions on the occurrence of microorganisms in sludge and their potential presence in crops if inadequately treated sludge is used for land application. However, the transmission of pathogens can be minimized by reducing the infectivity of sludges through effective treatment processes (Smith, 1996). Various techniques are used to eliminate or reduce the number of microorganisms to levels that do not threaten human health (EPA, 1999).

Many of these treatment processes are applied either to stabilize the sludge, i.e reduce its vector attraction potential and odour or render the sludge easier to handle, store and transport by reducing the volume or drying the wastewater sludge. Additional treatment technologies need to be employed to reduce the viable content. Some of these techniques recommended in the US Part 503 rule are indicated in Table 2.5.

If effective treatment is not available, long term storage could be used to accelerate inactivation and thus reduce the number of infective species before

sludge is spread onto soil (Jenkins *et al*., 1999). Jenkins and colleagues (1999) warned that although storing prior to spreading could be an effective management practice for reducing infective oocyst load, spreading of sludge during the cold season may have the opposite effect by sustaining the survival of *C. parvum* oocysts and positioning them for transport in surface runoff (Jenkins *et al*.,1999).

Table 2.5 Techniques listed in the 40 CFR Part 503 and their effectiveness in removing pathogens (EPA, 1999)

R = Reduction, E = Elimination, ✓ = effective in pathogen reduction/elimination and ✗ = not effective in pathogen reduction /elimination

Technique	Description	Effectiveness in Eliminating Pathogens					
		Viruses		Bacteria		Parasites	
		R	E	R	E	R	E
Aerobic Digestion	Sewage sludge is agitated with air or oxygen to maintain aerobic conditions	✓	✗	✓	✗	✗	✗
Air Drying	Sewage sludge is dried on sand beds or on paved or unpaved basins. The sewage sludge dries for a minimum duration of 3 months	✓	✗	✓	✗	✗	✗
Anaerobic digestion	Sewage sludge is treated in the absence of air at a specific temperature. The values of the temperature shall be between 15 days at 35 ^{o}C and 60 days at 20 ^{o}C	✓	✗	✓	✗	✗	✗
Composting	Using either the within-vessel, static aerated pile, or widow composting methods. The temperature of sewage sludge is raised to 40 ^{o}C or higher and remains at 40 ^{o}C or higher for 5 days. Fours in the 5 day period, the temperature in the compost pile exceeds 55 ^{o}C	✓	✗	✓	✗	✓	✗
Lime Stabilization	Sufficient lime is added to the sewage sludge to raise the pH of the sewage sludge to 12 for 2 hrs.	✓	✗	✓	✗	✗	✗
Thermal Treatment	Sewage sludge is heated to a temperature of 180^{o}C or higher for 30 minutes	✓	✗	✓	✓	✓	✗

2.6 Treatment and Sewage Sludge Classification in South Africa

Snyman and colleagues (2003) documented the treatment technologies employed by South African wastewater treatment plants. According to this study, 57% of the sludge that is produced employs anaerobic digestion of primary and humus sludge (Snyman *et al*., 2003). The sludge types generated from these plants are presented in figure 2.1.

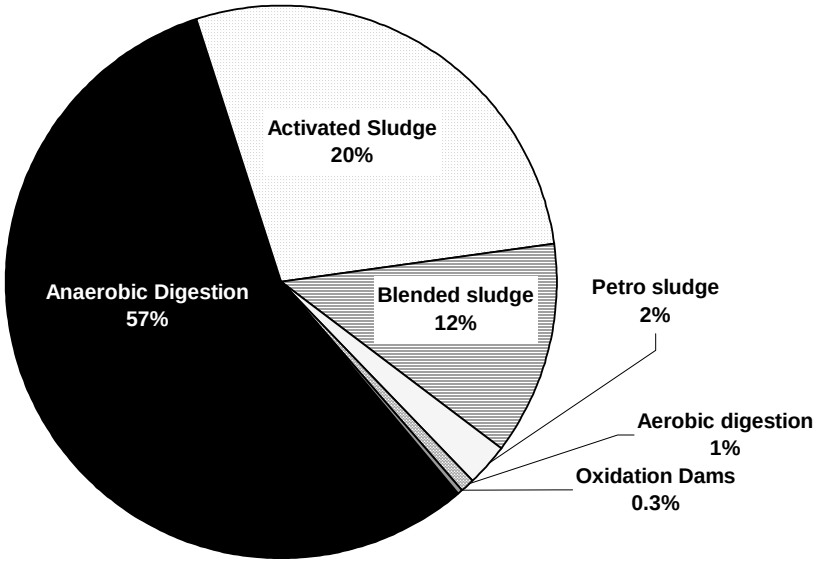

Figure 2.1 Sludge types produced by the wastewater treatment plants surveyed in South Africa on a mass percent basis. The blended sludge represents primary and activated sludge blended before or after digestion (Snyman *et al*., 2003).

Figure 2.2 illustrates the tertiary and additional stabilisation technologies employed by the wastewater treatment plants surveyed in South Africa. The majority (74% mass) of the sludge producing treatment plants surveyed do not treat the sludge further than the traditional anaerobic digestion and activated sludge treatment. Composting is used by both metropolitan city councils and plants in smaller town councils while pelletisation is only employed by large metropolitan councils (Snyman *et al*., 2003). Aerobic digestion is employed as an

additional treatment method after anaerobic digestion in one major site (Snyman *et al*., 2003).

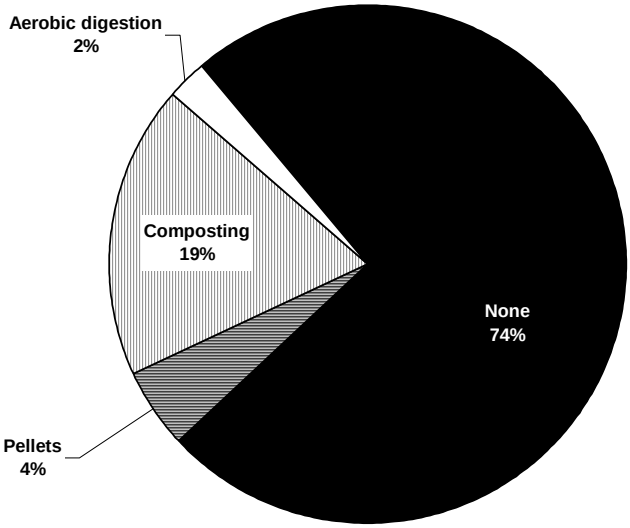

Figure 2.2 The tertiary and additional stabilisation technologies employed by the wastewater treatment plants surveyed in South Africa on a mass percent basis (Snyman *et al*., 2003).

The sewage sludge produced from treatment plants in South Africa is used for a number of activities, including application onto golf courses and use by municipalities for lawn cultivation, while some is collected by farmers for agricultural use. The disposal and beneficial use of sewage sludge in South Africa are summarized in figure 2.3.

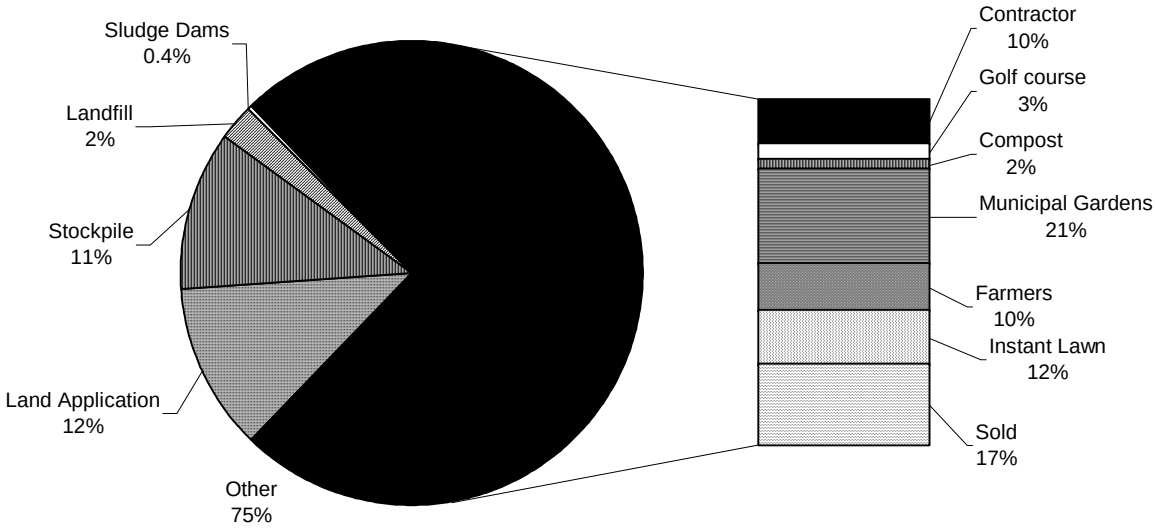

Figure 2.3 the major disposal methods employed by the wastewater treatment plants surveyed in South Africa on a mass percent basis (Snyman *et al*., 2003).

Table 2.6 summarizes the classification of sewage sludge indicated in the South African sludge guidelines. The South African guidelines classify sludge at three levels (Types A, B and C) and a fourth category (Type D) that stipulates ceiling limits for pollutants is added. Although the hygienic quality of Type D is similar to Type C, the Type D sludge is produced for unrestricted use on land at maximum application of 8 tonnes per hectare per year, the levels of metals and inorganic content are limited to acceptable low levels (WRC, 1997).

Table 2.6 Classification of Sewage Sludge to be used or disposed off on Land (WRC, 1997)

Sewage Sludge	Treatment	Characteristics-Quality of Sewage sludge
Type A Sludge	Cold digested sludge Septic tank sludge Oxidation tank sludge	Usually unstable and can cause odour nuisances and fly-breeding Contains pathogenic organisms Variable metal and inorganic content
Type B Sludge	Anaerobic digested sludge Surplus activated sludge Humus tank sludge	Fully or partially stabilized – should not cause significant odour nuisance or fly-breeding Contains pathogenic organisms Variable metal inorganic content
Type C Sludge	Pasteurised sludge Heat treated sludge Lime-stabilised sludge Composted sludge Irradiated sludge	Certified to comply with the following quality requirement: Stabilized – should not cause odour nuisances or fly-breeding Contains no viable *Ascaris* ova per 10 gram of dry sludge Maximum 0 *Salmonella* organisms per 10 gram dry sludge Maximum 1000 Faecal coliform per 10 gram dry sludge, immedialtely after treatment (disinfection/sterilization) Variable metal and inorganic content
Type D Sludge	Pasteurised sludge Heat-treated sludge Lime-stabilised sludge Composted sludge Irradiated sludge	Certified to comply with the following quality requirement: Stabilized – should not cause odour nuisances or fly-breeding Contains no viable *Ascaris* ova per 10 gram of dry sludge Maximum 0 *Salmonella* organisms per 10 gram dry sludge Maximum 1000 Faecal coliform per 10 gram dry sludge, immedialtely after treatment (disinfection/sterilization) Has specific limits for metal and inorganic content (summarized in WRC, 1997) Product must be registered in terms of Act 36 of 1947 if used for agricultural activities

2.7 Resistance of Microorganisms to Disinfection

The previous section discussed disinfecting techniques employed by wastewater treatment plants to reduce or eliminate the numbers of infective species. If the sewage sludge used is not adequately treated, there is potential for crop contamination.

Studies have shown that once fruits and vegetables have been contaminated, it may be difficult to disinfect them (Maxy, 1982; Takeuchi *et al*., 2000; Wachtel *et al*., 2002a; Wachtel *et al*., 2002b). Some microorganism such as *E. coli* show preferential attachment to the interior of damaged fruits and vegetables than on the surface (Takeuchi *et al*., 2000) as the juice within the vegetable provides good growth medium (Maxy, 1982). Itoh and coworkers (1998) found that *E. coli* was internalized when radish sprouts were produced from contaminated seeds and therefore would be protected from surface decontamination treatment. *E. coli* is capable of attachment to the interior of stomatal pores (Seo and Frank, 1999; Takeuchi and Frank, 2000; Takeuchi and Frank, 2001) and has a tendency to form aggregate associations (Wachtel *et al*., 2002a). These attachment sites and aggregation tendencies may cause bacterial resistance to physical methods of surface disinfection as well as chemical treatment such as chlorination (Wachtel *et al*., 2002b).

2.8 Protecting the Public and Environment through Regulatory Management

Most countries adopt a similar approach to protect the public from infection due to pathogens originating from wastewater sludge. The use of wastewater sludge is regulated and these regulations stipulate how the sludge should be disinfected and/or how to minimize the chance of infection through prescribed management practices. In the United States, the use and disposal of treated sewage sludge is regulated under CFR Part 503 (EPA, 1999).

The regulation protects public health and the environment through requirements designed to reduce the potential for contact with disease-bearing pathogens in

sewage sludge applied to the land or placed. These requirements are divided into:

- Requirements designed to control and reduce pathogens in treated sewage sludge and
- Requirements designed to reduce the ability of the treated sewage sludge to attract vectors (insects and other living organisms that can transport sewage sludge pathogens)

It includes both performance and technology-based requirements. Wastewater plants have the freedom to modify conditions and combine processes with each other to meet the requirements.

At present in South Africa humans and the environment are protected under the National Water Act 36 of 1998 (NWA), National Environmental Management Act 107 of 1998 (NEMA), Water Services Act 108 of 1997(WSA), the Constitution of the Republic of South Africa (Act 108 of 1996) and the Health Act 63 of 1977.

The Department of Water Affairs and Forestry is the custodian of water resources in South Africa. The guidelines for sewage sludge classification and application are summarized in a document on permissible utilization of sewage sludge (WRC, 1997; WRC, 2002). If the sludge reuse or disposal method does not comply with the requirements detailed for the applicable classification its reuse or disposal requires permission, which could be in a form of a licence or permit (WRC, 2002).

In South Africa, there aren't any specified restricted techniques for sludge treatment, but the chosen technologies need to yield the sludge quality as required in the guidelines (WRC, 1997).

2.9 Public Perception

The benefits of sewage sludge are well understood by the scientific community, and through consultation, most governments around the world recognize the benefits of using sludge in crop production. It is for this reason that a number of countries have since engaged in utilizing sewage for land application purposes.

Despite the advancements in sludge use in agriculture, the main recipients of these services have often been neglected. This often led to fear and rejection of sewage sludge among some members of the public as a result of misinformation due to media coverage (Sunday Times, 2003). Due to lack of scientific knowledge, the public will generally reject any association with a product or service if it is linked to odour or discolouration (Small Wright, 2002). Tyson (2002) reported that if sewage sludge did not smell, the public probably would not complain.

In a small preliminary survey done in South Africa, it has emerged that only a small percentage (39%) of low income earners were aware of what sewage sludge was (Snyman and Van der Waals, 2003). Snyman and Van der Waals (2003) also noted that the respondents did not understand the risks associated with using sewage sludge for agricultural soil amendment. Of the respondents from a higher income bracket, 79% were found to have knowledge of sewage sludge and its potential benefits. The majority of the respondents from this group also expressed their willingness to purchase vegetables from a sewage sludge fertilized farm, with 45% prepared to consume vegetables grown on sewage sludge (Snyman and Van der Waals, 2003). It appears from this survey that if members of the general public are informed of the benefits of sludge, reception of the use of sewage sludge might increase in the future. It is thus the responsibility of the sludge producers together with the governments to introduce mechanisms of educating the public of sewage sludge and its use in agriculture.

2.10 Assessing Human Risk Exposure

The sections preceding indicate that many wastewater plants generate sludges that still contain pathogens. However, these sludges are still used in agricultural practices. The question to address therefore is "What is the risk associated with this practice?" If the risk of using such sludge is unacceptable, what management practices should be adapted to reduce this risk to an acceptable level?

While complete elimination of pathogens from sludge is ideal, it has been indicated that if the numbers of pathogens in sludge are reduced to an acceptable level, the use of such sludge in agricultural land does not appear to result in unacceptable risk to human health (Apedaile, 2001; Tanner *et al*., 2003). According to Vesilind (2003), coming into contact with small doses of pathogens is the "sufficient challenge" our bodies need to stay healthy as our enhanced health comes not from zero exposure, but from a sufficient exposure to pathogens. Although this is true for healthy individuals, this could be different for the South African population, as a large percentage of the population is HIV positive and therefore immunocompromised (Dorrington *et al*., 2002).

One of the concerns often raised regarding sludge application is the emission of pathogenic aerosols during land application (Pillai *et al*., 1996). The risk of release rises as the pathogenic content in sludge increases. Raw sludge from municipal sewage would be more likely to release airborne pathogens than those that have been treated to reduce the pathogens (Straub *et al*., 1993). Tanner and colleagues (2003) evaluated the potential for bio-aerosols from sludge application, and concluded that the risk of adverse public health effects from bio-aerosols following land applied sludge is low. Forcier (2002) indicated that although quantities of bio-aerosols could be released during storage, loading and land application, they are diluted and scattered through atmospheric dispersion in ambient air. The survival of and the potential for infection from these organisms are lessened by the natural processes of attenuation such as ultra-violet radiation

and desiccation (Forcier, 2002). Bio-aerosol emissions are also lessened when applied sludge is subsequently incorporated into the soil (Straub *et al*., 1993). It appears that the methods used for sludge land application do not result in airborne release of biological agents to the same extent as in wastewater treatment facilities (Apedaile, 2001).

Tools exist to measure the risk to human health associated with the use of sewage sludge that contains pathogens in agricultural practices. The following section details one of the tools used in this thesis.

2.10 .1 Health Risk Assessment

The health risk assessment provides a means to estimate the probability of adverse effects associated with measured or estimated levels of the hazardous agents, and a tool for predicting the extent of potential or probable health effects. The protocol was originally developed for carcinogen assessments. However, current trends favour the application of similar procedure to establish the risk of microbiological hazards. The process as defined by the US EPA, is comprised of four distinguishable but interacting phases, namely:

- Hazard identification;
- Exposure assessment;
- Dose-response assessment and
- Risk characterisation (Zwietering and van Gerwen, 2001)

The interrelation of these phases is depicted in Figure 2.4.

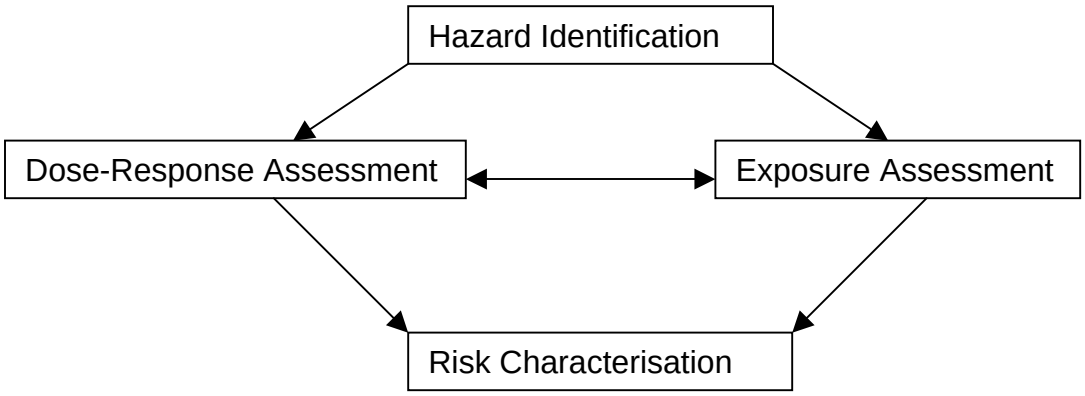

Figure 2.4 The interrelation of the risk assessment phases (Genthe 1998).

i Hazard Identification

This involves the identification of biological, chemical and physical agents capable of causing adverse health effects and that may be present in a particular food or group of foods (Rocourt *et al*., 2001). Once the health hazard has been identified, the remainder of the process encompasses the description of the properties of the hazardous agent and the identification of both acute and chronic health effects (Genthe, 1998).

ii Hazard Characterisation

This involves the qualitative and or quantitative evaluation of the nature of the adverse health effects associated with the hazard present in food. It provides description of the severity and duration of adverse effects that may result from ingestion of a microorganism in food. This involves a dose response assessment by establishment of a relationship between the dose of an agent and the rate of infection. Dose response assessment is considered a key ingredient of quantitative risk assessment as it is supposed to provide the link between exposure to a hazardous agent and the probability of ensuing health effects (Teunis and Havelaar, 2000).

