

The identification of biomarkers to assist in the hydrological characterisation of a chromium polluted mine

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“Water is the driving force of all nature.” Leonardo da Vinci

SUMMARY

Chromium is used in many processing applications, which has led to the formation of chromium(VI) waste. Cr(VI) is an unstable, mobile carcinogen, which is interchangeable with Cr(III) under certain environmental conditions. Management of this waste, however, is often not considered. Mine under investigation is an example of such historic mismanagement. During the second World War, Cr(VI) waste was transferred to the study site from areas where leather products were made for the war effort. This waste was not managed appropriately in the past and with time Cr(VI) leached into the groundwater and possibly surface water resources. As these water resources are used for domestic and agricultural water supply, this may have serious effects on the human and environmental health in the area.

Some of the major Cr(VI) effects on human and animal health include malignant tumours, skin irritation, respiratory and reproductive system damage. Lung cancer is of concern when Cr(VI) is inhaled and stomach tumours occur when this chemical pollutant is ingested. The effects of Cr(VI) on plants include the disruption of shoot and root elongation, and if it accumulates enough within the plant can cause ingested health problems for humans and animals.

Water quality guidelines state that the Target Water Quality Range for Cr(VI) in drinking water should not exceed 0.05 mg/L. Prolonged exposure of values higher than this target value has adverse health effects and may result in cancer. The study site has a scarcity in water sources and therefore requires good quality water resources.

This study aims to identify and use biomarkers to assist in the hydrological characterization of the mine. Available mitigation options can be implemented once it is known how the water in the area moves and distributes Cr(VI) pollution. A biomarker is an indicator of a biological state, which in turn can be used to assist in characterizing the chemical conditions of the sub-surface. Bacteria can aid as environmental biomarkers as they are sensitive and specific to the environmental conditions in which they flourish. In this way they give a good indication of the environmental condition and any possible pollution. Due to the fact that ground- and surface water are integrated resources, it is likely that if one is impacted by pollution, it will indirectly impact the other one. Therefore, the biomarkers identified can be used to characterize water pollutants that are present in ground- and some surface water resources.

A description of the study site is provided, wherein the climate, elevation, geology, land use, geohydrology, hydrochemistry and surface water are documented. These factors help to identify and clarify the sources and pathways that water and the pollution would follow.

Ten water samples, from surface and groundwater, were obtained in two separate sampling opportunities. The first analysis of the water samples included the determination of the chemical constituents. Two of these constituents analyzed were the total Cr and individual Cr(VI) levels. Six water samples had excessively high Cr values (exceeding the Water Quality Target Range of drinking water and water agricultural use). These values ranged from 0.1 – 3.9 mg/L. The 6 samples with excessive Cr(VI) values were used for the microbial analyses.

The microbial analyses consisted of DAPI (4',6-diamidino-2-phenylindole) staining, for cell enumeration, and molecular analyses. The molecular analyses included polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE) and sequencing applications concluded in the laboratory. Fifteen bands, representing different organisms, were removed from the DGGE polyacrylamide gel and processed for sequencing.

The organisms that were present in the sample were uncultured Cyanobacterium, *Sediminibacterium salmoneum*, uncultured Bacteroidetes bacterium, uncultured beta-proteobacterium, uncultured actinobacterium, uncultured Rhodocyclaceae, uncultured Chloroflexi bacterium and uncultured delta-proteobacterium. According to literature most of these organisms may adapt the ability to either reduce Cr(VI) or resist any effect of Cr(VI) in the environment. Two of the bands were highly unidentified organisms, which means that these organisms have not yet been cultured or identified in any sense. The reason for this is that most microorganisms have not yet been documented. This also makes it difficult to identify the exact bacterial strain present within the samples. The deoxyribonucleic acid (DNA) sequences of the different organisms were very different from each other, when compared by a dendrogram. This means that there was a diverse community present within the samples.

Electrical conductivity profiles were conducted in the monitoring boreholes to identify possible fracture positions. The total chromium and chromium(VI) levels were documented and compared.

Other chemical factors were analysed and those of high value, such as chloride, nitrate and chromium measurements, were used for statistical analyses and comparison with the biomarkers present in the sample. A positive correlation was found between the sample sites and the organisms present within each.

It was noted that different communities have different metabolic activities related to susceptibility and will therefore differ under specific environmental conditions. The microorganisms that were present in the 6 water samples all have the ability to either resist or reduce Cr(VI). This means that in Cr(VI) polluted areas they are more likely to flourish than organisms that do not possess this ability. Such susceptible, non-resistant organisms would otherwise occur naturally in a non-polluted environment. From the obtained results it was noted that microorganisms could aid as biomarkers when determining the environmental condition (with respect to Cr(VI) pollution). The bacteria analysed in the samples all indicate a level of chromium pollution, and aided in the determination of pollution sources. These biomarkers can therefore be used to determine the location of other chromium deposits not yet located.

Keywords: *Chromium(VI); groundwater; surface water; Target Water Quality Range; biomarkers; pollution; conceptual model; environmental condition; microorganisms; PCR; DGGE; electrical conductivity; bioremediation*

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LIST OF ABBREVIATIONS

$(\text{MgFe})_2\text{SiO}_4$	Magnesium iron silicate/ Olivine
A	Effective area of filtration
a	Reticule area
A260	Light absorbance at 260 nm
A280	Light absorbance at 280 nm
BLAST	Basic Local Alignment Search Tool
BOD	Biological oxygen demand
bp	Base pair
Ca	Calcium
CaCO_3	Calcium carbonate
$\text{CaMg}(\text{CO}_3)_2$	Calcium magnesium carbonate
Cl	Chloride
COD	Chemical oxygen demand
Cr	Chromium
Cr(III)	Trivalent chromium
Cr(VI)	Hexavalent chromium
CrO_4	Chromate
d	Dilution factor
DAPI	4',6-diamidino-2-phenylindole
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid

dNTPs	Deoxyribonucleotide triphosphates
EC	Electrical conductivity
Fe ₃ O ₂	Magnetite
GC	Guanine and Cytosine
gDNA	Genomic DNA
GIS	Geographic Information System
H ₂ O	Water
H ₂ S	Hydrogen sulphide
ha	Hectares
Mg	Magnesium
n	Number of cells per ml
Na	Sodium
NGA	National Groundwater Archive
NH ₄	Ammonium
NO ₃	Nitrate
nr/nt	Non-redundant database
PCA	Principle component analysis
PCR	Polymerase chain reaction
rDNA	Ribosomal deoxyribonucleic acid
Rf	An indication of the relative migration of bands of a DGGE
rRNA	Ribosomal ribonucleic acid
SANS	South African National Standards
SiO ₄	Silicate
SOP	Standard operating procedure

TAE	Tris Acetate EDTA
TDS	Total Dissolved Solids
TWQR	Target Water Quality Range
UFS	University of the Free State
UPGMA	Unweighted Pair Group Method with Arithmetic Mean

UNITS OF MEASUREMENT

°C	Degrees Celsius
μl	Microliter
μl/l	Microliter per litre
μm	Micron
km	Kilometre
m	Metres
M	Mole
Ma	Mega Annum
mamsl	Metres above mean sea level
mbgl	Metres below ground level
meq	Milli-equivalents
mg/L	Milligram per litre
ml	Millilitre
mm	Millimetre
mm/a	Millilitre per annum
Mm ³ /a	Thousand square metres per annum
mS/m	Milli-Siemens per meter
ng	Nanogram
nm	Nanometre
pH	A measurement of the hydrogen ion concentration
V	Volt

v/v

Volume per volume

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1 INTRODUCTION

1.1 BACKGROUND

In South Africa, the use of chromium in processing applications has led to the formation of chromium(VI) waste. Chromium(VI) and chromium(III) are used for chrome plating, dyes and pigments, leather tanning, and wood preserving. Unfortunately, in many instances, the management of this waste has been neglected. The mine under investigation (which is not named in this dissertation and is referred to as the study area, due to legal reasons) is an example of such historic mismanagement. During World War II, chromium(VI) waste was transferred there (mainly from Port Elizabeth) where leather products were made for the war effort. It was felt at the time that the chromium(VI) would be harmless when capped and placed in marble settings, hence the storage of this waste at a marble mine in Limpopo. With time this pollutant has leached into the groundwater and possibly surface water resources. These water resources are being used for domestic water supply and agriculture. It is now known that chromium(VI) can cause serious health problems in humans and animals (ATSDR, 2012), which include the following:

- Sperm damage and damage to the male reproductive system have been detected in laboratory animals exposed to chromium(VI).
- Skin contact with certain chromium(VI) compounds can cause skin ulcers. Allergic reactions consisting of severe redness and swelling of the skin have been noted.
- Breathing high levels of chromium(VI) can cause irritation to the lining of the nose, nose ulcers, runny nose, and breathing problems, such as asthma, coughing, shortness of breath, or wheezing.
- The main health problems seen in animals following ingestion of chromium(VI) compounds are irritation and ulcers in the stomach and small intestine and anemia.
- Inhalation of chromium(VI) has been shown to cause lung cancer in humans and animals. An increase in stomach tumors was observed in both humans and animals exposed to chromium(VI) contaminated drinking water.

1.2 PROBLEM IDENTIFICATION:

The current extent of the chromium(VI) pollution on site and in the vicinity of the site is unknown. However, there can be serious risks for humans and animals exposed to chromium(VI). As the water in the area is scarce it is imperative to ensure that it is suitable for use. It is important that these risks are identified and minimized to ensure the health and well-being of the community.

1.3 RESEARCH AIMS AND OBJECTIVES

This study aims to identify and use biomarkers to assist in the hydrological characterization of the mine. Once these sources and pathways are identified mitigation options, such as relevant bioreactors, can be implemented. A biomarker is an indicator of a biological state which in turn can be used to assist in characterizing the chemical conditions of the sub-surface water resources. However, it is important to note that surface water and groundwater are an integrated resource; therefore, it is likely that if one is impacted by pollution, it will indirectly impact the other one. Consequently, the biomarker can also be used to characterize pollutants that are present in some surface water resources. In order to achieve the aims stated above the following actions were completed:

- A literature survey of all relevant literature related to the proposed study.
- Collection and interpretation of all existing information influencing water conditions on site. This information included rainfall data, surface water flow and quality, groundwater levels and quality, infrastructure, geological conditions, and land use.
- Gaps in the existing data were identified and field studies were conducted to collect the relevant datasets.
- Laboratory work was conducted to identify biomarkers.
- The results of the biomarker assessment were validated by a hydrochemistry investigation.
- The results thereof were interpreted and conclusions were drawn.

1.4 LAYOUT OF DOCUMENT

This document consists of 9 sections which include the following:

- Section 1 comprises of the background of the study area, aims of this thesis and the layout of the document.
- Section 2 contains literature on the methods used during the study, as well as a detailed explanation of the problem statement.
- Section 3 describes the location, climate, geology, topography and drainage, and land cover of the study site.
- Section 4 describes the analytical methods used in the determination of the water quality on site and for the detection of a biomarker.
- Section 5 discusses the results obtained during the microbial analyses. Organisms that were present in the water samples were identified and discussed.
- The hydrochemistry and geohydrology of the study site are defined in Section 6. This is necessary as it aids in the understanding of the sources and pathways of the chromium pollution in the area.
- In Section 7 the hydrochemistry data is compared with the microbiological data.
- In Section 8 the conclusions and recommendations are drawn.
- Section 9 contains the references.

2 LITERATURE SURVEY

2.1 CARCINOGENIC METALS IN THE ENVIRONMENT

Many carcinogenic metals that are known to occur in the environment have immense adverse effects on the health of humans and animals. Some examples of such metals include arsenic, cadmium, nickel, beryllium, uranium and chromium (Guan *et al.*, 2010; Straif *et al.*, 2009). Numerous sources are responsible for these metals entering the environment. These sources include biogeochemical cycles, which occur naturally in the environment, and the metals from anthropogenic sources, such as industrial effluents, landfill leachate, and storm water runoff. The bioavailability and mobility of metals depends on the distribution mechanism of metal ions between the solid and fluid phases. Metals can be remobilized as a result of certain changes in the environment (for instance changes in pH or redox potential), which poses a great environmental risk (Trujillo-Cárdenas *et al.*, 2010).

Various metals that can occur in an abnormal oxidation state, with regards to the human body, have the possibility of becoming toxic. Chromium is such a metal, where chromium(III) is a trace element required by the body for lipid and carbohydrate metabolism, while chromium(VI) is a carcinogen (Fawcett, 1988).

Chromium exists in many oxidation states, from Cr(-II) to Cr(VI). From these, the most predominant states that occur naturally are the non-toxic trivalent (III) and toxic hexavalent (VI) chromium. Cr(III) has low solubility and good stability in slightly acidic, naturally occurring environments, rendering it immobile. Cr(VI), on the other hand, is extremely mobile and reactive (Barnhart, 1997; Dogan *et al.*, 2011). For the most part Cr(III) is the most dominant species. The pH and the oxidative properties of the environment determine the ratio between Cr(III) and Cr(VI) in the environment (Kotaś & Stasicka, 2000). Cr(VI) is predominant in groundwater when the oxidizing conditions are high and the pH is above 7 (alkaline conditions) (SWRCB, 2011). It is for this reason that more than 75% of the total chromium in groundwater of certain contaminated areas is Cr(VI) (Gonzalez *et al.*, 2005). By reducing Cr(VI) to Cr(III) the toxic properties and extreme mobility of Cr(VI) can be diminished (Dogan *et al.*, 2011). Cr(VI) is reduced to Cr(III) in the presence of organic matter, sulphide, ferrous iron, and by certain bacterial strains (Nkhalambayausi-Chirwa & Wang, 2004; SWRCB, 2011).

There is a wide variety where the applications of chromium are concerned, with the major utilization factor being the chemistry of chromium (Barnhart, 1997). Industrial activities dependent on the chemistry of chromium include chrome plating (which aids in protection of other metals), the treatment of wood with chromate copper arsenate, and the tanning of leather (Barnhart, 1997). Chromite is the form in which chromium is mined. South Africa produces approximately two-fifths of the world's chromite ores and concentrates (Papp, 2009).

2.2 EFFECTS OF HEXAVALENT CHROMIUM ON HUMAN HEALTH

It is well known that Cr(VI) is, to some extent, converted to Cr(III) in the stomach of humans after consumption. This is due to the acidic environment of the stomach. It is for this reason that many scientists in the past stated that the ingestion of Cr(VI) is harmless (Sedman *et al.*, 2006). Before the mid-1980s however, there was no evidence that indicated the stomach's reduction capacity. This left a great deal of uncertainty with regards to the risk involved with contaminated drinking water (Sedman *et al.*, 2006, Paustenbach *et al.*, 2003;).

