A QUALITATIVE STUDY OF SELECTED MICRO-ORGANISMS IN GEOPHAGIC SOIL FROM QWA-QWA

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DECLARATION OF INDEPENDENT WORK

I, Nellie Jacoba Smit, hereby declare that the dissertation submitted for the degree MAGISTER TECHNOLOGIAE in BIOMEDICAL TECHNOLOGY, at the Central University of Technology, Free State, is my own original work and has not previously been submitted to any other institutions by myself or any other person in fulfillment of the requirements for the attainment of any qualification.

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SIGNATURE OF STUDENT DATE

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SUMMARY

The existence of geophagia from as early as 460 BC up to now, makes it relevant to investigate all aspects related to geophagia. Geophagia is a direct route for potential transmission of pathogens to the human host, through the ingestion of soil. Soil-borne diseases in humans are causing growing concern as sewage disposal, which involve sewage sludge and waste water drainage from these plants, is on the increase. It is estimated that approximately seven million tons of sewage sludge is produced annually and that 54% of this sewage sludge is introduced into soil. Data on enteric infection in humans caused by contamination from soil is limited and need further investigation. The aim of the study was, therefore, to collect information on the microbiological presence in geophagic soil in the Qwa-Qwa district. Objectives included the collecting of information regarding various sampling sites in the Qwa-Qwa district and also soil samples sold by vendors, investigation of the prevalence of known human pathogenic bacteria and fungi in geophagic soil, investigating the culturability of *Salmonella enteritidis* in geophagic soil in comparison with the viability of these organisms in soil for long periods of time, investigating potential antimicrobial activity of geophagic soil, as some of the geophagists are convinced that the geophagic soils have medicinal properties, and to determine the microbial diversity of geophagic soils, which can not be accomplished by conventional microbial culturing methods.

The geophagia mines visited for samples collection were popular among the geophagic practicing people of Qwa-Qwa and varied from relatively neat to very dirty with rubbish and pieces of broken glass. Several mines were located close to houses and roads, raising concern about the health aspects of the soil and clays

from these mines. Collection and preparation practices of geophagic soil by the vendors have been found to be a potential contributing factor in the microbial safety of the soil, as the majority use their bare hands for collection and non-sterile bags for packaging. The soil obtained from the various mines displayed a wide range of colours and textures.

Bacillus cereus was commonly isolated from the majority of the soil samples. Two distinct species of anaerobic bacteria were identified as *Clostridium perfringens* and *Clostridium paraputrificum* and were isolated from eight of the 17 geophagic mining sites and from two of the five control mines. No anaerobic bacteria were isolated from the vendor soil samples. One Enteropathogenic *Escherichia coli* serotype was identified from a vendor soil sample and one from a control mine. Various fungi were isolated and identified. These included *Penicillium* spp., *Aspergillus fumigatus*, *Aspergillus flavus, Aspergillus* spp., *Alternaria* spp., *Paecilomyces* spp., *Mucor* spp., *Trychophyton rubrum* and *Candida albicans*. Vendor soil samples contained significantly less organisms than the majority of the other soil samples, which may be attributed to the processing of soil for consumption by heat treatment or baking. Although no definite correlation could be detected between the soil colour and the type or number of organisms isolated, one soil sample from a geophagic mine had a dark grey colour and three bacterial species as well as one fungus - the highest number of isolates from any soil sample.

The soil samples consisted of a wide range of colours and observed to present a wide range of culturability profiles. Survival time and culturability were lowest in the majority of the soil samples sold by the vendors, indicating a possible influence of treating soil samples on the sustainability for growth of bacteria. The longest periods of culturability were recorded in soil samples from the geophagic mining sites.

However, no definite correlation could be detected between soil colour or culturability. Soil or clay samples with gray, red or pink colours sustained the highest periods of culturability, which may be attributed to a high level of organic matter as well as ferric constituents, while survival rate was lower in the brown and darker soils. No antimicrobial activity was detected in any of the samples. It has been found that soil colour may play a role in antimicrobial activity, as white soil demonstrated more antimicrobial activity than any other soil colour. However, the majority of samples from the current study did not consist of white colour soils/clays.

The Quantity One software detected 431 bands in total and 54 different band positions. The number of bands that comprise the denaturing gradient gel electrophoresis (DGGE) patterns indicated that there is a high diversity of bacterial polymerase chain reaction (PCR) amplification products in all soil samples, except for the soil from mining site 2. However, the diversity profiles and clustering did not show any correlation with geographical location, soil colour or any other parameter considered in this study. A fundamental insight could be obtained of bacterial diversity in the geophagic soil from mining sites in the Qwa-Qwa region. This may be the first report of bacterial community diversity in soil consumed by humans, determined by a culture independent technique. More living organisms are found in soil than in any other ecosystem and these data may contribute to the initiation of an ongoing search into the actual biological content of soil, which could also include fungi, protozoa, viruses, prions, Archaebacteria, parasitic worm eggs, etcetera .

Although microbes in geophagic soil may be harmless and even beneficial to humans, there are serious risks involved in consuming soil contaminated with pathogenic bacteria. However, the prevalence of pathogenic bacteria was found to

be relatively low and the soil sold at the market was less contaminated than the soil mined directly from the popular geophagic mining sites.

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LIST OF ACRONYMS

ACRONYMS	DESCRIPTION
α	Alpha
β	Beta
ϑ	Gamma
µm	Micrometer
µl	Microlitre
API 20E	Analytical profile index 20E - Rapid identification of enterobacteriaceae
API10S	Analytical profile index 10 S - Simplified identification of gram negative rods
bp	Basis pair
CCDA	Charcoal-ceperazone-deoxycholate agar
CIBD	Chronic inflammatory bowl disease
cm	Centimeter
DGGE	Denaturing gradient gel electrophoresis
dNTPs	Deoxyribonucleotide trisphosphate
DNA	Deoxyribonucleic acid
DMP	Diagnostic Media Products
EDTA	Ethylene diamine tetraacetic acid
EPEC	Enteropathogenic *Escherichia coli*
G	Gram
GPS	Global positioning system
h	Hour
H_2S	Hydrogen sulphate
IMP	IMP calibration services
KCl	Potassium chloride
McC+CV	MacConkey agar with crystal violet
$MgCl_2$	Magnesium chloride
mm	Millimeter
mM	Millimolar
NLF	Non-Lactose Fermenters

^{0}C	Degrees Celsius
PAHs	Polycyclic aromatic hydrocarbs
PCR	Polymerase chain reaction
PCBs	Polychlorinated biphenyls
pH	Acidity or alkalinity
PHA	Polyhydroxyalkanoates
rDNA	Ribosomal deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
SS agar	*Salmonella Shigella* agar
Tris-HCl	Tris hydrochloride
UPGMA	Unweighted pair-group method with an arithmetic mean algorithm
UV	Ultra violet
VBNC	Viable but non culturable
XLD	Xylose lysine deoxycholate agar
YR	Yellow-red

Chapter 1

Introduction

1.1 Background to the study

Geophagia can be defined as the eating of soil or soil-like substances. Geophagia is most commonly seen among women and young children, but there are no age, race or sex boundaries for it. Geophagia in families and communities is often the result of the tradition continued from mother to daughter (Hunter, 2003). Daughters in these tribes will follow the same diet as their mothers, knowing that it had been successful in the past, especially at giving birth. In rural areas this is also practiced as a method of providing minerals needed by the body during pregnancy. In these geophagic practicing communities it is also possible to buy soil or clay samples in market areas.

Unfortunately, various dangers are associated with soil consumption. Studies showed that materials present in soil may influence mineral levels in humans consuming soil, while soils contaminated by industrial or human pollutants pose a considerable threat to anyone who eats them. These soils may contain parasites or other living organisms which may cause disease in humans (Callahan, 2003). Geophagia may also lead to different health complications such as constipation, cramping, pain, perforation of the intestines, intestinal obstruction, parasitic infestation and bacterial contamination (Onyekwelu, 2009).

More living organisms are found in soil than in any other ecosystem, with bacteria being the most abundant of soil organisms (>100 million per gram of soil) (Waltz et al., 2009). The majority of organisms are found in the top 15 cm of soil and are, therefore, influenced by soil temperature, water content and pH (Waltz et al., 2009). However, not all bacteria found in soil are beneficial.

Potential hazardous bacteria in soil are usually due to contamination of the soil with sewerage, drainage spills and animal fecal contamination. It has been suggested that some human pathogenic organisms may have the ability to survive in soil and become non-culturable, but may still be viable and able to cause disease (Someya, 2004). Against this backdrop various concerns have arouse about the safety of geophagic consumption of soils from the Qwa-Qwa region, known for being a geophagic practicing community.

1.2 Problem statement

A fascinating, but poorly understood aspect of soil is its living components, as more living organisms are found in soil than in any other ecosystem. The question, therefore, is: "How dangerous is eating dirt?" There are different dangers in soil consumption. For example, various materials present in soil may influence mineral levels in humans consuming soil. In addition, soil contaminated by industrial or human pollutants pose a considerable threat to anyone who eats it. These soils may contain parasites or other living organisms which may cause disease in humans.

There is a concern that the soil in Qwa-Qwa consumed by humans may be contaminated by pathogenic bacteria or fungi and that these could be harmful to geophagic consumers. It is, therefore, essential to investigate a selection of soil samples consumed in the area for the possible presence of pathogenic micro-organisms and to ascertain the risks associated with consumption of such contaminated soil.

1.3 Aims and Objectives

The aim of the study was to generate information on the diverse microbiological presence and antimicrobial ability of geophagic soil in the Qwa-Qwa district.

Objectives therefore, were:

1. To collect information regarding various sampling sites in the Qwa-Qwa district and also soil samples sold by vendors.
2. To investigate the prevalence of known pathogenic bacteria and fungi in geophagic soil collected from various mining sites and soil sold by vendors.
3. To assess the overall microbial diversity in soil originating from mining sites.
4. To investigate the culturability of *Salmonella* spp. in geophagic soil in comparison with the viability of these organisms.
5. To investigate the potential antimicrobial activity of geophagic soil.

1.4 Research Question

The question attempted to be answered with this investigation is about the safety of eating soil, and more specifically the possible risk of infectious diseases as a result of consumption of contaminated soil. Soil is exposed to various environmental hazards, of which human and industrial pollutants are the most

common. Geophagists are also often not informed or educated about the dangers that may be associated with the consumption of soil.

1.5 Hypothesis

It is hypothesised that soil consumed by geophagists in the Qwa-Qwa region may harbour some form of bacterial or fungal pathogen, originating from human or industrial contamination.

1.6 Rationale

There is concern about the soil in Qwa-Qwa consumed by humans being contaminated by pathogenic bacteria or fungi that could be harmful to geophagic consumers. It is, therefore, essential to investigate a selection of soil samples for the possible presence of pathogenic micro-organisms and to ascertain the risks associated with consumption of such contaminated soil.

1.7 Scope of the Study

All soil samples collected from the different sites were included in the study. Investigations on the prevalence of pathogenic micro-organisms were conducted by selecting for the most prevalent environmental human and faecal contaminants, as well as various fungal contaminants. The same specimens were used for all the tests conducted. These also included tests on culturability, antimicrobial activity and bacterial diversity. As the Qwa-Qwa region is well-known for geophagic practicing, only mines and soil selling vendors from this area were included in the study.

1.8 Study Site

The study was conducted on soils collected from different geographic sites in the Qwa-Qwa district in South Africa and included 17 known geophagic mining sites, five control sites and 13 vendors selling geophagic soil. Areas investigated included Qhelaphe, Nmahali, Phahameng, Kgubestwana, Mangaung and Madikwe in Phuthaditjhaba.

1.9 Significance of the Study

Results from this study provided valuable and novel information with regard to the prevalence and culturability of pathogenic micro-organisms in soil samples collected directly from the mines as well as those sold by vendors. The culture independent technique used to determine bacterial community diversity in the soil from the mines is not novel, but no previous reports could be found on such a procedure conducted on soil consumed by humans. Of specific interest is the lower microbial quality of the soil sold by vendors, as well as the information found by the DNA based technique on the bacterial diversity of the geophagic soil from the different mines. The information may be of importance in further studies on the safety of geophagic soil in the Qwa-Qwa region and may also be used in potential educational programmes on geophagic practicing.

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Chapter 2

Literature Review

2.1 What is Geophagia?

Geophagia can be defined as the eating of soil or soil-like substances and forms part of an eating disorder known as pica (Ella, 1990; Hooda et al., 2004; Shivoga and Moturi, 2009). This practice is wide spread throughout Africa and can be associated with medicinal treatments, ceremonial events and spiritual behaviours (Ghorbani, 2008). Geophagia is practiced for different purposes in humans, which include the body's instinctive demand, as a result of malnutrition, cultural habits, natural remedies or as psychological defect (Woywodt and Kiss, 2002). Western cultures also continue this practice, where it is disguised in the use of vitamins and minerals (Hunter, 2003). Geophagia is often considered to be a pathological consumption of non-food items such as rock, stone and soil. It is even considered by some as a disease (Callahan, 2003).

Soil intake in children of 12-18 months is seen as normal exploration of their environment, but it is commonly agreed that soil intake after this age is abnormal (Abrahams, 2002). Geophagia had previously been found in mentally retarded children where the occurrence was attributed to the fact that these children cannot distinguish between normal food and non-food items (Brand, De Jager and Ekosse, 2009). However, soil intake for a period longer than one month is considered as mentally inappropriate. This eating disorder is more common in the tropics among people living in poverty and in societies that still value these traditions (Ghorbani, 2008; Shivoga and Moturi, 2009).

Some authors state that Geophagia may develop as a result of physiological needs and that this could be traced back to periods of growth during pregnancy and childhood (Ghorbani, 2008; Shivoga and Moturi, 2009). The habit is widespread in Africa and is passed down by cultural beliefs. Here the habit also continues to exist because people enjoy eating soil rather than having a physiological need for it (Shivoga and Moturi, 2009). In some Non-Western cultures, soil has been believed to be a gift from the God(s). In these cultures geophagia is accepted into the market and families.

Geophagia is most commonly seen in women and young children, but has no age, race or sex boundaries (Abrahams, 2002; Hooda et al., 2004). Ongoing geophagia in families and communities may be the result of simple mother / daughter sharing traditions. Daughters in tribes will follow the same diet as their mothers, because they know that it had been successful in the past, for example at giving birth.

In rural areas this is an important practice to provide the minerals that the human body needs during pregnancy. In areas surrounding these cultures it is possible to buy soil or clay samples in market areas. The time of soil consumption and the amount consumed at a time may vary with tribes and individual persons, but soil is consumed consistently from specific sites. In some cultures there are well established trade routes. Clay traders provide rural soil and clays even in urban settings far from the actual mining sites. Clays from termite mounds are especially popular among geophagists, possibly because of their abundant calcium consistency (Hunter, 2003).

Although the most common reason for eating soil or soil-like substances in many societies is pregnancy, different clays are believed to have different medicinal advantages. Bentonite is available worldwide as a digestive aid, while Koalin is not only widely used as a digestive aid, but also forms the base for some medicines (Hooda et al., 2004). Palygorskite (commercially named Attapulgite) in the Southern United States is an active ingredient in many anti-diarrheal medicines. It is, however, still difficult to explain geophagia scientifically or to single out only one factor as the cause of geophagia. Geophagia is intertwined in sociology, medicine and religion and soil eating, therefore, needs an interdisciplinary approach (Hooda et al., 2004).

Wiley and Katz (1998) proposed that during pregnancy geophagia has different medical advantages. During the first trimester geophagia alleviates morning sickness by soothing stomach upset, while during the second and third trimester geophagia supplies nutrients which are needed during the forming of the fetal skeleton. Women often eat soil throughout the day, as a supplement rather than a meal during the first, second and third trimester or even throughout pregnancy. Most commonly consumed are subsurface clays, especially kaolin and montmorillonite, at volumes of 30-50 g a day (Callahan, 2003). However, eating soil is not always confined to pregnant women, even among the cultures of sub-Saharan Africa, nor is it limited to tribes with little or no access to dairy-derived calcium (Callahan, 2003).

Consuming 50 g of soil daily would be considered potentially pathological by some people. In June 2000, the US Agency for Toxic Substances and Disease

Registry set up a committee to investigate soil pica. The committee found pathological levels to be 500 mg or more soil consumed daily (Callahan, 2003). However, studies in 1971 by Vermeer et al. reported that in West Africa geophagic woman consumed 30 g of clay daily (Vermeer and Ferrel, 1971; Geissler et al., 1998).

Geophagia is also linked to socio-economic status in a population. This contradicts previous findings that geophagia is found in the mentally disturbed and the poor. These findings could not be explained in children, as it was noticeable that geophagic children came from higher socio-economic surroundings (Saathoff et al., 2002). Soils are collected from specific areas known to the geophagist. These areas include mines (areas where soil are constantly being removed for geophagic practices), termite mounds, riverbanks and even the walls of their own huts (Brand, de Jager and Ekosse, 2009).

2.2 History of Geophagia

Geophagia is defined in dictionaries as soil consumption as part of religion in some cultures, or because of the urge to eat soil due to tropical disease (HAT, 1985). Geophagia has been reported from as early as the 1800s by authors ranging from Roman physicians to explorers (Woywodt and Kiss, 2002; Ghorbani, 2008). Early in the 18th century the Sultan of Turkey ate clay from the island Lemnos. This started the Europeans in adopting these soils as health food (Ghorbani, 2008).

