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).

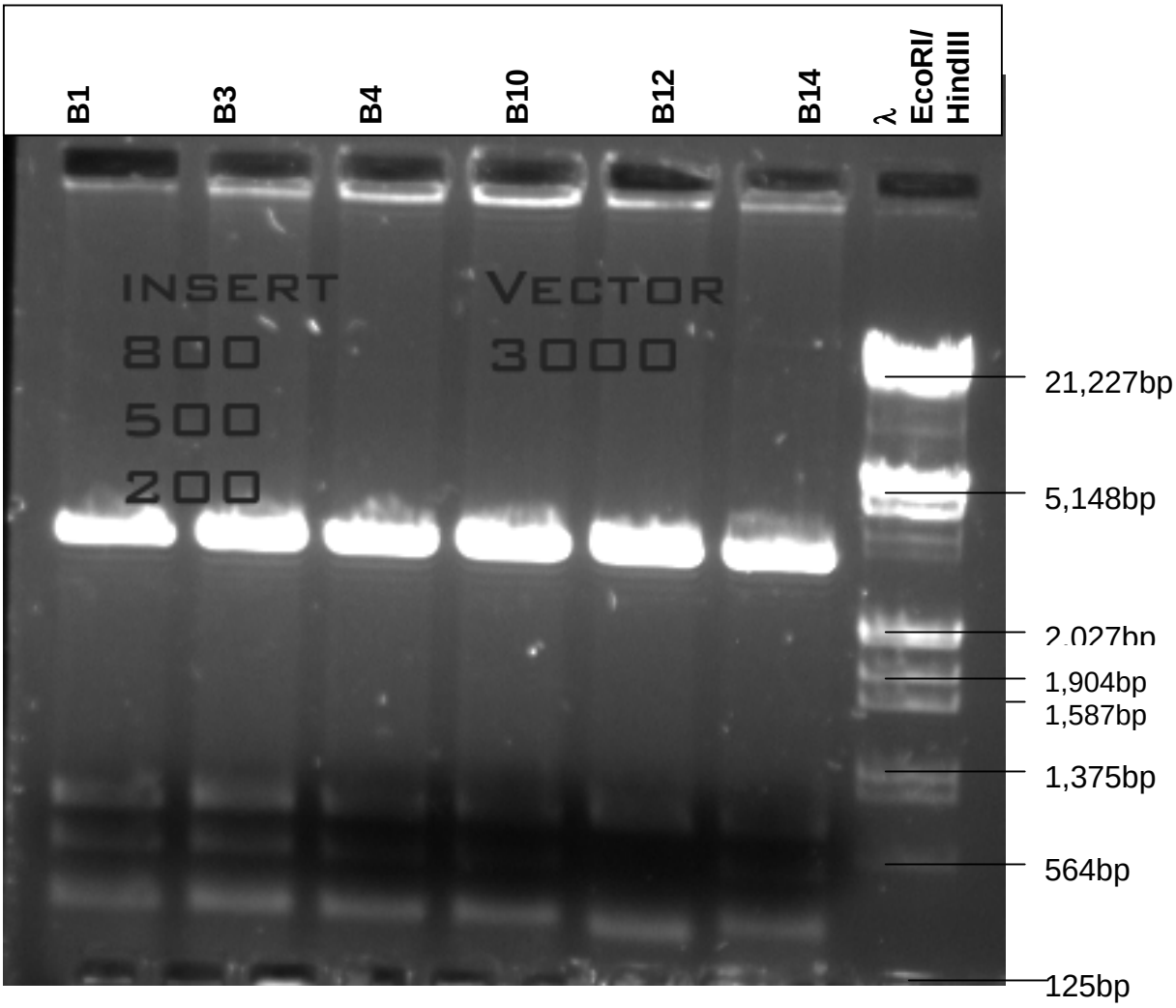

Figure 5.1 Agarose gel electrophoresis following restriction enzyme treatment.

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.

5.5 References

Amann, R. and Ludwig, W. 2000. Ribosomal RNA-targeted Nucleic Acid Probes for Studies in Microbial Ecology. *FEMS Microbiology Reviews*. **24**. 555-565

Ayyagari, A., Agarwal, J. and Garg, A. 2003. Antibiotic Associated Diarrhoea: Infectious Causes. *Indian Journal of Medical Microbiology*. **21**(1). 6-11

Beauchat, L.R. 1998. Surface Decontamination of Fruits and vegetables Eaten Raw: A Review. Food Safety Issues. World Health Organization. WHO/FS/FOS/98.2. 16pp

Boon, N., Marlé, C., Top, E.M. and Verstraete, W. 2000. Comparison of the Spatial Homogeneity of Physico-chemical parameters and bacterial 16S rRNA genes in Sediment Samples from a Dumping Site for Dredfing Sludge. *Applied Microbiology and Biotechnology*. **53**. 742-747