Studies focused on toxicokinetics and genotoxicity have revealed that a fraction of an ingested dosage of Cr(VI) is absorbed and taken up by the cells of numerous tissues, resulting in the damage of DNA. Oral exposure to Cr(VI) appears to pose a carcinogenic risk. This statement has been proven by the increased statistical amount of stomach tumours in humans and animals, and the mechanistic, genotoxic and toxicokinetic data obtained in studies (Sedman *et al.*, 2006). Additionally, the reduction of Cr(VI) to Cr(III) in biological systems generates free radicals. These free radicals have the ability to form complexes with intracellular target areas and may result in numerous DNA modifications and enhancement of lipid peroxidation (ATSDR, 2008; Flora, 2009).

Cr(VI) has been studied extensively due to its carcinogenic potential. The exact mechanisms of the carcinogenic activity, however, are not quite clear (Liu *et al.*, 2001; Sedman *et al.*, 2006). Cr(VI) mimics sulphate and phosphate salts, as it occurs naturally as a tetrahedral anion in the form of chromate. This means it is taken up readily by cells via active transport systems (Sedman *et al.*, 2006). A wide range of DNA damage is induced by Cr(VI) after it has been taken up by cells, which include DNA-DNA crosslinks, DNA adducts, mutations, abasic sites, micronuclei, DNA strand breaks, sister chromatid exchanges, chromosomal aberrations, and DNA-protein crosslinks (Holmes *et al.*, 2008; Mattagajasingh & Misra, 1996; Sedman *et al.*, 2006).

Some of the non-cancer effects of Cr(VI) compounds on humans and animals are common in the respiratory, hematological, gastrointestinal, and reproductive (particularly male reproduction) systems. Allergic contact dermatitis occurs from dermal exposure, resulting in an immunological response. Severe cardiovascular, respiratory, hematological, hepatic, gastrointestinal, renal, and neurological effects have been documented in patients after ingestion of incredibly high doses of Cr(VI), many of which lead to the death of the patients (ATSDR, 2008).

2.3 SOUTH AFRICAN WATER QUALITY GUIDELINES

According to the South African Water Quality guidelines (1996:a) Cr(VI) is one of the acutely toxic constituents. It has acute and irreversible effects at low concentrations on human health and water with concentrations above the Target Water Quality Range, or TWQR, should not be used (Department of Water Affairs & Forestry, 1996:a). The effects of various Cr(VI) concentration ranges on human health are stated in Table 2.1. These criteria were used as the standards for domestic water use. The risk levels that occur after oral intake of Cr(VI) require additional adequate experimental evidence, the criteria in Table 2.1 should therefore be considered as provisional.

Table 2.1: Effects of chromium(vi) on human health

Chromium(VI) Range (mg/L)	Effects
<i>Target Water Quality Range</i> <i>0 – 0.050</i>	<i>Risk of cancer induction insignificant</i> <i>No toxic or aesthetic effects</i>
As a precautionary measure it is recommended that the TWQR not be exceeded due to the potentially acute and/or irreversible effects of chromium(VI) on human health	
0.05 – 1.0	Possible risk of induction of gastrointestinal cancer following long-term exposure
1.0 – 5.0	Undesirable taste, slight nausea, and increasing risk of cancer induction
>5.0	Risk of acute toxicity

Table 2.1 is modified from the Department of Water Affairs & Forestry (Volume 1: Domestic use, 1996:a).

The South African National Standards (SANS) 241:1 (2011), as published by SABS, list chromium as a micro-chemical determinand that affects chronic health. This means that if it is consumed at concentrations beyond the quantified limits over a prolonged period, it causes a severe health risk. The standard limits of chromium in drinking water, as documented by SANS 241:1 (2011) is $\leq 50 \mu\text{L/L}$. This value is in accordance with the target water quality range for chromium(VI) specified by the Department of water Affairs & Forestry (1996:a) in Table 2.1, which has a maximum acceptable value of 0.05 mg/L, or 50 $\mu\text{L/L}$.

The World Health Organization, or WHO, (1996) documented international Cr(VI) standards in drinking water. The provisional guideline value WHO (1996) recommends is 0.05 mg/L. No additional toxicological data have indicated any need to change this value. This is consistent with the information from the Department of Water Affairs and Forestry (1996:a) and the more recent SABS (2011). This value was decided upon by WHO (1996) as no significant health risks were found with humans or animals exposed to Cr(VI) concentrations of 0.05 mg/L or less in drinking water.

Volume 3 of the South African Water Quality Guidelines (Industrial use) as issued by the Department of Water Affairs & Forestry (1996:b) does not mention any standard quantifiable measure of Cr(VI) for industries. This is of great importance where occupational exposure is of concern (United States Department of Labor, 2012). This indicates a shortcoming in the volume as many industries have waste containing Cr(VI) that should be measured. As mentioned, industrial sources play a role in Cr(VI) contamination of the environment (Barnhart, 1997).

The fourth volume of the South African Water Quality Guidelines (Agriculture use: Irrigation) as issued by the Department of Water Affairs & Forestry (1996:c) states that Cr(VI) is toxic to plants at high concentrations. Constant application of chromium to a source is strongly absorbed by soil and it accumulates in the surface layer. Cr(VI) however, is more mobile and leaches through to the underlying groundwater. It has been proven that a decrease in seed germination and root and shoot elongation occurs as the concentrations of the metal increases (Peralta *et al.*, 2001). The Department of Water Affairs and Forestry (1996:c) uses various norms for the effects of chromium on irrigation water. These norms are indicated in Table 2.2.

Table 2.2: Effects of chromium on irrigation water

Irrigation water uses	Norms for measuring water quality effects
Application to commercial crops	Crop yield as affected by crop sensitivity to chromium uptake through plant roots Crop quality as determined by chromium toxicity to consumers
Application to sustain suitability of irrigated soil	Accumulation in soil to concentrations where either crop yield or crop quality is affected
Maintenance of irrigation equipment	No known effects

Table 2.2 is modified from the Department of Water Affairs & Forestry (Volume 4: Agriculture use: Irrigation, 1996:c).

According to the Department of Water Affairs & Forestry (1996:c, vol 4) the toxic limit in soils for Cr(VI) ranges from 5 – 500 mg/kg and 50 – 5000 mg/kg for Cr(III). These values are as such due to the fact that Cr(VI) is mobile and therefore more available to plants in comparison to Cr(III) (Dogan *et al.*, 2011).

2.4 MICROBIAL REDUCTION OF CR(VI)

As previously mentioned, the highest oxidation state in which chromium occurs is Cr(VI) and is prevalent in nature as chromate (CrO_4^{2-}) (Chen & Hao, 1998; Dogan *et al.*, 2011). Cr(VI) has inhibitory consequences on biological water treatment methods. However, it has been proven that various microorganisms have the ability to reduce Cr(VI) within a broad variety of the metal's concentrations. Many environmental factors have an effect on microbial Cr(VI) resistance and reduction, some of which include pH, biomass and Cr(VI) concentration, temperature and oxidation-reduction potential (Chen & Hao, 1998; Fernández *et al.*, 2011; Silva *et al.*, 2009).

Bioremediation methods involve the metabolization of organic waste into nontoxic compounds, and finally carbon dioxide and water. This occurs as a result of the chemical oxygen demand (COD) and biological oxygen demand (BOD) of the microorganisms.

Metals can only be taken out of the environment biologically by sorption, precipitation, uptake, or valence state changes. Different bacterial strains have the ability to reduce Cr(VI) either aerobically or anaerobically. The reduction of Cr(VI) by bacteria that are known aerobes, such as *Thermus scotoductus*, is linked with soluble proteins and NADH, which acts as an electron donor (Opperman & Van Heerden, 2007). Cr(VI) reduction by anaerobic bacteria, such as *Enterobacter cloacae*, occurs as a result of the facilitation by the chromate reductase enzyme (soluble or membrane-bound) (Lovley, 1993; Opperman & Van Heerden, 2007). This bio-reaction is based on chromate acting as the final electron acceptor for the bacteria's biological system in the absence of oxygen (Lovley, 1993; Wang & Xiao, 1995). The ability of microorganisms to reduce Cr(VI) is usually plasmid- or membrane-associated (Chen & Hau, 1998; Mangaiyarkarasi *et al.*, 2011). Cr(VI) reduction is usually completed intracellularly by enzymes (reductases), which are located either in particulate fraction or in soluble fraction (Mangaiyarkarasi *et al.*, 2011; Opperman & Van Heerden, 2007; Wang & Xiao, 1995).

The remediation of Cr(VI) polluted areas require innovative technologies that are eco-friendly and of low cost. These methods can be used for the elimination of toxic heavy metals from water sources, rather than the accustomed physical-chemical methods (Chen & Hao, 1998; Fernández *et al.*, 2011). Such conventional methods include coagulation, precipitation, cementation, ion exchange, reverse osmosis, electro-winning, electro-dialysis, evaporation recovery, and membrane technologies (Mack *et al.*, 2004; Zahoor & Rehman, 2009). These methods are expensive and have several limitations, such as high energy necessities, deficient metal removal, and production of toxic sludge and other waste, making the more cost-effective, bioremediation technologies a requirement (Silva *et al.*, 2009). This is one of the reasons that the study of microorganisms that reduce Cr(VI) in alkaline conditions is of great importance for bioremediation, as Cr(VI) polluted environments have high pH ranges (Mangaiyarkarsi *et al.*, 2011).

According to the US Environmental Protection Agency or EPA (2012) a bioreactor is the biological degradation of organic waste by adding essential nutrients for the enhancement of microbial activity. In the case of an aerobic bioreactor oxygen is also added. Bacterial isolates that are proven to have the ability to reduce Cr(VI), such as *Thermus scotoductus* and *Enterobacter cloacae*, can be used for the bioremediation and elimination of Cr(VI) in polluted areas in well managed bioreactors (Macket *et al.*, 2004; Zahoor & Rehman, 2008). Such species include *Bacillus* sp. JDM-2-1 and *Staphylococcus capitis* (Zahoor & Rehman, 2009).

Measurable molecular, cellular or biochemical levels occur in the environment as a result of certain biological states. These measurable levels are called biomarkers and differ from organisms in different environments. The populations of organisms that occur in a certain environment can be determined by the use of biomarkers, such as lipids or fatty acids (Peters *et al.*, 2005). They can be used to determine environmental pollution and/or the response of the organism to the environmental condition (McCarthy & Shugart, 2008; Peakall, 1994). Biomarkers are of great significance as they aid in the detection and quantification of chemical compounds that occur in the environment. These biomarkers also play a role in the determination of possible human health hazards that are as a result of environmental pollutants (McCarthy & Shugart, 2008).

Molecular biological techniques are the most commonly used methods for the determination of the bacterial composition in environmental samples. These methods are culture-independent and allow analyses to be conducted directly from the samples (Ranjard *et al.*, 2000; Satokari *et al.*, 2001). The type of bacteria present in the environmental samples can give a good indication of the environmental conditions. Due to the fact that each community has its own specific activity arrangement, the composition of the microorganisms in the environment can be determined, which in turn may indicate the environmental conditions. In environmental samples, especially water samples, the microorganisms are indicative of certain environmental parameters (Kölbel-Boelke *et al.*, 1988; Ranjard *et al.*, 2000). For example, various microorganisms can be used as indicators of numerous types of pollution (Geldreich & Kenner, 1969; Selvin *et al.*, 2009; Whittenbury, 1964). When the environment is in a healthy, natural condition there are specific communities that flourish. Microorganisms are either very susceptible or resilient to changes in their habitat conditions, which brings about the reason for community changes due to environmental changes and resulting diversification. Different communities have different metabolic activities related to susceptibility and alternating species diversity of the community with regards to environmental stress (Aslan-Yilmaz *et al.*, 2004; Bloem & Breure, 2003; Roane & Kellogg, 1996).

Microorganisms can aid as a good biomarker in determining the condition of the environment. This is especially applicable where rehabilitation of an area is concerned.

Determining the microbial composition of that area will yield more accurate results when remediation is of concern (Aslan-Yilmaz *et al.*, 2004; Bloem & Breure, 2003).

Yarsan & Yipel (2013) states that the determination of aquatic organisms, such as algae and bacteria, in polluted water sources give a better indication of chemical pollution in those water sources. This study revealed that the mere analysis of the chemical constituents does not adequately reflect the extent of the pollution, nor its effect on the surrounding environment. The chemical analysis together with the determination of the bio-indicator organism (microbial species) may reflect the condition of the environment more accurately (Yarsan & Yipel, 2013). Although this study was published in 2013, the use of microbial indicators has been used to determine water quality for many years (Ashbolt *et al.*, 2001).

Microbes can be analysed to determine a variety of water pollutants. Although there is no universal bacterial indicator, specific strains may depict certain pollutants in water sources (Ashbolt *et al.*, 2001). A more accurate analysis of pollutants present in water sources may be ensured by the determination of these specific strains, which are dependent on the pollutant parameter in the water source.

2.6 MOLECULAR TECHNIQUES

2.6.1 POLYMERASE CHAIN REACTION

The polymerase chain reaction (PCR) is a molecular biological technique that amplifies a particular region of a strand of DNA and generates extensive amounts of replications of that target DNA sequence (Cheng *et al.*, 1994). Thermal cycling is the manner in which PCR commences and consists of 20-40 steps of heating and cooling. There are usually three temperature steps for each cycle. There are a number of parameters that determine the lengths and temperatures of each cycle, such as enzymes and melting temperatures of the primers (Rychlik *et al.*, 1990). The first step is the initialization step, which heats the reaction to approximately 95°C for 10 minutes. It is only used when a heat activation step is required by the DNA polymerases (Sharkey *et al.*, 1994). The initialization step is followed by the denaturing step, which causes melting of the DNA template and produces single-stranded DNA; the annealing step, where temperature decreases to permit the annealing of the single-stranded primers to the DNA templates; and the extension step, where a replicate DNA strand of the DNA template is synthesized by the addition of dNTPs (deoxynucleotide

triphosphates) from the 5' to 3' direction. The basic steps can be viewed in Figure 2.1. A final extension step is often included to ensure that all single-stranded DNA is completely elongated (Fankhauser, 2012; Sharkey *et al.*, 1994).