The first known medical mention of geophagia was made in a textbook by Hippocrates during 460–377 BC (Ghorbani, 2008). Hippocrates's first descriptions of geophagia were: "If a pregnant woman feels the desire to eat earth or charcoal and then eats them, the child will show signs of these things." Hippocrates's textbook was for centuries the cornerstone of medicine and it can be assumed that geophagia was a familiar phenomenon under Greek and Roman physicians. The Roman physician Soranus made early mentions of the use of geophagia to alleviate appetite and other symptoms associated with pregnancy (Ghorbani, 2008). Cornelius Celsus compiled the *De Medicina* medical textbook containing a passage which dealt with diagnostic signs of skin colour. Here it was stated that people whose colour is bad when they are not jaundiced are either sufferers from pains in the head or are earth eaters (Woywodt and Kiss, 2002). The American Medical Association reported in 1967 that at that time a significant number of people in the United States ate dirt (Hunter, 2003).

2.3 Geographic distribution

Geophagia is practiced in many parts of the world, from rural areas to the most populated areas, which may extend from Africa, Asia, Central America, Southern America, United States of America, Kenya and many more (Tateo et al., 2006; Odilon Kikouama et al., 2009). Hooda et al. (2004) reported on the collection of soil samples from geophagic sites in Tanzania (brown earth), Turkey (chalky clay), India (clay oven lining) and Uganda (brown earth, markets). In African counties such as Cameroon, Ghana, Sierra-Leone, Nigeria, Malawi, Zanzibar

and Zambia, geophagic practices among pregnant woman and children are a common phenomenon (Brand, de Jager and Ekosse, 2009). In South Africa clay consumption is mostly associated with pregnancy, but is also seen in young children. Studies by Ghorbani (2008) showed the prevalence for geophagia among black South African woman to be 38% in urban and 44% in rural communities as opposed to prevalence among coloured woman of 4.4%, Indian woman 2.2% and Caucasian woman 1.6%.

In a study done in 1958 on the clay trade from India to the East African coast, it was reported that the women buying these clay preferred the fine texture and smoothness of this particular clay (Hooda et al., 2004). South Asia is a common exporter of clay to Britain for consumption and use, while Germany is well known for using soil in commercial anti-diarrheal medicine.

2.4 Types of geophagic soil / clay

Three kinds of clay minerals are found in soil, namely kaolinite, illite and montmorillonite. These clay minerals differ in chemical composition, cohesion and cation adsorption in soil. Kaolinite has the lowest of each property and montmorillonite the highest of each property (Garcia and McKay, 1970). Kaolinite is a soft, earthy and usually white mineral and has a low shrink-swell capacity as well as a low cation exchange capacity (Pohl, 2011). It may also, in some parts of the world, have an orange-red colour because of iron oxide, while lighter concentrations of iron oxide may cause white, yellow or light orange colours. Kaolinite occurs in clays formed from chemical weathering of rocks in

hot, moist climates, such as in tropical rainforest areas. As the geographical climate becomes drier and cooler, the percentage kaolinite decreases, while the proportion of other clay minerals, such as illite, increases. Illite is a non-expanding, clay-sized mineral and is commonly found in sediments, soils and argillaceous sedimentary rocks. Glauconite is an iron rich member of the illite group (Mitchell, 1993).

Montmorillonite is found in green healing clay and is the main constituent of bentonite. The water content of montmorillonite varies and it expands greatly in volume when absorbing water. Montmorillonite often occurs intermixed with illite, kaolinite, chlorite, muscovite and cookeite (Hill and Forti, 1997). Koalinitic and montmorillonitic containing clays often contain organic material, including many live organisms.

2.5 Cultures and traditions associated with geophagia worldwide

Geophagic tendencies can be contributed to different causes such as medicinal, physiological, psychological, cultural and nutritional. A major concern with geophagia is whether these practices are beneficial or harmful to the practitioner's health (Brand, de Jager and Ekosse, 2009). Pregnant woman from the Southern parts of the United States of America traditionally believe that geophagia helps to prevent vomiting, cures swollen legs, causes babies to thrive and ensures beautiful babies (Ghorbani, 2008). In Malawi it is a tradition for pregnant woman to eat soil and in so doing show to the tribe that they are truly pregnant. Here clay eating is thus normal for pregnant woman, but not for the

rest of the tribe. Geophagia in many cultures is believed to have relations to fertility and reproduction (Ghorbani, 2008). The habit of soil eating is not generally seen in older boys and men, because they feel ashamed by it (Luoba et al., 2004).

Indigenous people have over centuries routinely used clays in food preparations. Clays were used during famine as food, during food preparation as spices or even to remove toxins from food, like aboriginal acorn bread. The amount of soil consumed and the time of consumption may vary from tribe to tribe, but the soil consistently came from known sites. Well established clay traders and trade routes exist in some cultures, which may account for the availability of geophagic clays in urban settings (Callahan, 2003).

2.6 Preferred soil type and preparation

Consumers usually take the colour, smell, flavour and texture of the soil into consideration when selecting soil for consumption. The colour of soil often provides an indication of the content of the soil to the consumer. White clays have the ability to absorb toxins and bacteria because of high kaolin content. This is one of the reasons why it is sold commercially as a cure for diarrhea. Aborigines from Australia prefer white clays for medicinal purposes, found in the billabongs of the coastal areas in the Northern parts, in river beds and fresh water springs (Ghorbani, 2008). Red clays are rich in iron oxides and aluminum, but the true value of these elements to human consumers is still under debate (Brand, de Jager and Ekosse, 2009).

After collection some people treat the soil before consumption. The soil is then usually dried in the sun or in an oven (depending on the socio-economic status of the consumer), which may either be a coal-stove, clay-oven, gas or electrical ovens. This is also the way vendors commonly prepare the soil before trade. However, some consumers prefer wet, cold clay and keep it in refrigerators or in cool places. Some consumers mix the soil with plant material or herbs (Callahan, 2003; Brand, De Jager and Ekosse, 2009).

2.7 Advantages and disadvantages of geophagia

Geophagia practices have been suggested to provide nutrient supplementation to the participant. However, it is also reported that soil removes nutrients from the human body rather than releasing minerals for absorption in the body. It was, therefore, concluded that soil ingestion can potentially reduce bio-available nutrient absorption in the human diet and lead to micronutrient deficiencies (Hooda et al., 2004; Brand, de Jager and Ekosse, 2009).

Studies done in Nigeria, Cameroon, Gabon, Kenya, Togo, Zaire and Zambia showed that edible clays have the ability to absorb tannic acids from the human system and to release calcium, magnesium, zinc, copper or iron into the system. This may lead to significant nutritional amounts of minerals being supplied by the clay (Brand, de Jager and Ekosse, 2009). In a study conducted in Iran, it was found that when clays with large cation exchange capacities are well saturated with macro minerals and trace elements, these elements may be released in humans on consumption of the soil. High cation-exchange properties of clays

may also account for the ability of these clays to absorb enteric toxins that cause diarrhea and in so doing alleviates diarrhea (Ghorbani, 2008).

Clay is also used by some cultures to prevent poisoning. In these cultures, clays are consumed before eating potentially poisonous food, such as some fish species. These cultures also consume clay to treat parasitic worm infestations (Ghorbani, 2008). In 2009, Brand et al. reported on clays being consumed for nutritional or medicinal purposes. Geophagia may even develop because of specific mineral deficiencies or cravings for specific nutrients. Earlier studies have found that 17-55% of the recommended mineral supplementation during pregnancy can be supplied by geophagic practices (Brand, de Jager and Ekosse, 2009).

Soil contains organic material, including many living organisms and regular soil consumption may, therefore, boost the mother's immune system, which may also benefit the fetus by receiving passive immunity from the mother. In a study done in 2003 by Callahan, it was found that monkeys eating soil regularly had lower parasite loads than monkeys from areas where they do not consume dirt. In some cultures clays are prepared before being eaten, which may involve many different procedures like sun drying, drying in coal stoves and boiling (Callahan, 2003). In many parts of the world women and children consume soil to still hunger (Giessler et al., 1998).

Soils are complex systems that interface with atmosphere, biosphere, hydrosphere, and lithosphere. True soil consists of gas, water, mineral and

organic components. Soils can be contaminated by rain, air-pollution, animal contact and human refuge contamination like sewerage. Potential human contact with soil includes inhalation, ingestion and dermal uptake (McKone and Maddalena, 1997). It is important that individuals practicing geophagia reveal their soil eating habit to their doctors and also from the doctor's side to take certain factors into account when treating such patients. Such factors may include possible poisoning with heavy metals, vitamin, mineral and iron deficiencies, electrolyte disturbances and also parasite and bacterial contamination (Brand, de Jager and Ekosse, 2009).

A study performed by Brand et al. (2008) on a patient from Cameroon with iron deficiency anemia aimed to determine whether the consumed koalin could induce her condition. She was treated with oral iron replacements, but the patient remained anaemic. She was convinced to stop practicing geophagia and was treated with intravenous iron replacement therapy. Her iron deficiency was consequently resolved and it was concluded that kaolin may interfere with iron absorption in humans (Johns and Duquette, 1991).

2.8 Other uses of geophagia

Reports from the early 20th century stated that Australian miners spread mountain tallow (soil) on their bread as a substitute for butter. In Southern Germany, quarrymen invented a food supplement from clay in the form of "stone butter" (Ghorbani, 2008). Poverty, starvation and famine are some of the main factors which may lead to geophagia, and soil is used as bulking agent to still

hunger during times of insufficient food availability (Ghorbani, 2008). In addition, urban South African women believe that soil digestion will give them a lighter skin complex and also a softer skin (Woywodt and Kiss, 2002).

2.9 Dangers associated with eating soil.

The Roman physician Soranus was one of the first people to warn others about the dangers of uncontrolled geophagia (Ghorbani, 2008). Studies showed that materials present in soil may influence mineral levels in humans consuming soil. Soils contaminated by industrial or human pollutants pose a considerable threat to anyone who eats them, as these soils may contain parasites or other living organisms which may cause disease in humans (Callahan, 2003). Health hazards due to geophagia were reported as early as 1825 in the Southern States of North America (Ghorbani, 2008). Geophagia may also lead to different health complications such as constipation, cramping, pain, intestinal obstruction, parasitic infestation, bacterial contamination and perforation of the intestines (Onyekwel, 2009). Geophagia has been associated with health problems, such as developmental problems, iron deficiency anemia, mechanical bowel disorders, nutritional dwarfism and parasitic infections (Hooda et al., 2004). Iron deficiency anemia as a result of soil ingestion has been widely reported from as early as 1956 by Tevetoglu, through to 1986 by Mokhobo. Hypokalaemia and hypozinceamia have also been associated with geophagia (Cheek et al., 1981).

In studies from as early as 1821 it was noted that humans will consume soil to try and correct mineral deficiencies such as iron and zinc (Hunter, 2003; Ghorbani,

2008). In 1981 a study reported on a 31 year old woman who was admitted to hospital with symptoms of weakness, nausea, pain, vomiting, fever and lack of bowel movement for two weeks. The patient was critically ill on admission and died soon after being admitted. The patient admitted to eating 200-300 g of soil daily during her pregnancies. This was the first case reported as a “maternal death from complications of geophagia”. The authors concluded that geophagia is “not an innocuous symptom or habit and must be handled aggressively” (Ella, 1990).

In 1687 it was reported that soil consumption was accountable for the death of about 50% of Jamaican slaves. During this time black slaves practiced geophagia because they believed that after death they would spiritually return to their native home. They consumed massive amounts of soil which consequently lead to their death. Harsh measures were put in place to control these geophagic practices. The measures included masks, iron gags and in some cases the person would be chained to the floor to prevent them from eating soil (Abrahams, 2002).

Excessive tooth wear may also be an indication of geophagia, while radiological examination of these people’s abdomens may reveal opaque soil masses in the colon. This may lead to constipation, which in turn leads to abdominal obstruction and pain as a result of the soil accumulation in the colon (Abrahams, 2002). A study conducted in Kenya and Costa Rica showed that people practicing geophagia in these regions preferred soil from termite mounds. Children and adults defecate behind the termite mounds and termites are known

to collect faeces for their mounds. This may lead to the high presence of parasitic eggs in termite mounds (Geissler et al., 1998). The practice of geophagia among children may lead to symptoms of malnutrition, anemia, constipation, diarrhea and worm infections. Diarrhea is annually held accountable for the deaths of about 1.6 million children under the age of five years in developing countries (Shivoga and Moturi, 2009).

2.10 Other therapeutic uses of eating soil.

Throughout history soil has been utilised medicinally. Two therapeutic uses of clays are the topical applications in spa centers as well as oral applications. The therapeutic use of clays is a spa tradition that has been in existence for centuries. Clay, mineral water and sea water are mixed and left for a long period of time to mature before being used as mud-therapy. The ingestion of clays include several practices, as clay is used in food preparation, as part of herbal remedies and as part of unintentional ingestion during low hygiene regimes. Healing uses of clays are wide spread, but knowledge of the different compounds that may be found in clays is barely considered as part of the healing process. These compounds can either be beneficial or detrimental to the user depending on the application (Tateo and Summa, 2006). Spas all over the world use special clays for different functions. The colour of clay identifies the use of the clay in these settings. Yellow clays are used against bacterial infection, red clays cleanse the skin and remove pains from joints, blue clays are believed to be active against acne, green clays are used to alleviate oily skin and black clays are used for general skin nutrition (Mpuchane et al., 2008).

Soil is still used to treat gastrointestinal disorders and poisonings. To date soil micro-organisms remain the main producers of natural antibiotics (Abrahams, 2002). Of all antibiotics prescribed, 50% are derived from one bacterial order namely Actinomycetales, with the strongest represented genus being *Streptomyces* (Abrahams, 2002). The laxative action of smectitic clays are contributed to the rich magnesium carbonate concentrations found in them. Soils with high clay content form cross links with intestinal mucosa glycoproteins and thus help to protect gastrointestinal epithelium (Brand, De Jager and Ekosse, 2009).

2.11 Supposed remedies for Geophagia

Daily ingestion of starch has proven a successful treatment against eating soil for pregnant woman and children in Iran (Ghorbani, 2008). Since soil eating is such a diverse practice and deeply rooted in certain communities, more can be gained from understanding geophagia rather than searching for a cure (Luoba, 2004).

2.12 Geophagia today

Geophagia remains a common practice in many cultures. However, people should be advised to try and find safe soil, or to treat the soil before ingestion, as it could be contaminated with faeces or organic material. Termite soil appears to be a better option, but the soil should still be cooked, baked or fried (Saathoff et al., 2002).

2.13 Soil bacteria

A fascinating but poorly understood aspect of soil is its living components. More living organisms are found in soil than in any other ecosystem and this living portion of soil is a complex collection of organisms. Some life can easily be seen with the naked eye, while the observation of others requires a high powered microscope (Waltz, Skipper and McCarty, 2009). Soil can be defined as aggregates of different substances such as minerals, water, humus and micro-organisms. The micro-organisms in soil do not exist as pure cultures, but are rather competitive populations with high diversity. Soil provides micro-organisms with varied physical and chemical properties, which they will not encounter in synthetic laboratory media (Garcia and McKay, 1970).

Soil bacteria form an integrated part of soil, and play an important role in decomposing compounds in soil to useful organic matter for utilization by plants (Ingham, 2009). However, not all soil bacteria are beneficial, and some may even be harmful to plants. Hazardous soil bacteria like *Salmonella spp*. can contaminate fruits and vegetables that are cultured in the soil and may cause disease to humans if consumed. Potential hazardous bacteria in soil are usually due to contamination of the soil with sewerage, drainage spills and animal faecal contamination (Falk, 1949).

Various bacterial species are found in abundance in soil, but not all have been identified. Soil also contains many different pathogenic organisms, such as *Clostridium tetanii* and *Clostridium perfringens*. Opportunistic human pathogens

such as *Pseudomonas aeruginosa* are also found in soil (Usui et al., 2009). Organisms living in soil may lead to emerging infectious diseases in humans and methods to effectively identify these pathogenic organisms in soil are continuously being developed (Usui et al., 2009).

Soil micro-organisms are microscopic in size and are morphologically different. These organisms consist of bacteria, algae, viruses, fungi, and protozoa. Nematodes and micro-arthropods are also small organisms that inhabit soil, but are not generally considered as microbes (Granatstein and Bezdicek, 2003). Soil bacteria may occur between the pores in soil aggregates and are embedded in the clay complex of soil.

Almost 75 years ago Waksman and Woodruff (1940) studied the survival of pathogenic micro-organisms in soil as a natural reservoir or as a temporary refuge site and reported on earlier studies which suggested that pathogenic bacteria will disappear soon after introduction to soil conditions (Garcia and McKay, 1970). The evaluation of soil factors that influence relative abundance and distribution of micro-organisms was difficult in the past due to failed cultivation and isolation techniques, as normal plating media favour the growth of fast growing cells at the cost of slow growing cells and fastidious organisms (Garcia and McKay, 1970). Direct bacterial observation by microscopic techniques yields much higher bacterial numbers from soil than culturing. The reason for this may be the fact that microscopic evaluation could not distinguish between dead and living organisms. In a study by Garcia and McKay (1970)

electron microscopic evaluation of soil led to the discovery of unusual microbial forms in soil.