Some microorganisms when present at sufficient levels are capable of causing disease, while others may produce toxins that contribute to the development of a

disease (Brooks *et al*., 1991). Toxins produced by bacteria are generally classified into two groups, exotoxins and endotoxins. Exotoxins are excreted by living cells, while endotoxins are released on bacterial death (Brooks *et al*., 1991).

iii Exposure Assessment

This involves the qualitative and or quantitative evaluation of the likely intake of biological, chemical and physical agents via food, as well as exposure from other sources if relevant (Rocourt *et al*., 2001). It is usually defined as a process of measuring or estimating the intensity, frequency and duration of human exposure to a contaminant. The task of exposure assessment is to provide the actual exposure conditions required to predict risk, and to identify and predict the effects of the proposed control options (Genthe, 1998).

iv Risk characterisation:

This involves the qualitative and or quantitative estimation, including attendant uncertainties of the probability of occurrence and severity of known or potential adverse health effects in a given population based on hazard identification, hazard characterization and exposure assessment (Rocourt *et al*., 2001). Risk characterisation has been defined as the process of calculating the incidence of the health effect under the conditions of exposure described in exposure assessment. A major component of risk assessment is an evaluation of all assumptions used and all sources of uncertainty (Genthe, 1998). In risk characterisation all results of the former steps are integrated, bringing together all inaccuracies from the former steps (Zwietering and van Gerwen, 2000). Thus risk characterization is defined as the process of estimating the likelihood or probability of experiencing the adverse effects of an identified hazard, the impact or consequences of those effects and describing the attendant uncertainty of the estimates.

2.11 Factors Affecting Management of Sewage Sludge Use in South Africa

South Africa has unique factors that could influence management of land application of sewage sludge. These factors include population density, high incidence of HIV/AIDS, unique climatic conditions and soil quality, amongst others. A detailed description of these factors will be provided in later sections to indicate how they influence management of sludge use in South Africa.

2.12 Conclusion

Sewage sludge could be used beneficially in agricultural practices, especially in South Africa's carbon depleted soils. It appears there are vast agronomic and economic benefits to sludge use, particularly as the cost of fertilizers are on the increase.

However, pathogens do occur in a large percentage in what is regarded as sewage sludge ready for agricultural use. In South Africa, little information is available on the risks associated with using sewage sludge that has not been disinfected.

International authors have investigated and quantified these risks. As a result of the factors that are unique to South Africa, it would not be appropriate to adopt work from other countries. These factors justify an investigation to assess the risks associated with the use of pathogen rich sewage sludge in agricultural practices.

A high risk crop was chosen to illustrate a worst case scenario. It was therefore decided to investigate the prevalence of microorganisms in a crop grown in sewage sludge amended soil. A risk assessment will provide a means of estimating the probability of adverse effects associated with measured or estimated levels of hazardous agents, and a tool for predicting the extent of potential health effects.

Based on our understanding and findings a functional management plan for sewage sludge application to agricultural land can be formulated.

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Chapter 3

The Microbiological Quality of Sewage Sludge in South Africa

3.1. Introduction

The practice of using sewage sludges in agricultural land is attractive to many farmers and water authorities (Carrington *et al*., 1982). It provides nutrients for crop growth as well as organic matter for soil conditioning (Melloul and Hassani, 1999). In the UK approximately 70% of sludge produced is deposited on land (Carrington *et al*., 1982). This is practiced primarily for economic reasons (Kelley *et al*., 1984; Bouwer, 1992) and also as an alternative means of disposal since the ban on sea disposal (EPA, 1999). In South Africa, agricultural soil is often degraded through erosion and the nutrient and carbon content are low. Land application therefore appears to be a beneficial and environmentally sustainable sludge management option (Sidhu *et al*., 1999) for South Africa.

However, sludge contains microorganisms that could pose a health hazard to humans. The types of organisms present in sludge are determined by the microbiological quality of wastewater from which the sludge is generated. These organisms include bacteria, viruses, protozoa and helminths (Burge and Marsh, 1978; Strauch, 1991).

At present, very few South African wastewater treatment plants disinfect their sewage sludge. Techniques commonly used in South Africa include aerobic stabilization by increasing the sludge age in the activated sludge process and anaerobic digestion in either mesophylic or heated digesters. These techniques are not capable of adequately disinfecting sewage sludge, resulting in a product that contains a large number of pathogens and can still have a high potential for vector attraction. Techniques such as lime stabilisation and composting which yield sewage sludge of improved microbiological quality (WRC, 1997), which can be safely applied to agricultural land are not common practice in South Africa.

The South African sewage sludge guidelines classify sewage sludge at four levels (Type A, B, C and D), rated in the order of improving microbiological quality. Type A is of low microbiological quality and may not be used for agricultural use. Type B sludge is typically an anaerobically digested sludge or waste activated sludge. This sludge type may be used in agricultural practice, but with strict control to minimize the exposure of humans to pathogens. As this sludge type is used extensively, the rest of the thesis focuses on the agricultural use of a type B sludge. The Types C and D are of acceptable microbial quality, with Type D being produced for unrestricted use, provided the levels of metal and inorganic content are kept at the limits set in the guidelines (WRC, 1997).

Most environmental concerns about land application of sewage sludge have focused on effects of nutrients especially nitrogenous (N) and phosphorus-containing (P) compounds and effects of heavy metals (Hyde, 1976). Microorganisms from sludge are often low on the priority list. To assess the threat posed by different microorganisms in sludge intended for soil conditioning, the types of organism present in the sludge must be determined. This chapter addresses the microbial quality of sewage sludge to be used for soil amendment purposes. The secondary aim of this chapter is to estimate the quantity of microorganisms (Faecal coliforms, *E.coli*, *Salmonella* spp and *Ascaris*) in sewage sludge, as the persistence of these organisms will be followed during the green house experiments.

3.2. Material and Methods

3.2.1 Sample Collection

Sludge samples (n=78) were collected at selected wastewater treatment plants. Three rounds of sampling were done to include seasonal variation, i.e winter, summer and autumn. These samples were analysed for microbiological content.

3.2.2 Microbiological Analysis

Two different South African laboratories were selected for the analyses: the East Rand Water Care Company Laboratory (ERLAB) and the Agricultural Research Council (ARC) Institute for Soil, Climate and Water (ISCW) laboratory. No accredited laboratory for the analysis of sludge could be found. For this reason, an inter-laboratory extraction and analysis train was set up. This was done to utilize the expertise of both laboratories to the optimum. Samples were collected and transported to the laboratories with a maximum delay of 72 hours. Organisms analysed were *Ascaris* ova (viability was not established), faecal coliforms and *Salmonella*, using methods as indicated in Table 3.1.

Table 3.1 Methods used in the analysis of microorganisms in sludge

Organism	**Method**
Ascaris ova	ERLAB Ascaris ova method. 2003
Salmonella spp	Bridson, 1998
Faecal coliforms	Difco Laboratories. 1998

3.2.3 Microbial Diversity

Additional sludge samples were obtained from two of the Waste Water Treatment Plants (WWTP) in the Gauteng province; namely Rondebult, a high metal sludge (HMS) and Olifantsfontein, a low metal sludge (LMS) (Table 3.2). These sludges are products of aerobic treatment (using aerator). Three samples were obtained for each of the sludge types. The sludges from these two plants were used as soil amendment in experimental trials. The microbial component in these sludge samples was determined using the Analytical Profile index and the Biolog technique.

Table 3.2 Metal content of sludge from Rondebult and Olifantsfontein (ERWAT, Sludge Analysis Report, 2003)

Metal	Rondebult (HMS) mg/kg	Olifantsfontein (LMS) mg/kg
Cr	308	31
Cu	167	42
Ni	138	21
Pb	155	47
Zn	1334	1036
Cd	11	5
Co	51	7

i Analytical Profile Index

Organisms present in each of the sludge samples (three HMS and three LMS) were identified using the Analytical Profile Index (API) according to the manufacture's instructions (BioMérieux, South Africa). The API system uses 21 miniature reaction compartments (cupules) that produce 23 biochemical reactions and is standardized for rapid identification of microorganisms. These tests and related reagents are indicated in Appendix A. The relevant API was chosen based on the Gram stain (-ve or +ve) reaction and the bacterial morphology.

The low metal sludge (LMS) and the high metal sludge (HMS) samples were collected from two wastewater treatment plants situated in the eastern Gauteng region, South Africa. Two samples comprising 1 g of each sludge type were emulsified in 9 ml of bacteriological peptone and incubated at 35 ^{o}C overnight. As the samples appeared concentrated, serial dilutions were made prior to transferring to petri dishes. A pour plate was made using plate count agar and incubated at 35 ^{o}C for 18 - 24 hrs. Following incubation, different colonies were picked up and transferred by streaking onto plate count agar, using an inoculating loop, and incubated at 35 ^{o}C for 18 - 24 hrs. Well-defined colonies were picked using an inoculating loop. Gram staining was done by using crystal violet dye, iodine and acetone (Eikelboom, 2000).

(a) Identification of the *Enterobacteriaceae*

The API 20E is an identification system for *Enterobacteriaceae* and other gram-negative bacteria (Juang and Morgan, 2001). Well-isolated colonies were picked off from the plate and suspended in 5ml sterile water. The suspension was carefully emulsified to achieve a homogeneous bacterial suspension. Bacterial identification tests were done according to the manufacturer's instructions (API 20E, BioMerieux, South Africa).

(b) Identification of the *Staphylococci*

For identification of the microorganisms belonging to the genus *Staphylococcus*, the API Staph was used (Ligozzi *et al*., 2002). Well-isolated colonies were picked off from the plate and suspended in 5ml API STAPH medium. The microtubes of the API Staph strip were filled with the bacterial suspension. The bacterial identification tests were done according to the manufacturer's instructions (API Staph, BioMerieux, South Africa).

ii Microbial Identification Using the Biolog Technique

The Biolog system was used to identity gram positive and gram negative bacteria isolated from the three LMS and three HMS sewage sludge samples. Bacteria typed by gram staining were inoculated onto appropriate Biolog media and subsequently onto specific Biolog 96-well microtiter plates for identification as outlined in the Biolog user manual. Gram postive cocci and rods were inoculated onto BUG (Biolog Universal Growth) + 5% sheep blood and BUG + glucose media, respectively prior to suspension in the supplied inoculating fluid. Gram negative bacteria were inoculated onto TSA with 5% sheep blood prior to suspension in the supplied inoculating fluid. Biolog microplates were inoculated with bacteria and incubated at 30 ^{o}C for 24 hrs according to the manufacturer's instructions. Colour formation in the individual cells of the microtitre plates was measured at 590 nm using microplate reader to determine the extent of reduction of the tetrazolium violet dye included with

the individual substrate in each microplate well. Readings were taken at 6 hrs and at 24 hrs following incubation.

3.3 Results and Discussion

3.3.1 Incidence of Organisms in Sludges from WWTPs in South Africa

Figure 3.1 indicates the faecal coliform counts from sludge samples studied between 2001 to 2003.

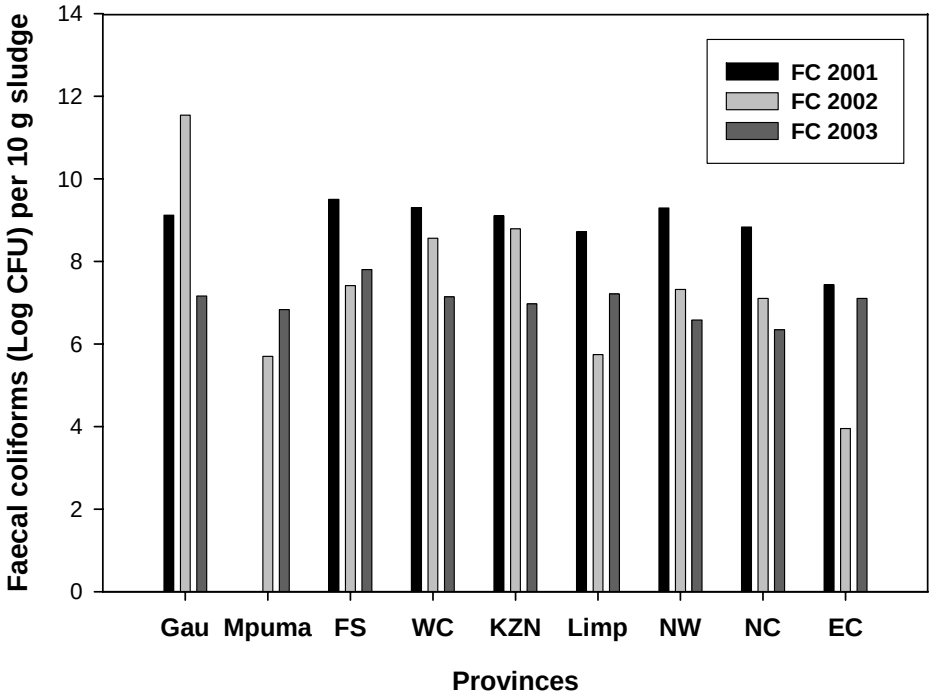

Figure 3.1 Incidence of faecal coliforms detected in sewage sludge from each of the South African provinces. Gau (Gauteng Province), Mpuma (Mpumalanga), FS (Free State), WC (Western Cape), KZN (KwaZulu-Natal), Limp (Limpopo), NW (North West), NC (Northern Cape) and EC (Eastern Cape).

Table 3.3 details the number of sludge samples that tested positive for *Salmonella* spp in each of the provinces. The results indicate that all provinces in South Africa need to manage or at least monitor prevalence of *Salmonella* spp in the wastewater sludges.

Figure 3.3 indicates the numbers of *Ascaris* ova detected in samples obtained from the WWTPs studied. Samples form Gauteng, Kwazulu-Natal and the Northern Cape had the highest number of *Ascaris* ova.

Table 3.3 Incidence of *Salmonella* spp in WWTPs at different South African Provinces.

Provinces	WWTP per Province	% WWTP with *Salmonella* 2001	% WWTP with *Salmonella* 2002	% WWTP with *Salmonella* 2003	% WWTP without *Salmonella* 2001-2003
Gauteng	20	60%	40%	30%	25%
Mpumalanga	2	0	50%	0	50%
Free State	5	40%	40%	80%	20%
Western Cape	15	80%	13%	13%	13%
KwaZulu-Natal	10	40%	40%	60%	20%
Limpopo	5	100%	60%	20%	0
North West	7	43%	57%	29%	14%
Northern Cape	4	75%	50%	50%	0
Eastern Cape	4	0	0	25%	75%

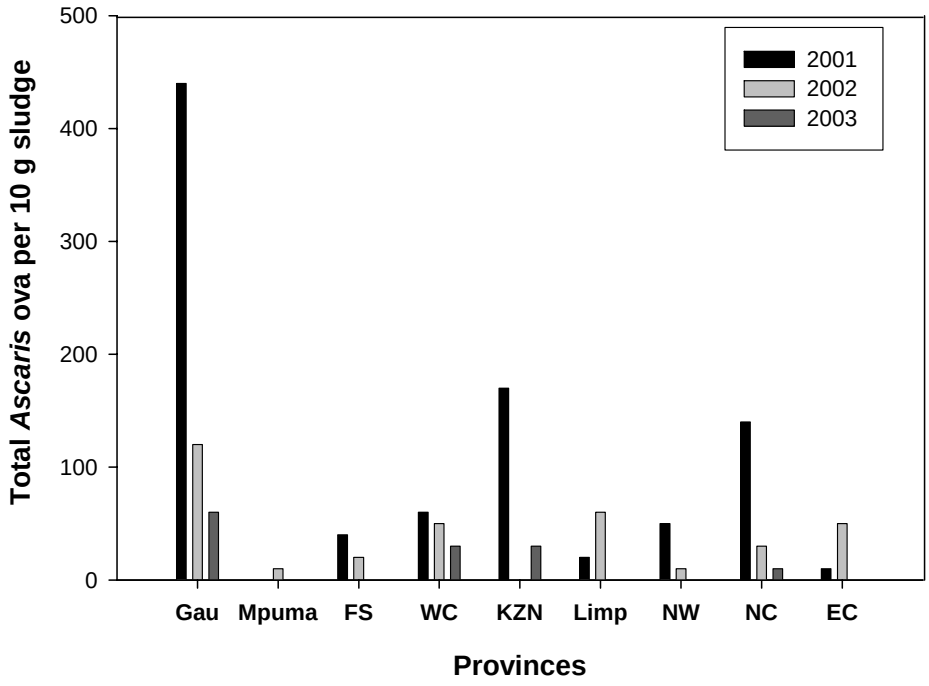

Figure 3.2 Incidence of *Ascaris* ova in sludge samples collected in all the provinces between 2001 and 2003.

The KwaZulu-Natal province has the largest number of people, with 20.3% of the population living in this area (Dorrington *et al*., 2002). However, a large proportion of people in KwaZulu-Natal live in rural areas which do not receive sanitation services. The WWTPs studied from this region service urban areas, which also yielded high prevalence of *Ascaris* ova and *Salmonella* spp.

Gauteng is the second largely populated province after KwaZulu-Natal, with 19.4% of people living in this province (Dorrington *et al*., 2002; Census, 2001). Gauteng is the heartland of the country's economy, and a province with the highest incidence of urbanization as people move into this area for improved quality of life. This migration results in increased number of informal settlements with poor sanitation facilities that may have resulted in the high incidence of *Ascaris* and *Salmonella* infections as noted in the sludge samples from the Gauteng region.

Other than a couple of belt press sludge samples, samples obtained from the Western Cape were mainly from drying beds or compost which show less

prevalence of microorganisms, as a result of the unfavourable dry conditions. Most of the WWTPs studied in the Western Cape service affluent communities. This could also explain the limited prevalence of *Salmonella* spp and *Ascaris* ova in this region. Samples from Mpumalanga were also collected from drying bed and showed limited prevalence. The prevalence of pathogens in the Eastern Cape appears limited, as a large proportion of people in this province live in rural dwellings that do not receive any sanitation services.

3.3.2 Microorganisms identified using API and the Biolog technique

Two known pure bacterial cultures were used as positive controls to validate the proficiency of the Biolog technique and were positively identified (Table 3.4).

Table 3.4 Validation of the proficiency of Biolog

Culture	Species
Control A	*Serratia marcescens*
Control B	*Photobacterium logei*

Table 3.5 Microorganisms occurring in sewage sludge, as found in this study and elsewhere

Microorganism	Reference	This Study Technique (Sludge Type)
Escherichia coli	Strauch, 1991	API (LMS and HMS)
Serratia spp.	Strauch, 1991	API (LMS and HMS)
Salmonella spp.	Carrington *et al*., 1982	API (LMS and HMS)
Citrobacter	Strauch, 1991	API (LMS and HMS)
Klebsiella ornilytica	Dudley *et al*., 1980	API (LMS and HMS)
Shigella spp	Strauch, 1991	**X**
Yersinia enterocolitica	Strauch, 1991, Pell, 1997	**X**
Clostridium spp	Strauch, 1991	**X**
Leptospira spp	Strauch, 1991	**X**
Mycobacterium spp	Strauch, 1991	**X**
Vibrio cholerae	Strauch, 1991	**X**
Streptococcus	Strauch, 1991	**X**
Enterobacter	Strauch, 1991	Biolog (LMS)
Serratia	Strauch, 1991	Biolog (LMS)
Proteus	Strauch, 1991	**X**
Providencia	Pelczar *et al*., 1993	**X**
Listeria monocytogenes	Strauch, 1991	**X**
Staphylococcus lentus	Dudley *et al*., 1980	API (LMS)
Achromobacter spp	Pelczar *et al*., 1993 Prazmo *et al*., 2003	Biolog (LMS)
Chromobacterium violaceum	Pelczar *et al*., 1993 Prazmo *et al*., 2003	Biolog (LMS)
Pseudomonas spp	Pelczar *et al*., 1993 Prazmo *et al*., 2003	Biolog (HMS)

Table 3.5 Continued

Microorganism	Reference	This Study Technique (Sludge Type)
Pseudomonas aeruginosa	Pelczar *et al*., 1993	Biolog (HMS)
Pantoea agglomerans	Pelczar *et al*., 2003	Biolog (HMS)
Serpens flexibilis		Biolog (LMS)
Oligella urethralis		Biolog (LMS)
Raoutella terrigena		Biolog (HMS)
Brevibacterium liquefaciens		Biolog (HMS)
B. mcbrellneri		Biolog (HMS)
B. linens		Biolog (HMS)
B. otitidis		Biolog (HMS)
Leclercia adecarboxylata		Biolog (HMS)
Rhodococcus australis		Biolog (HMS)
Cellulomonas hominis		Biolog (HMS)
Acitenobacter calcoaceticus		Biolog (HMS)
Exiguobacterium acetylicum		Biolog (HMS)

X = these organisms were not detected in the present study.