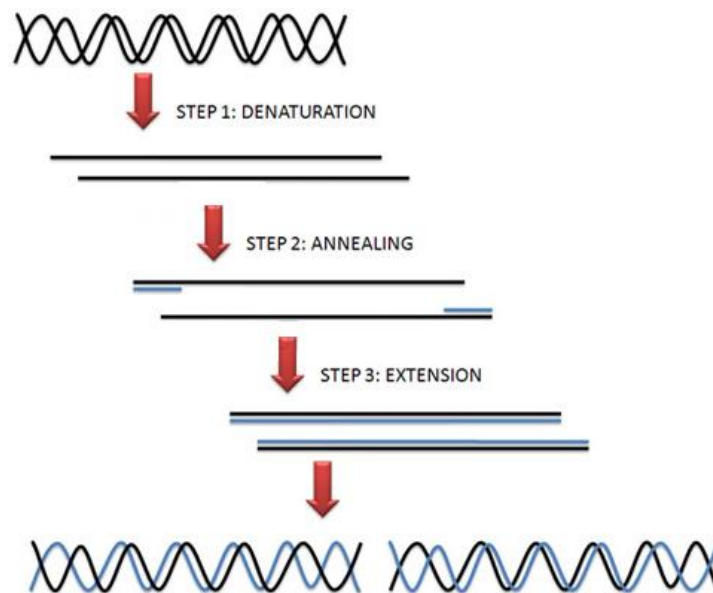


Figure 2.1: A simplistic example of the PCR cycle steps (Molecular Station, 2011)

The setup of a PCR comprises of a few reagents, which include the two single-stranded primers (forward and reverse) that bind to the template and initiate DNA replication; DNA polymerase (usually *Taq* polymerase); dNTPs for DNA synthesis (the DNA building blocks); a buffer solution to maintain an optimum environment for the reaction; magnesium or similar cations that aid as catalyst for the reaction; and the DNA template (Pavlov *et al.*, 2004).

rRNA (ribosomal RNA) and its encoding genes are one of the most broadly used methods in microbial ecology (Satokari *et al.*, 2001). Sequence specific primers target specific genes which can be used for the identification of organisms in a community. A fragment of rDNA is amplified when using a universal bacterial primer (16S primer). Various fragments generated by PCR can then be separated using denaturing gradient gel electrophoresis (DGGE) which separates the PCR products in a sequence specific manner. The DGGE profile will then characterize the protuberant bacteria in the community (Satokari *et al.*, 2001).

2.6.2 DENATURING GRADIENT GEL ELECTROPHORESIS

Denaturing gradient gel electrophoresis (DGGE) is a technique that separates rDNA fragments based on their base-pair sequences (even though they may have the same length). DGGE is used to determine the microbial composition and diversity of an environmental sample. The separation is based on the linear gradient of the denaturing agent across a polyacrylamide gel (Ranjard *et al.*, 2000). The DNA molecule partially melts in the gel and thus decreases its electrophoretic mobility. The fragments melt as they move down the gel gradient at sequences termed the melting domains. The fragment completely stops once the melting temperature of the melting domain with the lowermost melting temperature has been reached. It is the differences in the sequences within the different melting domains of the fragments that result in fragments halting at different positions in the gel. The different fragments are thus separated from each other based on their sequence. The separation is primarily based on the GC content and distribution within the microbial DNA (Hovda *et al.*, 2007). GC rich sequences have a high melting temperature and will migrate further down a loaded polyacrylamide gel gradient. GC clamps aid in the identification of small differences between bases in DNA sequences during DGGE, as the DNA can move further down the polyacrylamide gel (Wu *et al.*, 1999). Schwieger and Tebbe (1998) found that techniques such as DGGE require a PCR primer with high GC content (a GC clamp). The reason for this is to prevent total strand separation which may result during electrophoresis. This is especially important in community analyses, as such analyses may be meaningless if annealing occurs between similar, yet not identical, DNA strands which originated from different organisms.

Some of the samples contained some microorganisms that were the same. This is due to the conditions of the water body, and can be used for further interpretation.

The DNA fragments must be amplified with PCR before DGGE is performed. This technique can be used for many sequence based analyses, such as community composition determination, mutation analyses, determination of the presence of an organism in a sample, and the characterization of community structures (Muyzer *et al.*, 1993; Janse *et al.*, 2004, Motta *et al.*, 2006). DGGE has provided microbial ecology with a molecular fingerprinting technique that enables the study of the dynamics of microbes in complex environments (Chang *et al.*, 2008).

Some advantages of DGGE include its sensitivity to a variety of DNA sequences; many samples can be analysed at the same time; it can be applied to functional as well as phylogenetic genes; it allows the usage of universal primers; and changes in community structure can easily be detected (Patrinos & Ansorge, 2010). DGGE has its disadvantages as well. DGGE is quite time consuming; a large number of bands from diverse communities may look smeared on the gel; the rRNA encoding genes may result in a few bands within certain species, which may give rise to an overestimation of community diversity; PCR biases and introduction of contaminants; and it is not easy to reproduce the obtained results (Patrinos & Ansorge, 2010).

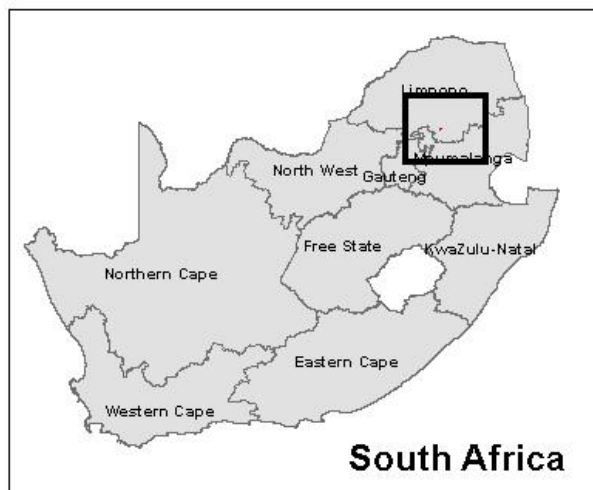
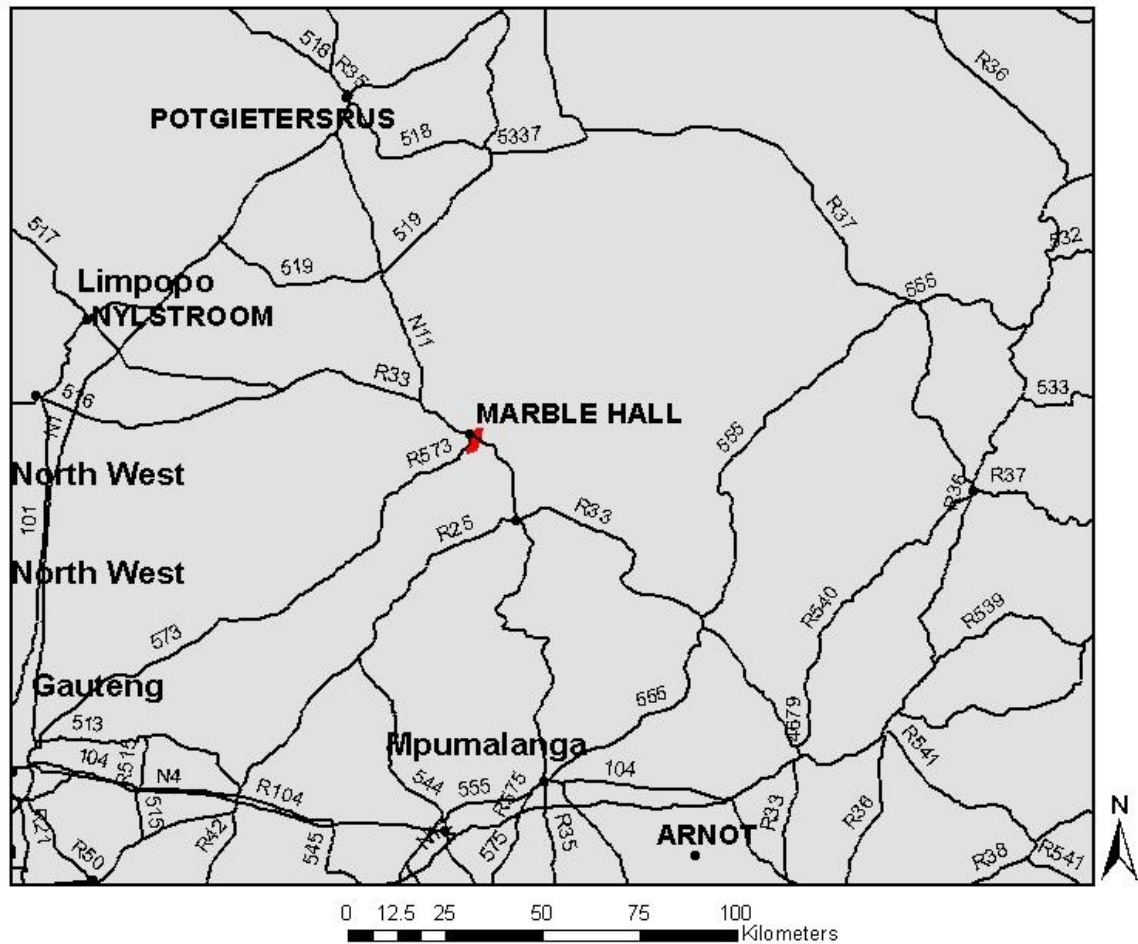
3 CASE STUDY OF THE AREA UNDER INVESTIGATION

3.1 LOCATION OF STUDY SITE

The study area is located in the Limpopo Province in South Africa. The National Road, the N11, runs to the south of the area. There are several rural villages in the vicinity of the area including Moganyaka and Manapsaue to the east of the site. Groblersdal is located 26 km southeast of the site.

Figure 3.1 illustrates the location of the study area in South Africa.

Location map of the study area in South Africa



Legend

- Towns
- Mainroads
- Study Area

Figure 3.1: The location of the study area in South Africa

3.2 HYDROLOGY

The study area is located between the Elands River and the Olifants River in a sub-catchment in the quaternary drainage region B31J, which forms part of the Olifants River catchment area. This area is east of the Middle Olifants dolomite (within the Chuniespoort Group) (eWISA, 2013; Department of Water Affairs, 2011:a).

Figure 3.2 illustrates the location of the Olifants River Basin in South Africa, as documented by the Water Resources of South Africa, 2005.

The Olifants River Basin

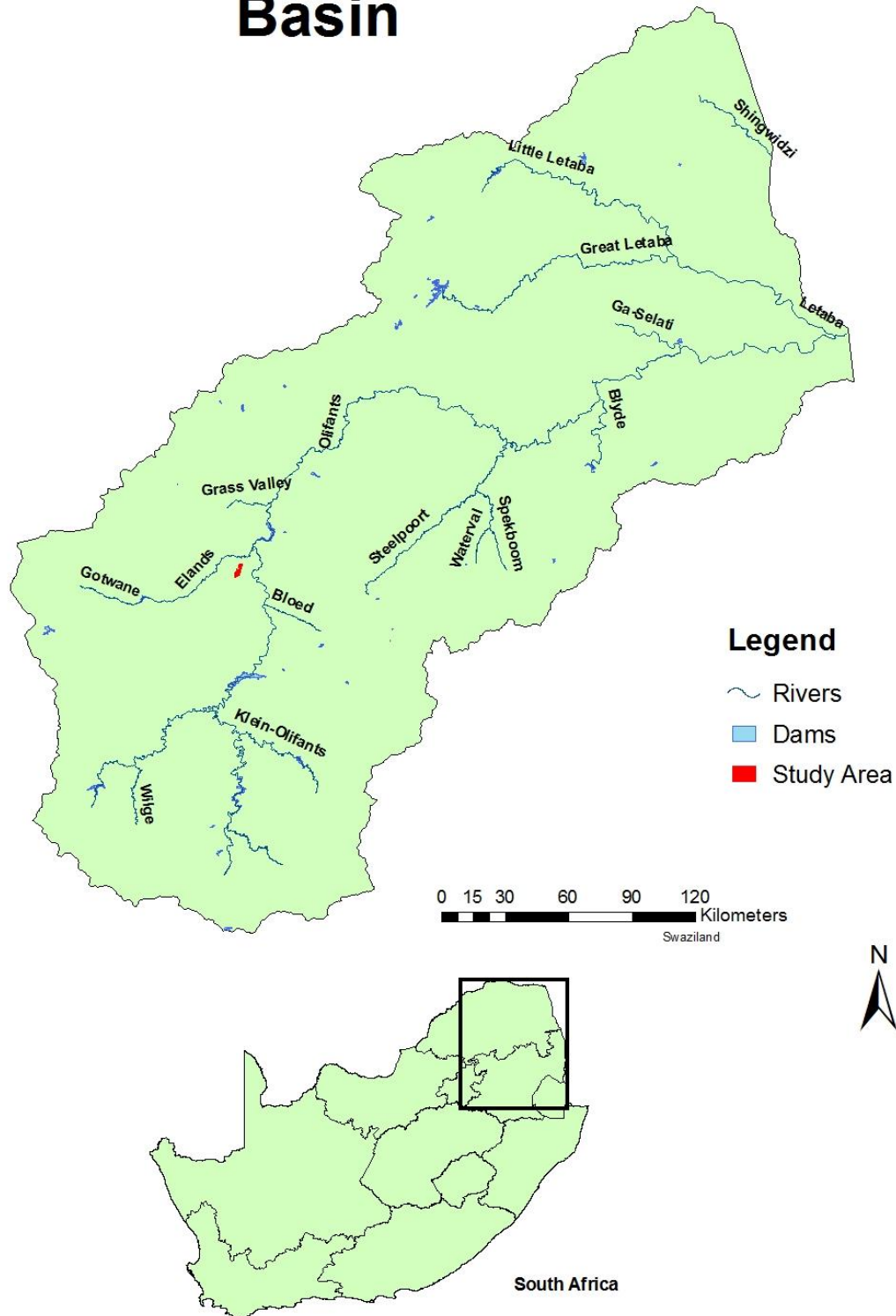


Figure 3.2: The location of the Olifants River Basin in South Africa (WR2005)

The temperature of study area is relatively cool in comparison with the rest of the basin, as it is located in the Highveld. Summer temperatures (September – March) reach average maximum temperatures of 30 – 34°C and average minimum temperatures of 18 – 22°C. Winter temperatures reach average maximum temperatures of 22 – 26°C and average minimum temperatures of 5 – 10°C (IWMI, 2008). The topography surrounding study area is relatively flat with lightly sloped hills. These slight slopes vary between 900 and 920 m above sea level (Department of Water Affairs, 2011:a; Infrasons Holdings Limited, 2012). Figure 3.3 indicates the topography and drainage of the study area. The water drains from higher regions to the lower regions. This has an effect on the movement of water, and therefore any pollution that may be present in the water sources.