Factors that influence soil formation also have a direct influence on the presence of soil bacteria (Granatstein and Bezdicek, 2003). These factors include parent material, time, climate, biota (plantsand animals, including man) (Garcia and McKay, 1970; Granatstein and Bezdicek, 2003). Anaerobic conditions that dominate soil conditions during waterlogged stages may give rise to the abundance of anaerobic organisms in soil. It was found that human pathogens can survive for up to ten months under swamp pasture and even longer under continuous waterlogged conditions (Garcia and McKay, 1970).

It is known that soil can easily be contaminated with high concentrations of enteric pathogens due to fecal matter produced by humans and animals, and also serves as a reservoir for bacterial pathogens. However, recent studies have shown that its role in transmission of enteric diseases may be greater than expected. Some of the organisms involved in gastrointestinal diseases include enteric bacteria such as: *Salmonella* spp., *Shigella* spp., *Yersinia* spp., *Vibrio cholera*, *Escherichia coli* 0157:H7 and *Campylobacter jejuni* (Santamaria and Toranzos, 2003). *E. coli* 0157:H7 is especially important, because of its small infectious dose. Only about 10 bacterial cells are required to cause infection in humans. The survival time of some human pathogens in soil has been investigated and for Enterovirusses and *Salmonella* spp. this was reported to be less than 20 days, while *V. cholerae* survived for less than ten days. Helminth

eggs on the other hand, were reported to survive for several months (Santamaria and Toranzos, 2003).

2.14 Soil factors influencing bacterial survival

Soil moisture may favour the survival rate of bacteria and viruses, although *Salmonella* cells were found to be able to withstand dry conditions better than other organisms. The clay content of soil also plays an important role in the survival of micro-organisms. Organisms tend to adhere better to clay molecules and clay molecules retain more water than sand molecules, resulting in a higher survival rate of organisms in clayey soil (Santamaria and Toranzos, 2003). Adsorption characteristics of soil are also influenced by soil pH, and increased cation exchange capacity will, therefore, result in increased cell adsorbtion of bacteria (Santamaria and Toranzos, 2003). Rainfall may also influence microbial movement and it has been found that during rain the organisms may spread more easily with the runoff of the rainwater. Underwater contamination with bacteria also increases during heavy rainfall (Santamaria and Toranzos, 2003).

The bacterial life cycle basically consists of five phases: the lag phase, exponential phase, stationary phase, death phase and the “period of prolonged decrease”. During the exponential phase bacteria are found in a state of balanced growth and nutrients, with a steady increase in the colony-forming units (CFUs). The lag phase is generally encountered after a time of starvation, and when the bacteria are reintroduced to nutrients and metabolic activities allow growth, a slow but steady rate of CFUs are encountered. The stationary phase is

entered when the CFUs in bacterial cultures show no increases. There is now a lack of sufficient nutrients to grow and bacteria may survive in this state for hours, days and even many years. Human pathogens found in soil environments are usually in the stationary phase due to a lack of sufficient nutrients. The death phase is where no CFUs are encountered, while the “period of prolonged decrease” is marked with the steadily decrease in CFUs over a long period of time. Bacteria surviving in soil for long periods of time without sufficient nutrients may encounter all of these phases (Storz and Hengge-Aronis, 2000).

2.15 Soil colour

The colour of clays plays a role in their classification. The Munsell Soil Color Book was developed to assist in the classification of soil samples. Soil classified according to this book gives a uniform interpretation of soil colour (Munsell, 2002). Soil colour gives an indication of nutrients and elements encountered in the soil and a correct classification of soil colour may assist in understanding the bacterial survival due to available nutrients. For a detailed discussion on soil colour please refer to Chapter 5.

2.16 Antimicrobial activity of soil

Antibiotics gain entry into the soil environment through sewage sludge, solid waste, municipal effluent and manure applications (Yanyu, Qixing and Yingying, 2009). Orally administered antibiotics may pass through animals unchanged at a rate of up to 80%. Chander et al. (2005) reported that antibiotic concentrations in soil are usually not present at therapeutic concentrations to inhibit bacterial

populations, but may cause antibiotic resistance selection by environmental bacteria (Chander et al., 2005).

The ability of soil organisms to produce antibiotics and related toxins has been well documented (Garcia and McKay, 1970) and even studies from the Amazon Basin on antimicrobial activity reported on the production of antimicrobial substances by some bacterial strains. The antimicrobial substances that were produced by Gram-positive bacteria are strictly directed against other Gram-positive bacteria. However, the potential of these micro-organisms to produce antimicrobial activity needs further investigation for future applications (Motta, Cladera-Olivera and Brandelli, 2004). Antimicrobial activities in clays were shown in earlier studies especially in clays with pH values lower than 4. Some of the clays that are commonly used as treatment for microbial diseases have been investigated, but did not demonstrate any antimicrobial activity. In a study carried out in 2009 it was found that only 9/102 clay samples demonstrated antimicrobial activities against different bacteria and yeasts (Mpuchane et al., 2010).

Groups of non-obligate bacteria that prey on other bacteria, fungi yeasts and protozoa have been found and are referred to as predator bacteria. These predator bacteria may be present in soil in even higher concentrations than the total soil bacteria measured by usual bacteriological methods. One of the currently top identified non-obligate bacterial predators in soil is *Burkholderia ambifaria* strain 679-2. This predator produces three antibiotic compounds which

interact to induce antimicrobial activity against certain bacteria, fungi and protozoa (Cain et al. 2003).

2.17 Conclusions

Disease Hypotheses may be formed when medical data are correlated with environmental factors of soil. However, it is important to keep in mind that statistical analysis may show correlation, but does not prove a cause. Soil contamination is a global problem and it is important to acquire new and adequate information on these problems and their involvement with humans (Abrahams, 2002).

Soil-borne diseases in humans are causing concern as sewage disposal, which involve sewage sludge and waste water drainage from these plants, are on the increase. It is estimated that approximately seven million tons of sewage sludge is produced annually and that 54% of this sewage sludge is introduced into soil (Santamaria and Toranzos, 2003). Data on enteric infection in humans caused by contaminants from soil is limited and need further investigation. There is also a need for the development of standardised methods for the detection of human pathogens from soil reservoirs (Santamaria and Toranzos, 2003).

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Chapter 3

Information on Geographic Soil Samples

3.1 Introduction

Geophagic soil or clays are being collected by individual consumers as well as soil vendors from various mining sites in the region under investigation. As a result of this, there are vast differences between the respective soils mined by individuals as well as those sold by vendors at the popular markets. In previous studies it was reported that soil samples were purchased from homes, shops and also open markets (Mpuchane et al., 2010). These samples were collected in sealed plastic bags and were then transported in large boxes by land-transport at ambient temperatures. Sample testing was carried out within one month.

In a report on a study performed in 2008 it was stated that *"the analytical results of a study are only as good as the samples on which they are performed".* It was emphasised during this study that the samples should be representative of those consumed by geophagists (Young et al., 2008). The sample size should be big enough to perform all relevant tests and it was suggested that approximately 100 g must be collected in polyethylene bags. It is also important to collect control samples from the same area as the geophagic samples.

The aim of this study was to gather information regading the sources of geophagic soil and to investigate the state of the soil prior to consumption. These include factors that could have an influence on the quality/cleanliness of the soil, such as pollution or faecal contamination.

3.2 Materials and Methods

3.2.1 Sampling sites

Soil samples were collected from different geographic sites in the Qwa-Qwa district and included 17 known geophagic mining sites, five control sites and 13 vendors selling geophagic soil.

3.2.2 Sampling method

Sample collection was done as described by Young et al. (2008), according to a set of guidelines for soil sample collection and analysis of geophagic material. These guidelines are set up for gathering of information on the type of soil, the area where it is collected from, special treatment of the soil prior to consumption, and the amount consumed. The guidelines are also set for the collection of control samples, archiving of samples, and shipment of samples.

GPS coordinates of each mining site were noted with a TomTom GPS device. The size measurements of each mine were recorded with a measuring tape. The width from side to side, the depth (measured from the centre front parameter of the mine to the centre back parameter of the mine), and the height were recorded. The amount of soil collected from each mining site varied from 200-300 g and was determined by the size of the mine. Soil was removed in such a manner not to cause major disturbance to the mine. Some mines were small and large amounts of soil collections would disturb the mining site. The soil samples were collected from the topsoil up to 10 cm deep with a steel shovel (Figure 3.1) and placed into a clean Ziploc bag. The shovels were cleaned prior to collection,

by spraying it with 70% ethanol and allowed it to air dry completely before the sample was taken. After sample collection the shovel was again soaked in 70% ethanol and wiped clean with paper towels (Figure 3.1). The colour of the soil was determined using the Munsell Colour Book (Munsell, 2002).

Figure 3.1: Shovel, spray bottle with 70% ethanol and paper towel

3.2.3 Specific location of the mines

The mining sites were located with the help of the vendors who supplied information on their location. Mining sites were also pointed out by local geophagists who collect their own soil for consumption.

3.2.4 Climate of mining area

Samples were collected during October 2009 just after the first seasonal rains. The annual rainfall in the Qwa-Qwa area varies from between 750-1000 mm and mainly occurs during the summer months from September to March. During

October 2009 the average mid-day tempratures were about 26^0 C with a humidity of 18 %.

3.2.5 Transportation and storage

The soil samples were transported from Qwa-Qwa to Bloemfontein in a polystyrene cooler box at ambient temperature. Soil samples were stored in the cooler box in the laboratory while samples were processed. The remainder of the samples was kept in the cooler box at ambient temperatures (20-26°C) until sample processing had been completed.

3.3 Results and Discussion

3.3.1 Type of soil collected

Different soil types were collected from the different mines, and varied from fine brown soil particles to lumps of yellow soil. Most of the samples taken from mines consist of damp soil particles, while samples collected from vendors consisted of dry soil mainly cured in the sun or in traditional ovens.

3.3.2 Mining sites

Mine 1:

Mine 1 is situated next to a main access road into Phuthaditjhaba (Figure 3.2). The road is tarred and carries heavy traffic during the day. The area where the mine is situated is known as Lusaka/Matsikeng. The mine is situated against a terrace approximately two meters from the road. On top of the terrace a water-

pipeline is situated just above the mine, a stop-valve is located on top of the mine, with about one and a half meters of soil between the stop-valve and the mine (Figure 3.3). The surrounding area is fairly neat with no rubbish near the mining site.

Figure. 3.2: Mine 1

Approximately 300 g of soil was collected from this site (Figure 3.4). At the time of sample collection the average temperatures ranged from 15°C in the morning with 74% humidity to 26°C at mid-day with 18% humidity.

Figure 3.3: Stop valve on top of mine 1

Figure 3.4: Soil sample from mine 1

Mine 2:

Mine 2 (Figure 3.5) is situated next to a main access road into Phuthaditjhaba opposite mine 1 in an area known as Lusaka/Matsikeng. The mine is part of a terrace approximately one and a half meters from the road. It is an open mine without any soil covering the mining area. During day-time the soil in the mine is exposed to direct sunlight and various other nature elements like rain and wind. Natural grasses were growing on the top soil surrounding the mining site. The surrounding area is fairly neat with no rubbish near the mining site. Approximately 300 g of soil was collected from this site (Figure 3.6).

Figure 3.5: Mine 2

Figure 3.6: Soil sample from mine 2

Mine 3:

Mine 3 (Figure 3.7) is an open mine situated near houses in the Qhelaphe area in Phuthaditjhaba. During day-time the soil in the mine is exposed to direct sunlight and other natural elements like rain and wind. Natural grasses were growing on the top soil surrounding the mining site. The surrounding area is fairly neat with no rubbish near the mining site. Approximately 300 g of soil was collected from this site (Figure 3.8).

Figure 3.7: Mine 3

Figure 3.8: Soil sample from mine 3

Mine 4:

Mine 4 (Figure 3.9) is situated near houses and free ranging animals in the Qhelaphe area in Phuthaditjhaba. This is a covered mine with some mining soil protected from contact with direct sunlight and some natural elements like rain and wind. Natural grasses were growing on the top soil surrounding the mining site and directly in front of the mine. The surrounding area is fairly neat with no rubbish near the mining site, although some animal dung could be seen near the mine. Approximately 200 g of soil was collected from this site (Figure 3.10).

Figure 3.9: Mine 4

Figure 3.10: Soil sample from mine 4

Mine 5:

Mine 5 (Figure 3.11) is situated in Namahali, Phuthaditjhaba, approximately 500 meters from the nearest houses, in an area that resembles a rubbish dumping site. This is an open mine with natural grasses growing on the top soil surrounding the mining site. The surroundings of the mine were very dirty, with bottle glass, stones, and other rubbish in close proximity to the mine site (Figure 3.11). It also appears as if the bottle glass pieces were used to collect soil for eating purposes. Approximately 200 g of soil was collected from this site (Figure 3.12).

Figure 3.11: Mine 5

Figure 3.12: Soil sample from mine 5

Mine 6:

Mine 6 (Figure 3.13) is situated under a tree in Namahali, Phuthaditjhaba. This mine is semi covered with overlap of soil. Natural grasses grow on the top soil surrounding the mining site. The surroundings of the mine resemble a field-like area. Some glass pieces were found near the mine. Approximately 200 g of soil was collected from this site (Figure 3.14).

Figure 3.13: Mine 6

Figure 3.14: Soil sample from mine 6

Mine 7:

Mine 7 (Figure 3.15) is situated in Namahali, Phuthaditjhaba, next to a dirt road. This mine is small and close to a pedestrian path and highway. Faint tyre tracks may be noted in the photograph next to the mine. Natural grasses grow on the top soil surrounding the mining site. This mine was pointed out by people staying in the area who use the mine when they do not have money to buy soil from vendors. They use sticks like those found on the photograph in Figure 3.17 to mine the soil. The mine is flat and extends downwards with no cover. Approximately 200 g of soil was collected from this site (Figure 3.16).

Figure 3.15: Mine 7

Figure 3.16: Soil sample from mine 7

Mine 8:

Mine 8 (Figure 3.17) is situated in Namahali, Phuthaditjhaba, beside a dirt road close to mine 7. This mine is also small close to pedestrian path and highway. Tyre tracks may be noted in front of the mine. This mine was pointed out by people staying in the area. The mine is used when they do not have money to buy soil from vendors. They use sticks like the ones in the photo in Figure 3.17 to mine the soil. The mine is flat and extends downwards with no cover. Approximately 200 g of soil was collected from this site (Figure 3.18).

Figure 3.17: Mine 8

Figure 3.18: Soil sample from mine 8

Mine 9:

Mine 9 (Figure 3.19) is situated in Phahameng next to the road and adjacent to houses. The mine surroundings are covered by natural grass and the area is neat. The people use sticks such as in Figure 3.19 to mine the soil. Approximately 200 g of soil were collected from this site (Figure 3.20).

Figure 3.19: Mine 9

Figure 3.20: Soil sample from mine 9

Mine 10:

Mine 10 (Figure 3.21) is situated in Phahameng next to the road about two meters from mine 9 and near houses. The mine surroundings are covered by natural grass and the area is very neat. The people use sticks such as the one in the photograph (Figure 3.21) to mine the soil. Approximately 200 g of soil was collected from this site (Figure 3.22).

Figure 3.21: Mine 10

Figure 3.22: Soil sample from mine 10

Mine 11:

Mine 11 (Figure 3.23) is situated in Kgubetswana next to a main road. The mine is situated against a steep hill. The mine has a pathway leading up to it against the steep sandstone hill. The mining site is covered with sandstone rock as a roof over the mine. Locals warn about touching the mine wall, as the sandstone "walks" when it is touched. Approximately 300 g of soil was collected from this site (Figure 3.24).

Figure 3.23: Mine 11

Figure 3.24: Soil sample from mine 11

Mine 12:

Mine 12 (Figure 3.25) is situated in Kgubetswana next to a main road. The mine is situated against a steep hill and has a very steep pathway leading up to it. The mine is covered with sandstone rock as a roof over the mine. Approximately 300 g of soil were collected from this site (Figure 3.26).

Figure 3.25: Mine 12

Figure 3.26: Soil sample from mine 12

Mine 13:

Mine 13 (Figure 3.27) is situated in Kgubetswana next to a main road and was pointed out by a local geophagist (Figure 3.28). The mine is situated halfway up a steep hill and is covered with sandstone rock as a roof over the mine. Approximately 300 g of soil were collected from this site (Figure 3.29).

Figure 3.27: Mine 13

Figure 3.28: Geophagist identifying mine

Figure 3.29: Soil sample from mine 13

Mine 14:

Mine 14 (Figure 3.30) is situated in Mangaung About 500 m from houses. The mine is in the field with a beautiful view of the landscape and the area around the mine is covered with natural grasses. A piece of aluminium was found near the mine, which is used to mine soil (Figure 3.30). Approximately 300 g of soil were collected from this site (Figure 3.31).

Figure 3.30: Mine 14

Figure 3.31: Soil sample from mine 14

Mine 15:

Mine 15 (Figure 3.32) is situated in Mangaung near a Valley where cattle graze. The area around the mine consist of plain natural grasses. Tree roots form part of the mine. The stick, piece of asbestos and glass bottle pieces were found near the mine, which are used to mine the soil (Figure 3.33). School children visit the mine after school to collect soil (Figure 3.34). Approximately 300 g of soil was collected from this site (Figure 3.35).

Figure 3.32: Mine 15

Figure 3.33: Utensils used to mine

Figure 3.34: Children practicing Geophagia

Figure 3.35: Soil sample from mine 15

Mine 16:

Mine 16 (Figure 3.36) is situated in Madikwe near a Valley where cattle graze. The area around the mine consist of plain natural grasses (Figure 3.37). A piece of aluminium was found near the mine, which is used to mine soil (Figure 3.38). Approximately 300 g of soil was collected from this site (Figure 3.39).