Bold = present in the current study, but not reported elsewhere.

This study used sensitive tests and detected microorganisms that are not commonly found in sludge. However these organisms are not indicated as human pathogens, but are mainly associated with the environment and, thus, may have originated from water or soil.

Most of the organisms identified using Biolog are not known to cause disease in healthy people. However, they may cause opportunistic infections in people who have weakened immune systems such as those undergoing antibiotic

therapy, cancer treatment or those with HIV/AIDS. For instance *Brevibacterium* strains usually present on the skin (Pelczar *et al*., 1993) have been implicated in bloodstream infections in HIV/AIDS patients (Brazzola *et al*., 2000).

Some organisms such as *Achromobacter*, *Chromobacterium* and *Pseudomonas* identified in this study, have been reported as frequently occurring in sewage (Pelczar *et al*., 1993; Prazmo *et al*., 2003). These bacteria are responsible for denitrification in soil (Drysdale *et al*., 1999).

Achromobacter xylosoxidans often found in aqueous environmental sources (Clermont *et al*., 2001) is an opportunistic pathogen that has been implicated in serious infections (Ramos *et al*., 1996; Hernandez *et al*., 1998). *Oligella urethralis* is an organism of the genital and urethral tracts and has been implicated in urinary tract infection (Mammeri *et al*., 2003) and meningitis (Graham *et al*., 1990), *Serratia marcescens* and *Pseudomonas aeruginosa* with respiratory infections (Kirschke *et al*., 2003), *Chromobacterium violaceum* is the causal agent of septicaemia (Perera *et al*., 2003) while *Acinetobacter* species are often associated with nosocomial infections (McDonald *et al*., 1998).

Children with chronic granulomatous disease are predisposed to infection caused by *Chromobacterium violaceum* (Macher *et al*., 1982). Although infections caused by *C. violaceum* are rare, when they occur they are responsible for a high incidence of mortality (Ti *et al*., 1993).

The presence of the opportunistic pathogens in sludge may have serious implications for the consumers if such sludges are used for soil amendment. This is particularly pertinent for young children, expectant women, the elderly and those infected with HIV/AIDS, as their immune systems are compromised.

The primary route of exposure to pathogens is by ingestion. If sludge is to be used in the production of food crops, then there is a chance of exposure

through ingestion. Consequently, there is a greater need to reduce pathogen numbers prior to soil application.

Biochemical profiling using API has become popular in recent years (Bezuidenhout *et al*., 2002). This technique although qualitative, identifies organisms based on their biochemical reactions and it provides rapid identification thereof (Juang and Morgan, 2001).

Organisms identified with API were common between the LMS and HMS except for the *Staphylococcus lentus*, which was only identified in the LMS (Table 3.3). Dudley *et al*., (1980) also found *Klebsiella* spp, *Salmonella* spp, and *Staphylococcus* spp in the Texas sewage sludge. *Salmonella* and *E.coli* are some of the most common organisms in sewage sludge (Jones, 1980; Carrington *et al*., 1982; Strauch, 1991; Bouwer, 1991; Jones 1999).

Bezuidenhout and colleagues (2002) in KwaZulu-Natal, South Africa also used API to identify similar species, namely *E.coli*, *Serratia* spp., and *Klebsiella* spp. in contaminated water. They however did not detect any *Salmonella* spp in this region. Generally *Salmonella* spp frequently occur in wastewater and sewage sludge and this organism has been reported to persist in various environments due to its ability to withstand stressful conditions (Strauch, 1991). For this reason outbreaks of Salmonellosis occur frequently worldwide (Melloul and Hassani, 1999). Carrington and colleagues (1982) have reported that *Salmonella* spp may multiply in sludge in the absence of competition from other microorganisms.

3.4 Conclusion

Testing of sludge samples showed large numbers of Faecal coilforms, indicating that intensive treatment of sludge from WWTPs across the country is required to meet the type C and D class South African guidelines.

It is likely that the large numbers of *Ascaris* in the Gauteng area could be related to increased urbanization in this province.

The microbial population determined for LMS was similar to the population of HMS except for the presence of *Staphylococcus lentus* in LMS.

Due to the presence of potentially noxious pathogens in the sewage sludge, it is recommended that sewage sludge need to be adequately disinfected prior to use in agricultural land.

Further research on the microbial quality in South African water and soil in the Gauteng region will be necessary to establish the types of organisms present in these environments.

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Chapter 4

Survival of Mircoorganisms in Soil Amended with Sewage Sludge, and their Subsequent Persistence in Crops.

4.1 Introduction

As indicated in Chapter 3, there are a number of microorganisms in a Type B sewage sludge produced by many of the wastewater treatment plants in South Africa. While the value of sludge use in agriculture is clearly understood, the potential persistence of microorganisms in agricultural soil has not been fully investigated. Studies done elsewhere on different crops have indicated the contamination of fruits and vegetables following irrigation with sewage sludge or wastewater (Rudolfs *et al*., 1951; Hyde, 1976; Bouwer, 1992; Armon *et al*., 1994; Wachtel *et al*., 2002; Petterson *et al*., 2001).

Measures to reduce pathogen load in sludge such as composting are not always successful in completely inactivating these microorganisms from sludge. For instance, *Salmonella* spp and *E.coli* can survive the composting process and then regrow in soil following amendment (Sidhu *et al*., 1999). As the regrowth potential is affected by a number of different inherent and environmental factors the regrowth of pathogens appears difficult to predict (Sidhu *et al*., 1999).

Kudva *et al*. (1998) reported that *E.coli* survived for more than a year in a non-aerated manure pile that was exposed to environmental conditions, and Jones (1999) pointed out that this organism is capable of surviving for four months in soil. *Salmonella* spp may survive over one year in slurry and may still be isolated in soil for up to 20 weeks following application to land (Jones, 1980). It has been shown that even processed sewage sludge still contains considerable proportions of viral, bacterial, protozoan and helminthic agents of disease (Burge and Marsh, 1978; Strauch, 1991).

One of the major routes of exposure to sludge is by ingestion, although other routes such as respiratory and ocular routes can be involved. If untreated or inadequately treated sewage sludge is used in the production of food crops, particularly those that are eaten raw, a chance of exposure to pathogenic microorganisms through ingestion exists.

In South Africa, most studies on sewage sludge focused on the effects of nutrients (Easton, 1983; Snyman *et al*., 1998; Henning *et al*., 1999) and heavy metals (Lotter and Pitman, 1997). The effects of sewage sludge-borne microorganisms have not been studied in detail. Apart from this study, there appears to be no other work done on survival of microorganisms in agricultural soil, under South African conditions. The research in this area was done in other countries with different climatic and socio-economic conditions compared to South Africa.

The aim of this chapter is to determine the prevalence of microorganisms in soil conditioned with sewage sludge and the persistence of these microorganisms in crops grown in this soil, following a single application of a Type B sewage sludge prior to planting. Faecal coliforms, *Salmonella* spp, *E. coli* and *Ascaris* were chosen as organisms to study, as they are used as indicators in the South African sewage sludge guidelines.

4.2 Materials and Methods

4.2.1 Green House Experiments

Potatoes (*Solanum tuberosum*) were obtained from a local farmer in the Tshwane area, South Africa. Potato was selected as the study sought for high risk crop that grows in contact with the soil, and the season was also appropriate. Samples of sludge representing the high metal sludge and the low metal sludge were obtained from the Eastern Gauteng region (South Africa). Experiments were done in

greenhouses under controlled conditions (temp 25 – 28 ^{o}C) for a three month period. The experimental layout is detailed in Table 4.1.

Table 4.1 Experimental lay-out of trials undertaken

Controls		**Trials**							
No Sludge		**Low Metal Sludge**				**High Metal Sludge**			
Controls		8 tons/ha		16 tons/ha		8 tons/ha		16 tons/ha	
8 pots	8 pots	8 pots	8 pots	8 pots	8 pots	8 pots	8 pots	8 pots	8 pots

Each pot contained approximately 4 kg of oven sterilised sandy loam soil. Prior to application, the sludge was sun-dried and crushed to achieve a fine product to ensure homogeneous mixing with the soil. For each trial and the controls there were duplicate pots dedicated for sampling (shaded cell in Table 4.1). These pots, although not planted, were subjected to the same conditions as the other pots. Soil samples were collected in a manner to avoid cross-contamination every second week, and analysed for microorganisms. The same amount of water (about a litre) was added to each pot every second day. At the end of the experiment, the potatoes were collected for microbiological analysis. Potatoes were harvested in a manner to avoid cross-contamination and placed in sterile bags. At least two potatoes were harvested per pot. In the laboratory, each potato was cleaned with sterile distilled water prior to microbiological analysis.

4.2.2 Microbiological Determinations

Microbiological analysis were carried out for both soil and potato samples.
Procedures for analyses of Faecal coliforms, *E.coli, Salmonella* and *Ascaris* ova are those adapted by the East Rand Water Care Company (ERWAT) in South Africa (ERWAT, 1996; Clesceri *et al* .,1998).

i *Salmonella* spp *analysis*

All chemicals used for this analysis were purchased from Oxoid. A 1 g of sample (soil or portion of potato) was placed in a 10 ml Buffered Peptone Water, mixed and incubated at 35 ^{o}C for 18 – 24 hrs. An aliquot (0.1 ml) of the mixture was transferred to 10 ml Rappaport VS Broth, and incubated at 44 ^{o}C for 24 hrs. The enrichment broth was subcultured by streaking the bacterial suspension onto the plates of Brilliant Green agar and incubated at 35 ^{o}C for 18 – 24 hrs. A presumptive positive result was suspected if red colonies grew. Selected colonies were then subcultured onto Xylose-Lysine-Desoxycholate (XLD) agar (Batch number 230180), and incubated at 35 ^{o}C for 18 – 24 hrs. Occurrence of black colonies confirmed the presence of *Salmonella* spp in the original sample.

ii *Analysis of faecal coliforms*

A subsample of 1 g (soil or potatoes at the end of experiment) from the experimental and control pots was added to 9 ml of peptone broth (Difco) and incubated overnight to resuscitate the microorganisms and serially diluted and filtered using sterile 0.45 μm gridded membrane filter (Sartorius). When filtration was completed, the membrane filter was removed with sterile forceps and rolled onto MFC agar (Difco) and incubated inverted at 44.5 ± 0.5 ^{o}C for 18 – 24 hrs. Using a colony counter, all blue colonies were counted. Results were expressed as colony forming units per gram (CFU/g).

iii *E. coli* analysis

The membrane from the faecal coliforms was transferred to the nutrient agar substrate containing MUG (4-methylumbeliferyl-β-glucoside) (Difco). The plates were then incubated together with one blank at 35 ± 0.5 ^{o}C for 4 hours. Colonies were observed using a long wavelength ultraviolet light source for the fluorescence on the periphery. Results were expressed as CFU/g.

iv *Ascaris analysis*

Before determining the amount of *Ascaris* ova, the moisture content of the sample was determined (ERWAT, 1996). Approximately 10 g of the sample was weighed into a beaker and treated with an alkaline soap while mixing the preparation with an orange stick. The sample was then washed through a treble Visser filter (comprising mesh sizes 100 μm; 80 μm and 35 μm), by rinsing repeatedly with a strong jet of tap water. The residue in the outer filter were rinsed with tap water and centrifuged at 3000 g for 3 minutes. The supernatant was removed using a Pasteur pipette, and the pellet was resuspended in $ZnSO_4$ (40%, 71 g/100ml H_2O) and centrifuged further for 3 minutes at 3000 g. The supernatant was transferred to a vacuum filtering system, using a filter of 12 μm (Millipore). The $ZnSO_4$ was rinsed off with distilled water to avoid recrystalization. The membrane filter was then placed in a glass petri dish and dried at 35 ^{o}C. A circular weight is usually placed around the edges of the membrane to prevent curling. Once dried, the filter was cut across its diameter and each of the half was placed onto a microscope slide, using a clear glue to hold it down. Using an orange stick, immersion oil was spread over the filter. *Ascaris* ova were counted using a phase light microscope (Olympus).

4.3 Results and Discussion

4.3.1 Microorganisms in Sludge

The quantity of microorganisms in sludge samples, together with amount expected to be present in the sludge applied to the pots are indicated in Table 4.2.

Table 4. 2 Microorganisms in sludge and expected quantities in the pot

Organisms in sludge	**LMS**		**HMS**	
Ascaris (per g)	2		1	
Faecal coliforms (CFU/g)	89 X 10^6		50 X 10^6	
E.coli (CFU/g)	89 X 10^6		49 X 10^6	
Salmonella	+ve		+ve	
Expected in the pots	**LMS 8**	**LMS 16**	**HMS 8**	**HMS 16**
Ascaris	14	28	7	14
Faecal coliforms	6.23 X 10^8	12.46 X10^8	3.5 X 10^8	7 X 10^8
E.coli	6.23 X 10^8	12.46 X10^8	3.4X 10^8	6.86 X10^8
Salmonella	+ve	+ve	+ve	+ve

4.3.2 Survival of Microorganisms in Contaminated Soil

All the control samples tested negative for all indicator organisms throughout the experiment. Descriptive statistics of the data used are shown in Appendix B. These values were generated using both the T-test and the Wilcoxon Signed Ranks test.

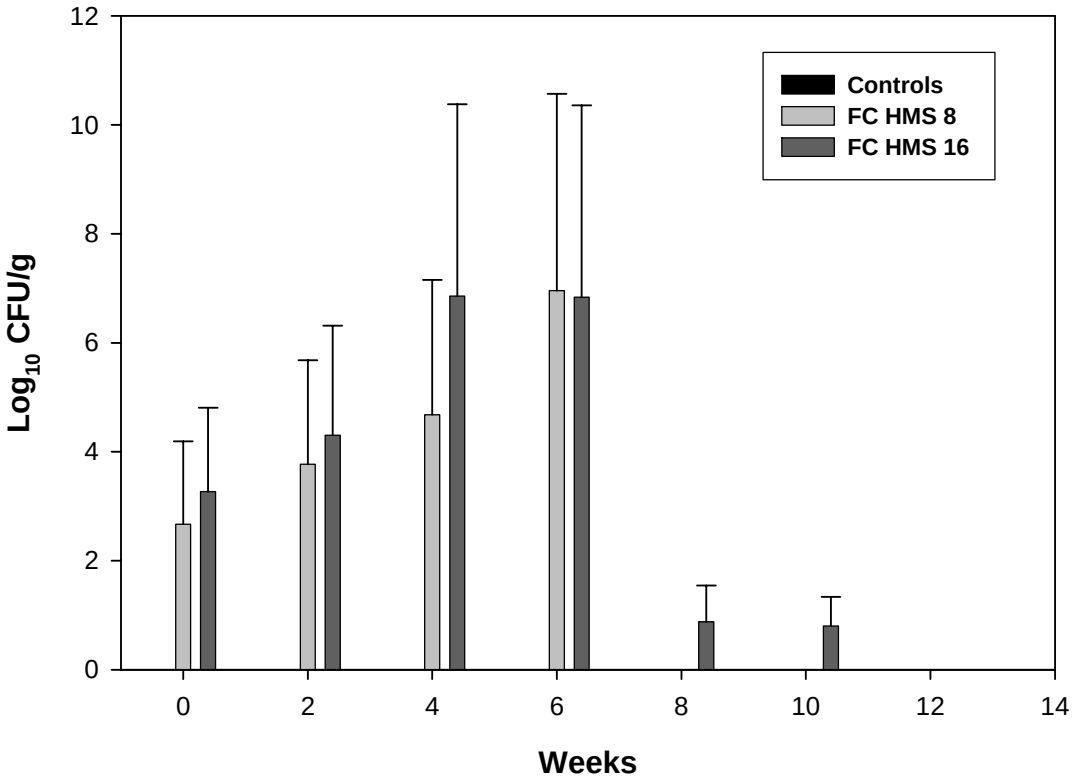

Figure 4.1 Faecal coliforms for HMS at application rates of 8 and 16 tons/ha.

As shown in Figure 4.1, pots amended with HMS 8 tons/ha, had fewer organisms than the HMS 16 tons/ha and these organisms were not detected from week eight until the end of the experiment. An increase of Faecal coliforms observed in both the 8 and 16 tons/ha pots up until week six was probably due to sufficient food and moisture as these pots were watered regularly.

There was a significant reduction in the number of organisms after week six. The organisms in the soil that received a dose of 16 tons/ha showed complete die-off after week ten.

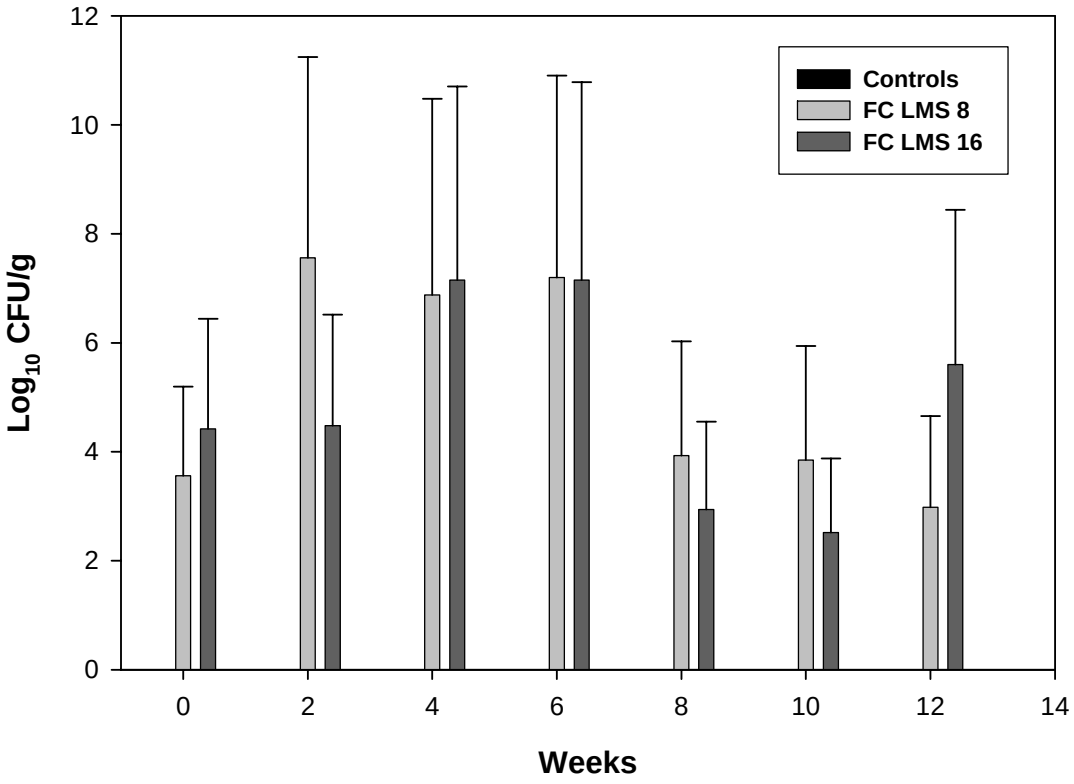

Figure 4.2 Faecal coliforms for LMS at application rates of 8 and 16 tons/ha.

LMS treatment showed persistence of Faecal coliforms throughout the duration of the experiment, although a decline was observed in weeks eight and ten for both 8 tons/ha and 16 tons/ha (Figure 4.2). The greatest survival of organisms was observed with LMS 16 tons/ha. In this treatment, although a decline in weeks eight and ten was noted, by the twelfth week, both faecal coliforms and *E. coli* had increased when compared to their initial values (week = 0) at the onset of the experiment (Figures 4.2 and 4.3). This could probably be due to competition between some microorganisms.