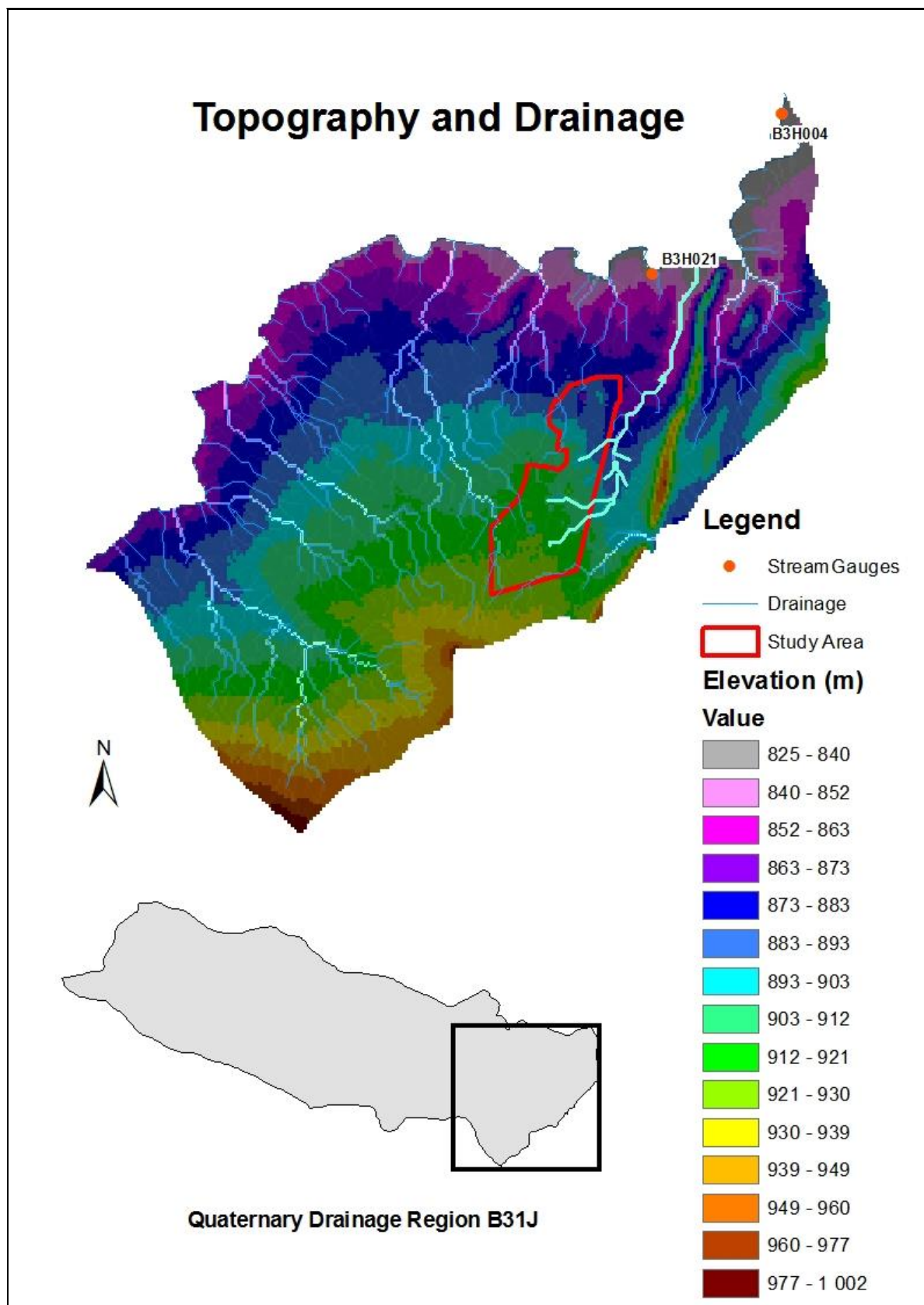


Figure 3.3: The topography and drainage of the study area (study area in red) – light blue highlighted drainage line flows from study area to river between stream gauges

Figure 3.4 indicates the EC logs of the stream gauges (as depicted in Figure 3.3) in the Elands River.

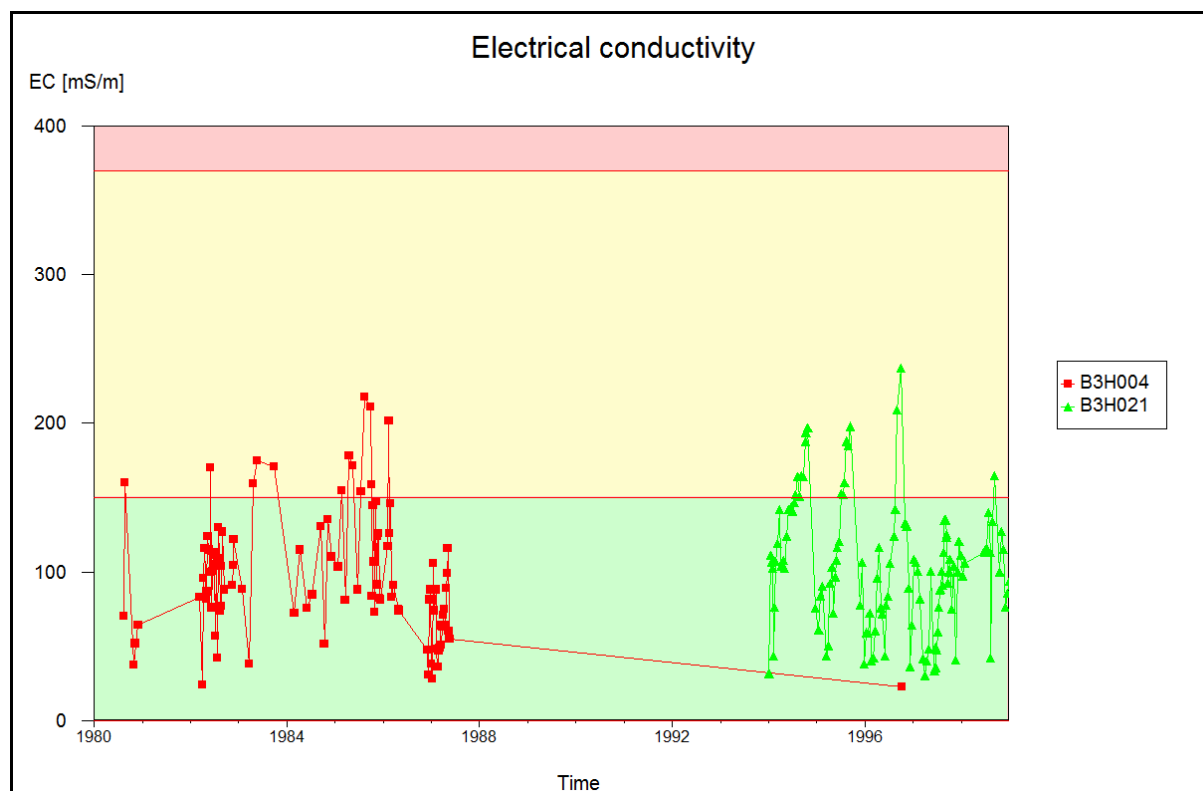


Figure 3.4: EC logs of the stream gauges in the Elands River

The stream gauges do not overlap in time, but certain similar traits can be visualized from Figure 3.4. The EC levels of the two stream gauges fluctuate often and in a similar fashion, but remain between 10 – 250 mS/m. The reason for the fluctuation may be due to various environmental influences, such as seasonal rainfall (more rainfall in summer may reduce EC levels due to dilution and vice versa). There is no evidence, however, on any pollutant infiltration. B3H004 would show higher EC levels if there were pollutants entering the river, as the drainage enters the river between these two gauges. The river is too far from the Cr(VI) polluted site to be effected (over 4 km).

According to the Department of Water Affairs (2011:a) the region wherein the study area is located, receives an average annual rainfall of approximately 500 mm. According to their report the possible evaporation of water is excessively higher than the precipitation. Rainfall data obtained from a weather station in the study area indicates that there is a mean annual rainfall of approximately 472.35 mm, which corresponds to the rainfall values documented by

the Department of Water Affairs (2011:a). Figure 3.5 indicates the average monthly rainfall for the study area from the year 1921 to 2004.

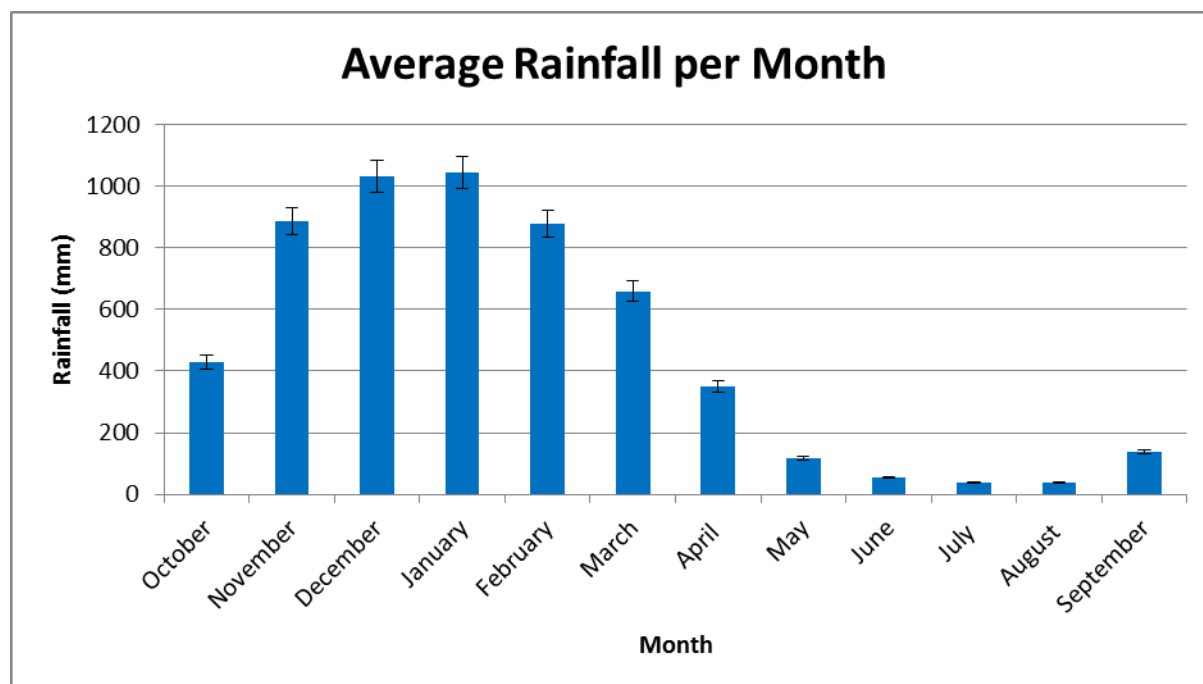


Figure 3.5: The average monthly rainfall of study area

3.3 RIVERS AND DAMS

The Olifants River flows from Loskop Dam through the smooth surface of the earth past the study area to Arabie Dam. It is here where the Olifants and Elands Rivers converge. Due to alluvial deposits the riverbed is quite sandy, especially where it meanders. The ecological state of water in this region is fair to poor according to the Department of Water Affairs (2011:b). The present ecological class for the quaternary catchment B31J is a D, which indicates Moderate levels of widespread impacts, which have limited but noticeable effects on the environment. This classification is conducted according to Chapter 3 of the National Water Act (1998:36). Figure 3.6 indicates the rivers and dams in the study area.

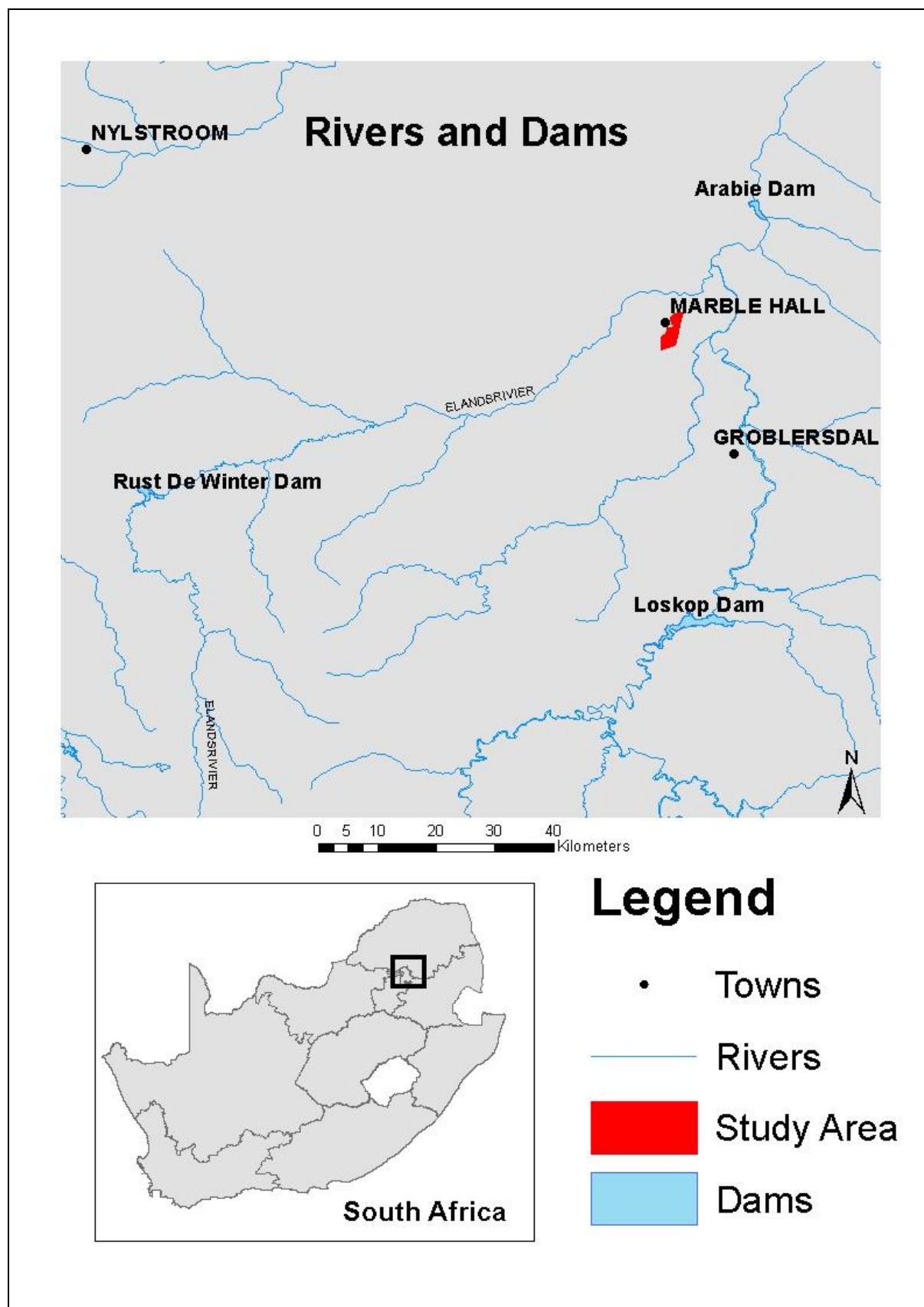


Figure 3.6: Rivers and dams located in the study area

3.4 GEOLOGY

The study area is in the Transvaal Supergroup, which includes intrusions of the Bushveld Complex. The Marble Hall Fragment is in the vicinity of the study area (within the Limpopo Province, South Africa) and is located approximately 150 km north-east of Pretoria (De Waal & Armstrong, 2000; Johnson *et al.*, 2006; Pitra & De Waal, 2001). This fragment is recognized as a window in the Early Proterozoic Transvaal Supergroup, which comprises of volcanic and sedimentary rocks, within the youngest constituent of the Bushveld Igneous Complex (Nebo granite of the Lebowa Granite Suite). Chuniespoort Group marble and meta-dolomite make up the centre of the fragment, which overlies complexes attributed to the Bloempoot Formation, namely gneiss, amphibolites and schist. The border of the fragment is made up of metamorphosed shale, basic volcanic rocks and quartzite from the Pretoria Group. The grade of metamorphism declines from the border towards the centre of the fragment (Pitra & De Waal, 2001). Quartzite from the Transvaal Supergroup lies on the Bloempoot rocks and is overlain by ferruginous shale, chert, dolomite, and shale that originates from the Malmani Subgroup (Chuniespoort Group) (De Waal & Armstrong, 2000).