Figure 3.36: Mine 16

Figure 3.37: View of mine 16 surroundings

Piece of alluminium

Figure 3.38: Utensils used to mine

Figure 3.39: Soil sample from mine 16

Mine 17:

Mine 17 (Figure 3.40) is situated in Madikwe in a scenic valley. The nearest house is approximately 700 m from the mining site. The area around the mine consists of plain natural grasses and vegetation. A stick was found near the mine, which is used to mine soil. The mine has been in use for a long time as the path from where it has started, has expanded over time. The mine was pointed out by a child who collects soil for his mother and friends on a regular basis (Figure 3.41). Approximately 300 g of soil were collected (Figure 3.42).

Figure 3.40: Mine 17

Figure 3.41: Soil collector

Figure 3.42: Soil sample from mine 17

A summary of the data on the 17 mining sites and the soil collected is presented in Table 3.1. At the time of sample collection the average temperature ranged from 15°C-17°C in the morning to 26°C-28°C at mid-day, while humidity ranged from 17-74% in the morning to 5-18% at mid-day.

Table 3.1: Summary of information gathered on the 17 geophagic mining sites

Mine no.	Location	GPS coordinates	Size of the mine	Description of the area	Soil colour analysis Hue Value Chroma	Soil colour
1	Phuthaditjhaba	S 28.51413° E 28.85304°	Width 280 cm Depth 28 cm Height 54 cm	Stop-valve on top of mine	2.5 YR 7 2	Pale red
2	Phuthaditjhaba	S 28.51396° E 28.85303°	Width 80 cm Depth 37 cm Height 25 cm	Open mine with natural grasses surrounding it	2.5 YR 7 1	Light reddish
3	Qhelaphe area in Phuthaditjhaba	S 28.51401° E 28.85285°	Width 250 cm Depth 110 cm Height 50 cm	Natural grasses surrounding the mine	7.5 YR 8 2	Pinkish white
4	Qhelaphe area in Phuthaditjhaba	S 28.51408° E 28.85285°	Width 90 cm Depth 49 cm Height 28 cm	Surrounding area fairly neat without rubbish	10 YR 8 2	Very pale brown
5	Namahali area in Phuthaditjhaba	S 28.51408° E 28.85289°	Width 30 cm Depth 15 cm Height 10 cm	Very dirty mine with glass, stone and rubbish	10 YR 8 1	White

Mine no.	Location	GPS coordinates	Size of the mine	Description of the area	Soil colour analysis			Soil colour
					Hue	Value	Chroma	
6	Namahali area in Phuthaditjhaba	S 28.55926° E 28.82986°	Width 30 cm Depth 15 cm Height 35 cm	Some glass pieces found near the mine	5 YR	7	1	Light gray
7	Namahali area in Phuthaditjhaba	S 28.55729° E 28.83263°	Width 35 cm Depth 5 cm	Mine is close to daily foot and vehicle traffic	5 YR	7	2	Pinkish gray
8	Namahali area in Phuthaditjhaba	S 28.56030° E 28.84315°	Width 37 cm Depth 5 cm	Mine is close to daily foot and vehicle traffic	Gley 2	6	5B	Greenish grey
9	Phahameng area in Phuthaditjhaba	S 28.52949° E 28.42187°	Width 14 cm Depth 5 cm Height 10 cm	Next to the road near houses	7.5 YR	4	6	Strong brown
10	Phahameng area in Phuthaditjhaba	S 28.52952° E 28.42185°	Width 17 cm Depth 10 cm Height 15 cm	Next to the road near houses	7.5 YR	4	6	Strong brown
11	Kgubetswana area near Clarens	S 28.53830° E 28.42515°	Width 120 cm Depth 20 cm Height 43 cm	Situated against a steep hill	5 YR	6	1	Gray

Mine no.	Location	GPS coordinates	Size of the mine	Description of the area	Soil colour analysis Hue Value Chroma	Soil colour
12	Kgubetswana area near Clarens	S 28.52937° E 28.43517°	Width 125 cm Depth 40 cm Height 68 cm	Situated against a steep hill	2.5 YR 7 1	Light reddish gray
13	Kgubetswana area near Clarens	S 28.52934° E 28.43527°	Width 70 cm Depth 69 cm Height 78 cm	Situated halfway up a steep hill	5 YR 7 2	Pinkish gray
14	Mangaung area in Phuthaditjhaba	S 28.52926° E 28.43538°	Width 51 cm Depth 30 cm Height 20 cm	Situated 500 m from nearest houses	5 YR 6 4	Reddish brown
15	Mangaung area in Phuthaditjhaba	S 28.57685° E 28.83201°	Width 85 cm Depth 47 cm Height 38 cm	Near a valley where cattle graze	7.5 YR 6 4	Light brown
16	Madikwe area in Phuthaditjhaba	S 28.58712° E 28.83615°	Width 120 cm Depth 65 cm Height 49 cm	Near a valley where cattle graze	10 YR 7 1	Light gray
17	Madikwe area in Phuthaditjhaba	S 28.58557° E 28.83837°	Width 53 cm Depth 64 cm Height 35 cm	Situated ±700 m from nearest houses	10 YR 5 4	Yellowish brown

3.3.3 Vendors:

All the vendors were women selling their product in the Setsing market area. Tasting samples were offered before purchasing.

Vendor 1:

Vendor 1 mines her own soil in a suburban area by using utensils. The soil is packed in a plastic carry bag at the mine and then transported to the market area. At the market area she processes the soil into smaller pieces by using a pair of scissors to break the soil lumps into smaller pieces. This process is performed on a “streep sak” on a pavement using her bare hands. The soil is packed onto small pieces of plastic (dry-cleaning plastic) and tied into bags (Figure 3.43).

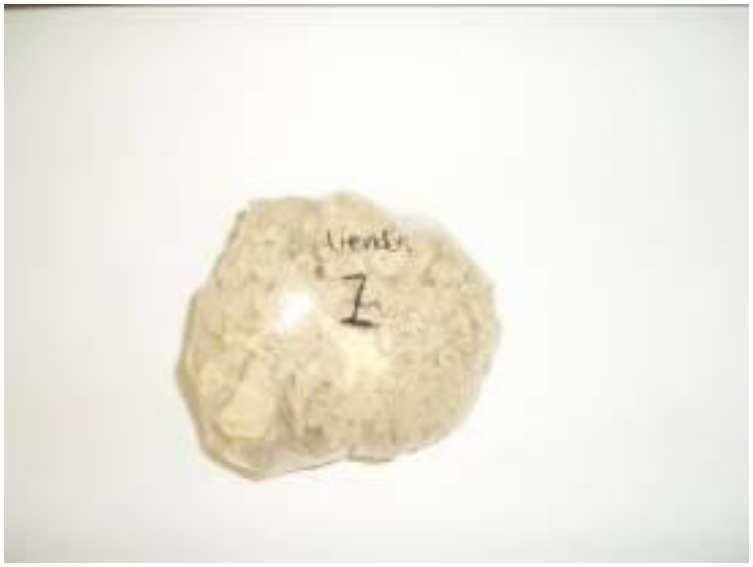

Figure 3.43: Soil sample from vendor 1

Vendor 2:

Vendor 2 mines her own soil in a rural area using utensils and her bare hands to pack the soil into a big plastic bag in which it is transported to her home. Soil is processed by placing it in a charcoal oven for 10 – 15 minutes to dry. The soil is then packaged into small non-sterile plastic bags (Figure 3.44).

Figure 3.44: Soil sample from vendor 2

Vendor 3:

Vendor 3 mines her own soil in a suburban area by using utensils and her bare hands to pack the soil into a big plastic bag in which it is transported to her home. The soil is processed by placing it in the sun to dry. The soil is then packaged into new clean plastic bags (Figure 3.45).

Figure 3.45: Soil sample from vendor 3

Vendor 4:

Vendor 4 mines her own soil in a rural area by using utensils and her bare hands to pack the soil into a big plastic bag in which it is transported to her home. The soil is processed by placing it in the sun to dry or places it in a coal-stove to dry. The soil is packaged into small non-sterile plastic bags (Figure 3.46).

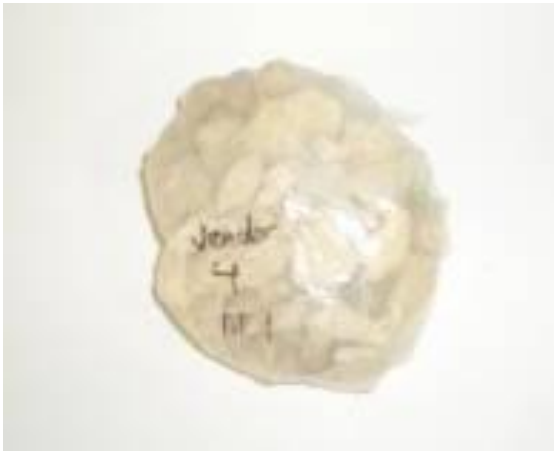

Figure 3.46: Soil sample from vendor 4

Vendor 5:

Vendor 5 mines her own soil in a rural area, but this sample was imported by herself from Johannesburg. The soil is packed into small non-sterile plastic bags (Figure 3.47). The vendor had no information on the prior preparation that this sample could be subjected to.

Figure 3.47: Soil sample from vendor 5

Vendor 6:

Vendor 6 mines her own soil in a rural area by using utensils and her bare hands to pack the soil into a big plastic bag in which it is transported to her home. The soil is processed by placing it in the sun or in a coal-stove to dry. The soil is packaged into small non-sterile plastic bags (Figure 3.48).

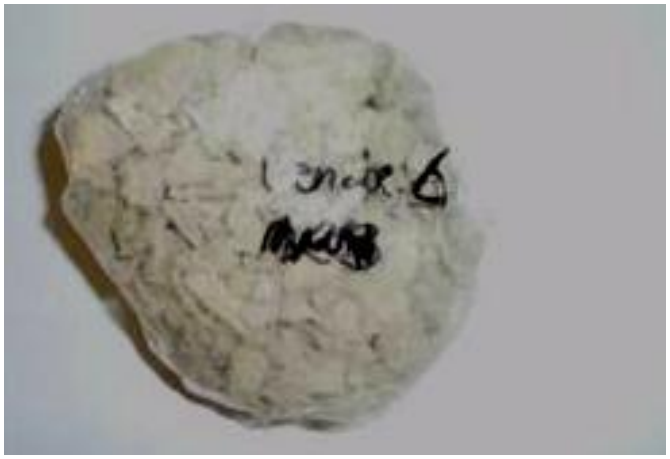

Figure 3.48: Soil sample from vendor 6

Vendor 7:

Vendor 7 mines her own soil in a rural area by using utensils and her bare hands to pack the soil into a big plastic bag in which it is transported to her home. The soil is processed by placing it in the sun or in a coal-stove to dry. The soil is packed into small non-sterile plastic bags (Figure 3.49).

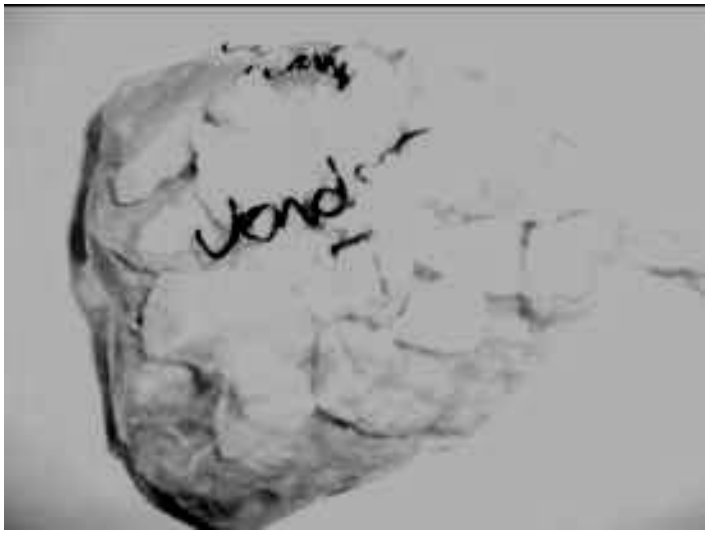

Figure 3.49: Soil sample from vendor 7

Vendor 8:

Vendor 8 mines her own soil in a suburban area by using utensils. The soil is packed into a big plastic bag in which it is transported to her home. The soil is processed by placing it in the sun to dry or she places it in a coal-stove to dry. The soil is packed into small non-sterile plastic bags (Figure 3.50).

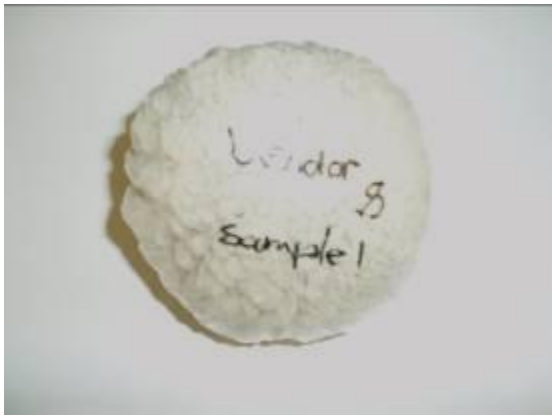

Figure 3.50: Soil sample from vendor 8

Vendor 9:

Vendor 9 mines her own soil in a rural area by using utensils and her bare hands to pack the soil into a big plastic bag in which it is transported to her home. The soil is processed by placing it in the sun or in a coal-stove to dry. The soil is packaged into small non-sterile plastic bags (Figure 3.51).

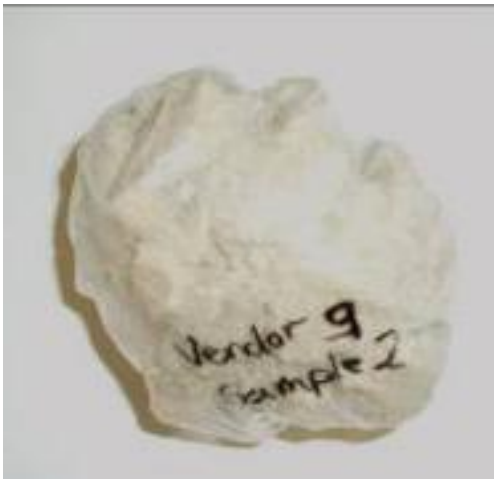

Figure 3.51: Soil sample from vendor 9

Vendor 10:

Vendor 10 mines her own soil in a rural area by using utensils and her bare hands to pack the soil into a big plastic bag in which it is transported to her home. Here she processes the soil by placing it in the sun to dry or she places it in a coal-stove to dry. The soil is packaged into small non-sterile plastic bags (Figure 3.52).

Figure 3.52: Soil sample from vendor 10

Vendor 11:

Vendor 11 mines her own soil in a rural area by using utensils and her bare hands to pack the soil into a big plastic bag in which it is transported to her home. Here she processes the soil by placing it in the sun to dry or places it in a coal-stove to dry. The soil is packaged into small non-sterile plastic bags (Figure 3.53).

Figure 3.53: Soil sample from vendor 11

Vendor 12:

Vendor 12 mines her own soil in a suburban area by using utensils and her bare hands to pack the soil into a big plastic bag, previously used for maize packaging, in which it is transported to the market area. She does not process the soil prior to packing it in the plastic bags (Figure 3.54).

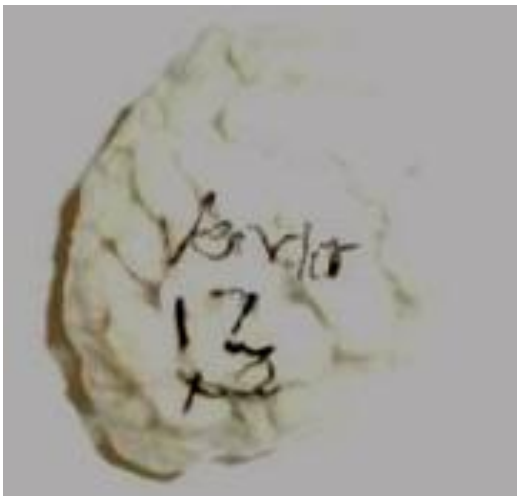

Figure 3.54: Soil sample from vendor 12

Vendor 13:

Vendor 13 mines her own soil in a rural area by using utensils and her bare hands to pack the soil into a big plastic bag in which it is transported to her home. Here she processes the soil by drying it in the sun prior to packing it into plastic bags (Figure 3.55). A summary of the data on the soil collected from vendors is presented in Table 3.2.