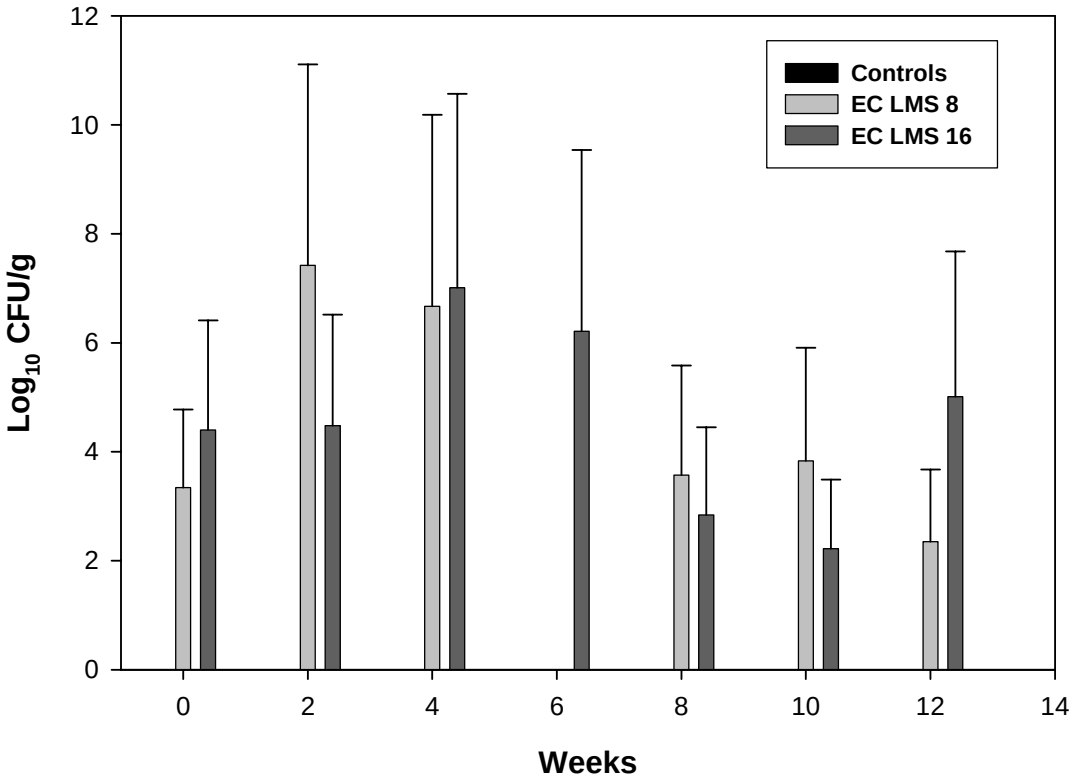

Figure 4.3 *E. coli* for LMS at an application of 8 and 16 tons tons/ha.

E. coli were detected throughout the study period with the exception of the LMS 8 samples taken in week 6 (Figure 4.3).

It appears that doubling the concentration of sludge (from 8 to 16 tons/ha) in the soil did not yield a large number of microorganisms from these pots. Instead, in some weeks there were more organisms in the 8 tons/ha than in the 16 tons/ha. For instance, the number of *E.coli* counted in LMS 8 tons/ha for weeks two, eight and ten were more than those counted in samples from pots containing LMS 16 tons/ha. However, the number of *E.coli* for LMS 16 tons/ha samples in the twelfth week were higher than the those counted in the LMS 8 tons/ha samples. In South Africa guidelines for use of sewage sludge require that the application rate should not exceed 8 tons/ha (WRC, 1997). Despite a decline in the number of microorganisms from the time of planting (time zero) to the harvest time (twelfth week), there was a

clear persistence of bacteria studied. Earlier Jones (1999) reported on the potential health risk associated with the persistence of *E. coli* in agricultural environment.

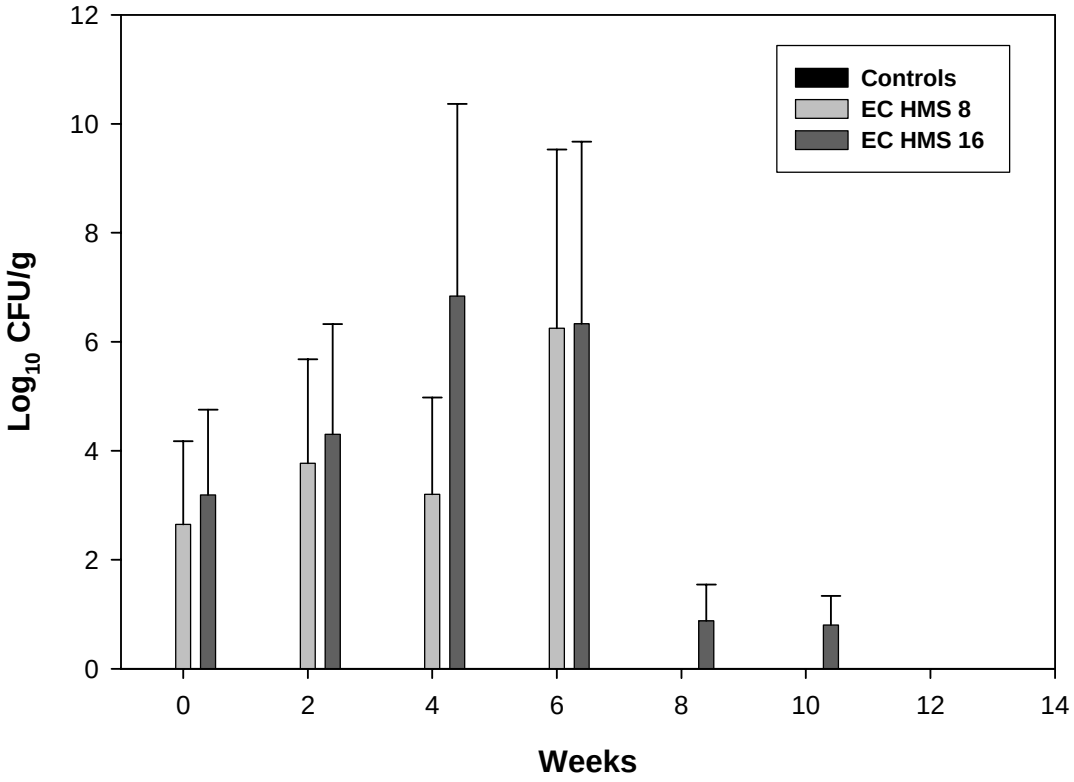

Figure 4.4 *E. coli* for HMS at application rates 8 and 16 tons/ha.

The number of *E.coli* for both the LMS16 (Figure 4.3) and HMS16 (Figure 4.4) pots peaked at week four and declined in the sixth week, although in the high metal sludge it is slightly lower than in the low metal sludge pots. By the twelfth week *E.coli* numbers in LMS16 were high, while in the HMS16 were very small.

In this study it was shown that microorganism can persist for a period of three months in soil amended with sewage sludge and thus may be a source of pre-harvest contamination of food crops growing in the field. In some countries such as the United States, sewage sludge is allowed to stand for up to three months before use to encourage bacterial die-off (EPA, 1999). Although counts were minimal by the

twelfth week (at harvest), it is likely that microorganisms will prevail in soil for a period well exceeding duration of the three months period (Strauch, 1991).

The decline in the number of microorganisms could be attributed to competition for food and space (Tester and Parr, 1983). As there was no nutritional addition made to any of the pots during the experimental period, these microorganisms were no longer able to reproduce at the rate observed in the earlier weeks (weeks two to six). As microorganisms grow, they tend to form colonies of millions of individual cells. As these colonies form, the food available to each cell becomes limited and excretions from these millions of cells become toxic to a microbe, such that some of the cells begin to die (Penner, 1998). Survival of bacteria is known to be influenced by a number of factors, which includes optimum temperatures and availability of organic matter (Bitton, 1994).

The HMS had far less persistence of both the Faecal coliforms and the *E.coli* as compared to the LMS. These microorganisms were only detected in soil up until the sixth week, with no further increase observed in subsequent weeks. This is probably due to the high concentrations of metals found in the HMS (Chapter 3; ERWAT, 2002). Metals have been reported to inhibit microbial growth (Tsai and Olson, 1990). Monpoeho *et al*. (2001) have pointed out that inorganic compounds such as heavy metals and polyphenols are toxic and cause lysis of the cells. The effect of metal-rich sludge on microbial community was also shown by Baath and colleagues (1998).

Table 4.3 provides an indication of whether *Salmonella* spp were found in the samples at each application rate for every week sampled. The presence of *Salmonella* spp was indicated with a positive sign, while the absence thereof was indicated with a negative sign. At time zero, *Salmonella* spp were only observed in the LMS at 8 tons/ha. All four treatments had *Salmonella* spp during weeks two and four. No *Salmonella* spp were detected in 8 tons/ha treatment for both LMS and HMS at week twelve.

Table 4.3 *Salmonella* found in sludge pots (+ = Presence, - = absence)

Weeks	Controls	LMS8	LMS16	HMS8	HMS16
0	-	+	-	-	-
2	-	+	+	+	+
4	-	+	+	+	+
6	-	-	+	+	+
8	-	+	+	+	+
10	-	+	+	+	-
12	-	-	+	-	+

The persistence of *Salmonella* spp throughout the experiment suggests their prolonged survival in soil. *Salmonella* spp have been indicated by other researchers as surviving in soil for a long period. For instance Strauch (1991) has reported that *Salmonella* spp could survive on and in the soil after a single application of sludge in summer for 424 to 820 days, and in winter the survival times were reported to be 104 to 350 days. Baloda *et al* (2001) also confirmed the prolonged survival of *Salmonella* spp, which he estimated to be about 299 days in soil. Sewage sludge spread on a hospital lawn has been implicated in an outbreak of salmonellosis in a hospital nursery (Burge and Marsh, 1978).

Table 4.4 shows the total number of *Ascaris* ova per gram of soil in all the pots for each application rate and sludge type for every week sampled. Other than at zero time and in the fourth week, there appeared to be no *Ascaris* detected in the soil sampled. Most *Ascaris* were found in samples collected in the fourth week. For instance, a total of four (4) *Ascaris* were counted in LMS 16 tons/ha samples. Although *Ascaris* samples were expected to occur in sewage sludge contaminated soil, this was not always the case. These pathogens might have been missed as a result of dilution caused by mixing of soil and sludge or they may only be unavailable in particular samples analysed. Although effort was done to ensure homogeneous mixing of sludge with soil, it is possible that there might have been islands/pockets of soil that might have not been affected.

Table 4.4 Numbers of *Ascaris* found in sludge pots per gram of contaminated soil

Weeks	Controls	LMS8	LMS16	HMS8	HMS16
0	0	1	0	0	2
2	0	0	0	0	0
4	0	2	4	1	0
6	0	0	0	0	0
8	0	0	0	0	0
10	0	0	0	0	0
12	0	0	0	0	0

4.3.3 Microorganisms in Potato

Indicated in Table 4.5 are the results of the analysis of microorganisms for the potato peel and the inside of the potato (core). None of the microorganisms tested were detected in the potato core. However, Faecal coliforms and *E.coli* were detected on the potato peel at the end of the experiment, in the treatment LMS at 16 tons/ha.

Table 4.4 Microorganisms found in potato in the 12th week

Sample	Microorganism	Control	LMS8	LMS16	HMS8	HMS16
Potato peel	Faecal coliforms **(CFU/g)**	0	0	2050	0	0
	E.coli **(CFU/g)**	0	0	1800	0	0
	Salmonella	-	-	+	-	+
	Ascaris ova	0	0	0	0	0
Potato core	Faecal coliforms **(CFU/g)**	0	0	0	0	0
	E.coli **(CFU/g)**	0	0	0	0	0
	Salmonella	-	-	-	-	-
	Ascaris ova	0	0	0	0	0

These are the mean values of all the eight repetitions carried out. Faecal coliforms, *E.coli* and *Salmonella* spp were found to be present on the outside (peel) of the cleaned potatoes at harvest time. Experiments done elsewhere on tomatoes have shown that even after field-grown tomatoes are washed with continuous vigorous agitation for as long as 15 minutes, the numbers of organisms remaining on tomatoes are essentially the same as on unwashed fruit (Rudolfs *et al*., 1951).

Although microorganisms studied were detected on the peel of the potato and none were found to be present in the inside of the potato, studies done elsewhere on other crops, have reported on the interior contamination of fruits and vegetables following irrigation with sewage or waste water (Wachtel *et al*., 2002; Petterson *et a*l., 2001). Organisms such as *E. coli* have been reported as capable of entering the plant (lettuce) through the root system and migrate to edible portion of the plant (Solomon *et al.,* 2002). It has also been indicated that *E.coli* can grow on raw salad vegetables (Adul-Raouf *et al*., 1993). Cieslak and colleagues (1993) previously reported case of outbreaks due to consumption of vegetables from a manured garden.

Another factor that should not be ignored is the possibility of cross contamination that could occur during preparation of contaminated vegetables, leading to a contaminated dish. Abdul-Raouf and colleagues (1993) demonstrated the ability of *E.coli* to grow on raw salad vegetables subjected to processing and storage conditions simulating those routinely used in commercial practice. However, through appropriate sewage sludge management practice, such contamination may be controlled.

Salmonella spp were only detected on the peel of potato samples from LMS 16 tons/ha grown in the 16 tons/ha for LMS and none of the core samples tested positive. Although Salmonellosis have previously and commonly been associated mainly with food of animal origin (Ayanwale *et al*., 1980; de Louvois, 1993; Blazer, 1996; Walls and Scott, 1997; Ebel and Schlosser, 2000; Sharma and Carlson, 2000), recent studies have shown that *Salmonella* contamination can be due to sewage

irrigation (Melloul and Hassani, 1999) which could lead to crop contamination (Asplund and Nurmi, 1991; Guo *et al*., 2000). A number of *Salmonella* species have been previously implicated in illness associated with the consumption of produce (del Rosario and Beauchat, 1995). The health threat of *Salmonella* and *E.coli* is also because the infectious dose of both these organisms is relatively low (Fratamico and Strobaugh, 1998). Potential infections due to these organisms necessitates that sewage sludge be appropriately treated before it is used as a soil conditioner.

Ascaris ova were not detected on the potato peel at the end of the experiment. Gaspard and Schartzbrod (1993) have shown that vegetables, namely, lettuce and tomato can be contaminated with *Ascaris* following irrigation. Ascaris have been reported to survive for up to two years in soil that has been irrigated with sewage sludge (Strauch, 1991), thus can lead to crop contamination (Gaspard and Schartzbrod, 1993) if untreated sewage sludge is used in agricultural land. *Ascaris* infections, especially in children are amongst the most common in the world (Carneiro *et al*., 2002). Blumenthal and colleagues (1996) could show doing experiments on lettuce, that the use of wastewater for irrigation causes transmission of nematode infections. Crop contamination with *Ascaris* was also reported by Ayres colleagues (1992). Considering that communities in developing countries such as South Africa are not in the habit of de-worming themselves, the use of untreated or inadequately treated sewage sludge, comprising viable *Ascaris* could result in serious infections. Although other microorganisms were not detected in the HMS at 16 tons/ha, *Salmonella* was present on the potato peel from this treatment.

4.4 Conclusion

It has been shown that *Ascaris* and microorganisms studied, namely faecal coliforms, *E.coli* and *Salmonella* spp will survive in soil for 3 months following a single application of sludge at planting.

The presence of *E.coli* and Faecal coliforms on the potato peel indicates that use of untreated sewage sludge for growing vegetables that come into contact with soil could be potentially hazardous to public health.

Bacteria cannot penetrate undamaged vegetable skin (Penner, 1998), but they can survive on the surfaces of vegetables, especially root vegetables such as potato.

Although there is a clear distinction between the LMS and HMS, there does not appear to be any appreciable difference in terms of the numbers of microorganisms between the two concentrations (8 tons/ha and 16 tons/ha) explored. It appears that doubling the application rate from the 8 tons/ha to 16 tons/ha does not significantly affect the persistence of microorganisms. The high metal sludge at an application rate of 8 tons/ha, had a quicker die off (week eight) of microorganisms. Generally microorganisms do not thrive in high metal sludge probably due to inhibition caused by these metals (Tsai and Olson, 1990).

Due to the presence of potentially dangerous pathogens in the sewage sludge, it is recommended that sewage sludge need to be adequately decontaminated prior to use in agricultural land If sewage sludge is to be used for soil amendment when growing crops meant for human consumption.

In this study, *Ascaris* viability was not investigated. Further research will need to determine viability of *Ascaris* throughout the experiment.
Further study in this subject should also pay attention to other parasites commonly found in sludge, such as *Taenia* spp.

Subsequent studies on this subject will need to include moisture content to evaluate as a variable.
This study recommends *E.coli* as a reliable indicator in sewage sludge microbiological investigations.

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Chapter 5

Identification of Pathogenic Bacteria from *Solanum tuberosum* Grown in Sewage Sludge Amended Soil.

5.1 Introduction

Control of microbiological quality of crops is important, since microorganisms may survive beyond the harvesting season and proliferate in crops during storage or processing (Strauch, 1991). If pathogenic microorganisms prevail in crops, it could become a source of microbial contamination that may eventually cause disease.

Bacterial communities have traditionally been compared by analysing isolates cultivated on media. However, a number of laboratories prefer the molecular techniques, due to the increased sensitivity of these methods (Wintzingerode *et al*., 1997; Boon *et al*., 2000; Amann and Ludwig, 2000). The Polymerase Chain Reaction (PCR) is the basis of molecular identification methods. The technique was developed to amplify DNA until there is enough to be detected, allowing even organisms occurring in small numbers in an environment to be detected (Wintzingerode *et al*., 1997).

The 16S rRNA gene, which codes for the small subunit of the ribosome is commonly used to identify organisms (Borneman *et al*., 1996). Ward and colleagues (1992) have illustrated the value of rRNA sequence analysis in the identification of bacteria . The rRNAs are universally distributed amongst cellular forms, and therefore useful for studies of all microorganisms (Brown, 1994). The functional constraints in this molecule result in a high degree of sequence conservation that permits bacterial characterization based on sequence information obtained from mixed communities (Klappenbach *et al*., 2001).

Molecular methods for microbial diversity assessment rely primarily on PCR-amplification of 16S rRNA genes from complex samples followed by cloning and sequencing (Brown, 1994; Brown, 1995; Klappenbach *et al*., 2001). The use of molecular techniques to investigate microbial diversity has been applied widely in environmental samples (Wintzingerode *et al*., 1997; Boon *et al*., 2000; Wattiau *et al*., 2001; Jeon *et al*., 2003). The ultimate goal of a PCR-mediated analysis of 16S rRNA genes is the retrieval of sequence information, which allows determination of microbial diversity (Wintzingerode *et al*., 1997).

This chapter investigated the bacterial community present in contaminated soil and potatoes using molecular techniques. The study sought to investigate the prevalence of pathogenic microorganism in crops grown in soil treated with sewage sludge in order to establish if these crops are potentially hazardous to human health.

5.2 Materials and Methods

5.2.1 Potato Samples

Low and high metal sludges were used at the application rates of 8 and 16 tons/ha to grow potatoes. This experiment was carried out in green houses under controlled conditions (the experimental layout was described in detail in Chapter 4). In chapter 4, the potatoes grown in LMS showed microbial contamination at harvest time. These potatoes and the sludge-treated soil in which they were grown (Chapter 4), warranted further study.

5.2.2 Extraction of Genomic DNA

Two (2) grams from each of the 3 soil samples and 2 g of the mashed potato peel, from each of the 3 contaminated potatoes used, were suspended in ringer solution. This was done in duplicate. As samples were concentrated, serial

dilutions were made. The suspension was plated out on nutrient agar and also on Chromocult agar and incubated for 18-24 hrs at 37 ^{o}C. Nutrient agar is a universal medium in which most bacteria would grow, and the Chromocult coliform agar is a selective culture medium for detection of *Enterobacteriaceae* (Byamukama *et al*., 2000). Single colonies were picked and transferred to LB (Luria Bertani) broth in Erlenmeyer flasks, and incubated at 37 ^{o}C for 18-24 hrs. The cell suspensions were transferred to sterile plastic tubes. The optical density (OD) of cell suspensions was measured at 620 nm. To calculate the number of cells needed for further use the following formula was used:

$$V\ (\mu l) = {}^{0.2}/OD_{260} \text{ X } 1000 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

The appropriate volume of cells was harvested and transferred to a clean Epperndorf tube and centrifuged at 12 000 g for 10 minutes.