According to Pitra & De Waal (2001) there is no available data relevant to the metamorphism of the meta-dolomites or marbles, even though they are known to contribute in part to the economy of the town. Numerous intrusions of diorite (sills) occur in the Marble Hall Fragment and are said to originate from the Rustenburg Layered Suite primary intrusion stages, as indicated by geochemical and geochronological statistics.

Figure 3.7 illustrates the various lithostratigraphic units that form part of the sub-catchment of the study area.

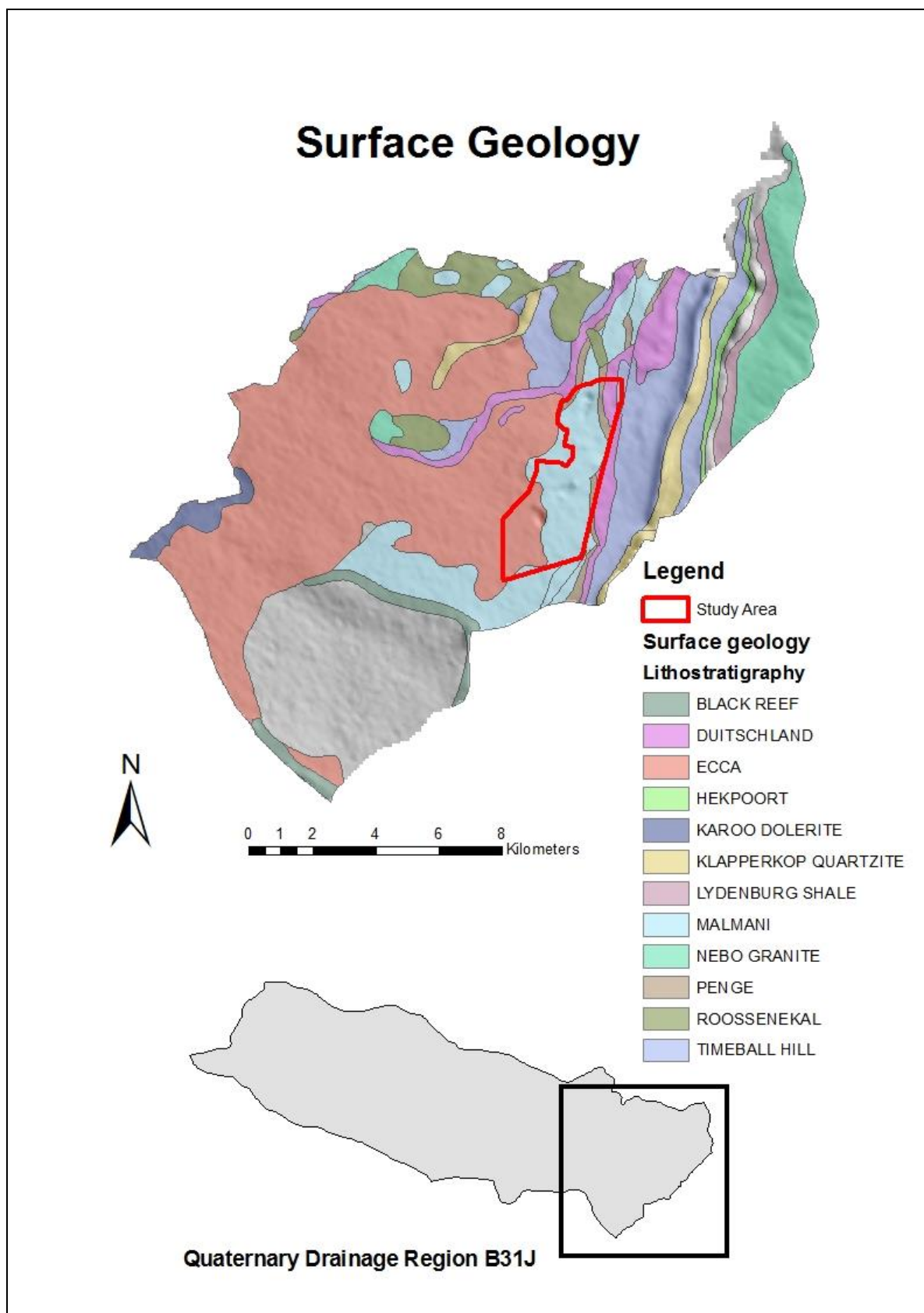


Figure 3.7: A lithostratigraphic map of the study area with its relevant reference map, indicating the location of the mine site (in red) within the quaternary drainage region B31J

According to obtained data, the following lithostratigraphic groups (Table 3.1) occur within and around the study area:

Table 3.1: Lithostratigraphic groups in study area

Lithostratigraphic group	Description (from ArcGIS 9.3 data system)
Duitschland (Small patches of this group found in the surroundings areas)	Dolomite/limestone, shale, subordinate quartzite, conglomerate, and diamictite
Ecca (Surrounds area on western side, covers portions of local farms)	Shale, with sandstone-rich units present towards the basin margins in the south, west and northeast and coal seams in the northeast
Roossenekal (Small patches of this group found further from study area)	Olivine diorite, magnetite gabbro, gabbronorite
Malmani (Covers mining area)	Dolomite, subordinate chert, minor carbonaceous shale, limestone and quartzite
Timeball Hill (Covers farms East of mine)	Mudrock, quartzite, minor diamictite

Following is a brief description of the characteristics of the minerals and rocks found in the lithostratigraphic groups from Table 3.1:

Dolomite:

This carbonate mineral is made up of calcium magnesium carbonate ($\text{CaMg}(\text{CO}_3)_2$) and is generally a sedimentary rock-forming mineral, primarily forming dolostone. Dolomite crystallizes as rhombohedrons, whereof a few may be curved (Amethyst Galleries' Mineral Gallery, 2012; Deer *et al.*, 1992).

Limestone:

This sedimentary rock comprises mostly of calcium carbonate (CaCO_3) as the mineral calcite (King, 2013:a; Smith, 1987). Limestone generally occurs together with dolomite and when mixed together in large amounts, are referred to as dolomitic limestone. Acids are

neutralized by the chemical composition of these basic minerals. Limestone is extracted from the ground by using open mine methods (Smith, 1987).

Quartzite:

This metamorphic rock is solid and non-foliated. Quartzite develops from sandstone through high pressure and heating, which is normally associated with tectonic compression. Many mineral impurities may occur in quartzite (iron oxide for example) and result in different colours (Powell, 1999).

Conglomerate:

This sedimentary rock consists of large (>2 mm) individual rounded clasts or particles, such as sand or pebbles, that have been cemented within a finer-grained matrix (King, 2013:b)

Diamictite:

Diamictite is a rock that is formed on land (terrigenous) and has a messy assortment. It consists of various sized, unsorted clasts, and is not a particularly arranged conglomerate. It represents rocks whereof the sediments and their source are close (Alden, 2013:a).

Shale:

This clastic sedimentary rock is fine-grained and consists of a mixture of mud containing clay minerals and minute clasts of various other minerals. Two of these minerals that are common are calcite and quartz. Breaks occur along planes of weakness less than a centimetre from one another, and are termed fissility (Blatt *et al.*, 2006).

Sandstone:

This clastic sedimentary rock can be formed from a variety of minerals, however they are limited to hardened sands that tend to cement together easily (of siliceous character). As sandstone is porous it can act as an aquifer by storing large amounts of water (Pettijohn *et al.*, 1987).

Olivine diorite:

Olivine is a mineral that has the formula $(\text{MgFe})_2\text{SiO}_4$, which means it is a magnesium iron silicate. Diorite is an intrusive igneous rock that consists of a variety of minerals, primarily of plagioclase feldspar, hornblende, pyroxene and biotite. As olivine is iron-rich, there is a possibility that the rock may grade into ferrodiorite. This rock occurs during the transition into gabbro (Blatt *et al.*, 2006.).

Magnetite gabbro:

Magnetite is the most important magnetic mineral and has the formula Fe_3O_2 . This gabbro occurs naturally in the environment (Harrison *et al.*, 2002). Gabbro is a plutonic rock that is low in silica. No quartz or alkali feldspar occurs in gabbro, only plagioclase with high amounts of calcium (Alden, 2013:b).

Gabbronorite:

This is a mafic intrusive igneous rock that contains plagioclase with high levels of calcium. Gabbronorite is a host rock for certain magmatic deposits and has extensive amounts of pyroxenes and hornblende (Himmelberg *et al.*, 1987).

Chert:

This sedimentary rock is a cryptocrystalline or microcrystalline that is rich in silicon dioxide. Chert is found naturally as nodules, concrete masses or as coated deposits. Often this rock has sharp edges due to conchoidal fracture breaks (King, 2013:c).

Minor carbonaceous shale:

These shales (see shale description) are from the Cretaceous period (approximately 145 to 66 Ma) and have carbon as a minor element, as it comprises of less than 1% organic carbon (Tourtelot, 1964).

Mudrock:

This sedimentary rock consists of fine grained fragments such as clay or silt. The fragments are often smaller than 0.0625 mm in diameter, which makes this rock quite difficult to study. Mudrocks may occur in the environment in combination with other minerals, such as quartz, dolomite, calcite and feldspars (Boggs, 2006).

3.5 LAND TYPES AND SOIL

The major land types and soil combinations that occur in the study area are listed in the Table 3.2 and Table 3.3. These tables just give an idea of the soil type of the surrounding areas. Soil type may have an effect on the movement of water through the area. The land types that occur in the study area, as documented by the Department of Agriculture and Land Administration (2005), are depicted in Table 3.2.

Table 3.2: Land types of the study area

Land Type	Area (ha)	%
Eutrophic; red soils not widespread (Bd)	19,574.6	17.7
Eutrophic; red soils widespread (Bc)	24,068.7	21.8
Exposed rock areas with miscellaneous soils (Ib)	5,275.6	4.8
Pedologically young soils (lime rare or absent) (Fa)	3,564.1	3.2
Red and yellow dystrophic and/or mesotrophic (Ac)	9,405.7	8.5
Red and yellow, high base status (Ah)	12,957.4	11.7
Red, high base status > 300 mm deep (no dunes) (Ac)	33,298.9	30.2
Vertic, melanic, and/or red structured diagnostic horizons (Ea)	340.8	0.3

Table 3.2 is modified from the Department of Agriculture and Land Administration (2005)

The values of Table 3.2 are depicted in the map of Figure 3.8 with their associated symbols.

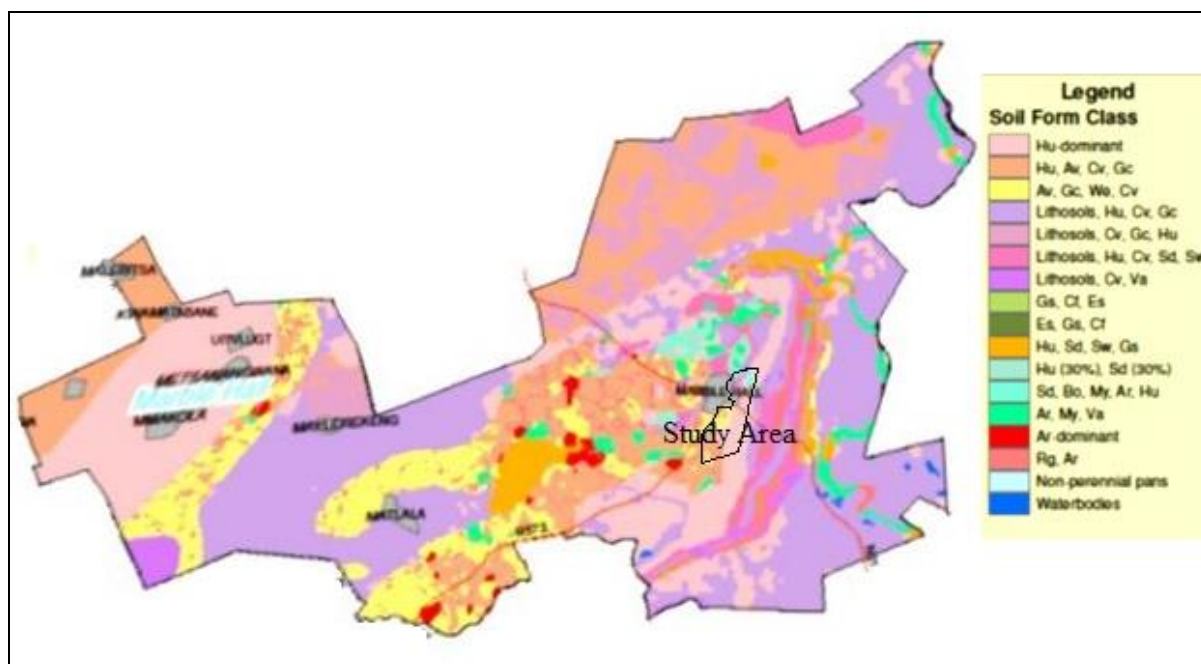


Figure 3.8: A soil map of the greater study area (modified from the Department of Agriculture & Land Administration (2005))

From Table 3.2 and Figure 3.8 it can be seen that the Ac (Lithosols) land type is predominant. These consist of red, brown or yellow coloured soils that are formed from strong weathering. They commonly have high clay content in their B horizons.