Figure 3.55: Soil sample from vendor 13

Table 3.2: Summary of information gathered on the 13 vendors selling geophagic soil

Vendor no.	Market area	Materials used for mining	Processing/handling	Packaging	Soil colour analysis	Soil colour	Price
1	Setsing market	Using utensils	Break into smaller pieces with scissors	Pieces of dry-cleaning plastic bags	Hue 10 YR 8/1	White	R2.00
2	Setsing market	Utensils and bare hands	Dry in a charcoal oven for 10 -15min.	Non-sterile plastic bags	Hue 10 YR 8/2	Very pale brown	R1.50
3	Setsing market	Utensils and bare hands	Dry in the sun	New clean plastic bags	Hue 10 YR 7/2	Light gray	R2.00
4	Setsing market	Utensils and bare hands	Dry in a coal-stove	Non-sterile plastic bags	Hue 10 YR 7/4	Very pale brown	R2.00
5	Setsing market	Imported from Johannesburg	No information on processing	Non-sterile plastic bags	Hue 10 YR 7/2	Light gray	R3.00
6	Setsing market	Utensils and bare hands	Dry in the sun or in coal-stove	Non-sterile plastic bags	Hue 10 YR 7/2	Very pale brown	R2.00

Vendor no.	Market area	Method of mining	Processing/handling	Packaging	Soil colour analysis	Soil colour	Price
7	Setsing market	Utensils and bare hands	Dry in the sun or in coal-stove	Non-sterile plastic bags	Gley 1 YR 8/1	Light greenish gray	R2.00
8	Setsing market	Utensils	Dry in the sun or in coal-stove	Non-sterile plastic bags	Gley 1 YR 8/2	Pale green	R2.00
9	Setsing market	Utensils and bare hands	Dry in the sun or in coal-stove	Non-sterile plastic bags	Hue 10 YR 7/1	Light gray	R2.00
10	Setsing market	Utensils and bare hands	Dry in the sun or in coal-stove	Non-sterile plastic bags	Hue 10 YR 6/2	Pale red	R2.00
11	Setsing market	Utensils and bare hands	Dry in the sun or in coal-stove	Non-sterile plastic bags	Hue 10 YR 8/1	Light white	R2.00
12	Setsing market	Utensils and bare hands	No processing prior to selling	Non-sterile plastic bags	Hue 7.5 YR8/1	White	R2.00
13	Setsing market	Utensils and bare hands	Dry in the sun	Non-sterile plastic bags	Hue 7.5 YR6/1	Gray	R2.00

3.3.4 Control Samples

Approximately 300 g of soil was collected from each of the five control site (Appendix). A summary of the data on the control mining sites and the soil collected is presented in Table 3.3. At the time of sample collection the average temperature ranged from 17°C in the morning to 27°C at mid-day, while humidity ranged from 17% in the morning to 5% at mid-day.

3.4 Conclusions

The mines that were visited and where the samples were collected, appeared to be popular among the geophagic practicing people of Qwa-Qwa. The mines varied from relatively neat to very dirty with pieces of glass and other rubbish. Many of the mines were found to be located close to houses and even roads, which raises concern about the health aspects of the soil and clays obtained from these mines. In addition to this, the collecting and preparation practices of the vendors selling geophagic soil may play a role in the microbial safety of the soil, as the majority use their bare hands for collection and non-sterile bags for packaging. Apart from bacterial or fungal contamination, parasites may be a serious concern. However, these were not under investigation in the current study.

The soil obtained from the various mines displayed a wide range of colours and textures and it would be interesting to conduct further investigations on the preference of the local geophagists for a specific type of soil or clay from a specific mining site.

Table 3.3: Summary of information gathered on the five control mining sites

Control No.	Location	GPS coordinates	Description of location	Soil colour analysis	Soil colour
1	Phahameng area in Phuthaditjhaba	S 28.56027° E 28.84316°	2 m from mine 10	Hue 7.5 YR 5/2	Brown
2	Kgubetswana area near Clarens	S 28.53830° E 28.42515°	30 cm from mine 11	Hue 2.5 YR 7/1	Light reddish gray
3	Kgubetswana area near Clarens	S 28.53831° E 28.42515°	50 cm from mine 11	Hue 5 YR 6/1	Gray
4	Mangaung area in Phuthaditjhaba	S 28.52926° E 28.43538°	Surrounding area fairly neat without rubbish	Hue 5 YR 5/4	Reddish brown
5	Madikwe area in Phuthaditjhaba	S 28.58712° E 28.83614°	Very dirty mine with glass, stone and rubbish	Hue 10 YR 8/1	White

3.5 References

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Chapter 4

Isolation of Selected Micro-organisms

4.1 Introduction

Geophagia is a direct route for potential transmission of pathogens to the human host, through ingestion of soil (Bisi-Johnson et al., 2010). Such eating of large quantities of soil increases exposure to biological pathogens. Many of the major enteric pathogens are transmitted via the faecal-oral route and the deposition of faeces from human and other animal sources can potentially contaminate soil with bacteria, protozoa and viruses (Abrahams, 2002). Other findings have also shown that soil may have a larger role in the transmission of enteric diseases than previously thought (Santamaria and Toranzos, 2003).

Bacteria are the most abundant of soil organisms, as more than 100 million organisms can be found per gram of soil (Waltz, Skipper and McCarty, 2009). The Actinomycetes are a broad group of bacteria responsible for the distinctive smell of freshly exposed, moist soil. The majority of organisms is found in the top 15 cm of soil and are influenced by soil temperature, water content and pH. Most soil bacteria require temperatures between $10^{o}C$ to $35^{o}C$ and adequate soil moisture while the optimal pH for bacteria ranges from 5.5 to 8.0. These factors causes fluctuations in bacterial content with seasonal changes. The largest populations are, therefore, found during spring, early summer and fall. Bacteria have a short generation time (20 minutes between cell divisions) and can rapidly colonise the soil in optimal conditions (Burmolle et al., 2009; Waltz, Skipper and McCarty, 2009).

Routine procedures to identify human pathogenic organisms in soil samples depend on the cultivation of the bacteria on selective agar media. Soil bacteria have mainly been cultured on plate count agar (Mpuchane et al., 2008). From the counted plates representative colonies were inoculated onto nutrient agar for further identification. Corn meal agar has been used for the cultivation of yeasts. Bacterial isolates were identified using morphological and biochemical tests, while yeasts were identified microscopically (Mpuchane et al., 2010). Pathogenic bacteria and fungi often found in soil are discussed below as well as the diseases associated by these organisms.

Bacillus cereus is associated with food-poisoning in humans and can cause onset of symptoms within a few hours of ingestion. Small numbers of *B. cereus* are commonly present as harmless contaminants in the environment and only large numbers in food will lead to food-poisoning. *B. cereus* is a large Gram positive rod with square-ends. These bacilli may produce central spores that are seen in the Gram stain as unstained areas in the bacilli. *B. cereus* produces large grey colonies with β-haemolysis on blood agar. *B. cereus* also produces a positive lecithin reaction, which distinguishes it from other *Bacillus* spp. except *B. anthracis* which also produces a weak lecithin reaction. *B. cereus* and *B. anthracis* are distinguished on grounds of their motility as *B. cereus* is motile and *B. anthracis* is non-motile (Colleen et al., 1996).

Salmonella spp. are common human pathogens that may cause diarrhea on ingestion. These organisms are non-capsulating, non-sporeforming, Gram negative rod-shaped bacilli. *Shigella* spp. are also common human pathogens

that may cause diarrhea after ingestion. *Shigella* spp. are non-capsulating, non-sporeforming, Gram negative rod-shaped bacilli (Colleen et al., 1996).

Enteropathogenic *Escherichia coli* (EPEC) is a serotype of *E. coli* exhibiting primary pathogenicity in the intestine and may cause gastroenteritis. Community outbreaks were in the past mainly prevalent among the lower socio-economic groups and groups with poor personal hygiene. However, only certain strains of EPECs are able to cause disease (Colleen et al., 1996).

Campylobacter spp. are small curved, comma formed Gram negative rods with the appearance of “gull-wings”. These organisms require aerophilic growth conditions for optimal growth, and growth is generally slow from 2-5 days. *Yersinia entercolitica* is a Gram negative cocco-bacillus with motility at 22°C, but not at 37°C. These organisms grow slowly on blood agar as non-haemolytic small colonies at aerobic and facultative anaerobic conditions. *Nocardia* spp. are normal inhabitants of soil (Colleen et al., 1996).

Some anaerobic organisms such as *Clostridium perfringens* are abundantly present as part of the normal flora of the human gastrointestinal tract, oropharynx and female genital tract. These organisms may cause disease when entering sterile sites other than these known normal flora sites (Colleen et al., 1996).

Fungi are abundantly represented in soil and different fungi may cause disease in humans, especially in hosts that are immune-compromised (Colleen et al., 1996).

Ingestion of soil, or geophagia, is another way in which humans can get infected with enteric pathogens (Santamaria and Toranzos, 2003). However, limited data is available on the risk of infection by these pathogens discussed above when ingesting soil. The aim of the investigation was, therefore, to determine the presence of selected pathogenic bacteria *Listeria* spp., *Bacillus cereus*, *Salmonella* spp., *Shigella spp*. Enteropathogenic *E. coli*, *Campylobacter* spp., *Yersinia enterocolitica*, *Nocardia* spp., anaerobic bacteria and fungi, specifically *Candida* spp. in the soil samples collected.

4.2 Materials and Methods

4.2.1 Bacterial Isolation

Each soil sample (1 g) was suspended in 7 ml Brain-heart Infusion broth (BHI, DMP, South Africa). BHI broth with soil suspensions were incubated aerobically for 24 h at 37^{o}C. After incubation the suspension was inoculated in duplicate onto 5% blood agar (Oxoid Base Nr 2, DMP, South Africa), CCDA agar (DMP, South Africa), MacConkey with crystal violet (McC+CV) agar (DMP, South Africa), XLD agar (DMP, South Africa), *Listeria* selective agar (DMP, South Africa) and Saboroud Dextrose agar (DMP, South Africa). Selection for anaerobic isolates was done by placing a metronidazole disc onto the inoculum on a 5% blood agar plate and incubation under anaerobic conditions for 24 h at 37^{o}C. All the other agar plates were incubated aerobically for 24 h at 37^{o}C, except for the CCDA agar plates, which were incubated under microaerophilic conditions for 48 h at 37^{o}C.

The 5% blood agar was used for the isolation of *B. cereus*, *Norcardia* spp. and *Yersinia enterocolitica*. After 18-24 h incubation the 5% blood agar plates were inspected for growth. Colonies suspected to be *B. cereus* were observed on MacConkey agar as large, irregular, pale and non-lactose fermenting. Colonies on 5% blood agar were grey and irregular, displaying clear zones of β-haemolysis. Gram stains of these colonies that revealed Gram positive bacilli with central spores, were subcultured onto egg yolk agar, which is routinely used to distinguish *B. cereus* and *B. anthracis* from other *Bacillus* species. These two species produce a phospholipase which splits the lecithin in the egg yolk agar to form a zone of opacity around the colonies. *B. cereus* colonies produce a much stronger and thus bigger zone of opacity around the colonies. *B. cereus* is further distinguished from *B. anthracis* on grounds of motility, as *B. cereus* is motile and *B. anthracis* is non-motile (Colleen et al., 1996).

The 5% blood agar pates were also examined for *Norcardia* spp., observed as white pitting colonies, and re-incubated for a further 24 h. Blood agar (5%) and McC+CV agar were used for detection of *Yersinia enterocolitica,* recognised as shiny grey colonies on blood agar and colourless colonies (non-lactose fermenting) (NLF) on the pink background of the McC+CV agar. Non-lactose fermenting colonies on the McC+CV agar were subjected to the API 20E system (BioMèrieux, South Africa) for identification of suspected *Y. enterocolitica* .

Cultures from anaerobically incubated blood agar plates presenting with a zone of inhibition around the metronidazole disc were further identified by Gram staining and final identification with the API 20NE BioMèrieux system.

Enteropathogenic *E. coli*

Lactose fermenting colonies selected from the McC+CV agar were sub-cultured on Mueller-Hinton agar and incubated for 18 h. After incubation the colonies were suspended in sterile water to obtain a milky suspension. These suspensions were then typed with *E. coli* polyvalent 2, *E. coli* polyvalent 3, and *E. coli* polyvalent 4. After obtaining a positive reaction in any of the three typings, the original milky suspension was boiled in a water bath for one hour. The suspension was then cooled to room temperature and re-typed with the previous positive polyvalent. If the suspension was again positively typed the specific sample was noted as Enteropathogenic *Escherichia coli* (EPEC) positive.

Detecting the presence of *Salmonella* spp. and/or *Shigella* spp.

One gram of each soil sample was suspended in 7 ml Selenite-F broth, vortexed and incubated under aerobic conditions for 24 h at 37^{o}C. Cultures were streaked onto *Salmonella-Shigella* (SS) agar (DMP, South Africa) and incubated again for 24 h at 37^{o}C. After incubation the plates were examined for the presence of possible *Salmonella* spp. and/or *Shigella* spp. colonies. *Salmonella* spp. appears as colourless colonies with a black centre, while *Shigella* spp. appears as colourless colonies.

Xylose lysine deoxycholate (XLD, DMP, South Africa) agar was also used for the detection and isolation of *Salmonella* spp. and *Shigella* spp. On the XLD agar *Salmonella* spp. would appear as pink colonies with a black centre. This is caused by the hydrogen sulphide (H_2S) produced by these organisms when

reacting with the ferric ammonium citrate in the medium. *Shigella* spp. would appear as pink colonies, as they do not produce acid from the xylose, lactose or sucrose in the medium. Both colony types would need further identification with the API 10S (BioMèrieux, South Africa) system and sero typings for final identification. Other enterobacteria form yellow colonies on the XLD agar (Colleen et al., 1996).

Charcoal-cefoperazone-deoxycholate agar (CCDA) (DMP, South Africa) was used for the isolation of *Campylobacter* spp. Cultures were incubated in a micro-aerophilic gas pot for 48 h at 37°C and the plates examined for shiny grey colonies.

4.2.2 Isolation of Fungi

Saboroud Dextrose agar was used for the isolation of fungi, more specifically *Candida* spp*.,* incubated at 33°C for up to 14 days. Agar plates were examined daily for growth. Gram staining was performed on growth suspected to be *Candida* spp*.* to confirm the presence of yeast cells. A serum test was used on a suspected colony to identify as *Candida albicans*. Presumptive positive cultures were subjected to a lacto phenol cotton blue (DMP, South Africa) stain to investigate the characteristics of the hyphae and spores for classification to species level. Identification criteria for fungal isolates are provided in Table 4.1.

Table 4.1: Identification criteria for fungal strains.

Fungus	Colony morphology		Microscopy	
	Surface	Lower part	Hyphae	Spores
Trichophyton rubrum	Fluffy, white becoming pink	Pink or red	Long hyphae	Spore are bared alongside the hyphae and Chlamydospores are present in large numbers
Penicillium sp.	Green / pink			
Alternaria sp.	Brown / black	Brown	Segmented hyphae	Conidia alongside the hyphae Macroconidia are present
Mucor sp.	Initially colourless, becoming yellowish- brown	Colourless	Sporangiophores With columellated sporangia	Sporangiospores +-3-5 µm in diameter
Aspergillus flavus	Dark green black		Conidiophores with round vesicle and sterigmata over entire surface	Conidia
Aspergillus fumigates	Green	Yellow	Conidiophores with flask shape vesicle and sterigmata on the upper two-thirds of the vesicle.	Conidia
Paecilomyces sp.	Pink			
Candida albicans	White colonies			Budding sells Serum test positive

4.3 Results and Discussion

4.3.1 Bacteria isolated

In Tables 4.2 to 4.4 the strains isolated from all the soil samples are summarised. Information on the mines and vendors were derived from Chapter 3. *Bacillus cereus* was commonly isolated from the majority of the soil samples. These include all the samples from the geophagic mining sites, two of the five control mine samples and 10 of the 13 vendor soil samples. Two distinct species of anaerobic bacteria were identified as *Clostridium perfringens* and *Clostridium paraputrificum* and were isolated from eight of the 17 geophagic mining sites and from two of the five control mines. *Clostridium* spp. are often encountered in the surface layers of soil, as a result of, amongst others, human and animal excreta (Abrahams, 2002). No anaerobic bacteria were isolated from the vendor soil samples. This may be due to the fact that many of the samples have been treated before selling. One Enteropathogenic *Escherichia coli* serotype was identified from a vendor soil sample (Table 4.4) and one from a control mine (Table 4.3).

Table 4.2: Summary of all the micro-organisms isolated from all 17 the mining sites.

Mining site	Soil colour	Bacteria isolated	Fungi isolated
1. Stop-valve on top of mine	Yellow	*B. cereus*	*Penicillium* sp.
2. Open mine with natural grasses surrounding it	Grey	*B. cereus* *C. paraputrificum*	*Penicillium* sp.
3. Natural grasses surrounding the mine	Grey	*B. cereus*	*Penicillium* sp.
4. Surrounding area fairly neat without rubbish	Yellow	*B. cereus* *C. paraputrificum*	*Penicillium* sp.
5 Very dirty around mine, looks like a dumping site	Grey/white	*B. cereus* *C. paraputrificum*	*Penicillium* sp.
6 Under a tree	Grey/white	*B. cereus*	*Aspergillus flavus*
7 Small, next to path, mine with sticks	Yellow	*B. cereus* *C. paraputrificum*	*Penicillium* sp.
8 In a walking path	Dark grey	*B. cereus* *C. paraputrificum* *C. perfringens*	*Alternaria* sp.
9 Next to the road	Red	*B. cereus* *C. paraputrificum*	*Mucor* sp.
10 Next to the road	Red	*B. cereus*	
11 Sandstone mine, high on a hill, next to the road	Grey/Yellow	*B. cereus*	
12 High on a hill	Grey/yellow	*B. cereus*	

13.Situated halfway up a steep hill	Grey/yellow	*B. cereus*	
14. Situated 500 m from nearest houses	White/yellow	*B. cereus*	*Aspergillus flavus*
15. Near a valley where cattle graze	Yellow	*B. cereus*	*Aspergillus fumigatus*
16. Near a valley where cattle graze	White	*B. cereus* *C. paraputrificum*	*Paecilomyces* sp.
17 500 m from houses	Grey/red	*B. cereus* *C. perfringens*	*Tricophyton rubrum*

Table 4.3: Summary of all the micro-organisms isolated from the control mining sites.

