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.

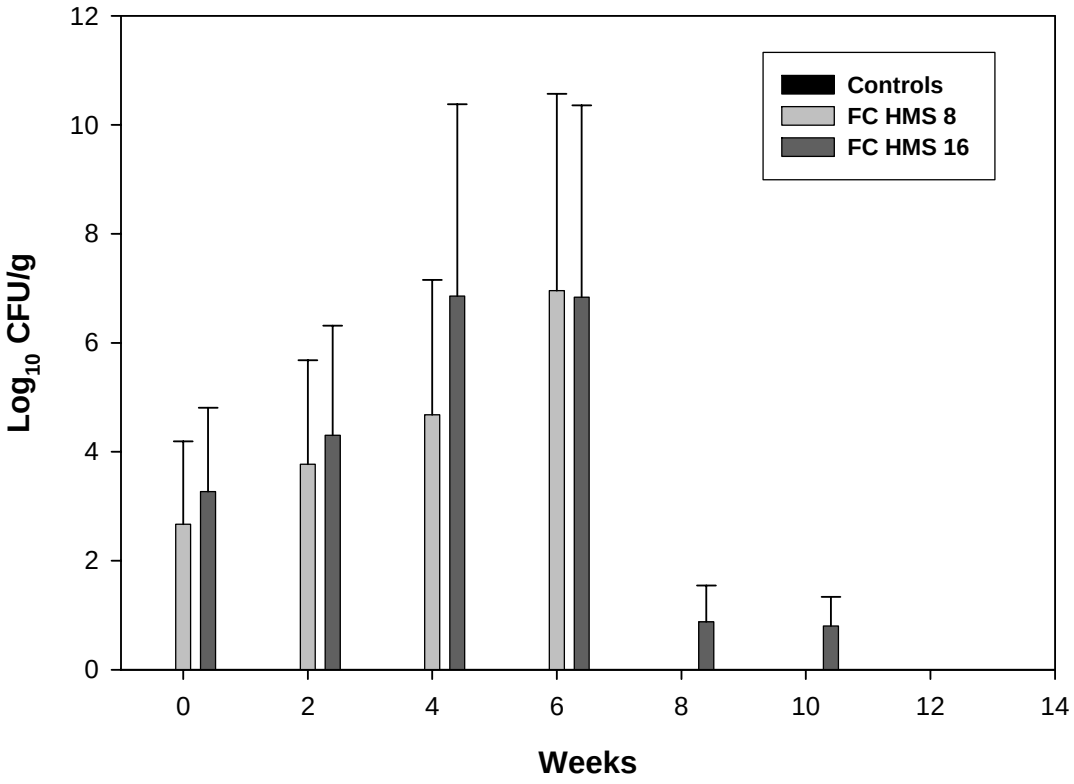

Figure 4.1 Faecal coliforms for HMS at application rates of 8 and 16 tons/ha.

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.

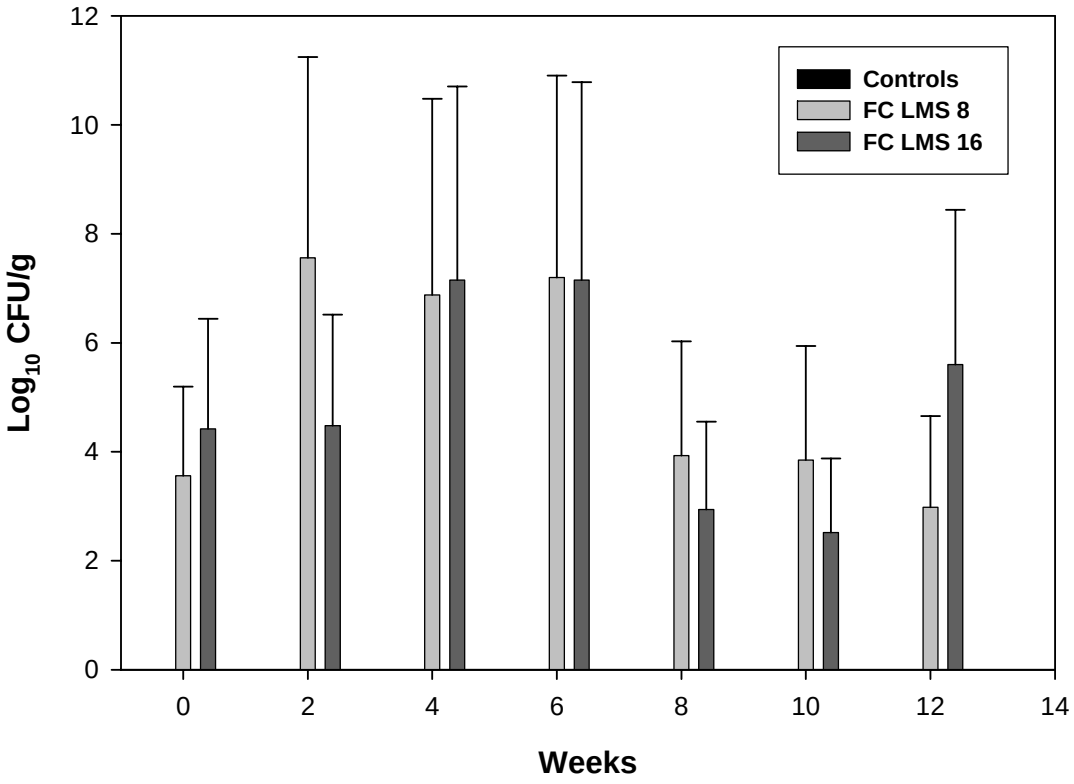

Figure 4.2 Faecal coliforms for LMS at application rates of 8 and 16 tons/ha.

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.