The soil clay contents that are prevalent in the study area, as documented by the Department of Agriculture and Land Administration (2005), are depicted in Table 3.3. Higher clay contents result in less flow of water through the area.

Table 3.3: Soil clay contents of the study area

Clay class	Area (ha)	%
Clay	2,612.0	2.4
Clayey	2,556.8	2.3
Loam	22,451.9	20.4
Loam & clay	10,712.6	9.7
Loamy sand	39,865.0	36.1
No Data	0.8	0.0
Sandy	2,416.7	2.2
Sandy loam	27,646.3	25.1
Very clayey	280.6	0.3
Very sandy	1,623.0	1.5

Waterbodies	149.6	0.1
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Table 3.3 is modified from the Department of Agriculture and Land Administration (2005)

From Table 3.3 it can be seen that the prevalent clay classes are loamy sand and sandy loam. From Table 3.2 and Table 3.3 is noted that there is a level of clay content. The amount, however, is quite low and the flow of water through these soils may be permitted.

3.6 LAND COVER

The greater study area is known for its widespread irrigation farming of citrus fruits and vegetables which plays a large role in the economy of neighbouring towns, and of the country. The irrigation farms obtain water from groundwater resources and from the Loskop Dam via the Loskop Dam irrigation canal (Department of Water Affairs, 2010). Figure 3.9 depicts the land cover of the study area. Figure 3.10 indicates these land cover values in a histogram. Figure 3.11 shows the widespread agricultural land use of the greater study area.

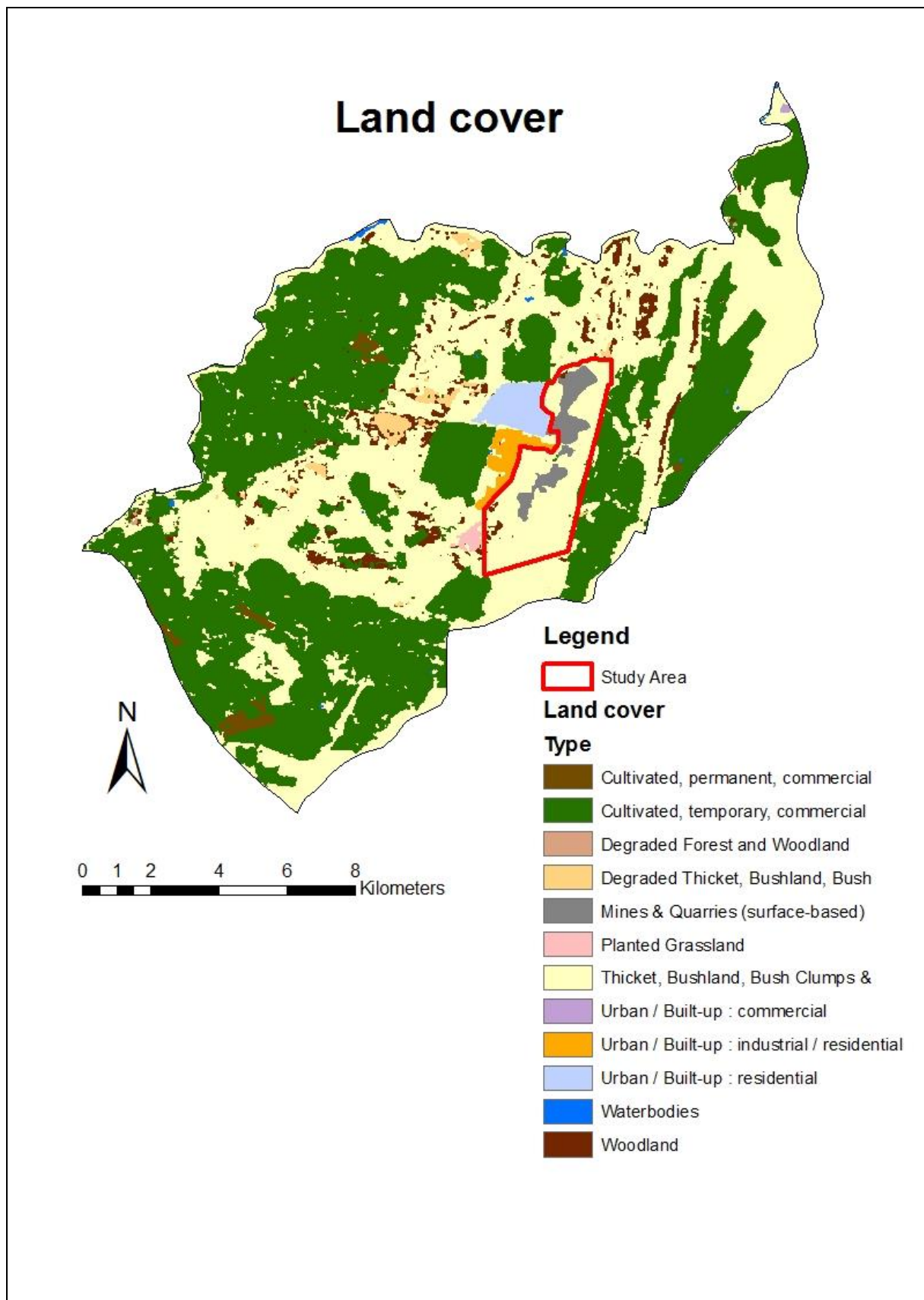


Figure 3.9: The land cover of the boundary around the study area

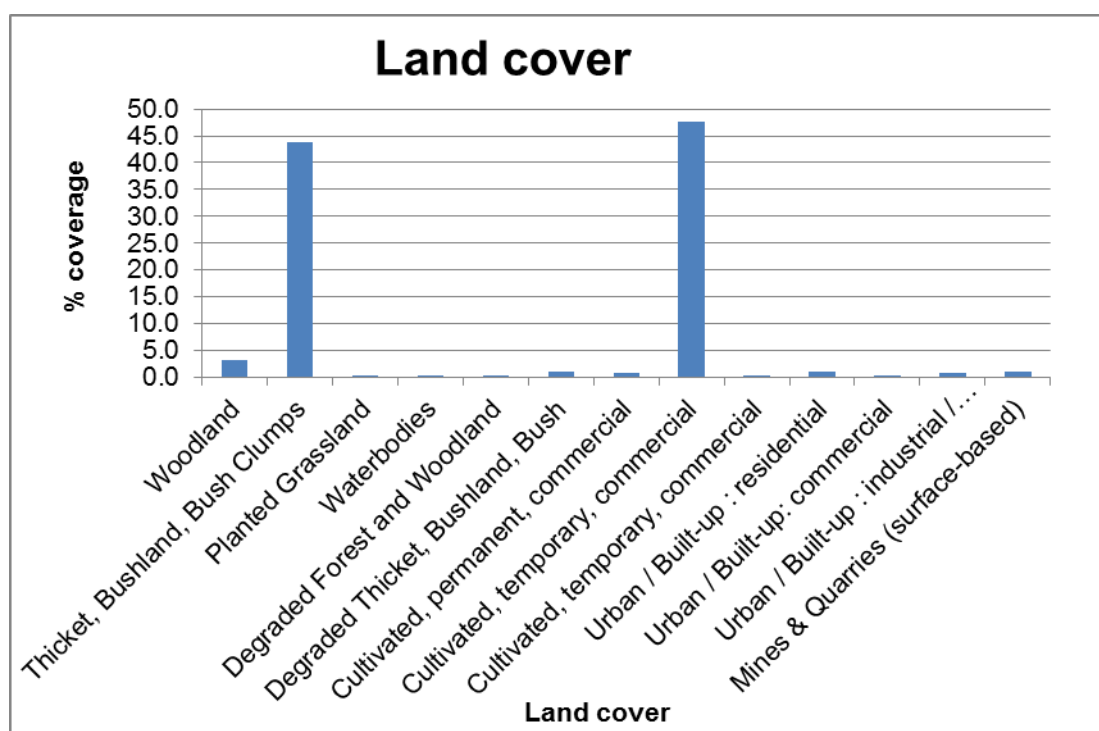


Figure 3.10: The individual land cover values of the area surrounding the study site

The land covers cultivated commercial (farms) and thicket and bush land covers most of the area. This is depicted in the map in Figure 3.9 and the aerial photograph of Figure 3.11. Surface mines and thicket and bush land cover most of the study area.



Figure 3.11: An aerial photograph depicting the widespread agricultural land use of the greater study area (mine lease in red)

3.7 THE MINING SITE

The mine is situated to the east of the town Marble Hall. The topography of the mining area is relatively flat, with an underlying ore-body that consists of a metamorphosed dolomite. This dolomite comprises of high levels of calcium carbonate (CaCO_3) and low levels of silica (in the form of SiO_2) (Infrasors Holdings Limited, 2012).

The mine started operations in the year 1919 by the mining of marble blocks. Currently, a milling section exists that produces powders. Additionally, there is an industrial plant on the mining site that aids in the production of aggregate that is then used in the steel and local construction industry. Bulk powders are mainly supplied to the agriculture and coal mining sectors. The mining activities are based on standard open pit principles (Infrasors Holdings Limited, 2012).

The study area is shown on an aerial photograph in Figure 3.12. The proximity of the mine to the neighbouring town is also indicated.

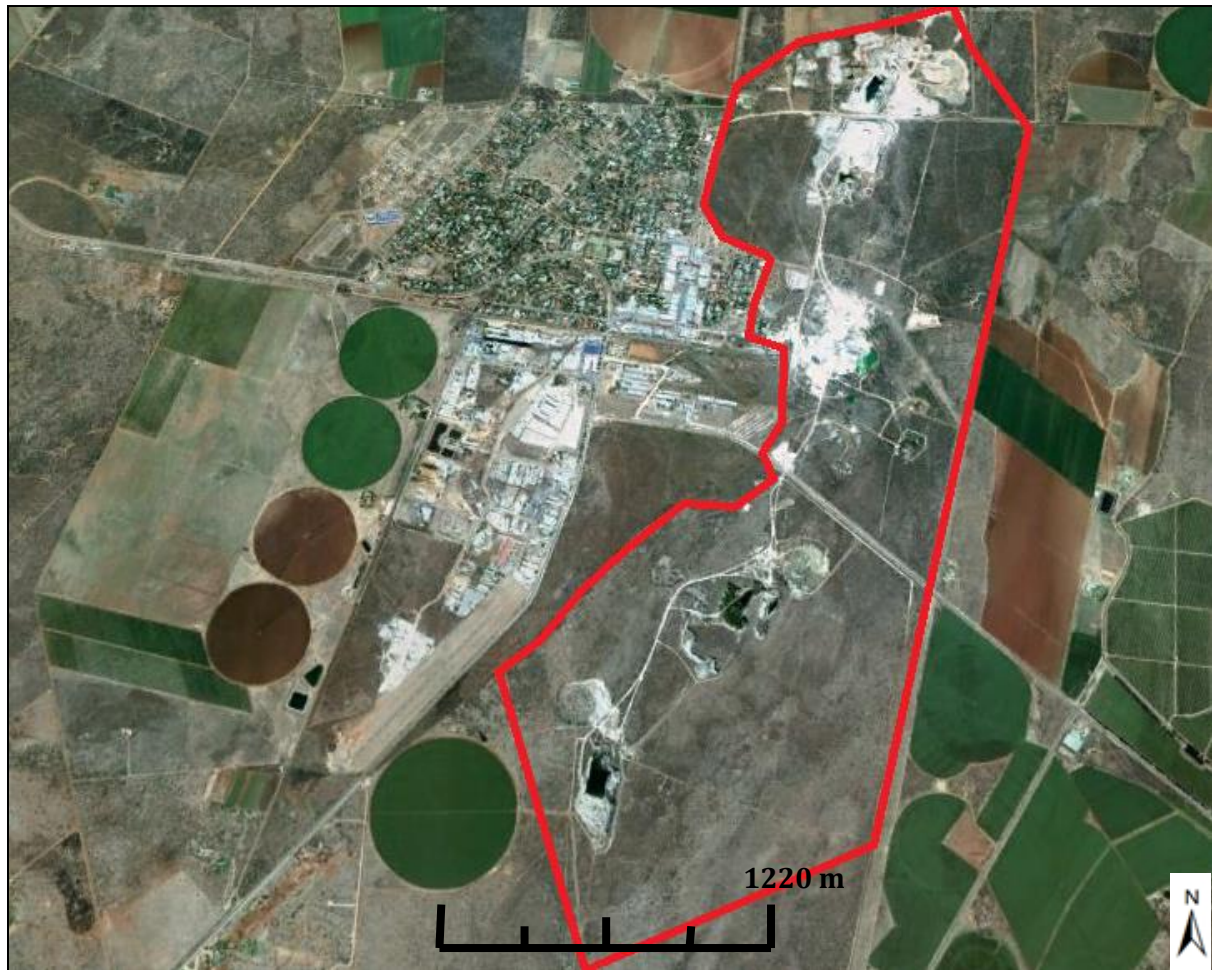


Figure 3.12: The extent of the study area (red zone)

4 APPROACH TO STUDY

Figure 4.1 indicates the sampling areas. Figure 4.1 depicts these areas in an aerial photograph.



Figure 4.1: A Google Earth aerial map of the sampling areas (depicted as flags)

4.1 FIRST ROUND SCREENING

Sampling

Two sampling opportunities were conducted throughout the period of the study, where ten water samples were obtained from groundwater (8) and surface water bodies (2) and analyzed. The sampling equipment used was rinsed thoroughly with the corresponding sampled water as to avoid contamination. Groundwater samples were obtained at positions with maximum electrical conductivity, based on EC logging. The electrical conductivity was measured at various depths in the boreholes on site and documented.

The physical-chemical properties of the samples (using ICP-MS), including chromium(VI) concentrations, were analyzed and documented in an Excel spreadsheet (which was used in the GIS mapping of the catchment areas). Before the water analyses commenced, the

water samples were prepared by lowering their pH to 3. This step was conducted to ensure that all the suspended solids within the samples were dissolved. Thereafter, the parameters in the water samples were determined by measuring the anions on the 1C 761 Metrohm, and on the Agilent ICP-MS 7500 CE (inductive plasma coupled spectroscopy) (by Eco-Analytica, Unit for Environmental Sciences and Environmental Management, Faculty of Natural Sciences, Potchefstroom Campus, NWU). The inductive plasma coupled spectroscopy method is a spectroscopic method that can determine any elements within a sample, excluding argon. The source of the atomic radiation is inductively coupled argon plasma (Boumans, 1987).