Control site	Location	Soil colour	Bacteria isolated	Fungi isolated
1	2 m from mine 10	Red	*B. cereus* *C. paraputrificum*	*Paecilomyces* sp.
2	30 cm from mine 11	Grey	*B. cereus* *E. coli* (EPEC)	
3	50 cm from mine 11	Purple		*Penicillium* sp.
4	100 cm from mine 15	Yellow	*C. paraputrificum*	*Penicillium* sp. *Aspergillus* sp.
5	1 m from mine 16	Yellow	*C. paraputrificum*	*Penicillium* sp.

Table 4.4: Summary of all the micro-organisms isolated from the vendor soil samples.

Vendor	Soil origin	Soil colour	Soil preparation	Packaging	Bacteria isolated	Fungi isolated
1	Own soil, suburban	Yellow	Use utensils to mine. Process pieces on a "streep sak" into smaller bits. Use bare hands to pack.	Drycleaner bags		
2	Own soil in rural area	White	Use utensils to mine. Transfer with bare hands to large plastic bag. Process in charcoal oven 10-15 min.	Small, non-sterile bags	*B. cereus* *E. coli* (EPEC)	
3	Own soil, suburban	White	Use utensils to mine. Transfer with bare hands to large plastic bag. Samples dried in sun before selling.	New plastic bags	*B. cereus*	
4	Unknown	Grey	Samples dried in sun before selling.	Old plastic bags		
5	Unknown	White/pink	Unknown	Old plastic bags	*B. cereus*	
6	Bought from city	Yellow	Unknown	Old plastic bags	*B. cereus*	
7	Unknown	Grey	Unknown	Old plastic bags	*B. cereus*	*Candida albicans*
8	Unknown	Grey	Unknown	Old plastic bags	*B. cereus*	
9	Unknown	Grey	Unknown	Old plastic bags	*B. cereus*	*Aspergillus* sp. *Mucor* sp.
10	Unknown	Grey/purple	Unknown	Old plastic bags		
11	Unknown	Grey/purple	Unknown	Old plastic bags	*B. cereus*	*Penicillium* sp.
12	Own soil, suburban	White	Use utensils to mine. Transfer with bare hands to large plastic bag (Maize).	Plastic bags	*B. cereus*	
13	Own soil in rural area	White	Use utensils to mine. Transfer with bare hands to large plastic bag. Samples dried in sun before selling.	Plastic bags	*B. cereus*	

4.3.2 Fungi isolated

Various fungi were isolated and identified from 13 of the geophagic mining sites, four of the control mines and only from three of the vendor soil samples (Tables 4.1 - 4.3). The fungi isolated include *Penicillium* spp. (10), *Aspergillus fumigatus* (1), *Aspergillus flavus* (20) *Aspergillus* spp. (4), *Alternaria* spp. (1), *Paecilomyces* spp. (2), *Mucor* spp. (2), *Trychophyton rubrum* (1) and *Candida albicans* (1).

It was overall obvious that the vendor soil samples contained less organisms than the majority of the other soil samples. The processing of soil for consumption by heat treatment or baking has been reported to render the soil pathogen free, but the effectiveness of such treatment has not yet been specifically determined (Bisi-Johnson et al., 2010). This may be dependent on the type of organism present, since spore formers, such as *Clostridium* are often resistant to heat treatment.

No definite correlation could be detected between the soil colour and the type or number of organisms isolated. However, one soil sample from geophagic mine no. 8, which was situated in a walking path, had a dark grey colour and three bacterial species as well as one fungus were isolated (Table 4.1). This is the highest number of isolates from any soil sample.

4.4 Conclusions

Although it has often been reported that the microbes in geophagic soil may be harmless and even beneficial to humans, there are also serious risks in consuming soil contaminated with pathogenic bacteria. However, the prevalence and frequency of pathogenic bacteria in the current study was relatively low, specifically with regard to organisms often associated with faecal contamination. Although no definite relevance could be established with regard to the conditions of the geophagic mining environment and the pathogens isolated, it was demonstrated that the soil sold at the market was less contaminated than the soil mined directly from the popular geophagic mining sites.

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Chapter 5

Culturability of *Salmonella enterica* sv. enteritidis and Antimicrobial Activity of Geophagic Soil

5.1 Introduction

It is commonly known that faecal pathogens may survive in soil for long periods of time. Earlier studies showed the survival of coliform bacteria in soil for between 16 to 72 days, while *Salmonella* spp. survived for 4-61 days in soil under laboratory conditions (Someya et al., 2004). However, the question arises if human pathogens in soil may sometimes be missed by conventional culturing methods, as a result of becoming non-culturable in soil environments. This does not mean that they are not viable and may even still be capable of causing disease in humans. The statement of "viable but non-culturable" (VBNC) has evolved over two decades. Recognised pathogens among the VBNC bacteria are *Escherichia coli* and *Salmonella* spp. Some human pathogenic organisms may, therefore, have the ability to survive in soil and still be viable and able to cause disease, but may not be culturable (Someya et al., 2004).

Studies by Bakhrouf (2008) showed that *Salmonella enterica* serovar Agona may be resuscitated after 13 years of incubation in natural dry soil. This organism was found culturable after 24 hour incubation under suitable conditions. The normal biochemical profile and microscopic appearance of the cells were initially different from the original character but returned to normal after six months (Bakhrouf et al., 2008).

Studies by Dhiaf, Addallah and Bakhrouf (2010) reported on the resuscitation of *Salmonella typhimurium* after five years of incubation in seawater microcosms. These organisms were resuscitated within 24 hours and recuperated their original biochemical character after five months of

resuscitation. The survival of *Salmonella typhimurium* for long periods in natural soil microcosms confirmed the adaptive profile of this bacterium under starving conditions and these results were similar to those found in *Vibrio cholerae* 01, enteropathogenic *Escherichia coli*, *Shigella dysenteriae* and *Vibrio alginolyticus* (Bakhrouf et al., 2008).

In 1993 Morita found that several bacteria, such as *Salmonella* can survive in stressing environments for long periods of time due to sequential changes in cellular physiology and gradual morphology changes. In addition to this, in 1996 Colwell found that VBNC bacteria were still viable, with metabolic activity and respiration, but were not able to show colony forming units during conventional plate counts and thus remain hidden (Bakhrouf et al., 2008).

Soil exhibit a wide range of colours. These may vary from gray, black, white, various shades of reds, various browns to yellow and sometimes even green (Brady et al., 2006). Development and distribution of colour in soil result from chemical and biological changes, especially redox reactions. The presence of specific minerals can also affect soil colour. For example, manganese oxide causes a black colour, glauconite renders the soil green, while calcite can cause soil in dry regions to appear white (Brady et al., 2006). Soil colour is also a good indicator of soil quality and shows changes in humus content of soils (Shepherd et al., 2008). A darker coloured soil is usually rich in organic matter and would, therefore, be expected to sustain the life of microbes. Soil rich in inorganic matter, such as ferric oxide, would have a more reddish colour. Red or yellow soils are often high in iron content that is less capable of supporting growth. It is also believed by geophagic individuals that reddish soil are rich in iron and could be used to supplement iron.

Soil colour can also provide information on the drainage, biotic activity and fertility of the soil (Soil Types and Testing, 2010). Grey/blue colours would indicate that the soil is poorly drained and poorly aerated, while brown-yellow, brown, reddish-brown and red soils usually indicate well-aerated, well drained conditions (Shepherd et al., 2008). To be healthy, soil needs to breath and water needs to be able to move through it easily. Sandy soils are light and typically very free draining, usually holding water very poorly due to very low organic content, while clay soils are not typically free draining.

It has been reported that up to 80% of orally administered antibiotics may pass through animals without any changes to the antibiotics. These antibiotics are then excreted with the animal manure (Thiele-Bruhn, 2003). Antibiotic concentrations in most soils are not at therapeutic levels, although this may cause inhibitor effects on bacteria, which can select for antibiotic resistance in soil bacteria (Gavalchin and Katz, 1994). Some antibiotics, such as tetracycline and tylosin, have the ability to strongly bind to different soils or clays (Yanyu et al., 2009). Studies have shown that antibiotic absorption depends on different factors in soil, which include pH, cation exchange capability, Al/Fe hydrous oxides and clay components. Antibiotic absorbtion tends to decrease with an increase in pH (Yanyu et al., 2009).

Studies have also reported that the chemistry of clay and the surface properties that affect pH and oxidation state are important factors in antibacterial properties of clays (Williams et al., 2008). In a study done in 2002 it was found that there existed an interaction between modified clay and bacteria. This then led to the assumption that clay disrupts the cell wall of some bacteria, causing the bacteria to leak until death (Bowen et al., 2002).

In a study conducted in South Africa to demonstrate antimicrobial activity of some clays, organisms were selected on grounds of their ability to cause human disease, their presence in food and the environment and their applicability as indicators of contamination. Nine out of 102 clay samples indicated antimicrobial activity (Mpuchane et al., 2008).

The aim of this chapter was to determine the culturability of *Salmonella enteritidis* inoculated in a wide range of soil samples (from Chapter 3), to determine the possible influence of soil colour and to investigate possible antimicrobial activity of geophagic soil. *Salmonella enteritidis* was selected because if its pathogenicity to humans on consumption.

5.2 Methods and Materials

5.2.1 Culturability

Each soil sample was weighed and 50 g of soil was placed into a 200 ml sample container. A suspension of *Salmonella enterica* sv. enteritidis equal to a 0.5 McFarland standard was prepared in 10 ml distilled water. The suspensions were mixed thoroughly and then introduced into the different soil samples. The soil and suspension mixtures were mixed thoroughly with a sterile swab and then incubated at room temperature. After incubation a swab was dipped into sterile selenite broth (Oxoid, Media Madge, South Africa), stabbed into several areas of the soil suspension and inoculated onto *Salmonella-Shigella* agar (Oxoid, Media Madge, South Africa). The agar plates were incubated at 37°C for 18-24 hours. This stabbing procedure was repeated every seven days. Agar plates were examined for growth every seven days until no *S. enteritidis* was recovered from the different soil

samples. The exact procedure as mentioned above was carried out for a second time to validate the reproducibility of the experiment.

5.2.2 Antimicrobial activity of soil samples

One gram of each soil sample was weighed using a Sartorius Scientific Balance (IMP, South Africa). Soil samples were placed into 5 ml sterile water and the mixtures vortexed until a uniform suspension was obtained. The mixtures were then left to stand for half an hour to allow for the soil sediment to move to the bottom of the tube. McFarland standard (0.5) suspensions of *Salmonella* spp., Enteropathogenic *Escherichia coli*, *Bacillus cereus* and *Candida albicans* were prepared in sterile distilled water. A Densicheck apparatus (BioMèrieux, Italy) was used to confirm the suspension density of the different organisms. The microbial suspensions were evenly spread over Mueller-Hinton agar. The end opening of sterile pasteur pipettes were used to make six boreholes in each of the agar plates. Each borehole was inoculated with 20 µl of the different soil supernatants. After inoculation the plates were incubated in an inverted position at 37°C under aerobic conditions for 18-24 hours. After incubation the plates were examined for any indication of a zone of inhibition around the wells, which would indicate antimicrobial activity against the specific test organism. After 24 h the procedure was repeated.

5.2.3 Soil slurry used to test antimicrobial ability

A similar test was done as performed above by using the slurry of each suspension to test for antimicrobial activity against the test organisms. One gram of each soil sample was weighed and placed into 5 ml sterile distilled water. The mixtures were vortexed until a uniform suspension was obtained

and left to stand for half an hour to allow for the soil sediment to move to the bottom of the tube. The different organisms as mentioned above were used to prepare a 0.5 McFarland suspension in sterile distilled water and the densities of the organisms were confirmed using a Densicheck apparatus (BioMèrieux, Italy). Suspensions were spread evenly over Mueller-Hinton agar. Slurry of each soil sample was inoculated directly onto the agar plates by using sterile cotton swabs. After inoculation the plates were incubated in an inverted position at 37°C under aerobic conditions for 18-24 hours. Plates were examined for an indication of a zone of inhibition around the inoculation points, which would indicate antimicrobial activity against the specific test organism.

5.3 Results and Discussion

The soil samples consisted of a wide range of colours. Soil samples from the various mining sites were observed to present a wide range of culturability profiles. This was also found for the various colours of the soil samples. Culturability of the *Salmonella* spp. recovered from the various soil samples is summarised in Tables 5.1 to 5.3. Soil colours are indicated on the tables.

It was interesting to note that survival time and culturability was lowest in the majority of the soil samples sold by the vendors. It appears that the treatment of soil samples before selling may have an influence on the sustainability for growth of bacteria (Table 5.2).

The soil from the control mines were found to sustain culturability for up to three weeks in some of the gray samples (Table 5.3). This was also lower than the culturability found in the soil samples from the geophagic mines

(Table 5.1). The longest periods of culturability were recorded in soil samples from the geophagic mining sites. However, it was evident that no definite correlation could be detected between soil colour or culturability.

Table 5.1: Culturability of *Salmonella enteritidis* from the 17 geophagic mining sites.

Mine site	Soil colour	Recovery (weeks) First investigation	Recovery (weeks) Second investigation
1	Pale red	9	9
2	Light reddish gray	8	4
3	Pinkish white	6	7
4	Very pale brown	2	4
5	White	2	2
6	Light gray	First day only	First day only
7	Pinkish gray	2	2
8	Greenish gray	1	1
9	Strong brown	1	1
10	Strong brown	First day only	First day only
11	Grey	9	7
12	Light reddish gray	6	5
13	Pinkish gray	7	5
14	Light reddish brown	1	1
15	Light brown	First day only	First day only
16	Light gray	First day only	First day only
17	Yellowish brown	First day only	First day only

Table 5.2: Culturability of *Salmonella enteritidis* from the 13 vendor soil samples.

Vendor site	Soil colour	Recovery (weeks) First investigation	Recovery (weeks) Second investigation
1	White	First day only	First day only
2	Very pale brown	First day only	First day only
3	Light gray	First day only	First day only
4	Very pale brown	First day only	First day only
5	Light gray	First day only	First day only
6	Very pale brown	First day only	First day only
7	Light greenish gray	5	4
8	Pale green	2	2
9	Light gray	First day only	First day only
10	Pale red	First day only	First day only
11	Light white	First day only	First day only
12	White	First day only	First day only
13	Gray	First day only	First day only

Table 5.3: Culturability of *Salmonella enteritidis* from the five control mines.

Control site	Soil colour	Recovery (weeks) First investigation	Recovery (weeks) Second investigation
1	Brown	First day only	First day only
2	Light reddish gray	3	3
3	Gray	3	3
4	Reddish brown	First day only	First day only
5	Light brown	1	1

The longest period of survival and recovery time (nine weeks) was recorded in pale red and gray soils/clays. This was followed by a light reddish gray soil (eight weeks), a pinkish gray soil (seven weeks), a light reddish gray and a pinkish white soil (five weeks), while a light greenish gray soil allowed for culturing after four weeks (Table 5.4).

Shorter survival and recovery times were found in gray soil/clay and light reddish gray soil/clay (three weeks), followed by very pale brown, pinkish gray, pale green, while culturability was achieved after two weeks in white soils or clays (2 weeks). All the other soil samples showed a recovery rate of a week or less than one week (Table 5.4).

The results indicate the diversity of the culturability of *Salmonella enteritidis* in different soil samples from different origins and colour. There were indications that geographic location, soil colour as well as soil treatment all play an important role in the ability of soil to sustain culturability of bacterial life. In Figure 5.1 it can be visually observed that soil or clay samples with gray, red or pink colours sustained the highest periods of culturability, which

may be attributed to a high level of organic matter as well as ferric constituents. It was also evident that survival rate of the organism was lower in the brown and darker soils.

Table 5.4: Survival rate of *Salmonella enteritidis* in specific soil colours.

Soil colour	First investigation	Second investigation
BROWN	1 day	1 day
BROWN (Light)	1 day, 1 week	1 day, 1 week
BROWN (Strong)	1 day, 1 week	1 day, 1 week
BROWN (Very pale)	1 day, 2 weeks	1 day, 4 weeks
BROWN (Light reddish)	1 week	1 week
BROWN (Reddish)	1 day	1 day
BROWN (Yellowish)	1 day	1 day
GRAY	1 day, 3 weeks, 9 weeks	1 day, 3 weeks, 7 weeks
GRAY (Light)	1 day	1 day
GRAY (Light reddish)	3 weeks, 6 weeks, 8 weeks	3 weeks, 4 weeks, 5 weeks
GRAY (Pinkish)	2 weeks, 7 weeks	2 weeks, 5 weeks
GRAY (Greenish)	1 week	1 week
GRAY (Light greenish)	4 weeks	5 weeks
GREEN (Pale)	2 weeks	2 weeks
RED (Pale)	1 day, 9 weeks	1 day, 9 weeks
WHITE	1 day, 2 weeks	1 day, 2 weeks
WHITE (Light)	1 day	1 day
WHITE (Pinkish)	6 weeks	7 weeks

In Figure 5.1 the culturability of *Salmonella enteritidis* is illustrated graphically, showing the differences between the various soil coloured samples.