The pellet was suspended in 100 μl Tris-HCl buffer (10mM, pH 8.2). DNA extraction from the bacterial cultures was carried out using the DNA extraction kit purchased from Qiagen and conducted according to the manufacturer's instructions.

5.2.3 PCR Amplification of the 16S rRNA Gene

The 16S rRNA gene was amplified using primers rP2 and fD1 as described by Weisburg and colleagues (1991). Sequences for these primers are indicated in Table 5.1. PCR amplification was carried out in 50 μl mixtures that comprised the following: 5 μl template, 1.5 mM $MgCl_2$, 1.5 mM dNTP, 12.5 ρmole FD_1, 12.5 ρmole rP_2 and 0.5U Taq DNA polymerase (Southern Cross Biotechnologies) and 10 mM Tris-HCl pH 9.0. Sterile distilled water was used to make the mixture up to a volume of 50 μl.

Table 5.1 Sequences of Primers used

Primer	Sequence	Reference
rP_2	5' ACGGCTACCTTGTTACGACTT 3'	Weisburg *et al* (1991)
FD_1	5' AGAGTTTGATCCTGGCTCAG 3'	Weisburg *et al*. (1991)

Amplification was carried out on a Perkin Elmer GeneAmp PCR System 2400 thermocycler using the following thermal profile: initial denaturation step at 95 ^{o}C for 3 minutes, thirty cycles denaturation (94 ^{o}C for 30 seconds), annealing (55 ^{o}C for 30 seconds), and extension (72 ^{o}C for 1 minute). An additional extension step of 7 minutes was performed after completion of the thirty cycles.

5.2.4 Agarose Gel Electrophoresis

To evaluate the success of amplification, the PCR product was electrophoresed through a 1% agarose gel (containing 3 μl ethidium bromide (10 mg/ml)) suspended in 1x TAE buffer (40mM Tris-HCl, 20 mM NaOAc and 1 mM EDTA, pH 8.5) for 30 minutes at a current of 42 Amps and a voltage of 100 V. The gel was assessed under UV for the presence of bands.

5.2.5 DNA Purification

Since residual reaction components, such as unincorporated dTNPs, primers and residual enzyme can interfere with subsequent DNA sequencing methodologies, PCR product was purified. This purification was done using the Qiagen PCR Purification Kit (Southern Cross Biotechnologies, South Africa) according to the manufacture's instructions. To assess the purity and concentration of the purified product, 1 μl was subjected to electrophoresis on a 1% agarose gel [Promega].

5.2.6 Cloning

The PCR products were cloned into the pDrive cloning vector supplied in the Qiagen PCR cloning kit (Southern Cross Biotechnologies) according to the manufacture's instructions. Plasmids were introduced into competent *E. coli* DH5α cells. Both a negative and a positive control were prepared**.** About 200 µl drawn and plated on the AMP plates (smeared with 10 µl IPTG and 40 µl XGAL) and incubated for 18 -24 hours at 37 ^{o}C. Recombinants were isolated according to standard protocols (Saambrook *et al*., 1989).

5.2.7 Plasmid Extraction

About 1.5 ml cell suspension was centrifuged for 3 minutes. The pellet was resuspended in 100 µl of Solution I and left on ice for 5 minutes. About 200 µl of Solution II was added to the mixture and left on ice for a further 5 minutes. Solution III (150 µl) was added and left on ice another 5 minutes. The mixture was centrifuged at high speed for 5 minutes and transferred to a new microcentrifuge tube. Two volumes of 100% EtOH were added and incubated at room temperature for 1 hour. This mixture was centrifuged for 15 minutes at high speed. The pellet was washed with 1 ml of 70% EtOH, centrifuged for 5 minutes and air dried to remove excess EtOH. The pellet was dissolved in 30 µl TE buffer.

5.2.8 Plasmid Purification

Sterile dH_2O was added to the sample to a final volume of 200 µl. Phenol (200 µl) was added and centrifuged for 5 min. Chloroform-isoamyl alcohol (24:1) (200 µl) was added and centrifuged for 5 min at full speed. Two volumes of 100% Ethanol (EtOH) and Sodium acetate (NaoAc) to a final concentration of 1.8 mM, were added to the supernatant and this was left on ice for 1 hour. The precipitate was washed with 70% EtOH, and suspended in 15 µl sterile distilled water.

5.2.9 Restriction enzyme

The restriction enzyme reaction was carried out to determine successful cloning. The reaction used the restriction enzyme, EcoRI (Roche Molecular Diagnostics, South Africa) according to the manufacture's instructions. Electrophoresis was also carried out to evaluate the action of the restriction enzyme using the λ EcoRI / Hind III (Roche Molecular Diagnostics, South Africa) as a molecular weight marker.

5.2.10 Sequencing

Sequencing was carried out in 10 μl reaction volumes that comprised of the following: 2 μl of purified plasmid, 2 μl ready reaction pre-mix (supplied with the sequencing kit, containing dye terminators, dNTPs, Taq DNA Polymerase, MgCl2, and Tris-HCl buffer pH 9.0), and 10 ρmol of rP2 primer (Weisburg *et al*., 1991). The reactions were carried out in a Perkin Elmer GeneAmp PCR System 2400 thermocycler and comprised of 25 cycles of denaturation (96 ^{o}C for 5 seconds), annealing (50 ^{o}C for 5 seconds) and extension (60^{o}C for 4 minutes). At the end of the cycles, the reactions were kept at 4^{o}C until needed.

5.2.11 DNA Precipitation

Products of the sequencing reactions were precipitated with 60% (v/v) ethanol at room temperature for 15 minutes, centrifuged at 12 000 g for 15 minutes, washed with 70% (v/v) ethanol, vacuum dried and stored at -20 ^{o}C until needed.

5.2.12 Sequence Determination

Sequencing samples were run overnight on an ABI 377 Automated Sequencer at the sequencing facility at the University of Pretoria, South Africa. Sequence identity was determined using the BLAST search tool.

5.3 Results and Discussion

5.3.1 DNA Extraction and PCR Amplification of the 16S Gene.

In this chapter, DNA was extracted from sewage contaminated soil and potatoes grown in such soil, and the 16S rRNA genes of viable bacteria amplified. Representative colonies from Nutrient agar and Chromocult coliform agar were used for DNA extraction. The DNA extraction method described earlier resulted in pure DNA suitable for PCR amplification. The PCR product showed sufficient DNA amplification, which was subsequently cloned. The white colonies obtained following plating of the ligation reaction, suggested successful cloning. Restriction enzyme treatment confirmed DNA transfer to the vector (Figure 5.1).

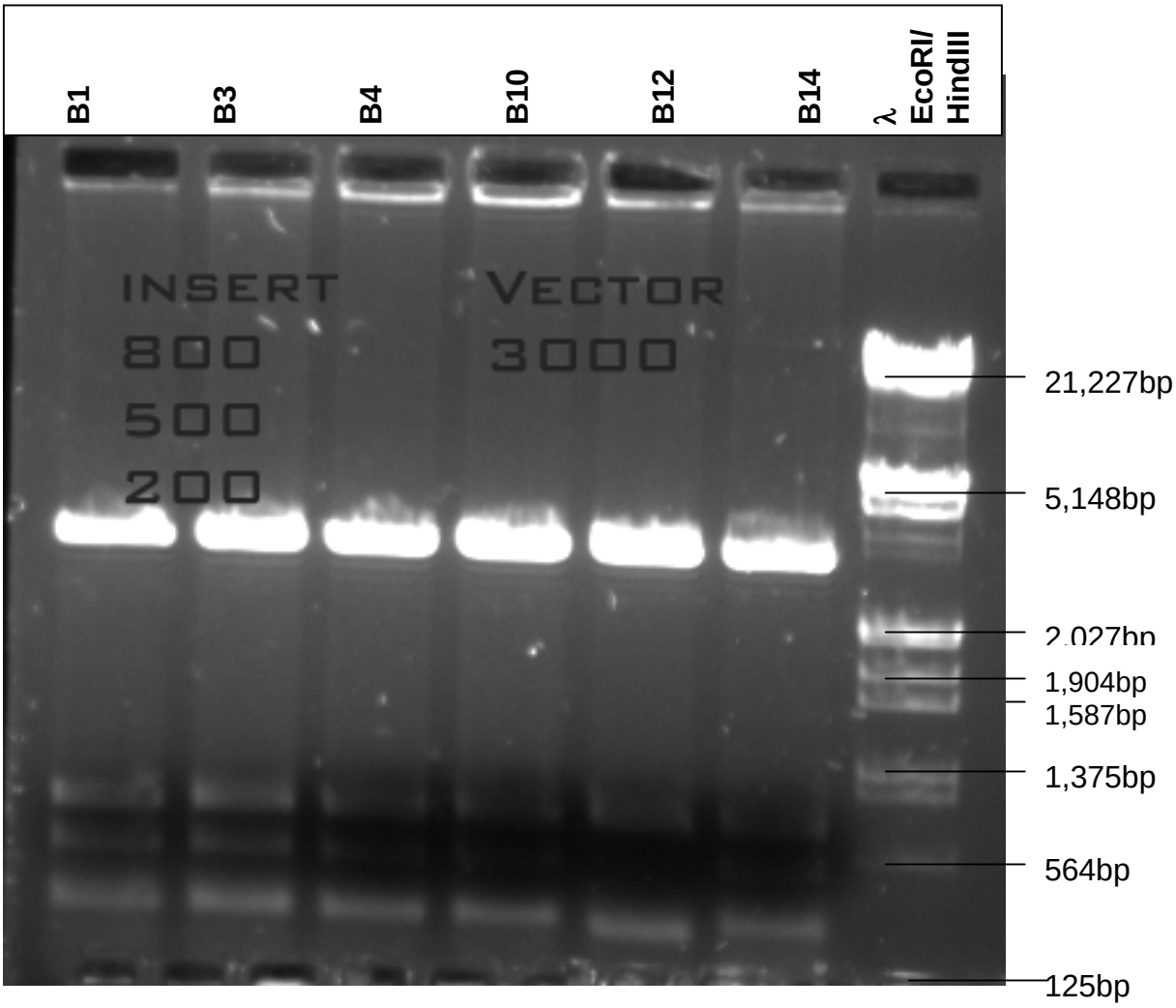

Figure 5.1 Agarose gel electrophoresis following restriction enzyme treatment.

Three bands were visualized from each of the samples. These were estimated to be in the regions of 200, 500 and 800 bp (Figure 5.1), which are fragments resulting from the restriction of the 1,5 kb PCR amplicon of the 16S rRNA molecule (Weisburg *et al*., 1991). These estimates are based on the comparison with the band sizes of the molecular weight marker (λ EcoRI/ HindIII). This marker yields fragments ranging in size from 21kb to 125 bp. The PCR amplication of the 16S rRNA gene using primers fD1 and rP2 resulted in the detection of an amplified fragment of about 1500 bp for all isolates. This

corresponds to the size of 16S rRNA genes previously determined by Brosius and colleagues (1978).

5.3.2 Homology Searches Using the BLAST.

Each 16S rRNA sequence was compared with the sequence in Genbank according to the BLAST search tool. Organisms identified from the matched sequences are tabulated below (Tables 5.2 and 5.3). Sequence analyses of the 16S rRNA gene remains one of the most reliable indicators for revealing the identity of the organisms (Wintzingerode *et al*., 1997; Amann and Ludwig, 2000). In this study, generated sequencing data of studied isolates, yielded unique matches for most isolates, with the Genbank sequence database. However, sequence similarity was observed between some isolates.

Most of the microorganisms identified in this study were found not to be primary human pathogens, but those that normally exist in the environment, in the soil, water or in plants. Plant pathogens in sewage sludge may originate from washing of vegetable and fruit (Beauchat, 1998). These organisms may cause opportunistic diseases in individuals with suppressed immune systems (Greenwood, 1997).

As indicated in Table 5.2, according to the sequencing data, the sludge contaminated soil yielded a variety of microorganisms. Other than the *Klebsiella* spp., *Enterobacter* sp*, Proteus* sp and *Escherichia coli*, which are enteric organisms found commonly in the gastrointestinal tract of humans and animals, bacteria identified were predominantly *Bacillus* spp, which are usually found in soil, water and rarely in plant material (Greenwood *et al*., 1997).

Infections associated with sewage sludge use may result from contaminated crops (Rudolfs, 1951; Pahren *et al*., 1979; Cieslak *et al*., 1993), airborne particles (Dutkiewicz, 1997) or unintentional ingestion of pathogens from contaminated

hands, utensils or surfaces. Farm workers may also be infected (Pande *et al*., 2000). Although agricultural application of sewage sludge on food crops has been used by some countries over the years (Rudolfs, 1951; Dorn *et a*l., 1985; Strauch, 1991), it has been reported that use of pathogen containing sludge could result in a broad variety of infections (Burge and Marsh, 1978; Pell, 1997; NRC, 1996).

Table 5.2 Organisms identified in contaminated soil

Organism	Percentage match
Bacillus firmus	97%
Bacillus pumilis	99%
Enterobacter aerogenes	98%
Proteus mirabilis	100%
Klebsiela oxytoca	99%
Bacillus sphaericus	99%
Bacillus luciferensis	99%
Klebsiella pneumoniae	98%
Bacillus niacini	99%
Bacillus drentensis	99%
Pantoea sp.	98%
Klebsiella fusiformis	98%
Escherichia coli	99%
Klebsiela ornithinolytica	99%

Bacteria of the genus *Klebsiella* are opportunistic pathogens that can lead to severe diseases such as septicemia, and urinary tract and soft tissue infections (Jonas *et al*., 2004). For instance *Klebsiella oxytoca* is one of the organisms often implicated in antibiotic associated diarrhoea (Ayyagari *et al*., 2003), while *K. oxytoca* and *K. pneumoniae* have been associated with outbreaks in newborn babies (Westbrook *et al*., 2000). These pathogens are also capable of being airborne and have been implicated in respiratory problems that occurred

following land application of sludge (Dutkiewicz, 1997). Small Wright (2002) recently reported the occurrence of three deaths in Pennsylvania, USA that occurred as a result of exposure to sludge spread fields.

Bacillus spp are usually implicated in food poisoning. They are capable of forming endospores during unfavourable conditions, whereby the interior of the cell transforms into a multi-layered structure around the bacterial DNA (Walker, 1998). The spores can survive adverse environments and grow again when conditions improve. If contaminated food is cooled slowly or kept warm before serving they will germinate (Walker, 1998). Some species such as *Bacillus licheniformis* have been implicated in nosocomial infections (Matsumoto *et al*., 2000), while *B. fusiformis* is the causative agent for noma (Deeb *et al*., 1999).

Bacteria detected in potato samples were mostly plant pathogens or environmental organisms (Table 5.3). *Erwinia* spp are responsible for plant diseases such as soft rot (*Erwinia carotovora*), vascular wilts (*Erwinia stewartii*) and fire blight (*Erwinia amylovora*), especially in potato (Pérombelon and Kelman, 1980; Cappellini *et al*., 1984, Prescott *et al*., 2002). Although these are primarily plant invaders, some *Erwinia* spp such as *E. amylovara* are opportunistic pathogens implicated in cases of septicimia, urinary tract infections, conjunctivitis and endophthalmitis (Faulde *et al*., 2001). *Pectobacterium* spp are also plant pathogens, known to cause blackleg and soft rot (Toth *et al*., 2003). Four *Buttiauxella* spp were isolated from the potato samples. Of these, *Buttiauxella agrestis*, *B. noackie* and *B gaviniae* have been implicated in the urinary bladder infection of a spinal cord patient. The frequent occurrence of *Buttiauxella* spp is normally in mollusks, mainly snails and slugs, and they have been isolated from soil but rarely from humans (Muller *et a*l., 1996).

Plant pathogens are not known to cause disease in humans with competent immune systems. However they can provide a route of entry for human pathogens as they cause lesions for easy entry. Earlier, Wells and Butterfield

(1997) indicated that the incidence of *Salmonella* spp on fruits and vegetables affected by bacterial soft rot is far greater than in healthy produce as this provides favourable environment for replication.

Several *Pantoea* spp and an *Enterobacter* spp were identified in the samples. *Pantoea spp* are coliform bacteria that are often isolated from the environment (Greenwood *et al*., 1997). Strauch (1991) also reported on the presence of *Enterobacter spp* in sludge. Most *Enterobacter* spp are enteric organisms that make up the normal flora of the human gastrointestinal tract. These species can cause urinary tract infections and other opportunistic infections on various parts of the body (Greenwood *et al*., 1997). Rolph and colleagues (2001) also using the molecular technique found some *Pantoea* spp and *Enterobacter* spp in endodontic infections.

Recently, Staskawicz and his colleagues (2001) have reported on the ability of some bacteria to harm both animal and plant hosts. However, as their common habitat is not directly linked to humans or animals, but the environment, their presence in contaminated crop may not necessarily implicate sewage sludge as the source.

Enterobacter agglomerans also referred to as *Pantoea agglomerans,* is found in water and soil and has only occasionally been isolated from humans. *P. agglomerans* is a causative agent for allergic alveolitis in workers exposed to sewage sludge (Dutkiewicz, 1997). This organism has also been implicated in neonatal meningitis and sepsis (Greenwood *et al*., 1997).

Table 5.3 Organisms identified from potatoes following sequencing

Organism	Percentage closeness
Pantoea agglomerans	99%
Enterobacter agglomerans	99%
Pantoea agglomerans	99%
Erwinia carotovora	99%
Pantoea ananatis	98%
Pantoea toletana	98%
Erwinia amylovora	97%
Pectobacterium carotovorum	99%
Pectobacterium chrysanthemi	98%
Buttiauxela agrestis	98%
Buttiauxela ferragutia	97%
Buttiauxela noackiae	97%
Buttiauxela gaviniae	98%

5.3.3 Bacteria Associated with Sewage Sludge Use

Table 5.4 details the organisms that are usually found in sludge and organisms detected in this study following sludge use. In this study, human pathogens known to be sludge borne (Chapter 2) were mostly detected in the sewage sludge samples as indicated earlier in Chapter 3. However, most of these organisms were not detected in the soil or potato samples.

Table 5.4 Bacteria associated with sludge use

Organism	Sludge borne (Chapter 2)	Sewage sludge (Chapter 3)	Sewage sludge treated soil (Chapter 4 & 5)	Potatoes from sludge treated soil (Chapter 4 & 5)
Achromobacter spp	✓	✓	×	×
Acitenobacter calcoaceticus	×	✓	×	×
Bacillus spp	✓	×	✓	×
Brevibacterium spp	×	✓	×	×
Buttiauxela spp	×	×	×	✓
Cellulomonas hominis	×	✓	×	×
Chromobacterium violaceum	✓	✓	×	×
Citrobacter spp	✓	×	×	×
Clostridium spp	✓	×	×	×
Enterobacter spp	✓	✓	✓	✓
Erwinia spp	×	×	×	✓
Escherichia coli	✓	✓	✓	✓
Exiguobacterium acetylicum	×	✓	×	×
Klebsiella spp	✓	✓	✓	×
Leclercia adecarboxylata	×	✓	×	×
Leptospira spp	✓	×	×	×
Listeria spp	✓	×	×	×
Mycobacterium spp	✓	×	×	×
Oligella urethralis	×	✓	×	×
Pantoea spp	✓	✓	✓	✓
Pectobacterium spp	×	×	×	✓
Proteus spp	✓	×	✓	×
Providencia spp	✓	×	×	×
Pseudomonas spp	✓	✓	×	×
Raoutella terrigena	×	✓	×	×
Rhodococcus australis	×	✓	×	×
Salmonella spp	✓	✓	✓	✓
Serpens flexibilis	×	✓	×	×
Serratia spp	✓	✓	×	×
Shigella spp	✓	×	×	×
Staphylococcus spp	✓	✓	×	×
Streptococcus spp	✓	×	×	×
Vibrio cholerae	✓	×	×	×
Yersinia enterocolitica	✓	×	×	×

Organisms detected from soil or potato samples using molecular techniques were mostly opportunistic pathogens that may cause infection at the advent of limited immune capacity. They take advantage of weakened host defense systems to colonize and elicit a variety of disease states. Thus, their presence in crops could lead to adverse effects in individuals with compromised immune system such as pregnant women, children, the elderly, cancer patients and those suffering from HIV/AIDS (Greenwood *et al*., 1997). Considering the high incidence of HIV infection in South Africa (Dorrington *et al*., 2002), the use of inadequately treated sludge could result in a large number of the population being sick.