The bacterial composition was determined using molecular methods. Six of the samples were of great interest as their Cr(VI) concentrations were extremely high. The samples were labeled as in Table 4.1.

Table 4.1: Sample types

Sample	Site type	Cr(VI)
MH1	Groundwater	
MH3	Groundwater	
MH4	Groundwater	
Tap	Groundwater	
Greencoy	Surface water	High Cr(VI) concentrations (3.954 mg/l)
Quarry	Surface water	High Cr(VI) concentrations (0.2821 mg/l)
Spring 50/1	Groundwater	High Cr(VI) concentrations (3.658 mg/l)
MH2	Groundwater	High Cr(VI) concentrations (0.1213 mg/l)
Trench	Groundwater	High Cr(VI) concentrations (1.919 mg/l)
MH5	Groundwater	High Cr(VI) concentrations (0.2856 mg/l)

The last six samples were those with high concentrations of Cr(VI).

4.2 BACTERIAL ENUMERATION (ONLY FOR LAST SIX SAMPLES)

4.2.1 DAPI STAINING

The DAPI (4',6-diamidino-2-phenylindole) staining was conducted using the standard operating procedure (SOP) for the enumeration of bacterial cells (modified from Karner & Fuhrman, 1997). Two milliliters of the samples was filtered through a Millipore GTTP type filter, with a pore size of 0.22 μm . The cells were fixed in 4% formaldehyde final concentration at 4°C for 1 hour and thereby filtered using 0.2 μm Millipore GTTP type filters. DAPI stain (0.1 $\mu\text{g}/\text{ml}$ final concentration) was added to each filter. This was allowed to react with the cells by incubation in the dark at room temperature for 2 minutes. A drop of anti-fading solution (10 μl citifluor) was then added on the slides with their filters. The filters were viewed under a 100x oil immersion lens with a mercury light that ensured fluorescence of the stained cells. The filter of the mercury light was set at 0.2, where after cell enumeration commenced.

4.3 MOLECULAR TECHNIQUES (ONLY FOR LAST SIX SAMPLES)

4.3.1 GENOMIC DNA ISOLATION

In order to ensure that sufficient genomic DNA was isolated from the environmental samples, 500 ml of each sample was filtered through 0.2 μm filters. After filtering, the filters were sliced into small portions and placed into a microfuge tube. The Fast DNA[®] Spin Kit for Soil from MP Biomedicals (US) was used for DNA isolation. The DNA was finally eluted with autoclaved deionized water.

The concentration and purity of each genomic DNA extract was determined using the Nanodrop Spectrophotometer 3300 (Thermo Scientific, US). Gel electrophoresis was performed on the genomic DNA to evaluate the quality of the genomic DNA extracts as well as to confirm the concentrations of the extracts. Each sample (7 μl gDNA with 2 μl loading dye) was loaded on a 0.8% agarose gel. Electrophoresis was performed at 90V for 1 hour using a Bio-Rad electrophoresis system for the determination of the gDNA quality. A 10000 bp, GeneRuler™ DNA ladder (Fermentas, US) was used to compare the individual bands with.

4.3.2 PCR AMPLIFICATION

PCR (polymerase chain reaction) was performed on all 6 genomic DNA isolates (technique modified from Braker *et al.*, 1998). The PCRs were performed in a final volume of 50 µl with the amount of gDNA varying for each sample as a result of their different concentrations, which are indicated in Table 4.2.

Table 4.2: DNA volumes of the samples

Sample	Volumes
Greencoy	2 µl
Quarry 50/1	2 µl
Spring 50/1	3 µl
MH2	1.5 µl
Trench	2 µl
MH5	1.5 µl

The 16S rRNA primers that were used during the PCR were the 341F (containing the GC clamp at the 3' terminal) and the 908R (Federici *et al.*, 2011) (Bio-Rad, US). The reaction started with a denaturing step of 95°C for 5 minutes, where after 25 cycles commenced. These cycles consisted of denaturing at 94°C for 45 seconds, annealing at 55°C for 45 seconds, and extension at 70°C for 2 minutes. The reaction was concluded by an additional extension at 70°C for 10 minutes (Thermocycler, Bio-Rad, US).

Gel electrophoresis was performed on the PCR products on a 1% agarose gel. 5 µl of each of the products were mixed with 2 µl loading dye and loaded on the gel and run for 1 hour at 90V using a Bio-Rad electrophoresis system for the determination of the product quality. A 10000 bp ladder (Bio-Rad, US) was used to compare the individual bands with.

4.3.3 DGGE

The standard operating procedure of DGGE (denaturing gradient gel electrophoresis) was performed on the PCR products for the fingerprinting analysis (modified from Muyzer *et al.*, 1993). Since the principle of separation of DNA mixtures of the same length is based on their sequence-specific melting point in a polyacrylamide gel, with a gradient of denaturants such as urea and formamide (Tezeneva *et al.*, 2008), 40-60% urea and formamide polyacrylamide gel was prepared by use of a gradient maker. In this case, 100% denaturant is defined as 7 M Urea and 40% v/v formamide (McCaig *et al.*, 2001). A 1 x TAE was used as a buffer for the reaction. Pipetted amounts of 25 µl of the PCR products were each mixed with 10 µl dye and 20 µl of each mixture was loaded onto the gel. The DGGE was performed for 14 hours with a ramp of 50 at 60°C at 100V using the Bio-Rad DCode™ Universal Mutation Detection System.

The prominent bands that developed on the polyacrylamide gel were visualized using the Bio-Rad Chemidoc MP, excised and numbered 1-15 and placed in microfuge tubes. A following 50 µl of deionized water was added to each tube and incubated overnight at 55°C.

4.3.4 DNA SEQUENCING AND ANALYSIS

Re-amplification of excised DGGE products was performed after the overnight incubation. The 16S rRNA primers used were the 341F (without the GC clamp) and the 908R. The same PCR conditions were performed during the sequencing PCR as the initial PCR, with the only difference being the annealing temperature. The annealing temperature during the sequencing PCR was 52°C, as there were no GC clamps.

Purification of PCR products was performed using BioSpin Gel Extraction kit (BioFlux, Bioer Technology Co., Ltd, China) following manufacturers' protocol instructions. Concentrations of the purified amplicons were determined using a Nanodrop 3300 spectrophotometer (Thermo Scientific, US). This was followed by sequencing PCR and purification of the PCR products using the ethanol extraction method. Chain termination sequence analysis (ABI Prism) was performed in-house (UFS Biotechnology department). A BLAST (Basic Local Alignment Search Tool) was performed on the sequences obtained and compared against a database. This allowed for the possible identification of the origin of the sequences. In other words, the organisms to which these specific sequences originated from were identified.

A phylogenetic tree was drawn from the sequenced data by using the MEGA 5.10 software. An UPGMA (Unweighted Pair Group Method with Arithmetic Mean) dendrogram was generated based on the DGGE profile. This was done using the Phoretix 1D software. These diagrams indicate the relationship between the different organisms based on their DNA sequences and position in the gel, respectively.

The following forward (F) and reverse (R) primers were used during the analyses:

317F: 5'-CCTACGGGAGGCAGCAG-3'

908R: 5'-CGTCAATTCMTTGTGAGTT-3'

5 MICROBIAL ANALYSES - RESULTS AND DISCUSSION

The sampling areas are depicted in a Google Earth aerial map (Figure 5.1), as to locate them in the mining area.



Figure 5.1: A Google Earth aerial map of the sampling areas (depicted as flags)

5.1 BACTERIAL ENUMERATION

The DAPI method was used to determine the cell count. For this method cells were counted in six consecutive reticules for each sample, where after the average cells per ml were calculated. The formula of the calculations was as follows:

$$n = \frac{YAd}{av} \text{ where:}$$

n = the number of cells per ml of sample

Y = the mean cell count per reticule area used

A = the effective area of filtration ($2 \times 10^8 \mu\text{m}^2$)

d = the dilution factor ($V_{\text{final}} / V_{\text{sample}}$)

a = the reticule area ($10^5 \mu\text{m}^2$)

v = the final volume filtered (2 ml)

(Moffitt, 1993)

Figure 5.2 contains the enumeration results obtained the DAPI staining.

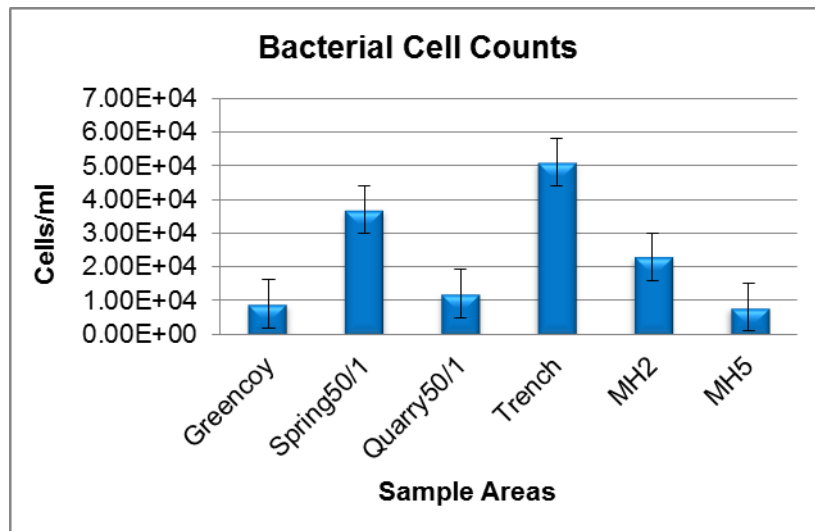


Figure 5.2: A graph of the bacterial cell counts as determined by DAPI staining

Cells were present in different amounts in all the samples, where genomic DNA could be isolated from. Sample Trench had the most cells per ml ($>5.00\text{E}+04$), while sample MH5 had the least ($<1.00\text{E}+04$).

Two photographs of the DAPI stains were included. The bright, fluorescent areas against the dark blue background are the bacterial cells as depicted in figures 5.3 and 5.4. The scale specifies a distance of $2 \mu\text{m}$.



Figure 5.3: Second reticule area counted of sample Spring50/1



Figure 5.4: Forth reticule area counted of sample Trench

5.2 MOLECULAR ANALYSIS

5.2.1 DNA EXTRACTION AND SPECTROPHOTOMETRY

The DNA concentration and purity is documented in Table 5.1.

Table 5.1: Quantity and Purity of the genomic DNA as determined by Nanodrop (spectrophotometry)

Sample	Concentration (ng/μl)	Purity (A260/A280)
Greencoy	2.25	1.98
Spring 50/1	10.48	1.82
Quarry 50/1	6.19	1.75
Trench	8.57	1.53
MH2	28.64	1.79
MH5	33.81	1.94

The genomic DNA was of good quality as the 260/280 nm values were approximately between 1.6 and 2.0. Values below 1.6 indicate protein contamination and values above 2.0 indicate RNA contamination (Clark & Christopher, 2000). The concentrations given by the Nanodrop were extremely low. Gel electrophoresis (Figure 5.5) was performed to confirm the concentrations and purity of the genomic DNA extracts. Although sample MH5 may be a bit sheared (as indicated with a red box), the results obtained in Figure 5.5 showed the suitability of each genomic DNA extract for further downstream applications. From Figure 5.5 it can be noted that the Greencoy and MH2 samples were sheared somewhat as well. This did not affect the suitability of the genomes either.

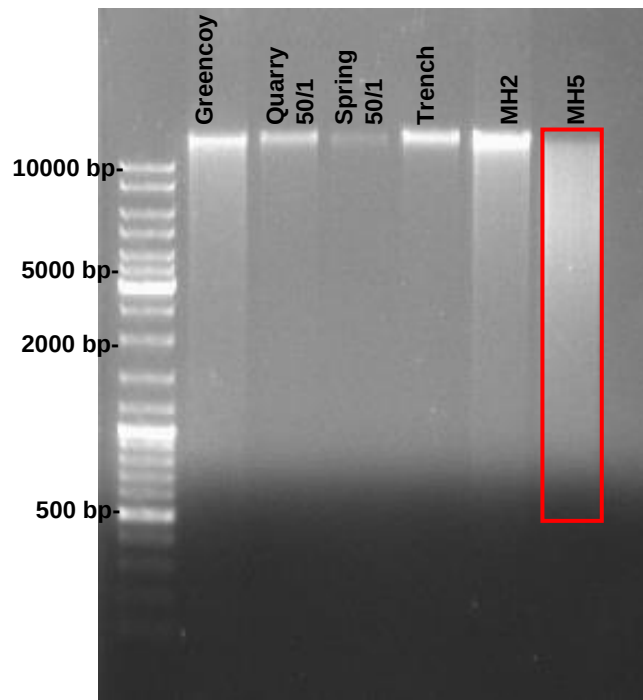


Figure 5.5: Gel electrophoresis of the genomic DNA

The electrophoresis gel did not exactly correlate with the concentrations in Table 5.1, which gave extremely low values for the different samples. The gel in Figure 5.5 indicates that there were higher concentrations of genomic DNA. The reason for this is that the Nanodrop is manually calibrated, which may mean that it was not calibrated correctly at the time of measurement. This shows the importance of comprehensive evaluation of genomic DNA concentration and purity before downstream applications.

5.2.2 PCR AMPLIFICATION

Gel electrophoresis was performed on the PCR products for the determination of their quantity and quality. Figure 5.6 shows fluorescent bands corresponding to the expected product size (over 500 bp) which encodes the ribosomal subunit.

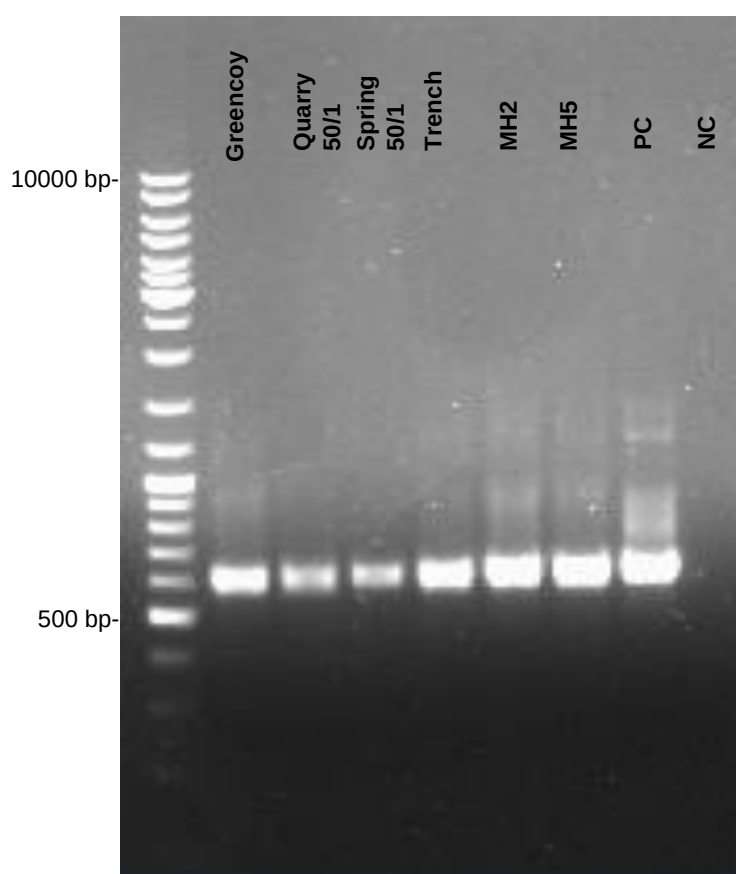


Figure 5.6: Gel electrophoresis of the PCR products

The PC is the positive control, where *E. coli* DNA was used, and the NC is the non-template control, where deionized water was used (no DNA). They both gave the expected results as the positive control had DNA amplification and the negative control had none. The negative control having no amplification indicates that there was no contamination.