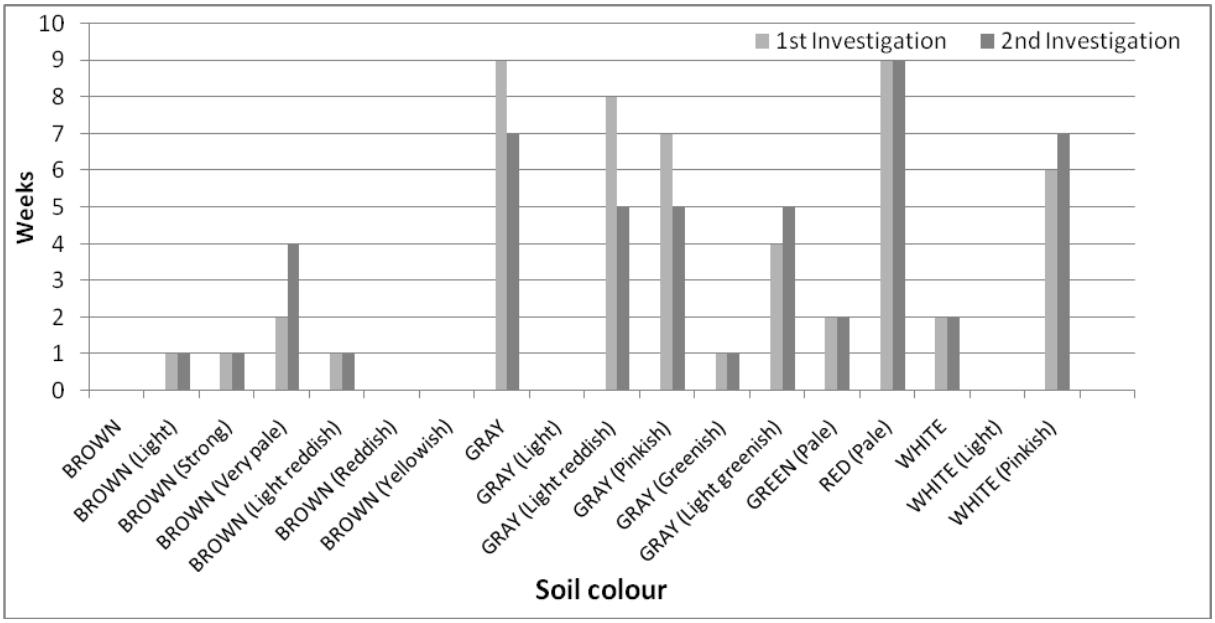

Figure 5.1: Highest culturability of *Salmonella enteritidis* in various coloured soil samples

No antimicrobial activity was detected in any of the samples by using either of the two methods. However, the majority of samples from the current study did not consist of white colour soils/clays. In previous studies done in South Africa, it was found that soil colour may play a role in antimicrobial activity (Mpuchane et al., 2008). In their study white clays demonstrated antimicrobial activities against all the isolates. A larger sample size may, therefore, also be important for antimicrobial activities. Testing Clay particles are known to have the ability to absorb antibiotic molecules into their inter-clay spaces (Kumar et al., 2005). However, during this absorption to clay some antibiotics may lose their antibacterial activity.

5.4 Conclusions

Although the colour of soils or clays may play a role in predicting the possibility of recovering bacterial pathogens such as *Salmonella enteritidis* from a specific soil or clay type, definite correlation was not evident in the current study. It is known that geophagic practicing persons have a preference to specific coloured soil or clay samples. If culturability of a bacterial pathogen would be higher in such a preferred geophagic sample, this may have a significant influence on the safety and also the quality of the soil being consumed. It may even become necessary to educate geophagic practicing communities from rural areas with regard to the possible risks in consuming certain coloured soils or clays.

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Chapter 6

Bacterial Diversity in Soil from Geophagic Mining Sites

6.1 Introduction

Reports of health hazards due to geophagia date back to as early as 1825 (Ghorbani, 2008). Materials present in soil may influence mineral levels in the consumer, while pollutants (industrial and human) and parasites or other living organisms may cause disease in humans (Callahan, 2003). Several researchers have investigated the heavy metal content, mineralogy and chemistry of geophagic soil (Ekosse and Jumbam, 2010; Kutalek et al., 2010; Ngole et al., 2010). A fascinating, yet poorly understood aspect of soil is its living (microbial) component; one which may very well influence the prevalence of heavy metal content, mineralogy and chemistry. To date a selective culturing based approach to search for a select few microbes have been the focus of microbiological investigation of Geophagic soils with a great deal of emphasis on improving the culturablility of soil bacteria (Hayakawa et al., 1991; Burmolle et al., 2009).

Cultured micro-organisms present in soil represent only a small fraction of natural microbial communities and hence the microbial diversity in terms of species abundance is grossly underestimated. Microbial communities differ in both qualitative and quantitative composition and are subject to environmental physico- chemical changes as well as physiological and metabolic changes caused by the organisms. Furthermore, some micro-organisms that are abundant and culturable under certain conditions may develop into dormant and possibly uncultured forms (von Wintzingerode et al., 1997).

The aim of this chapter was, therefore, to assess the bacterial diversity of soil from established geophagic mining sites in the Qwa-Qwa region using a culture independent method.

6.2 Materials and Methods

6.2.1 Sampling and storage

Soil samples were gathered from 17 different mining sites in the Qwa-Qwa area. The geographic location, soil colours and sampling protocol was discussed in Chapter 3, Table 3.1. A small fraction of each soil sample was stored at -80°C for later analysis.

6.2.2 Total community DNA extraction and PCR amplification

Total genomic DNA was extracted from *ca.* 0.272g ± 0.018 g of each soil sample using the ZR Soil Microbe DNA Kit™ according to the manufacturers' instructions (Zymo Research). This protocol employs a filter process to remove typical PCR inhibitors such as humic acids and polyphenols. DNA concentrations were determined spectrophotometrically with the NanoDrop Spectrophotometer (Thermo Scientific) and used as template in subsequent PCR amplifications performed in the G-Storm GS482 thermal cycler (Gene Technologies).

A two step nested amplification approach was followed where the first PCR, targeting 1 300 bp of the 16S rRNA gene, was performed using primer set 63-F (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 1387-R (5'-GGG CGG WGT GTA CAA GGC-3') (Marchesi et al., 1998). The PCR was carried out in a total volume of 25 µl, containing 5–15 µl template DNA, 2.5 µl reaction

buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3), 0.2 mM dNTPs, 0.5 µM of each primer (Integrated DNA Technologies) and 1 unit Supertherm Taq ploymerase (Southern Cross Biotech). Reaction conditions included an initial denaturation step at 94°C for 3 min, 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. Final elongation was performed at 72°C for 10 min. PCR products were separated on a 1% agarose gel, stained with 0.05% Goldview (Guangzhou Geneshun Biotech) and visualised under UV light.

Stabs from this gel served as template for the amplification of a second, shorter fragment of 233 bp nested within the same target gene using primer set 341-F^{GC} (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCC CCG CCC CCG CCC CCT ACG GGA GGC AGC AG-3') with incorporated GC-clamp at the 5'-end and 517-R (5'-ATT ACC GCG GCT GCT GG-3') (Muyzer et al., 1993). The PCR was performed in a total volume of 50 µl and contained 5 µl reaction buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3), 0.2 mM dNTPs, 0.5 µM of each primer (Integrated DNA Technologies) and 1 unit Supertherm Taq ploymerase (Southern Cross Biotech). Initial denaturation was performed at 95°C for 5 min, 30 cycles of 95°C for 45 sec, 55°C for 45 sec and 72°C for 1 min. Final elongation was performed at 72°C for 10 min. In order to reduce possible inter-sample PCR variation, the PCR was performed in duplicate and pooled before loading on the DGGE gel. DNA fragments were separated on a 2% agarose gel and stained and visualised as previously described.

6.2.3 Denaturing gradient gel electrophoresis (DGGE)

Analysis was performed on 30 µl of the 233 bp GC-clamped PCR fragments with 8 µl added 6X Loading dye solution (10 mM Tris-HCl [pH 7.6] 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol 60 mM EDTA) using the D-Code Universal Mutation Detection system (BioRad). Sequence specific separation of the amplicons was obtained in a 8% (w/v) polyacrylamide (Acrylamide/Bis 37.5:1) gel in 1X TAE buffer (40 mM Tris-HCl, 20 mM glacial acetic acid, 1 mM EDTA) containing a 40 – 60% linear denaturant gradient. The 100% denaturant solution contained 40% (v/v) formamide and 7 M urea (Muyzer et al., 1993). Electrophoresis was performed with a constant voltage of 130 V at 60°C for 4.5 h. Gels were stained with 0.05% GelStar® (Lonza) for 15 min, rinsed with ultra-pure water and photographed under UV light. At least two representatives of each band position was excised from the gel on a DarkReader (Clare Chemicals Research), each band incubated in 5 µl ultra-pure water at 60°C for at least 5 h and 5 µl used as template for re-amplification. The re-amplified fragments (0.5 µl) were used as template for direct sequencing.

6.2.4 Sequencing analysis

Sequencing of the re-amplified DGGE bands was performed on the ABI Prism 3130 XL genetic analyser using the Big Dye® Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems). DNA for sequencing was precipitated with the EDTA-ethanol method (125 mM EDTA, 100% ethanol). Sequencing data were applied to the National Centre for Biotechnology Information (NCBI)

for identification. Only similarities with a Blast index of 90% and above were considered for identification to genus level.

6.2.5 Data processing

Quantity One software (BioRad) was used to identify different band positions and peak areas were used to indicate the intensities. Bands with a relative intensity of less that 1% of the sum of all band intensities were discarded. Species abundance was calculated as a percentage of the total amount of bands detected. Cluster analysis describing pattern similarities among different soil samples was performed using an unweighted pair-group method with an arithmetic mean algorithm (UPGMA).

6.3 Results and Discussion

The 16S rRNA gene was targeted for bacterial diversity analysis. Representatives of the amplified 1 300 bp fragment from each soil sample are shown in Figure 6.1A. Where no amplification was obtained the PCR was repeated with an increased volume of template genomic DNA, until amplicons were obtained for all soil samples. GC clamped DGGE-PCR products of 233 bp were amplified using the nested approach (Figure 6.1B) and analyzed by DGGE.

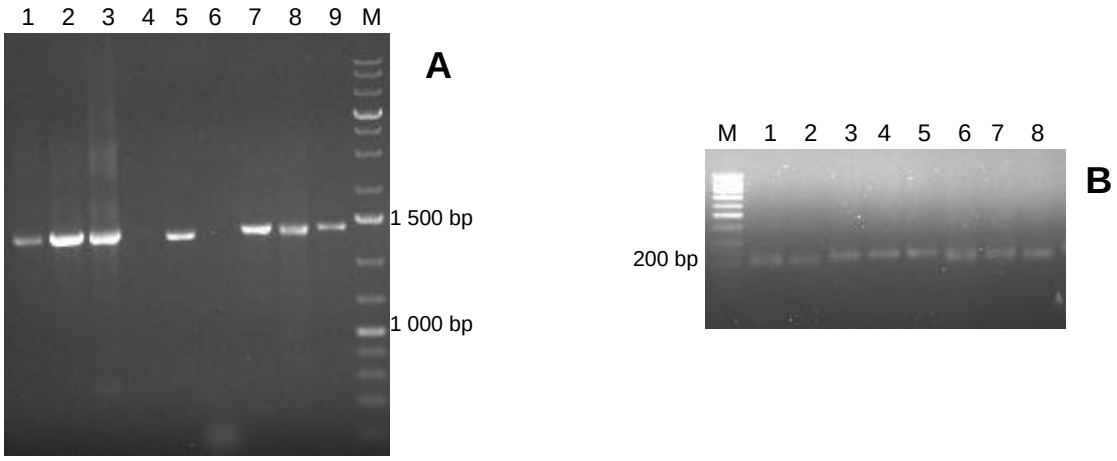

Figure 6.1: (A) Representative agarose gel electrophoresis of the 16S rRNA gene products (1 300 bp) amplified from different soil samples. Lane M: GeneRuler 1 kb DNA ladder plus (Fermentas). (B) nested 233 bp GC-clamped amplicons. Lane M: GeneRuler 50 bp DNA molecular weight marker (Fermentas).

The Quantity One software detected 431 bands in total and 54 different band positions (Figure 6.2). The number of bands that comprise the DGGE patterns indicated that there was a high diversity of bacterial PCR amplification products in all soil samples, except for the soil from mining site 2. Representative bands of each position were recovered from the gel (81%), re-amplified and sequenced for identification. As this is a DNA based technique it is important to note that the results do not reflect microbial viability.

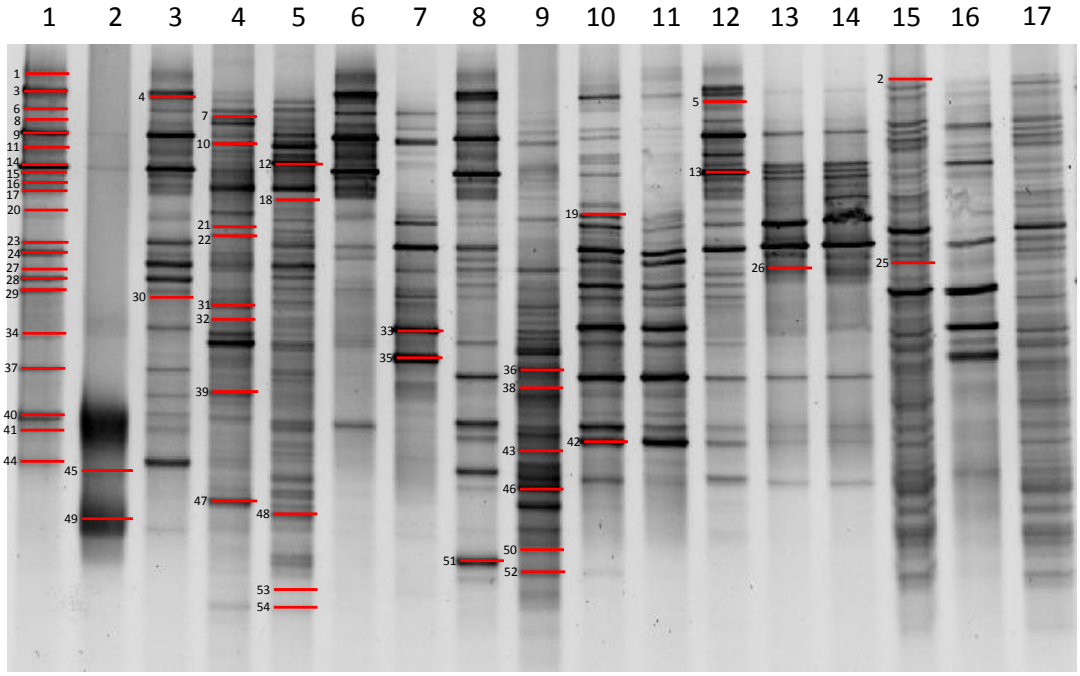

Figure 6.2: Bacterial communities in soil samples profiled by DGGE (8% polyacrylamide with a 40-60% urea gradient) of amplified 16S rDNA fragments. Lane numbers indicate the mining sites, while band positions detected by Quantify One software are indicated as numbered red bars.

Bacterial diversity across all soil samples are shown in Figure 6.3. Representatives of three of the 22 known bacterial phyla were identified belonging to Proteobacteria (32.9%), Firmicutes (13.5%), Actinobacteria (17.2%), unknown/unidentified bacteria (17.4%) and unrecovered bands amounting to 19%. The most abundant genera corresponded to the Actinobacteria of which six different families were identified, each represented by a single genus. The phyla α-proteobactria and Firmicutes were both represented by three families and γ-proteobacteria by only the two families Enterobacteriaceae and Xanthomonadaceae. Interestingly, the respective

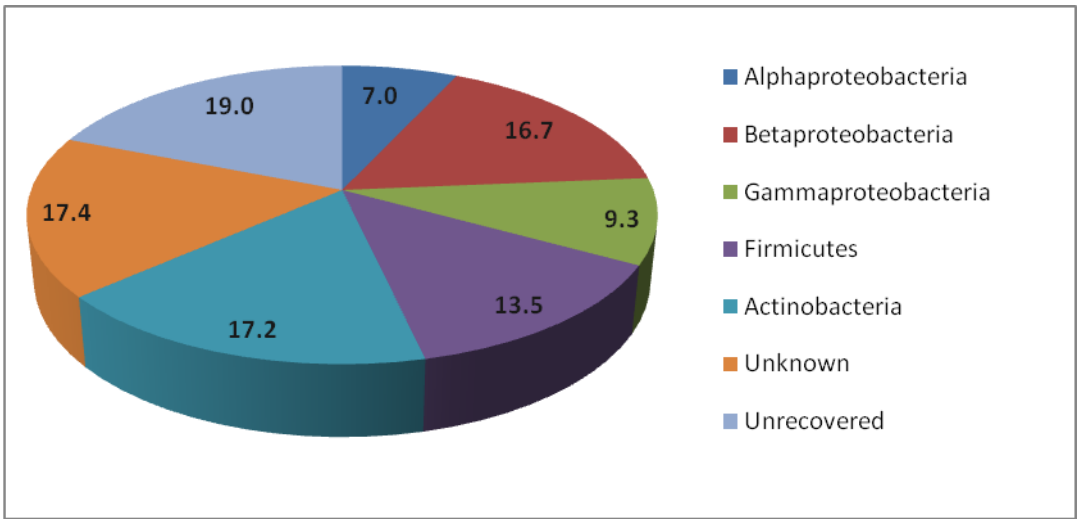

Figure 6.3: Bacterial phyla detected in the soil samples from 17 different mining sites identified from the sequencing data. Values on the chart were calculated as a % of the total number of bands detected by PCR-DGGE.

genera representative of each family, namely *Pantoea* and *Stenotrophomonas* were dominant in many of the soil samples and will be discussed. Two families belonging to the phylum β-proteobacteria, namely Rhodocyclaceae and Oxalobacteraceae were also detected, of which the latter was represented by three different genera.