Borneman, J., Skroch, P.W., O'Sullivan K.M., Palus, J.A., Rumjanek, N.G., Jansen, J.L., Niehuis, J. and Triplett, E.W. 1996. Molecular Microbial Diversity of an Agricultural Soil in Winsconsin. *Applied and Environmental Microbiology.* **62**(6). 1935-1945

Burge, W.D. and Marsh, P.B. 1978. Infectious Disease Hazards of landspreading Sewage Wastes. *Journal of Environmental Quality*. **7**(1). 1-9

Brosius, J., Palmer, M.L., Kennedy, P.J. and Noller, H.F. 1978. Complete nucleotide Sequence of a 16S Ribosomal rRNA gene from *E.coli*. *Proceedings of the National Academy of Sciences* USA. **75**. 4801-4805

Brown, T.A. 1994. DNA Sequencing. Oxford University Press. New York. 101pp

Brown, T.A. 1995. Gene Cloning. 3rd Ed.Chapman and Hall. New York. 334pp

Byamukama, D., Kansiime, F., Mach, R.L. and Farnleitner, A.H. 2000. Determination of *Escherichia coli* Contamination with Chromocult Coliform Agar Showed a High Level of Discrimination Efficiency for Differing Fecal Pollution Levels in Tropical Waters of Kampala, Uganda. *Applied and Environmental Microbiology*. **66**(2). 864-868

Cappellini, R.A., Ceponis, M.J. and Lighter, G.W. 1984. Disorders in Potato Shipments to the New york Market, 1972-1980. *Plant Disease*. **68**. 1018-1020

Cieslak, P.R., Barrett, T.J., Griffin, P.M., Gensheimer, K.F. Beckett, G., Buffington, J. and Smith, M.G. 1993. *Escherichia coli* O157:H7 Infection from a Manured garden. *The Lancet*. **342**. 367

Deeb, G.R., Yih, W-Y., Merrill, R.G. and Lundeen, R.C. 1999. Noma: Report of a Case Resulting in bony ankylosis of the Maxilla and Mandible. *Dentomaxillofacial Radiology*. **28**. 378-382

Dorrington, R., Bradshaw, D. and Budlender, D. 2002. HIV/AIDS Profile in the Provinces of South Africa. Indicators for 2002. Medical Research Council, South Africa. 31pp

Dorn, C.R., Reddy, C.S., Lamphere, D. N., Gaeuman, J.V. and Lanese, R. 1985. Municipal Sewage Sludge Application on Ohio Farms: Health Effects. *Environmental Research*. **38**. 332-359

Dutkiewicz, J. 1997. Bacteria and Fungi in Organic Dust as Potential Health Hazard. *Annals of Agricultural and Environmental Medicine*. **4**. 11-16

EPA, 1999. Environmental Regulations and Technology. Control of pathogens

and vector attraction in sewage sludge. U.S. Environmental Protection Agency. EPA/625/R-92-013. 111pp

Faulde, M., Sobe, D., Burghardt, H. and Wermter, R. 2001. Hospital Infestation by the Cluster Fly, *Pollenia rudis sesu stricto Fabricus* 1794 (Diptera: Calliphoridae), and its Possible Role in Transmission of Baterial Pathogens in Germany. *International Journal of Hygiene and Environmental Health*. **203**. 201-204

Greenwood, D., Slack, R.C.B. and Reutherer, J.F. 1997. Medical Microbiology. A Guide to Microbial Infections: Pathogenesis, Immunity, Laboratory Diagnosis and Control. 15th Ed. Churchill Livingstone. London. 690pp

Jeon, C.O., Lee, D.S. and Park, J.M. 2003 Microbial communities in Activated Sludge Performing Enhanced Biological Phosphorus Removal in a Sequencing Batch Reactor. *Water Research*. **37**. 2195-2205

Jonas, D., Spitzmuller, B., Dashner, F.D., Verhoef, J. and Brisse, S. 2004. Discrimination of *Klebsiella pneumoniae* and *Klebsiella oxytoca* Phylogenetic Groups and othe Klebsiella species by use of Amplified Fragment Length Polymorphism. *Research Microbiology*. **155**(1). 17-23

Klappenbach, J.A., Saxman, P.R., Cole, J.R. and Schmidt, T.M. 2001. Rrndb: the Ribosomal RNA Operon Copy Number Database. *Nucleic Acids Research*. **29**(1) 181-184

Matsumoto, S. Suenaga, H., Naito, K., Sawazaki, M, Hiramatsu, T. and Agata, N. 2000. management of Suspected Nosocmial Infection: An Audit of 19 Hospitalized Patients with Septicemia Caused by *Bacillus* Species. *Japanese Journal of Infectious Diseases*. **53**. 196-202

Muller, H.E., Brenner, D.J., Fanning, G.R., Grimont, P.D. and Kampfer, P. 1996. Emended Description of *Buttiauxella agretis* with Recognition of Six New Species of *Buttiauxella* and Two New Species of Kluyvera: *Buttiauxella ferragutiae* sp. nov., *Buttiauxella gaviniae* sp. nov., *Buttiauxella brennerae* sp. nov., *Buttiauxella izardii* sp. nov., *Buttiauxella noackie* sp. nov., *Buttiauxella warmboldiae* sp. nov., *Kluyvera cochleae* sp. nov., and *Kluyvera Georgiana* sp. nov. *International Journal of Systematic Bacteriology*. **40**(1) 50-63