Although a number of viable bacteria belonging to the *Enterobacteriaceae* were found in the potato, neither *E.coli* nor *Salmonella* spp (also members of this group) were identified from the sequencing results. *Salmonella* spp and *E.coli* are amongst organisms of major concern with regards to sludge use (EPA, 1999). The absence of *Salmonella* spp and *E.coli* in potato samples reserved for molecular studies may be as a result of the unfavourable environmental and refrigerator conditions.

Although the types of organisms identified in this study may not necessarily present a complete community due to the cost of the molecular technique, they however provide a representation of the types of pathogens in this environment.

5.4 Conclusion

Bacteria identified in the sludge-contaminated soil were predominantly non-enteric and of environmental origin, probably out-competing the enteric pathogens, as enteric organisms survive well in the human and animal gut and not in the environment.

It appears that growing even high risk crops such as potato using sewage sludge contaminated soil may not lead to a high infestation of produce with primary human or animal pathogens. However, even though limited, the presence of human pathogens detected at harvest may cause infection if ingested.

Considering the opportunistic tendency of the secondary pathogens and the prevailing state of weakened immune systems of the South African population, proper treatment of sewage sludge prior to use in agriculture is essential.

Organisms identified from potatoes were mainly plant pathogens. Bacterial soft rot in crops caused by plant pathogens such as *Erwinia* spp could lead to interior contamination of crops with human pathogens, if untreated sewage sludge is used.

If sewage sludge is used in agricultural land, routine analysis of harvested crops has to be in place for quality assurance purpose.

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Chapter 6
Microbial Risk Assessment of Using Sewage Sludge for Soil Enrichment

6.1 Introduction

Changes in agricultural practice have over the years raised concern regarding the risk of ingesting pathogens from vegetables irrigated with wastewater or sewage sludge (Rudolfs *et al*., 1951; Brent *et al*., 1995; Shuval *et al*., 1997). The occurrence of foodborne diseases remains a widespread problem in both the developing and developed world (Zwietering and van Gerwen, 2000). A systematic evaluation of safety is therefore important to control the risk of foodborne diseases. It is for this reason that worldwide, many initiatives are being taken to develop and apply microbial risk analysis (Blumenthal *et al*., 1989; Rose and Gerba, 1991; Zwietering and van Gerwen, 2000). While sewage sludge contaminated soil has been shown to potentially lead to contaminated crops (Rudolfs *et al*., 1951), in parts of Africa including South Africa, contaminated soil on its own is a hazard as deliberate and direct soil ingestion is common in these areas, especially by pregnant women in rural and peri-urban communities (Hunter, 1993; Walker *et al*., 1997).

There is an increasing interest in the application of quantitative risk analysis in the production of microbiologically safe products (Notermans and Teunis, 1996; Petterson *et al*., 2001). However, the quantitative evaluation of food safety is very complex, especially since in many case specific parameter values are difficult to obtain (Zwietering and van Gerwen, 2000). Scarcity of data often leads to qualitative assessments.

Individual adverse health effects related to microbial pathogens usually result from a single acute exposure, rather than long term chronic exposure (Farber *et al*., 1996). Attempts of microbial risk assessment have generally used thermotolerant

faecal coliforms (Al-Nakshabandi *et al*., 1997; Shuval *et al*., 1997). However, unlike many other hazards, risk assessment of bacterial pathogens is influenced by a number of factors, including growth and possible inactivation from processing steps such as cooking in the case of vegetables or desiccation in the case of soil. Microorganisms are dynamic and adaptable. They can lose or acquire virulence-associated characteristics and can also adapt to the control measures set to manage microbial risks (Voysey and Brown, 2000). Also, consumption patterns may vary between individuals. These differences may have strong demographic components such as sex, age, culture and health status (Farber *et al*., 1996).

A risk assessment provides a means of estimating the probability of adverse effects associated with measured or estimated levels of hazardous agents, and a tool for predicting the extent of potential health effects (Genthe, 1998). It involves a process that scientifically evaluates the probability of occurrence and severity of known or potentially adverse health effects resulting from human exposure to foodborne hazards (Zwietering and van Gerwen, 2000).

Risk assessments normally consist of four distinguishable but interacting phases generally referred to as:

- Hazard identification;
- Exposure assessment;
- Dose-response assessment and
- Risk characterization.

These were detailed in Chapter 2 section 2.10.1

6.2 Health Considerations for Consumption of Contaminated vegetables

In assessing microbial risk, the benchmark of 1 infection in 10 000 people per year is regarded as an acceptable level (Haas, 1996). This estimation is also supported by the United States Environmental Protection Agency (Rose and Gerba, 1991; Macler and Regli, 1993).

Infection with microbial hazard is complicated by a number of factors, that include the fact that:

- Microorganisms are capable of replicating;
- The virulence and infectivity of microorganisms can change depending on their interaction with the host and the environment;
- Genetic material can be transferred between microorganisms leading to the transfer of characteristics such as antibiotic resistance and virulence factors;
- Microorganisms can be spread through secondary and tertiary transmission;
- The onset of clinical symptoms can be substantially delayed following exposure;
- Microorganisms can persist in certain individuals leading to continued excretion of the microorganism and continued risk of spread of infection and
- Low doses of some microorganisms can cause a severe effect (Buchanan *et al*., 2000).

Although sewage sludge contains various microorganisms as was indicated in earlier chapters, for the purpose of this study, the risk assessment was carried out only for *Escherichia coli*, *Salmonella* spp and *Ascaris* based on the laboratory analysis outlined in Chapter 4.

6.3 Assumptions

The approach used in the health risk assessment in this study involved a descriptive approach, which relies on estimating the frequency and severity of exposure to health hazards. Some common assumptions include:

i The population is equally susceptible to an exposure.

ii Exposure is from consumption of contaminated crops (potatoes as a worst case scenario) grown in sewage sludge amended soil. Individuals may also be exposed to pathogens by accidentally or deliberately ingesting contaminated soil (Walker *et al*., 1997).

iii Pathogenic microorganisms from sludge used are homogeneously distributed in the soil.

iv It was assumed that there would be a certain degree of pathogen die-off and/or removal from the sludge and soil until the final ingestion by an individual in the home. These factors include settling, adsorption into soil, biological competition, UV irradiation from sunlight and a degree of removal and/or inactivation by washing of the vegetables. While other workers have indicated a rapid die-off of microorganisms following wastewater irrigation of soil (Rudolfs *et al*., 1951), a possible re-growth of bacteria on vegetables have also been reported (Armon *et al*., 1995).

v While cooking or boiling of vegetables would reduce microorganisms, it was assumed that cross contamination could occur during food preparation.

vi The risk of being infected by microbiological pathogens correlates with the level of contamination and the amount of contaminated vegetables

consumed. Higher numbers of microorganisms will indicate a higher risk of contracting microbial infection.

vii All microorganisms ingested with the vegetable (or with the soil) are infective.

6.4 Methodology

Due to the scarcity of epidemiological data, assessment of the risk to health from the use of sewage sludge is based on a potential risk. This is based on accidental consumption of contaminated soil during the growing season and also on detection of microorganisms on the crops at the time of harvesting. The application rates of 8 tons/ha in high metal sludge and 16 tons/ha in low metal sludge were used.

Models used in this study are the beta-distribution infectivity probability model for bacteria and the single hit exponential model for parasites (Rose and Gerba, 1991).

ß-distribution infectivity model

$p = 1- (1 + (N/\beta))^{-\alpha}$ …… (1)

Single-hit exponential model

$p = 1 – \exp(-rN)$ …… (2)

where p = probability of infection from a single exposure or daily risk of infection
N = exposure or number of organisms ingested per exposure
α,β,r = parameters characterized by dose response curves

In addition to the single exposure risk or daily infection, weekly, monthly and yearly risk were calculated as 7, 30 or 365 days of exposure respectively, where

$P\,t = 1- ((1-Pcalc)^{t})$

t = 7, 30 or 365

A risk of 1 in 10 000 per year is considered acceptable risk of infection.

6.4.1 *E. coli*

The presence of *E. coli* was established by culture method. The quantity of *E. coli* present in 1 g of sample (soil or potatoes) was determined (Chapter 4). Contaminated potatoes (16) were those obtained from the LMS16 in chapter 4. This concentration was used to determine the *E.coli* present in 200 mg soil.

Risk estimation was based on the Beta–ditributed "infectivity probability" model. The α and β values (0.1705 and 1.16) for *E. coli* are those proposed by Pepper *et al*., 1996). Hypothetical values were also used to assess what levels of exposure are associated with certain levels of risk, and to estimate what quantity of *E. coli* in a (1) gram of potato or in 200 mg of soil would constitute acceptable risk.

6.4.2 *Salmonella* spp

The presence of *Salmonella* spp was determined by making use of culture method as described in Chapter 4. Results were based on the presence (positive test) and absence (negative test) of the organisms.

As the method used for *Salmonella* spp identification did not involve enumeration, hypothetical numbers were used in the analysis. The α and β values (0.33 and 139.9) for *Salmonella* spp are those proposed by Rose and Gerba (1991).

6.4.3 *Ascaris ova*

At present, there appears to be no information on the dose-response data for *Ascaris*. Therefore, there are no estimates for the r value for this organism. The value (r = 0.0199) for *Gardia* (Rose and Gerba, 1991), another protozoan was used in estimating the probability of infection as they have the same infective dose (Brooks *et al*., 1991). Hypothetical numbers were used in analysis.

6.5 Results and Discussion

Although the present study assumed that all exposed individuals stand an equal chance of infection, risk of infection will vary between individuals depending on a number of factors. That is, a particular meal may pose no risk or a very high risk to an individual depending on the processing and handling of contaminated crops. Also factors such as the age, sex, previous exposure and immunocompetence of an individual influence the risk of infection (Buchanan *et al*., 2000). Carneiro *et al* (2002) could establish a link between rates of infection and socioeconomic status. In their study, children with less intense infection came from affluent households with higher socioeconomic and schooling profiles, while children from crowded dwellings had most infections (Carneiro, *et al*., 2002). Farm workers could be among those at a high risk of infection, as a result of continued exposure at the work place.

6.5.1 *E. coli*

Of the 224 contaminated soil samples studied throughout the experiment, 71 were found not to have *E.coli*. Estimates of risk of infection from accidentally or deliberately ingesting 200 mg of soil contaminated with sewage sludge at different treatment options are tabulated in Tables 6.1 to 6.4. For 8 tons/ha high metal sludge (HMS), the risk is reduced by week six due to pathogen die-off (Table 6.1). The HMS at an application rate of 16 tons/ha shows far less risk than both LMS 8 tons/ha and 16 tons/ha (Tables 6.3 and 6.4). Application rate of 16 tons/ha LMS

shows a greater risk of infection when compared the other treatment options (HMS 8 tons/ha, HMS 16 tons/ha and LMS 8 tons/ha).

Using high metal sludge at 8 tons/ha resulted in a quick pathogen die-off as there were no microorganisms detectable after the sixth week of planting. All the subsequent weeks showed no risk of infection (Table 6.1). At harvest, no microorganisms were detected from potatoes grown in the HMS for both 8 tons/ha and 16 tons/ha. The HMS shows low probability of infection, probably due to the inhibitory role of heavy metals in this sludge (Tsai and Olson, 1990). Although pathogen die off appears an answer for controlling crop contamination, some researchers (Byrd *et al*., 1991; Amman and Ludwig, 2000; Buchanan *et al*., 2000) argue that for microorganisms that release toxins and those that may be non-culturable, the absence of viable pathogens may not necessarily imply microbiologically safe produce.

Table 6.1 Risk of ingestion of *E.coli* associated with accidental or deliberate ingestion of 200 mg soil contaminated with High Metal Sludge applied at 8 tons/ha

Time (Weeks)	Organisms CFU/200 mg	P(Single Exposure)	P(Weekly)	P(Monthly)	P(Yearly)
0	89.25	5.24E-01	9.94E-01	1.00E+00	1.00E+00
2	1172.25	6.93E-01	1.00E+00	1.00E+00	1.00E+00
4	314.5	6.16E-01	9.99E-01	1.00E+00	1.00E+00
6	352500	8.84E-01	1.00E+00	1.00E+00	1.00E+00
8	0	0	0	0	0
10	0	0	0	0	0
12	0	0	0	0	0

Table 6.2 Risk of ingestion of *E.coli* associated with accidental or deliberate ingestion of 200 mg soil contaminated with High Metal Sludge applied at 16 tons/ha

Time (Weeks)	Organisms CFU/200 mg	P(Single Exposure)	P(Weekly)	P(Monthly)	P(Yearly)
0	309.5	6.14E-01	9.99E-01	1.00E+00	1.00E+00
2	3982.5	7.50E-01	1.00E+00	1.00E+00	1.00E+00
4	1398250	9.08E-01	1.00E+00	1.00E+00	1.00E+00
6	427511.2	8.88E-01	1.00E+00	1.00E+00	1.00E+00
8	1.5	1.32E-01	6.29E-01	9.86E-01	1.00E+00
10	1.25	1.17E-01	5.82E-01	9.70E-01	1.00E+00
12	0	0	0	0	0

Of the 64 potatoes studied from all the treatment options, 16 (25%) were found to be contaminated. These potatoes were grown in LMS 16 tons/ha. Risks of infection for exposure to *E. coli* calculated based on consumption of 1 g of contaminated potato grown in LMS 16 tons/ha soil are shown in Table 6.3. The average of the counts obtained from cleaned potato peels was 18 000 CFU/g. This number of organisms yielded a high probability of exposure of 8.07 E01 for a single exposure. The weekly, monthly and yearly exposures yielded even higher risk of infection (100% probability). At harvest, none of the potatoes from HMS 8 tons/ha, HMS 16 tons/ha or LMS 8 tons/ha had any *E. coli*. The probability of exposure to microorganisms from potato using these application rates is zero.

Table 6.3 Risk of ingestion of *E. coli* associated with accidental or deliberate ingestion of 200 mg of soil contaminated with Low Metal Sludge applied at 16 tons/ha

Time (Weeks)	Organisms CFU/200mg	P(Single Exposure)	P(Weekly)	P(Monthly)	P(Yearly)
0	5050	7.60E-01	1.00E+00	1.00E+00	1.00E+00
2	5987.5	7.67E-01	1.00E+00	1.00E+00	1.00E+00
4	2040000	9.14E-01	1.00E+00	1.00E+00	1.00E+00
6	325895	8.82E-01	1.00E+00	1.00E+00	1.00E+00
8	136.9	5.57E-01	9.97E-01	1.00E+00	1.00E+00
10	32.9	4.38E-01	9.82E-01	1.00E+00	1.00E+00
12	20325	8.11E-01	1.00E+00	1.00E+00	1.00E+00
12	***18 000 (in 1 g potato)***	***8.07E-01***	***1.00E+00***	***1.00E+00***	***1.00E+00***

Table 6.4 Risk of ingestion of *E.coli* associated with accidental or deliberate ingestion of 200 mg soil contaminated with Low Metal Sludge applied at 8 tons/ha

Time (Weeks)	Organisms CFU/200mg	P(Single Exposure)	P(Weekly)	P(Monthly)	P(Yearly)
0	435.75	6.36E-01	9.99E-01	1.00E+00	1.00E+00
2	5272500	9.27E-01	1.00E+00	1.00E+00	1.00E+00
4	925000	9.01E-01	1.00E+00	1.00E+00	1.00E+00
6	0	0	0	0	0
8	750	6.68E-01	1.00E+00	1.00E+00	1.00E+00
10	1355	7.00E-01	1.00E+00	1.00E+00	1.00E+00
12	45.25	4.67E-01	9.88E-01	1.00E+00	1.00E+00

Hypothetical numbers of *E. coli* were fitted to the model to predict what level of contamination would produce a corresponding risk of 1 infection in 10 000 (Table 6.5). These numbers were used to determine risk of infection ranging from a single

exposure to yearly exposure, and to estimate the quantity of organism that would constitute acceptable risk (Rose and Gerba, 1991).

Table 6.5 Risks associated with hypothetical exposures to potato or soil contaminated with *E. coli* (Beta Distribution Model)

N (Number of organisms)	P(Single Exposure)	P(Weekly)	P (Monthly)	P (Yearly)
10 000	7.87E-01	1.00E+00	1.00E+00	1.00E+00
1 000	6.84E-01	1.00E+00	1.00E+00	1.00E+00
100	5.33E-01	9.95E-01	1.00E+00	1.00E+00
10	3.20E-01	9.33E-01	1.00E+00	1.00E+00
1	1.01E-01	5.24E-01	9.58E-01	1.00E+00
0.1	1.40E-02	9.40E-02	3.45E-01	9.94E-01
0.01	1.46E-03	1.02E-02	4.30E-02	4.14E-01
0.001	1.47E-04	1.03E-03	4.40E-03	5.22E-02
0.0001	1.47E-05	1.03E-04	4.41E-04	5.35E-03
Number of organisms required for an acceptable risk				
$6.83X10^{-4}$	**1.00E-04**			
$9.75X10^{-5}$		**1.00E-04**		
$2.27X10^{-5}$			**1.00E-04**	
$1.87X10^{-6}$				**1.00E-04**

The present study has shown that for a daily consumption of vegetation grown from contaminated soil (or ingestion of soil), the number of *E. coli* should be less than $6.83X10^{-4}$ (Table 6.5) for the risk of infection to meet the requirements suggested by US EPA of 1 in 10 000 acceptable risk (Rose and Gerba, 1991; Macler and Regli, 1993). For the annual risk of infection to be less than 1 in 10 000, the number of organisms should be less than $1.87X10^{-6}$. The presence of 1 CFU/g *E. coli* is likely to bring about a probability of infection of 1.01E-01, which is approximately 1 in 10. This risk increases for a weekly, monthly and yearly infection

with corresponding risks of 5.24E-01 (5 in 10), 9.58E-01 (9 in 10) and 100% probability respectively (Table 6.5).

Handling of potatoes has been associated with *E. coli* infection in the United States (Armstrong *et al*., 1996). Jones (1999) also indicated the potential health risks associated with the persistence of *E. coli* in agricultural environments, yielding a high incidence of human infections in the UK.

Figure 6.1 shows that even small numbers of bacteria could result in a high probability of infection. Considering the high prevalence of HIV/AIDS in South Africa (Dorington *et al*., 2002), the use of inadequately treated sludge could pose serious health hazards in individuals with compromised immune systems.

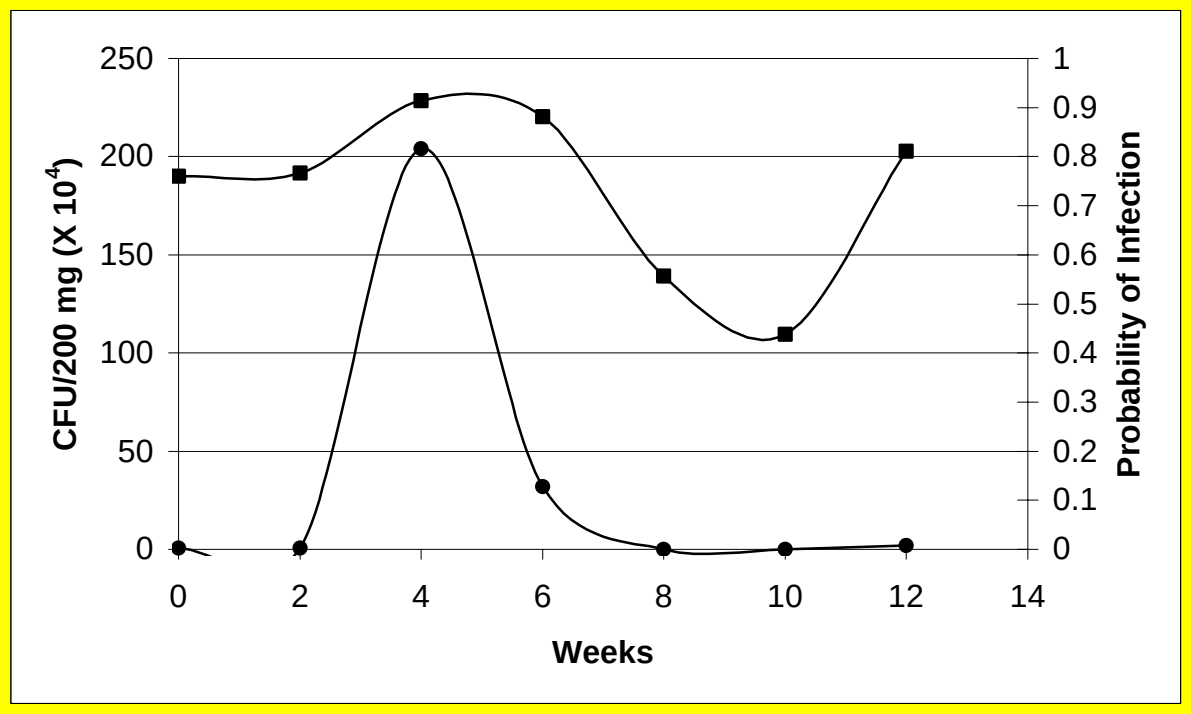

Figure 6.1 The relationship between the number of bacteria in 200 mg of contaminated soil amended with 16 tons/ha LMS, and the probability of infection (• = CFU/200 mg; ■.= Probability of infection).