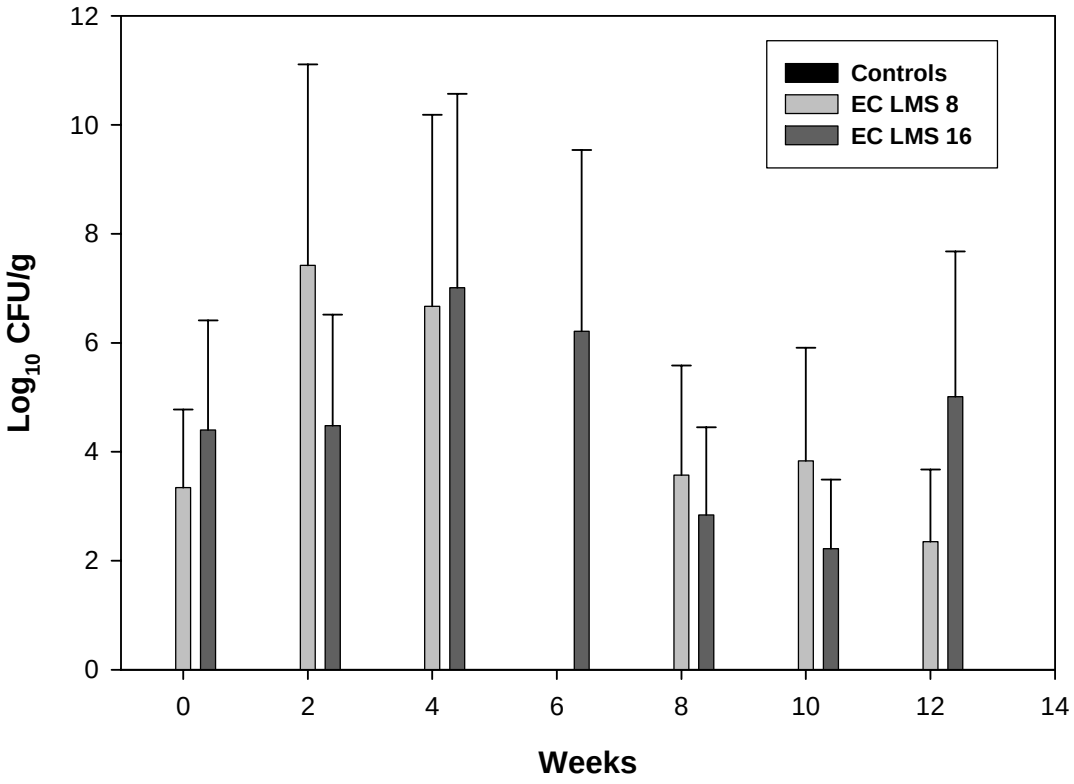

Figure 4.3 *E. coli* for LMS at an application of 8 and 16 tons tons/ha.

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.

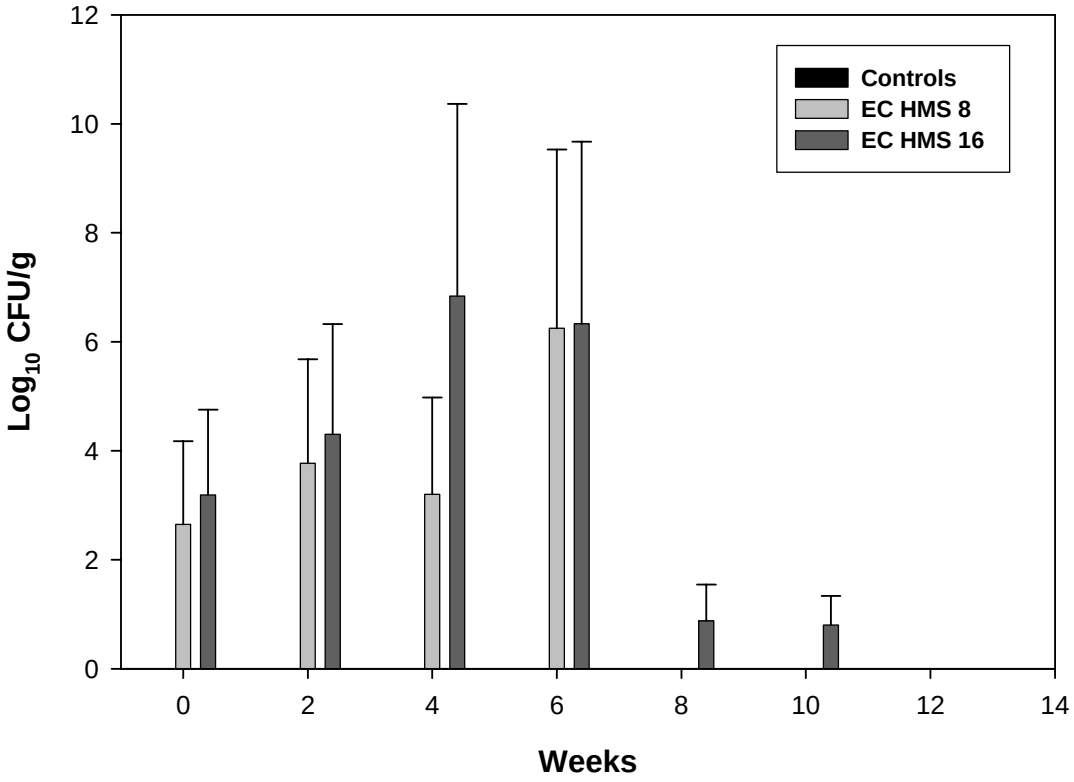

Figure 4.4 *E. coli* for HMS at application rates 8 and 16 tons/ha.

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.

4.5 References

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