NRC, 1996. Use of Reclaimed Water and Sludge in Food Crop Production. Committee on the Use of Treated Wastewater Effluents and Sludge in the Production of Crops for Human Consumption. Water Science and Technology Board. Commission on Geosciences, Environment and Resources. National Research Council. National Academy of Press, Washington, DC

Pande, B. N., Krysinska-Traczyk, E., Prazimo, Z., Skorska, C., Sitkowska, J. and Dutkiewicz, J. 2000. Occupational Biohazards in Agricultural Dusts from India. *Annals of Agricultural and Environmental Medicine*. **7**. 133-139

Pahren, H.R., Lucas, J.B., Ryan, J.A. and Dotson, G.K. 1979. Health Risks Associated with Land Application of Municipal Sludge. *Journal of Water Pollution Control Federation*. **51**(11) 2588-2601

Pell, A.N. 1997. Manure and Microbes: Public and Animal Health Problem? *Journal of Dairy Science*. **80**. 2673-2681

Pérombelon, M.C.M. and Kelman, A. 1980. Ecology of the Soft Rot *Erwiniae*. *Annual Reviews of Phytopathology*. **18**. 361-387

Prescott, L.M., Harley, J.P. and Klein, D.A. 2002. Microbiology 5th ed. McGraw-Hill. New York. 1026pp

Rolph, H.J., Lennon, A., Riggio, M.P., Saunders, W.P., MacKenzie, D., Coldero, L. and Bagg, J. 2001. Molecular Identification of Microorganisms from Endodontic Infections. *Journal of Clinical Microbiology*. **39**(9) 3282-3289

Rudolfs, W., Falk, L.L. and Ragotzkie, R.A. 1951. Contamination of Vegetables Grown in Polluted Soil. *Sewage and Industrial Wastes* . **23**. 992 -1000

Saambrook, J.E., Fritsch, E.F. and maniatis, T.A. 1989. Molecular Cloning: A Laboratory Manual. 2nd Ed. Cold Spring Harbor Laboratory, New Yrok. Cold Spring Harbor laboratory

Small Wright, M. 2002. Sewage Sludge/Biosolids. A health and Environmental Crisis and Scandal. Perelandra Center for Nature Reseach. Perelandra Health Watch http://www.prelandra-itd.com/AB1473/webpage.cfm

Staskawicz, B.J., Mudgett, M.B., Dangl, J.L. and Galan, J.E. 2001. Common and Contrasting Themes of Plant and Animal Diseases. *Science*. **292**. 2285-2289

Strauch, D. 1991. Survival of pathogenic Microorganisms and Parasites in Excreta, Manure and Sewage Sludge. *Revue Scientifique et Technique office International des Epizooties* .**10**(3). 813-846

Toth, I.K, Bell, K.S., Holeva, M.C. and Birch, P.R.J. 2003. Soft Rot *Erwiniae:* From Genes to Genomes. *Molecular Plant Patholgy*. **4**(1). 17-30

Walker, T.S. 1998. Microbiology. W.B. Saunders Company, London. 504pp

Wattiau, P., Renard, M.E., Ledent, P., Debois, V., Blackman, G. and Agathos, S.N. 2001. A PCR Test to Identify *Bacillus subtilis* and Closely Related Species and its Application to the Monitoring of Wastewater Biotreatment. *Applied Microbiology and Biotechnology*. **56**. 816-819

Ward, D.M., Bateson, M.M., Weller, R. and Ruff-Roberts, A. 1992. Ribosomal RNA Analysis of Microorganisms as they Occur in Nature. In: *Advances in Microbial Ecology*. Marshall, K.C. (Ed.) Plenum Press. New York. Vol **12**. 219-287

Weisburg, W.G., Barns, S.M., Pelletier, D.A. and lane, D,J. 1991. 16S Ribosomal DNA Amplification for Phylogenetic Study. *Journal of Bacteriology*. **173**. 697-703

Wells, J.M. and Butterfield, J.E. 1997. *Salmonella* Contamination Associated with Bacterial Soft Rot of Fresh Fruits and Vegetables in the Marketplace. *Applied Environmental Microbiology*. **62**. 2567-2570

Westbrook, G.L., O'Hara, C.M., Roman, S.B. and Miller, J.M. 2000. Incidence and Identification of *Klebsiella planticola* in Clinical Isolates with Emphasis on Newborns. *Journal of Clinical Microbiology*. **38**(4). 1495-1497

Wintzingerode, F.V., Gobel, U.B. and Stackebrandt, E. 1997. Determination of Microbial Diversity in Environmental samples: Pitfalls of PCR-based rRNA analysis. *FEMS Microbiology Reviews.* **21**. 213-229