Low metal sludge yielded high risk of infection throughout the duration of the experiment based on the accidental ingestion of 200 mg of contaminated soil (Figure 6.1; Table 6.3 and 6.4). This study assumed that people might be infected from accidentally ingesting soil (WRC, 1997) or deliberately and willingly ingesting soil (Hunter, 1993; Walker *et al*., 1997; Smith, 2002). In Africa, eating soil (geophagia) dates from the 18th century as observed in Nigeria, Ghana and Sierra-Leone (Hunter, 1993). It has become a common practice and spread to other countries, namely Malawi, Zambia, Swaziland and South Africa (Walker *et al*., 1997). Although the main reason for consuming soil is uncertain, it has been associated with poverty and poor eating habits, and has been found to be prevalent in pregnant women, those with poor nutrition and those with a family history of geophagia (Geissler *et al*., 1998; Smith, 2002). The use of sewage sludge either in their gardens or in the farms could have serious health effects for communities who practice geophagia. Geissler and colleagues (1998) studied a relationship between geophagy in school children in Western Kenya, and helminthes, and found that 77% of the children ate soil daily and 48% of the soil samples they tested were contaminated with *Ascaris*.

Potatoes are rarely consumed raw, however there is evidence that contaminated potatoes can lead to serious infections (Seals *et al*., 1981; Brent *et al*., 1995). Although some organisms such as *Clostridium botulinum* can survive baking (Brent *et al*., 1995) the prevalence of microorganisms in potato dishes is likely to be due to a number of factors that include handling which could lead contamination of utensils and surfaces. As microorganisms have a potential to replicate, such cross contamination may eventually contaminate the finished product.

6.5.2 *Salmonella spp*

As the results for *Salmonella spp* were only based on the presence and absence of this organism, risk assessment based on the beta distribution model for consumption of *Salmonella* spp was based only on hypothetical numbers (Table 6.6). For this organism, to attain a risk of infection to be less than 1 in 10 000, there need to be less than $4.25X10^{-2}$ organisms per g for daily exposure and $1.17X10^{-4}$ for yearly exposure. If one ingests a single organism, the probability of infection is about 2 in a 1000 (2.35E-03) for a single exposure and about 2 in 100 (1.63E-02), 7 in 100 (6.81E-02) and 6 in 10 (5.76E-01) for weekly, monthly and yearly exposures respectively.

Table 6.6 Risks associated with hypothetical exposures to *Salmonella*

(Beta Distribution Model)

N (Number of organisms)	P(Single Exposure)	P(Weekly)	P (Monthly)	P (Yearly)
10 000	7.57E-01	1.00E+00	1.00E+00	1.00E+00
1 000	5.00E-01	9.92E-01	1.00E+00	1.00E+00
100	1.63E-01	7.12E-01	9.95E-01	1.00E+00
10	2.25E-02	1.47E-01	4.95E-01	1.00E+00
1	2.35E-03	1.63E-02	6.81E-02	5.76E-01
0.1	2.36E-04	1.65E-03	7.05E-03	8.25E-02
0.01	2.36E-05	1.65E-04	7.07E-04	8.57E-03
0.001	2.36E-06	1.65E-05	7.08E-05	8.61E-4
0.0001	2.36E-07	1.65E-06	7.08E-06	8.61E-05
Number of organisms required for an acceptable risk				
$4.25X10^{-2}$	**1.00E-04**			
$6.08X10^{-3}$		**1.00E-04**		
$1.42X10^{-3}$			**1.00E-04**	
$1.17X10^{-4}$				**1.00E-04**

Risk from infection due to *Salmonella spp* contaminated food was indicated by Walls and Scott (1997) in USA where 1.2 million cases of *Salmonella spp* infection are reported per year. Based on their assessment and the number of cases reported, they concluded that on any given day, 3190 individuals might become infected with *Salmonella* (Wall and Scott, 1997).

6.5.3 *Ascaris*

Ascaris ova were detected in the soil only at the beginning of the experiment (1/g soil for HMS in week 2, and 4/g soil for LMS in week 4) (Chapter 4). No *Ascaris* were detected in samples analysed from the sixth week of the experimental period.

Table 6.7 Risks associated with hypothetical exposures to *Ascaris*

(Exponential Model)

N (Number of organisms)	P(Single Exposure)	P(Weekly)	P (Monthly)	P (Yearly)
10 000	1.00E+00	1.00E+00	1.00E+00	1.00E+00
1 000	1.00E+00	1.00E+00	1.00E+00	1.00E+00
100	8.50E-01	1.00E+00	1.00E+00	1.00E+00
10	1.73E-01	7.36E-01	9.97E-01	1.00E+00
1	1.88E-02	1.25E-01	4.34E-01	9.99E-01
0.1	1.90E-03	1.32E-02	5.54E-02	5.00E-01
0.01	1.90E-04	1.33E-03	5.68E-03	6.70E-02
0.001	1.90E-05	1.33E-04	5.70E-04	6.91E-03
0.0001	1.90E-06	1.33E-05	5.70E-05	6.93E-04
Number of organisms required for an acceptable risk				
$5.27X10^{-3}$	**1.00E-04**			
$7.55X10^{-4}$		**1.00E-04**		
$1.76X10^{-4}$			**1.00E-04**	
$1.45X10^{-5}$				**1.00E-04**

According to the model, using hypothetical numbers, if only a single *Ascaris* is consumed, there is a probability of infection is 1.88E-02 (approximately 2 in 100) for daily exposure and 1.25E-01 (1 in 10), 4.34E-01 (4 in 10) and 9.99E-01 (9 in 10) for weekly, monthly and yearly exposures respectively (Table 6.7). If less than $5.27X10^{-3}$ organism is consumed (from soil or vegetable), the risk of infection is acceptable (< 1 in 10 000). For yearly exposure, infection will be less than 1 in 10 000 if less than $1.45X10^{-5}$ organisms are consumed.

Although no *Ascaris* ova were detected on the potato peel at the time of harvest, the possibility of infection from these organisms may not be ignored. Recently, Carneiro *et al*. (2002) reported prevalence of *Ascaris lumbricoides* infection from consumption of contaminated water in Brazil. It has been earlier reported that *Ascaris* infects approximately 25% of the world's population annually (Crompton, 1988).

6.6 Conclusions and Recommendations

This risk assessment was based on the accidental or deliberate ingestion of contaminated soil during the planting, growing or harvesting following sewage sludge application, and also on the consumption of contaminated potato post-harvest. Risk estimation was based on the quantity of organisms in the soil and on the surface of potato.

It has clearly been shown that even very low numbers of pathogens may present a high risk of infection from *E. coli*, *Salmonella* spp and *Ascaris* to those individuals exposed to these pathogens. Risk assessment for these organisms required that their numbers present on the crop should be extremely low to correspond to a less than 1 in 10 000 annual risk of infection. Haas and colleagues (2000) who validated their results with reference to two outbreaks have pointed out that comparison of real world situations with the predictions these models are highly plausible.

This study recommends the use of *E. coli* as an indicator for safe use of sewage sludge in agricultural land.

Using HMS at 8 tons/ha resulted in limited risk of infection as pathogens die long before crops are harvested. However, other agents such as heavy metals and organic chemicals should be put into consideration when there is intended use of sewage sludge.

Considering the risk associated with exposure to heavy metals, the LMS at 8 tons/ha appears a better option to use, if preceded by intense treatment.

The risk estimated in this study is based on the pathogens studied. Considering that sewage sludge contains numerous pathogens (Chapters 2 and 3) including viruses, undoubtedly the potential for sludge use in agricultural land to cause gross health effects far exceeds the estimates made in this study.

The risk assessment is a useful tool to illustrate that management practices could play an important role in reducing the health risk associated with the use of sewage sludge on agricultural land.

Due to the prevalence of HIV/AIDS in the country and the poor hygienic practices of most people including those living in informal settlements, use of inadequately treated sludge in agricultural land used for crops meant for human consumption holds potential to yield countless infections, and could pose a serious health hazard for such communities.

Intensive pathogen reduction in sewage sludge will be necessary prior to using the product as soil conditioner. This will ensure that sewage sludge to be applied to land starts with low numbers that may eventually die off.

As this study was based on conservative assumptions, and has estimated a high risk, further studies especially those based on epidemiological data are recommended.

This study was based on potato, which is one of the high risk crops, further research on other crops will need to be investigated for evaluation of risk of infection.

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Chapter 7

Management Practices Regarding Sewage Sludge Use in Agricultural Land

7.1 Introduction

The preceding sections have revealed the types of microorganisms found in sludge, their potential to persist in soil and crops as well as the risk associated with this persistence. This section focuses on managing the risk through appropriate management practices.

This chapter sought to indicate that if farmers adhere to the regulations stipulated regarding application of sewage sludge to agricultural land, application of sewage sludge should not present a risk to food safety. This would ensure that noxious pathogens such as *E.coli* and *Salmonella* spp would not be transferred into the food chain when using sewage sludge in selected agricultural practices.

7.2 International Trends Regarding Microbiological Sludge Quality

The US Part 503, subpart D pathogen reduction requirements for sewage sludge are divided into two categories, namely Class A and Class B. The implicit goal of the Class A requirements is to reduce the pathogens in sewage sludge (including enteric viruses, pathogenic bacteria and helminth ova) to below detectable levels. The goal of Class B requirements is to reduce pathogens in sewage sludge to levels that are unlikely to pose a threat to public health and the environment. Another category, exceptional quality (EQ) refers to sewage sludge that has met the Part 503 pollutant concentration limits. While Both A and B have site restrictions, EQ may be land applied without site restrictions (EPA, 1999).

Mexico adopted the US guidelines, with a few modifications to suite their environment and sludge quality (Jimenez *et al*., 2003). They use similar limits for

heavy metals and Faecal coliforms and modified the limits for *Salmonella* spp and helminth ova (Jimenez *et al*., 2003).

In Australia, regulatory responsibility is carried out by individual states. Recently, the national guidelines (National Water Quality management Strategy (NWQMS): *Draft Guidelines for Sewage Management – biosolids Management*) have been drafted. These draft guidelines define three pathogen grades (P1, P2 and P3), which are based on prescribed treatment processes and microbiological standards. Vector attraction reduction measures are also detailed.

Grade P1 biosolids are considered suitable for unrestricted use, and grades P2 and P3 have increasing degrees of restrictions (Reid, 2003). The P1 grade includes microbiological criteria of <1 *Salmonella* per 50 grams dw and <100 *E.coli* (or thermotolerant coliforms) per gram dw. The microbiological standards for grade P2 are <10 *Salmonella* per 50 grams dw and <1000 *E.coli* (thermotolerant coliforms) per gram dw (Reid, 2003).

In South Africa, sewage sludge is classified into three types, namely A, B and C. This classification is based on the decreasing order of potential to cause odour nuisances and fly breeding as well as to transmit pathogenic organisms to humans and the environment (WRC, 1997). There is an additional type D, which is similar in hygienic quality to Type C. However, as Type D is produced for unrestricted use, the metal and inorganic content are limited to acceptable low levels.

Sewage sludge generally contains a number of pathogenic microorganisms (as indicated in Chapters 2 and 3). As it is impossible to analyse for all pathogenic organisms, only the numbers of *Ascaris* ova, *Salmonella* spp and *Faecal coliforms* are included in the analysis as indicator organisms for determining hygienic quality of Type C and type D sludge.

7.3 Factors that can Influence Sludge Management Practice in South Africa

There are a number of factors that affect the South African population and need to be taken into consideration to ensure adequate protection of human health with regard to sewage sludge use. These include the following:

i Compromised Immune Systems

A large number of South Africans are immuno-compromised as a result of the high incidents of HIV/AIDS, which translates into diverse disease profiles such as cholera, tuberculosis and more recently meningitis. Cancer patients, as a result of the treatment they receive, tend to have suppressed immune systems. Other groups with weak immune systems include children and the elderly. Appropriate management of land application of sewage sludge has to place these individuals into consideration.

ii Poverty and Unemployment

Large areas in South Africa are rural with the majority of people living in these areas being unemployed and consequently living below the poverty line (Parliamentary Bulletin, 1996). Due to limited skills and illiteracy of a large fraction of the population in the country, the rate of unemployment has increased in recent years. These populations have to be taken into consideration when formulating management practice for land application of sewage sludge. Sludge producers and farmers should communicate at a level so that communities will comprehend the risks and benefits of sludge use.

iii Population density

There is an increase in population size as a result of social behaviours, religions, teenage pregnancies and immigration particularly from neighbouring countries. There is also a tendency for people from rural areas to move to cities for an

improved quality of life, resulting in an upsurge of urbanization, especially in Provinces with better socio-economic status such as Gauteng, Western Cape and KwaZulu-Natal. This resulted in dense informal settlements with limited hygiene practices. Sewage sludges from these areas are likely to have high incidence of pathogens (Chapter 3).

iv Sparse Sanitation

Adequate sanitation in South Africa is still limited to cities, with the majority of people living in rural areas having no access to sanitation. People in these communities rely on surface water or water from wells for all household activities including drinking, bathing and cooking. If sludge is not adequately treated, runoff from the land to which sewage sludge is applied could lead to contaminated wells, and eventually infecting the waters from which these communities drink.

v Cultural diversity

South Africa is home for a number of diverse cultures. Some groups have developed a habit of deliberately ingesting soil, a practice that has to be taken into consideration with regard to using sewage sludge for soil amendment. If adequately treated sludge is used, the chances of individuals ingesting soil to be infected will be reduced or eliminated. Management practice may also prohibit soil ingestion in these areas through warnings.

vi Climatic conditions

The survival of microorganisms depends on the surrounding temperature and humidity conditions. South Africa is a semi-arid country. The survival of and the potential infection of microorganims in sludge will be reduced by the high ultra-violet radiation and desiccation, as most microorganisms will not proliferate under these conditions.

vii Soil structure

Agricultural land in South Africa are carbon depleted in some areas as a result of high microbial activity. As a result, the number of pathogens from humans sources added to soil from sewage sludge will be relatively small compared with the densities of pathogens present in soil. Thus introduced pathogens into soil have a minimal chance of survival as a result of competition (Apedaile, 2001; Forcier, 2002).

7.4 Exposure pathways

The U.S EPA used various risk assessment procedures to develop exposure pathways to establish the risk factor to humans and the environment (Table 7.1). The risk assessment section discussed in Chapter 6 has demonstrated the possibility of infection through some of these pathways if inadequately treated sludge is used in agricultural land. An effective management plan is necessary to protect the public from infection through these pathways. Pathways 1, 2, 3, 11 and 13 (Table 7.1) are of particular concern regarding human health safety.

Risk is defined as follows:

Risk = Hazard X Probability of infection

As shown in chapter 6, the risk of infection regarding sewage sludge use is regarded as acceptable if 1 in 10 000 ($1:10^4$) infections occurs per year (Haas, 1996). Models computed in this section, have shown that the risk of infection from contaminated crops become reduced as the period between application and harvest is increased.

Table 7.1 Exposure pathways for land applied sludge (WRC, 1997)

No.	Pathway	Description
1	Sludge-Soil Plant-Human	Consumers in regions heavily affected by spreading of sludge
2	Sludge-Soil Plant Human	Farmland converted to residential home garden five years after reaching maximum sludge application
3	Sludge-Soil Human	Farmland converted to residential use five years after reaching maximum sludge application with children ingesting sludge-amended soil
4	Sludge-Soil-Plant-Animal-Human	Households producing a major portion of their dietary consumption of animal products on sludge-amended soil
5	Sludge-Soil-Plant-Human	Households consuming livestock that ingest sludge-amended soil
6	Sludge-Soil-Plant-Animal	Livestock ingesting food or feed crop grown in sludge-amended soil
7	Sludge-Soil-Animal	Grazing livestock ingesting sludge/soil
8	Sludge-Soil-Plant	Crops grown on sludge-amended soil
9	Sludge-Soil-Soil Biota	Soil biota living in sludge-amended soil
10	Sludge-Soil-Soil Biota-Biota Predator	Animals eating soil living in sludge amended soil
11	Sludge-Soil-Airborne Dust-Humans	Tractor operator exposed to dust from sludge-amended soil
12	Sludge-Soil-Surface Water/Fish-Humans	Humans eating fish and drinking water from watersheds draining sludge-amended soils
13	Sludge-Soil-Air-Human	Humans breathing fumes from any volatile pollutants in sludge
14	Sludge-Soil-Groundwater-Human	Humans drinking water from wells surrounded by sludge-amended soils

7.5 Ranking of the Exposure Pathways for South African Conditions

Both the healthy individuals and those with compromised immune systems need consideration. Healthy individuals are however less susceptible to infection. People closely affected by the agricultural sludge application are the farm family, as they live on the farm, and people living close to such farms (EPA, 2003).

In chapter 6, it was shown that the ingestion of crops grown in sewage-amended soil may pose minimal probability of infection (pathways 1,2, 4, 6, 8), as a result of the advantageous climatic conditions. Thus, adequately treated sewage sludge is less likely to pose any unacceptable risk with regard to exposure pathways for both the healthy and the immuno-compromised individuals, as the pathogen load in this sludge is expected to be minimal. Safety for using sludge can be enhanced by following the recommendations regarding its application (WRC, 1997). The restrictions require that sludge is mixed or covered with soil (WRC, 1997), reducing the pathogen load per area as a result of dilution.

Sludge use is regarded as yielding an unacceptable exposure if the risk of infection or consequent is greater than 1: 10^4 (Haas, 1996). For instance if sewage sludge use result in 1:10 deaths or acute infections leading to disease profiles such as hemolytic uremia, the risk is unacceptable. The probability of such infection is high (Table 7.2). If sludge use result in sporadic ailments or occasional symptoms, the probability of infection does not pose an unacceptable risk (Table 7.2).

Table 7.2 Generic risk rating matrix based on human health. Numbers 1 to 10 indicate the probability of a hazard occurring

Hazard	Probability of hazard occurring					
Loss of life	10	8	6	4	2	1
Acute illness	8	6	4	2	1	0
Chronic illness	6	4	2	1	0	0
Sporadic ailments	4	2	1	0	0	0
Occasional symptoms	4	2	1	0	0	0
No effect	0	0	0	0	0	0
The risk	$1:10^1$	$1:10^2$	$1:10^3$	**$1:10^4$**	**$1:10^5$**	**$1:10^6$**

If type A or B sludges are used, the probability of infection may be increased. Tables 7.3 and 7.4 provides the risk ranking for both healthy and immuno-suppressed individuals respectively, with regard to application of the three sludge types. Individuals with weakened immune systems present a high probability of infection. For this reason, the ranking was quantified to reflect high probability of infection when using type A and type B sludges compared to using type C or D. This will be a problem, particularly for exposed crops such as root crops, including carrots and potatoes. However these risks can easily be managed by prohibiting the use of type A and B wastewater sludge on such vegetable types and also on public parks or recreational facilities.

Some members of the population may be exposed to multiple pathways (Harrison, 1999). For instance, some adults and children who have a habit of ingesting soil, may be exposed to pathway 3 in addition to other exposure pathways, while this may not be the case in adults who do not practice geophagia in their cultures (Hunter, 1993). The concentration of pathogens in sludge-amended agricultural soil can be reduced by mixing the sludge and soil

properly (effecting a dilution). If sludge is applied during warm summer days, a rapid pathogen die-off can be encouraged such that soil ingestion would not lead to serious infections (pathway 3, 7).