Cluster analysis was used to study the similarities between the banding patterns generated by PCR-DGGE as shown in Figure 6.4. The software considered both band patterning and intensity and used an unweighted pair-group method with an arithmetic mean algorithm to produce the dendrogram. Three main clusters (A, B, C) and four sub-clusters (C1, C2, C3, C4) were

identified. The lanes of the DGGE gel (Figure 6.2) were rearranged according to the suggested clustering and pattern similarities among the different soil samples clustering together are clearly distinguishable. Table 6.1 shows the identities of dominant bands (based on band intensity) indicated in Figure 6.4 which forms part of further discussion in terms of represented genera and significance. Although some sequences showed very high homology up to species level, the data was only discussed to genus level, since the short fragment used for sequencing could lead to misinterpretation.

Cluster A which consisted only of mine 2 shows limited diversity and the prominent bands represent *Rhodococcus* (41) and an unidentified/unknown bacterium (49). The mentioned numbers corresponded to the band positions indicated in Table 6.1 and Figure 6.4. *Rhodococcus* was also prevalent in cluster B (mine 11) and sub-clusters C2 and C4. The genus consists of 34 species of which some have been recognised as human pathogens with increasing frequency, especially in immune-suppressed/compromised individuals, and is often confused with *Mycobacterium tuberculosis* (Bell et al., 1998). Others have considerable potential for bioremediation, since they can degrade a range of environmental pollutants and toxins. They could also be useful in other biotechnology applications for their ability to synthesise several surfactants, flocculants, enzymes and pigments (Martinkova et al., 2009). Cluster B, which consists only of mine 11 showed five prominent bands; two of which represent *Rhodococcus* (37/42), *Stenotrophomonas* (23), *Duganella* (24) and *Pantoea* (33). The genus *Stenotrophomonas* was also characteristic of sub-clusters C3 and C4 and contains 13 species, some potential human

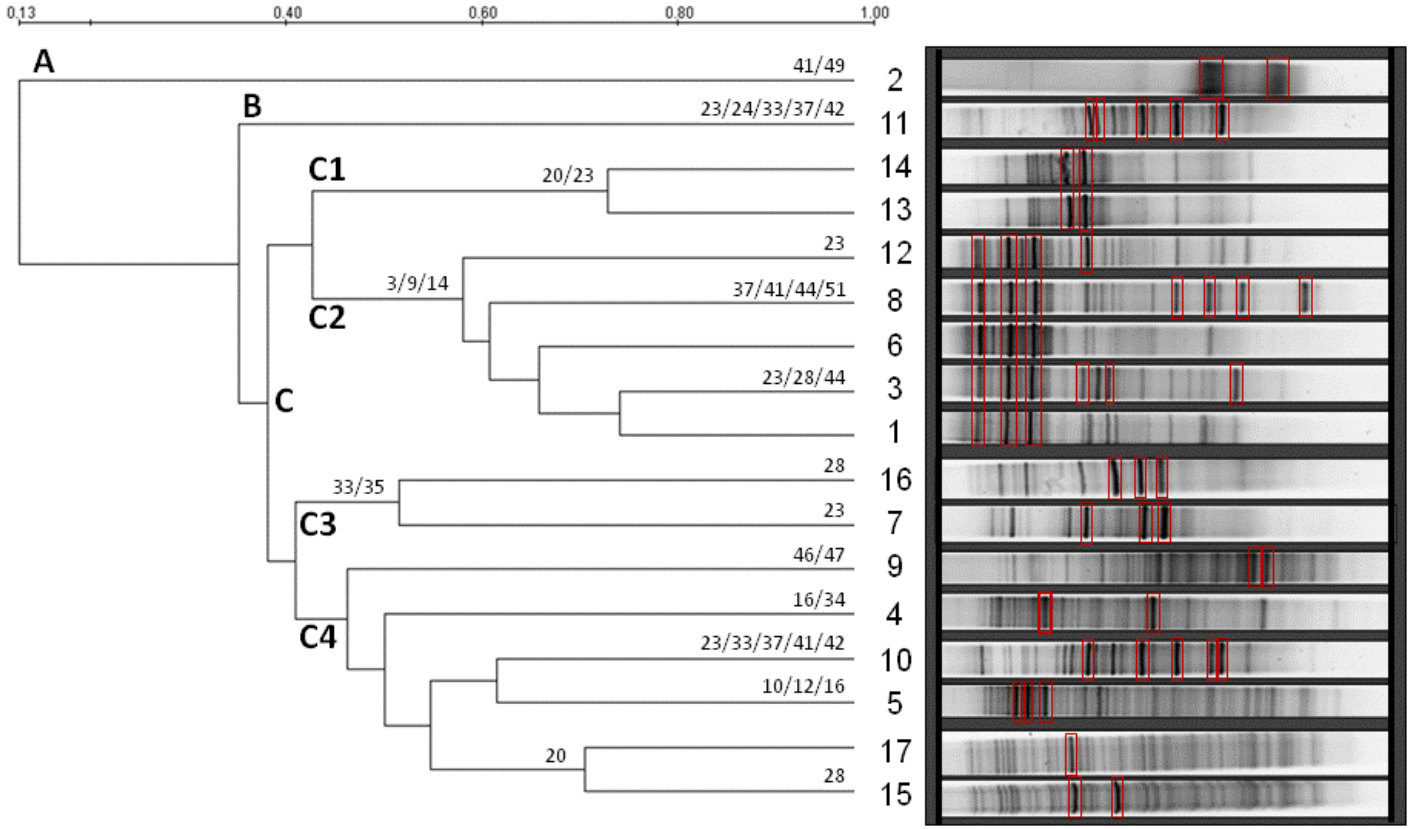

Figure 6.4: UPGMA cluster analysis of DNA band fingerprints obtained by PCR-DGGE. A-C and C1-C4 indicated clusters and sub-clusters, respectively. Mining sites/mines are indicated by large numbers. The numbers on the dendrogram show the band position of the specific dominant bands (indicated in red on the gel). These numbers correspond to the band numbers in Table 6.1.

Table 6.1: Summary of the sequences obtained from the respective dominant bands in the DGGE gel and the closest match from the GenBank database.

Band	Database match with accession number in parentheses	% identity	Family	Phylogenetic group
3	Uncultured Oxalobacteraceae bacterium (GU300362.1)	95	Oxalobacteraceae	β-proteobacteria
9	*Listeria monocytogenes* 08-5578 (CP001602.1)	94	Listeriaceae	Firmicutes
10	Uncultured bacterium clone TX4CB_131 (FJ153000.1)	98		
12	*Bacillus* sp. MYL-9 16S (HQ738480.1)	99	Bacillaceae	Firmicutes
14	*Massilia* sp. II_Gauze_W_12_10 (FJ67554.1)	95	Oxalobacteraceae	β-proteobacteria
16	*Rhodocyclus* sp. oral taxon 028 clone AV097 (GU397939.1)	98	Rhodocyclaceae	β-proteobacteria
20	*Massilia* sp. MJBC20 (HM026213.1)	95	Oxalobacteraceae	β-proteobacteria
23	*Stenotrophomonas maltophilia* (DQ103763.1)	95	Xanthomonadaceae	γ-proteobacteria
24	*Duganella* sp. AT1-1 (GU332616.1)	91	Oxalobacteraceae	β-proteobacteria
28	*Methylobacterium* sp. F05 (D32231.1)	91	Methylobaceriaceae	α-proteobacteria
33	*Pantoea agglomerans* strain new*16 (AF130895.1)	97	Enterobacteriaceae	γ -proteobacteria
34	*Cellulomonas* sp. KAR12 (EF451642.1)	96	Cellulomonadaceae	Actinobacteria
35	*Pantoea agglomerans* strain EhY112-9/86 (FJ756356.1)	99	Enterobacteriaceae	γ-proteobacteria
37	*Rhodococcus kyotonensis* strain DS472 (NR 041512.1)	94	Nocardiaceae	Actinobacteria
41	*Rhodococcus* sp. 4A-4 (AY197005.1)	95	Nocardiaceae	Actinobacteria
42	*Rhodococcus* sp. MJBC36 (HM026224.1)	99	Nocardiaceae	Actinobacteria
44	*Brevibacterium healii* (AY017117.1)	95	Brevibacteriaceae	Actinobacteria
46	*Dactylosporangium* sp. NMS-14 (FN662892.1)	98	Micromonosporaceae	Actinobacteria
47	Uncultured bacterium (AB363536.1)	95		
49	Uncultured bacterium clone RW5983 (GU641327.1)	99		
51	*Geodermatophilus* sp. 0708S6-1 (HM222666.1)	96	Geodermatophilaceae	Actinobacteria

pathogens demonstrating multidrug resistance, but cause mostly nosocomial infections. They can be the causative agent of diarrhea in immune-suppressed/compromised individuals and have been isolated from patients with chronic inflammatory bowel disease (CIBD) (Apisarnthanarak et al., 2003). Certain species have antifungal activity against *Candida* and *Aspergillus* species (Denton and Kerr, 1998) and others show the potential to remediate soil contaminated with petroleum products (Verma et al., 2010). The genus *Duganella* consists of only three species, able to degrade Poly (β-hydroxy-alkanoate) polyesters (PHA) promising materials for the production of biodegradable plastics (Suyama et al., 1998; Li et al., 2004). The genus *Pantoea* has 24 known species of which *P. agglomerans* is a known opportunistic human pathogen in the immune-compromised individuals, but rarely cause disease in otherwise healthy individuals (Cruz et al., 2007; Liberto et al., 2009).

Prominent bands in sub-cluster C1 (mines 13 and 14) represented *Massilia* (20) and *Stenothophomonas* (23). *Massilia* was also present as a dominant genus in sub-clusters C2 and C4. This genus comprises 17 species often associated with solid waste landfill sites containing contaminants such as hydrocarbons, polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) (Perez-Leblic et al., 2010).

An uncultured Oxalobacteriaceae bacterium (3) was characteristic of sub-cluster C2 and all the representatives of this sub-cluster demonstrated a prominent band identified as *Listeria* (9). Interestingly, this genus was represented by three distinct band positions (4/5/9), although not the same intensity, suggesting three different species. It was also detected in soil from every mining site, except mines 7 and 9. The genus *Listeria* has eight species of which the major human pathogen is *L.*

monocytogenes, the causative agent of listeriosis, a serious infection caused by eating food contaminated with the bacteria. This disease affects primarily pregnant women, newborns and immune-compromised/suppressed individuals. *L. ivanovii* predominantly causes infections in ruminants, but has also been associated withh rare infections in humans (Low and Donachie, 1997; Ryser and Marth, 1999; McLauchlin et al., 2004; Schmid et al., 2005; den Bakker et al., 2010).

Prominent bands in the sub-cluster C2 (mines 8 and 3) represent the genus *Brevibacterium* (44) consisting of 28 known species reported to produce a variety of bacteriocins and antimicrobial substances inhibitory towards food borne pathogens such as *Staphylococcus aureus* and *Listeria monocytogenes* (Collins, 2006). Mining site 8 also contained *Geodermatophilus obscurus* (51), the only species in the genus *Geodermatophilus,* associated withh harsh environments, UV-C resistance and manganese oxidation (Ivanova et al., 2010). Sub-cluster C2 (mine 3) shows a prominent band representing the genus *Methylobacterium* (28), which has 37 species and this genus also formed part of the dominant profiles of sub-clusters C3 and C4, in mines 16 and 15, respectively. *Methylobacterium* are known as indicators of soil pollution and are able to break down polycyclic aromatic hydrocarbons (PAHs) to their less toxic metabolites (Andreoni et al., 2004).

Prominent bands in mine site 9 represent an uncultured/unknown bacterium (47) and the genera *Dactylosporangium* (46) which consist of nine species and are rare Actinobacteria often outnumbered by other bacteria in soil. They are important sources of industrially useful metabolites, notably antibiotics (Hayakawa et al., 1991). Mines 4 and 5 in sub-cluster C4 contained the genus *Rhodocyclus* (16) of which there are only two known species associated withh uranium repository sites (Islam et al., 2011). The soil from mine site 4 also presented the genus *Cellulomonas* (34)

which demonstrates multiple mechanisms of uranium immobilisation (Sivaswamy et al., 2011).

Finally, the sequencing data from the prominent bands in mining site 5 represent the extremely diverse group of bacteria from the genus *Bacillus* (12) of which there are 57 species - several of which synthesise important enzymes and antibiotics and others that cause an array of infections (McKillip, 2000; Haki and Rakshit, 2003). An uncultured/unidentified bacterium (10) was also present in the soil from this mine.

The diversity profiles and clustering did not show any correlation with geographical location of soil colour or any other parameter considered in this study. In fact, mining sites located literally right next to each other had significantly different bacterial community profiles.

6.4 Conclusions

The reported results produced a fundamental insight into the bacterial diversity in the geophagic soil from mining sites in the Qwa-Qwa region. This is, to our knowledge, the first report of bacterial community diversity in soil consumed by humans, determined by a culture independent technique. More living organisms are found in soil than in any other ecosystem and this data, therefore, merely scratched the surface of the true biological content which could also include fungi, protozoa, viruses, prions, Archaebacteria, parasitic worm eggs, etc.

Although this DNA based technique does not provide information on bacterial viability, it does present a snapshot of the community diversity and provides valuable information pertaining to environmental health and safety. Sequencing results suggested the presence of 19 different genera of bacteria as recognised or emerging

human pathogens, producers of beneficial products (enzymes, bacteriocins, antibiotics), indicators of pollution and candidates for biotechnology/bioremediation applications. These essential insights provide the platform for adjusting culturing strategies to isolate specific bacteria, further phylogenetic and functional analysis studies as well as microbial-mining prospect for bacterial species of possible economic importance. The presence of numerous unidentified/uncultured bacteria also leaves the door open for the isolation and characterisation of new species.

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Chapter 7

General Conclusions

7.1 Concluding remarks

Soil samples were collected from various popular geophagic mines in the Qwa-Qwa district. It was obvious that most of these mines were well known to the geophagic practising communities in Qwa-Qwa and were frequented for collection of geophagic soil, even by school children. Soil samples collected indicated a wide range of colours and textures, providing for the different preferences of various geophagists. Mines were often found to be located near houses and areas where pollution is obvious. It was also obvious that collection of soils was often done by bare hand and unclean utensils - sometimes mining utensils consisted of pieces of glass! Much research is necessary to ascertain the safety of practising geophagia, with regard to microbial contamination of soil of clay.

Although the microbes in geophagic soil may be harmless and even beneficial to humans, there are also serious risks in consuming soil contaminated with pathogenic bacteria. However, the prevalence and frequency of pathogenic bacteria in soil samples in the current study was relatively low, especially those often associated withh faecal contamination. Soil sold at the market was also less contaminated than the soil mined directly from the popular geophagic mining sites.

The colour of soil may be a contributing factor in survival of a microorganism. Geophagic practicing persons also have a preference to specific coloured soil or clay samples. It may, therefore, become necessary to educate geophagic practicing communities from rural areas with regard to the possible risks in consuming certain coloured soils or clays. However, in the current study no correlation was evident between soil colour or texture and recovering bacterial pathogens such as *Salmonella enteritidis* from a specific soil or clay type.

An attempt to report on the microbial diversity using a DNA based technique has succeeded in providing additional information on the bacterial content in the geophagic soil from mining sites in the Qwa-Qwa region. However, this technique does not provide information on bacterial viability, but did provide valuable information pertaining to environmental health and safety. Nineteen different genera were detected with this method, recognised or emerging human pathogens, or alternatively indicators of pollution and candidates for biotechnology/bioremediation applications. The presence of such numerous uncultured bacteria may provide new gateways for further research on the microbial aspects and importance of microbes in geophagic practicing.

7.2 Future Research

- The soil obtained from the various mines displayed a wide range of colours and it would be interesting to conduct further investigations on the preference of the local geophagists for a specific type of soil or clay from a specific mining site.
- Data on enteric infection in humans caused by contamination from soil is limited and need further investigation. There is also a need for the development of standardised methods for the detection of human pathogens from soil reservoirs.
- Assessment of diversity using RNA based methods to reflect microbial viability.
- Investigations on the influence of human handling on the bacterial diversity in soil sold by vendors.
- Investigating gene/plasma transfer into the ecosystem.

Appendix

Control Sample 1:

Control sample 1 is situated in Phahameng 2 m from mine 10. The mine surroundings are covered by natural grass and the area is neat. The miners use sticks to mine the soil (Figure A.1).

Figure A.1: Soil sample from control sample 1

Control 2: Control sample 2 is situated in Kgubetswana 30 cm from mine 11.

Figure A.2: Soil sample from control sample 2

Control 3: Control sample 3 is situated in Kgubetswana 50 cm from mine 11.

Figure A.3: Soil sample from control sample 3

Control 4: Control sample 4 is situated in Mangaung 100 cm from mine 15.

Figure A.4: Soil sample from control sample 4

Control 5: Control sample 5 is situated in Madikwe 1 m from mine 16.

Figure A.5: Soil sample from control sample 5