Some pathogens are capable of being air borne, often influenced by windy dry days. Farm workers are at the risk of inhaling dust borne pathogens during application, which can result in infection of the respiratory tract. This exposure can be prevented by ensuring that each farm worker is provided with a protective clothing such as a mask capable of covering both the nostril and mouth area, during sludge application. People neighbouring the farms can be protected from aerosols by irrigating the agricultural land following sludge application (Apedaile, 2001). Also, wetting the treated dry sludge prior application may reduce emission of bio-aerosols. This would ensure that few particles are suspended in the air (pathway 11 and 13).

Pathogens in the soil can enter a water body through runoff and erosion. However, the concentration of pathogens in the leachate from agricultural land may be diluted in the watersheds/groundwater system before reaching a nearby well used for drinking (EPA, 2003). Regular monitoring of such water bodies will ensure that the number of pathogens present in such water is kept at acceptable levels (pathway 12 and 14), such that these waters would not pose a health risk for rural communities who using the resource.

Ecological receptors are also exposed to contamination through ingestion of terrestrial or aquatic food items. Their food chain include vegetation, soil and prey items in their diet that they obtain from the farm field where sewage sludge is applied (EPA, 2003). These receptors include beef or diary cattle raised by the farm family. Protecting these receptors will ensure that humans feeding on their products (such as meat or milk) are protected. However, as the pathway between sludge and the ecological receptor is often long (4,5, 6, 9, 10 and 12), pathogen load may well be reduced to such an extent that the risk is negligible. Human enteric pathogens such as *Salmonella* spp are capable of surviving in

warm-blooded animals (Jones, 1980). Humans can be protected from infection by avoiding raw products from such animals. For instance, by employing adequate cooking of meat products and effective pasteurization of milk.

Table 7.3 Risk ranking per pathway for sludge types with regard to Healthy individuals

Pathways	Type A Sludge	Type B sludge	Type C/D Sludge
1	6	4	0
2	6	4	0
3	8	6	0
4	4	2	0
5	4	2	0
6	4	2	0
7	4	2	0
8	6	4	0
9	6	4	0
10	6	4	0
11	4	2	0
12	4	2	0
13	4	2	0
14	6	4	0

Table 7.4 Risk ranking per pathway for sludge types with regard to immuno-compromised individuals (including HIV/AIDS and cancer patients)

Pathways	Type A Sludge	Type B sludge	Type C/D Sludge
1	8	6	0
2	8	6	0
3	10	8	0
4	8	6	0
5	8	6	0
6	8	6	0
7	8	6	0
8	8	6	0
9	8	6	0
10	8	6	0
11	10	8	0
12	10	8	0
13	10	8	0
14	10	8	0

7.6 Risk Management

The main challenge in risk management is not in predicting potential infection due to pathogens in sewage sludge, but being able to introduce the interventions necessary to prevent the occurrence of such infections. In most countries around the world, recycling sewage sludge to agricultural land is still regarded as the best practical environmental option. Understanding the pathways and the fates of contaminants derived from sewage sludge, and their ultimate effect on the environment and on human health is a useful tool in designing safety procedures regarding sewage sludge use in agricultural land.

Table 7.6 provides a generic presentation of probability of exposure and severity of hazard. Description of hazard severity is provided in Table 7.5.

Table 7. 5. Pathogen potential rating

Pathogen load	Description
High	Pathogens are present in sufficient quantities to cause concern
Medium	Pathogens could be present at levels of concern
Low	Pathogens present in sludge, but monitoring indicates minimal levels
Negligible	Pathogens not present in sufficient quantities in sludge to cause concern

Table 7.6 Risk ranking based on the probability of exposure and severity of hazard

		Probability of exposure			
		Frequent	***Reasonably probable***	***Occasional***	***Remote***
Hazard severity	***High***	Higher risk			
	Medium		Medium risk		
	Low			Lower risk	
	Negligible				Acceptable risk

The quality of sewage sludge and the probability of exposure of humans to the sludge determine the risk of contracting an infection. The sludge quality in this study is determined by the concentration of pathogens in sewage sludge. In raw (untreated) sludge, it is expected that there will be large numbers (high concentration) of disease causing pathogens. Application of such sludge would certainly pose a 'higher risk' of infection. Type B sewage sludge (WRC, 1997) could yield 'medium risk'. Types C and D are likely to yield 'lower risk' as they contain limited pathogenic organisms (WRC, 1997). As indicated in Table 7.2,

the frequency of exposure and the pathogen content determines the extent of the risk of infection. This implies that if sewage sludge is properly treated prior to application to land, and the periods between applications, and between application and harvesting are managed properly, the risk of contracting infection becomes an 'acceptable risk'. If farmers adhere to the 8 tons/ha application and the sludge is well mixed with the soil and evenly spread, this will result in dilution of the sewage sludge. The natural die-off of the microorganism will occur (as demonstrated in Chapter 4) placing the hazard severity on the lower or negligible end.

Although there are presently no known cases of infection or illness implicating sewage sludge use in South Africa, considering the country's large population of immuno-compromised individuals as a result of the high incidence of HIV/AIDS, it is necessary to introduce advanced precautionary steps to prevent any occurrence of such infections.

Proper training in taking precautionary measures can reduce the chances of infection during sludge handling by farm workers and personnel working at the WWTPs. Continued proper management of sludge application to agricultural land will require that effective skills transfer is implemented to increase the pool of personnel knowledgeable regarding sludge use and management.

Scientific community need to work closely with sludge producers providing advise on efficient but cost effective techniques that can be used to reduce pathogen load.

Adequate sewage sludge treatment should ensure that offensive odours are not generated from the final product, reduce vector attraction and that pathogen regrowth is controlled (EPA, 1999).

Direct soil ingestion by toddlers or people who have adopted this habit represents a risk of infection for this group if sludge is inadequately treated.

Management may reduce a risk of infection by not allowing entry into the premises or by educating these individuals.

7.7 Conclusion and Recommendations

Use of untreated (raw) sludge should not be allowed on any exposed crops or root crops.

Farmers who are the recipients of sludge, have a responsibility to adhere to proposed application rates and to educate farm workers on the precautionary measures necessary for sludge handling.

For type B sludge, the period between sludge application and harvesting should be such that pathogen reduction in soil is ensured. This study has shown that significant pathogen reduction can occur in 12 weeks (3 months) following application (Chapter 4). Prolonging this period will reduce the risk of infection. EPA (1999) recommend a 14 month harvest restriction for crops that touch the soil.

Sludge application may also be done well in advance (3 months) prior to planting thus ensuring that the period between harvesting and application is long.

Access to land applied with sewage sludge may also be prohibited through fencing and/or penalty for those who do not comply. In this way, the receptor will be removed from the pathway, thus the risk of potential infection will be reduced.

Comprehensive management plan that involves regular monitoring processes and public awareness campaigns needs to be in place to ensure understanding by the public of the benefits of sewage sludge and steps taken to ensure sludge safety.

Monitoring techniques need to be well documented, rapid, less complicated, cost effective and enforced.

Sludge producers may enhance safety use by supplying information to farmers indicating the product quality and emphasizing the necessary precautions to be taken.

7. 8 References

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Chapter 8

Concluding Remarks

8.1 Introduction

As sewage sludge comprises high levels of organic matter and nutrients such as N and P, it can be used as soil conditioner, particularly in developing countries. In South Africa one of the serious problems is the widespread degradation of agricultural soil due to erosion and nutrient depletion.

Unfortunately, utilization of sewage sludge for agricultural purposes has disadvantages as well. Apart from aesthetic reasons, the principal disadvantage of the agricultural use of wastewater sludge is the potential of the transmission of human bacterial and parasitic infections through consumption of produce, if the sludge used was not adequately treated (Rudolfs *et al*., 1950).

While benefits that result from using sludge as soil conditioner have been studied widely, knowledge on its limitations regarding microbial pathogens is limited. This study sought to establish the potential prevalence of pathogenic microorganisms in a high risk crop grown from soil amended with sewage sludge, and to provide suggestions on management practice for beneficial use of sludge.

8.2 Research Findings

The two types of sludge, namely low metal (LMS) and high metal sludge (HMS) were found to have several human pathogenic microorganisms, including the *E.coli* and *Salmonella* spp (Chapter 3). This is consistent with other studies on sludge pertaining to the types of microbes present in sludge (Strauch, 1991; Juang and Morgan 2001).

These organisms persisted in soil for a period of three months during planting (Chapter 4). Earlier, other researchers (Strauch, 1991; Baloda *et al*., 2001) reported on the survival of *E.coli* and *Salmonella* spp. Culture based technique showed the prevalence of these microorganisms on the potatoes grown in 16 tons/ha LMS. None of the potatoes from other treatment options (LMS 8 tons/ha, HMS 8 tons/ha and HMS 16 tons/ha) had any of these organisms (Chapter 4).

Further analysis of contaminated potatoes using molecular technique yielded no viable primary pathogenic organisms (Chapter 5). Although some species of the *Enterobacteriaceae* were found to be present in the potato, none of these were indicated as primary pathogens to humans. None of the 16S rRNA sequences from potatoes matched any of the *Salmonella* spp or *E.coli* from the data bank. However, *E.coli* and other enteric pathogens, namely, *Proteus sp, Enterobacter sp* and several *Klebsiella* spp were isolated from the sewage sludge-contaminated soil in which these potatoes were grown. Organisms isolated from the crops were mainly plant organisms that are known to be responsible for diseases such as rotting in potatoes.

Observations from this study suggest that if a single application of sludge is made during planting, and crops remain in the field for a period no less than three months, it is possible that pathogen load will be significantly reduced (Chapter 4). However, proper sludge treatment and management prior to use is essential. These management practices require taking into consideration the unique prevailing conditions that affect agriculture in South Africa (Chapter 7).

8.3 Sewage Sludge Management Requirements

This study has shown that while pathogens are present in sewage sludge, this can be managed to maximize on the benefits associated with land application of sewage sludge can be realized. The underlying basic requirements are proposed

to assist sludge producers, regulators and handlers with regard to sewage sludge management:

8.3.1 Developing the awareness, understanding and commitment of the community

If community groups and farmers are not completely convinced of the benefit of land application of sewage sludge, the utilization system will not be successful. The communication strategy should be open and transparent. In a country such as South Africa with different cultural backgrounds and languages, communication can be achieved by providing brochures and seminars in languages spoken by predominant groups such as isiZulu, Afrikaans, seSotho, isiXhosa and English. Citizen commitment can also be enhanced by site visits and encouragement of community participation in advisory forums.

Farmers, constitute a group that will easily appreciate using sewage sludge in fertilizing agricultural soil (Snyman and Van der Waals, 2003), but they still need to comprehend the importance of adhering to code of practice regarding sludge application.

8.3.2 Recruitment and training of competent people

Training of competent people to operate and manage the system will ensure effective management.

8.3.3 Securing long-term support of politicians

In a country of widespread political diversity as is the case in South Africa, it is necessary to reach all spheres of political interest, such that once the system is in place it will remain sustainable irrespective of changes in the political interest

of the ruling body. Political support can be gained through educating politicians regarding the needs and benefits of the country's people.

8.3.4 Foster knowledge and understanding by way of capacity building

By reaching the educational infrastructure, through awareness in undergraduate and postgraduate programmes in the fields of science and engineering focusing on specialized training in sewage sludge management. This will ensure that there is always an available pool of individuals who appreciate the benefits of sewage sludge utilization, who will eventually contribute to effective management of the system.

8.3.5 Monitoring systems necessary to compliance

The quality of sewage sludge to be used for land application must be closely monitored to ensure acceptable quality and to determine appropriate application rates. Parameters of concern, both biological and chemical need to be regularly checked to protect both human health and the environment.

8.4 Future Trends

Utilisation in agricultural land will continue to grow as the preferred sewage sludge management practice because it is based on the fundamental concept of sustainable waste management. Societies worldwide are recognizing that there is an urgent need to change our thinking from resource consumption to ecosystem protection, and from disposal of what is regarded as waste materials to the extraction and reuse of the valuable portion of these resources.

The need for effective sewage sludge management systems in developing countries is particularly urgent. In order to accelerate their process of formulating management systems, some developing countries have adapted the experiences

of existing systems to meet their local needs. For instance, Mexico adopted the US limits for heavy metals and Faecal coliforms and modified the limits for *Salmonella* spp and the *Ascaris* ova (Jimenez *et al*., 2003). Coupled to the necessary management systems, is changing the perceptions of environmental groups and improving the knowledge of the general public regarding sludge use.

8.5 References

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Chapter 9

Appendices

Appendix A

Table A.1 API 20E TESTS FOR *ENTEROBACTERIACEAE*

ABREVIATION	TEST
ADH	Arginine
AMY	Amygdalin
ARA	Arabinose
CIT	Sodium citrate
GEL	Kohn's gelatin
GLU	Glucose
H_2S	Sodium thiosulphate
IND	Tryptophane
INO	Inositol
LDC	Lysine
MAN	Mannitol
MEL	Melibiose
ODC	Ornithine
ONPG	Ortho-nitro-phenyl-β - galactopyranoside
OX	Oxidase
RHA	Rhamnose
SAC	Sucrose
SOR	Sorbitol
TDA	Tryptophane
URE	Urea
VP	Creatine

Table A.2 API STAPH FOR STAPHALOCOCCI AND MICROCOCCI

ABREVIATION	TEST
ADH	Arginine
FRU	Fructose
GLU	Glucose
LAC	Lactose
MAL	Maltose
MAN	Mannitol
MDG	α- methyl-glucoside
MEL	Melbiose
MNE	Mannose
NAG	N-acetyl-glucosamine
NIT	Potassium nitrate
PAL	β-naphthyl-acid phosphate
RAF	Raffinose
SAC	Sucrose
TRE	Trehalose
URE	Urea
VP	Sodium pyruvate
XLT	Xylitol
XYL	Xylose

Table A.3: Results of the API tests undertaken for microbial identification. API Staph was used to identify Isolate in column labeled 6. Tests are indicated in braces

TEST	Isolates					
	1	2	3	4	5	6
ONPG	+	+	+	+	+	(O) -
ADH	-	-	-	+	-	(ADH) -
IDC	+	+	+	-	+	(FRU) +
ODC	+	+	+	+	+	(MNE) +
CIT	-	+	+	+	+	(MAL) +
H2S	-	-	-	-	-	(LAC) +
URE	-	-	-	-	+	(URE) -
TDA	-	-	-	-	-	(TRE) +
IND	+	-	-	+	+	(XLT) -
VP	-	+	+	-	+	(VP) +
GEL	-	+	+	-	-	(NIT) +
GLU	+	+	+	+	+	(GLU) +
MAN	+	+	+	+	+	(MAN) +
INO	-	+	+	-	+	(PAL) -
SOR	+	+	+	+	+	(RAF) +
RHA	+	-	-	+	+	(XYL) +
SAC	-	+	+	-	+	(SAC) +
MEL	+	+	+	-	+	(MEL)+
AMY	-	+	+	+	+	(MDG) -
ARA	+	-	-	+	+	(NAG) +
OX	-	-	-	-	-	

Appendix B

Data generated from Statistical analysis

Table B.1: Summary of Descriptive Statistics for Faecal coliforms

Time (weeks)	00	02	04	06	08	10	12
N	**8**	**8**	**8**	**8**	**8**	**8**	**8**
LMS 8 tons/ha							
Mean	3628.75	36575000	7525000	15775074	8525	7049.63	955
SD	1873.66	23419757	15785595	25629047	15533	14967	2245.78
P	0.0009	0.0031	**0.2196**	**0.1252**	**0.1645**	**0.2245**	**0.2682**
Median	2750	34000000	500000	3400000	0	0	0
P	0.0078	0.0078	0.0078	0.0156	**0.2500**	**0.2500**	**0.5000**
LMS 16 tons/ha							
Mean	26125	29937.5	14250000	14213225	870	327.87	395375
SD	10881.8	11724.33	12739590	18453896	1666.97	507.52	474287
P	0.0003	0.0002	0.0158	**0.0658**	**0.1834**	**0.1104**	**0.0505**
Median	31000	33500	11700000	3250000	30	69.5	195000
P	0.0078	0.0078	0.0078	0.0078	**0.0625**	0.0313	0.0313
HMS 8 tons/ha							
Mean	470	5861.25	47455	9175000	0	0	0
SD	1105.74	6546.56	89300.87	16803890	0	0	0
P	0.2684	0.0391	**0.1765**	**0.1664**	-	-	-
Median	75	3550	665	2800000	0	0	0
P	0.0313	0.0078	0.0078	0.0313	-	-	-
HMS 16 tons/ha							
Mean	1848.75	20162.50	7303750	6975056	7.5	6.25	0
SD	1195.04	10810.04	11065447	10933623	21.21	11.88	0
P	0.0033	0.0012	**0.1042**	**0.1141**	**0.3506**	**0.1803**	-
Median	1575	17600	4200000	2900000	0	0	0
P	0.0078	0.0078	0.0078	0.0313	**1.0000**	**0.5000**	-

Table B.2: Summary of Descriptive Statistics for *E. coli*

Time (weeks)	**00**	**02**	**04**	**06**	**08**	**10**	**12**
N	**8**	**8**	**8**	**8**	**8**	**8**	**8**
LMS 8 tons/ha							
Mean	2178.75	26362500	4625000	0	3750	6775	226.25
SD	736.91	23884959	10825730	0	10606.6	14315	447.63
P	0.0001	0.0168	**0.2667**	-	**0.3506**	**0.2225**	**0.1959**
Median	2000	20000000	0	0	0	0	0
P	0.0078	0.0078	**0.2500**	-	**1.0000**	**0.5000**	**0.5000**
LMS 16 tons/ha							
Mean	25250	29937.5	10200000	1629475	684.5	164.38	101625
SD	10361.47	11724.33	13249690	459475	1676.66	348.04	216955.5
P	0.0002	0.0002	**0.0659**	**0.3492**	**0.2861**	0.2234	**0.2268**
Median	29000	33500	5500000	0	5	6.5	0
P	0.0078	0.0078	0.0078	**0.2500**	**0.1250**	**0.1250**	**0.2500**
HMS 8 tons/ha							
Mean	446.25	5861.25	1572.5	1762500	0	0	0
SD	1113.64	6546.55	3575.49	3624495	0	0	0
P	0.2944	0.0391	**0.2536**	**0.2114**	-	-	-
Median	50	3550	315	0	0	0	0
P	0.0313	0.0078	0.0313	0.5000	-	-	-
HMS 16 tons/ha							
Mean	1547.5	19912.5	6991250	2137556	7.5	6.25	0
SD	1359.76	11092.78	11149352	4914364	21.21	11.88	0
P	0.0147	0.0014	**0.1194**	**0.2583**	**0.3506**	**0.1803**	-
Median	1450	17600	3600000	0	0	0	0
P	0.0078	0.0078	0.0078	**0.2500**	**1.0000**	**0.5000**	-

Appendix C

Reagents

LB Medium

10g Bacto-tryptone

5g Bacto-yeast extract

5g NaCl

Adjust pH to 7.0 with NaoH

Ampicilin

Ampicilin 0.25g

Sterile dH20 5ml

Filter sterilise, make aliquots and store at –20 ^{o}C

LB plates with Ampicilin

Add 12g of agar to 1L of LB medium

Autoclave

Allow the medium to cool at 50^{o}C before adding ampicilin to a final concentration of 100 μg/ml

Pour 30-35 ml of medium into 85 mm petri dishes

Can be stored at 4 ^{o}C for up to a month or at room temp for a week

Nutrient Agar

20 g of nutrient agar in 1L of distilled water. Autoclaved at 121 ^{o}C

TE Buffer

10 mM Tris.HCl

1mM EDTA

pH 8.0

50X TAE Buffer

40 mM Tris.HCl

20 mM NaoAc

1mM EDTA

pH 8.5

Dilute 1:50 in dH_2O before use (1 X TAE)

IPTG stock

1.2g IPTG

Add water to 50 ml final volume

Filter sterilise and store at -4^oC

X-Gal

100 mg 5-bromo-4-chloro-3-indolyl-β -D-galactoside

Dissolve in 2 ml N,N'-dimethyl-formamide

Cover with aluminium foil and store at -20^oC.

Solution I

50 mM Glucose

10mM EDTA

25 mM Tris.HCl

pH 8.0

Solution II

0.2 N NaoH

1% SDS

Must be prepared fresh

Solution III

3 M NaoAC

pH 4.8