5.2.3 DGGE POLYACRYLAMIDE GELS

DGGE was performed on the PCR products and the polyacrylamide gels were viewed under UV light as shown in Figure 5.7.

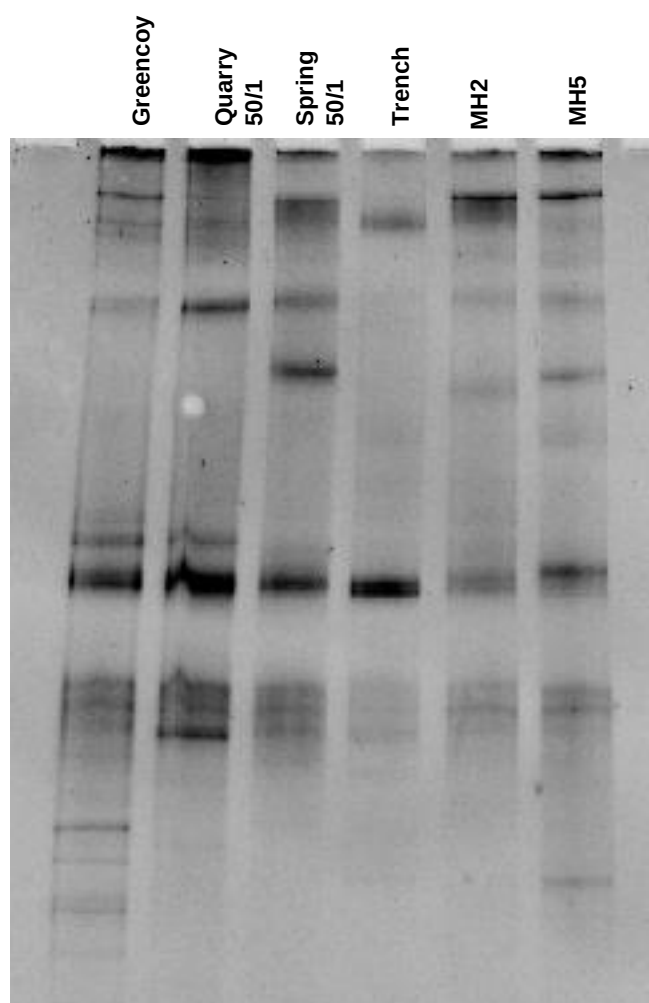


Figure 5.7: Inverted image of DGGE gel of PCR products

Each sample is represented as a vertical column and each bacterial phylotype as a horizontal band which has been separated based on the various melting domains (Muyzer *et al.*, 1993). A selection of prominent bands were excised and prepared for sequencing. Figure 5.9 indicates and numbers the bands that were excised from the DGGE gel.

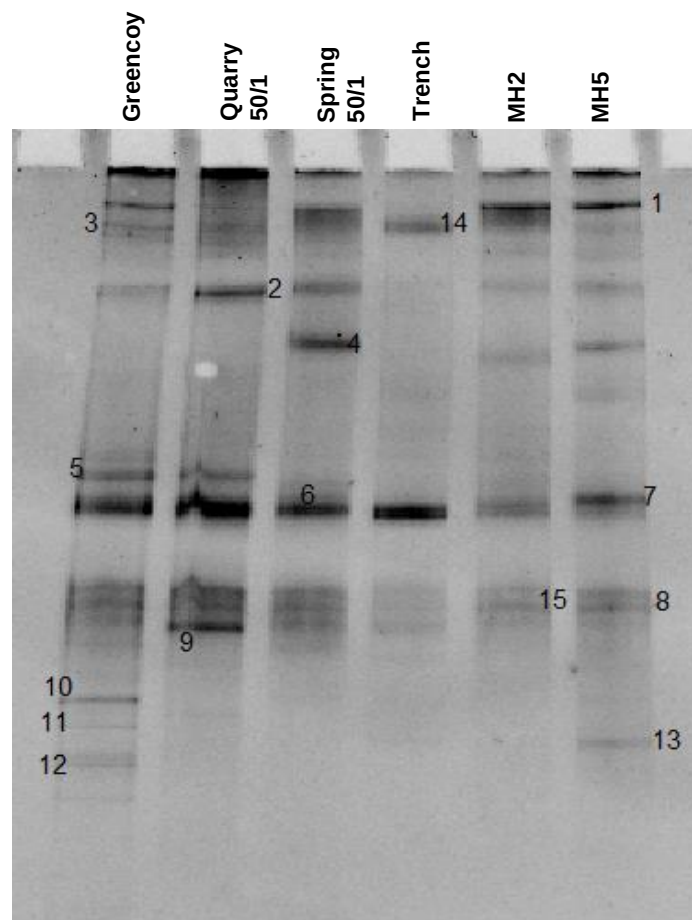


Figure 5.8: Numbered excised bands from the DGGE gel

The excised bands were numbered 1-15 as shown in Figure 5.9. After preparation and re-amplification of the excised DNA, the samples were sequenced in-house (UFS department of Biotechnology). FinchTV was used to BLAST (Basic Local Alignment Search Tool) the sequences to determine the organism to which to sequences belong. The Rf values of the bands is documented in Appendix A.

5.3 MICROBIAL DIVERSITY

5.3.1 BLAST RESULTS

The BLAST results of the 15 excised bands were compared to the Nucleotide collection (nr/nt) database and optimized for highly similar sequences (Megablast). E values (error) approaching zero indicate minimum error. The Max identity percentage indicates the similarity of the genetic sequence to sequences in the database that it is compared to, with highest similarity having a value of 100%. The BLAST results of the 15 excised bands are given in Table 5.2, while their characteristics are documented in Table 5.3.

Table 5.2: The BLAST results of the excised bands

Band ID	Name
1	Uncultured Cyanobacterium 16S rRNA gene, clone HG314
2	<i>Sediminibacterium salmoneum</i> strain NJ-44 16S rRNA gene
3	-
4	Uncultured Bacteroidetes bacterium clone H0.2-0h-16 16S rRNA gene
5	Uncultured beta-proteobacterium partial 16S rRNA gene, DGGE band
6	Uncultured beta-proteobacterium clone B3-18 16S rRNA gene
7	Uncultured Actinobacterium clone WA0.2-0d-33 16S rRNA gene
8	-
9	Uncultured Rhodocyclaceae clone KRA34 16S rRNA gene
10	Uncultured delta-proteobacterium clone JL-ETNP-S57 16S rRNA gene
11	Uncultured Chloroflexi bacterium clone Z53M75B 16S rRNA gene
12	Uncultured delta-proteobacterium gene for 16S rRNA
13	Uncultured Cyanobacterium clone SM2C12 16S rRNA
14	Bacteroidetes bacterium TQQ6 partial 16S rRNA gene, strain TQQ6
15	Uncultured Cyanobacterium clone SM2C12 16S rRNA

Table 5.3: Characteristics of excised bands as documented by BLAST results

Band ID	Rf position	Sequence size (bp)	Accession number	Max score	Query coverage	E value	Max ident	Source
1	0.055	551	FN646728.1	867	96%	0	96%	Environmental sample
2	0.171	545	NR044197.1	898	98%	0	97%	Environmental sample
3	0.086	495	Highly unidentified	-	-	-	-	-
4	0.235	552	JN183361.1	959	98%	0	99%	Environmental sample
5	0.414	554	FN429757.1	606	97%	5.00E-170	87%	Environmental sample
6	0.453	524	JN371460.1	869	90%	0	99%	Environmental sample
7	0.442	480	HM153619.1	824	95%	0	99%	Environmental sample
8	0.582	550	Highly unidentified	-	-	-	-	-
9	0.613	552	AY689089.1	547	98%	3.00E-152	85%	Environmental sample
10	0.743	560	AY726868.1	651	95%	0	89%	Environmental sample
11	0.782	557	FJ484546.1	503	93%	6.00E-	84%	Environmental sample

						139		mental sample
12	0.796	559	AB247832.1	737	97%	0	91%	Environmental sample
13	0.761	535	AF445719.1	728	97%	0	92%	Environmental sample
14	0.08	554	HE793031.1	497	98%	3.00E-137	83%	Environmental sample
15	0.581	541	AF445719.1	405	98%	2.00E-109	81%	Environmental sample

The Rf position (Appendix A) is an indication of the relative migration as it is the position of the bands on the DGGE gel once they have stopped migrating downward with the current. These values were then used to draw a dendrogram, which was used to determine the correlation between the different bands.

From the BLAST results it was noted that bands 3 and 8 were highly unidentified sequences as the error value was too high and the query coverage and max identity values were too low. Therefore, the organisms from which these sequences originated could not be determined.

The sequences of bands 1, 13 and 15 had the most correlation to uncultured species of Cyanobacterium 16S rRNA genes. Cyanobacterium is also known as blue-green bacteria or algae and occurs in most aquatic and terrestrial habitats (Waterbury *et al.*, 1979). These are photosynthetic prokaryotes that use sunlight, water and carbon dioxide to synthesize chlorophyll while releasing oxygen into the atmosphere (Whitton & Potts, 2000). Cyanobacteria can occur in numerous habitats in the environment, which include planktonic cells or phototrophic biofilms in water sources and certain strains form endosymbiotic relationships with hosts such as plants, sponges, lichens and numerous protists. The aquatic cyanobacteria, in both marine and fresh water environments, are infamous for the immense blooms they develop. These blooms are coupled with toxicity that creates health risks (California Department of Public Health, 2012).

Unicellular as well as colonial species (as filaments or sheets) of cyanobacteria exist in the environment. Filamentous species are able to differentiate into various types of cells, which range from vegetative cells to akinetes (resistant spores) and thick-walled heterocysts. Such a filament is termed a heterocystous trichome. Heterocysts have the ability to fix nitrogen due to the presence of the nitrogenase enzymes. Species that form heterocysts are able to fix nitrogen gas (N_2) from the atmosphere into ammonia (NH_3), which in turn can be converted into nitrites (NO_2^-) and finally nitrates (NO_3^-). Nitrates are absorbed by plants from the soil and water and used for the synthesis of proteins and nucleic acids. This is a very important process as atmospheric N_2 is not biologically available for plants (Vitousek *et al.*, 2002; Whitton & Potts, 2000).

The Proterozoic Era (2500-570 Ma) was named as the Age of Cyanobacteria due to their great quantity in fossil reports. This evolutionary history of cyanobacteria plays a large role in the reason for their present success. Resistance and resilience aided these organisms to adapt to changing environments. Metal resistance was an adaptation adopted when metal concentrations in the environment were increased. The reason for this adaptation is the metal-utilizing and metal resistance proteins that may have evolved as a response to the environmental change (Whitton & Potts, 2000). Some other adaptations that are well documented are the tolerance of many species to low oxygen levels. Another is the fact that sulphide can be better tolerated by cyanobacteria than naturally occurring eukaryotes. Certain strains are able to use H_2S as a hydrogen donor instead of water (H_2O) (Whitton & Potts, 2000), while various strains of cyanobacteria are acidophilic and can withstand extreme temperatures (Nancucio & Johnson, 2012; Whitton & Potts, 2000).

Sulphate-reducing bacteria release H_2S into the environment while the majority of the sulphate precipitates as metal sulphides. These bacteria grow in specific environments, which are anaerobic, alkaline and comprise of enough electron donors (Whitton & Potts, 2000; Nancucio & Johnson, 2012). These environmental conditions are created by the acidophilic algae that are in that environment. They deplete the oxygen, thereby producing an anoxic, alkaline environment. Metal removal from water sources is increased by sulphidic precipitation. The reason for the increased removal is that the solubility of sulphide is smaller than that of hydroxide in a variety of pH (Das *et al.*, 2009). Several industrial processes may result in the generation of increased amounts of metals and metalloids in the environment, and such microbiological and biogeochemical processes may aid in the remediation of such contaminated environments (Das *et al.*, 2009; Trujillo-Cárdenas *et al.*, 2010).

Cr(VI) can readily be absorbed by various microbial species, one of which is cyanobacteria. The absorption may be as a result of metabolism (actively) or through chemical and physical processes (passively) (Gupta & Rastogi, 2008). Cyanobacteria, such as *Nostoc muscorum* from fresh water sources, is better equipped for toxic chromium absorption due to its large surface area, simple nutrient requirements and increased binding affinity of the mucilage (Gupta & Rastogi, 2008). This filamentous cyanobacteria has the ability to survive in high pH environments and presents a way of resolving situations where hazardous wastes occur in the environment (Gupta & Rastogi, 2008). It is therefore probable that certain cyanobacterium strains may occur in the samples taken during this study, which the results of the molecular analysis have supported.

The sequences of bands 4 and 14 had the most correspondence to an uncultured *Bacteroidetes* bacterium clone 16S rRNA gene. The sequence of band 2 had the most correspondence to the *Sediminibacterium salmoneum* NJ-44 16S rRNA gene, which is a member of the phylum *Bacteroidetes*. *S. salmoneum* is an aerobic, light pink-pigmented, single celled, gram-negative Rod. It is motile by way of gliding in its environment and has been isolated from reservoir sediments and marine waters (Qu & Yuan, 2008).

Bacteroidetes is a phylum that comprises three classes of anaerobic, non-spore-forming, gram-negative bacteria. These microbes occur extensively in the environment, ranging from marine water, soil, sediments and the intestines of warm blooded animals. Certain strains from the *Bacteroides* genus are opportunistic pathogens (Krieg *et al.*, 2010). As many types of *Bacteroidetes* occur in animal intestines, it can be used as some indication of faecal contamination in the environment (Dick & Field, 2004). Many gut microbes have the ability to become resistant and/or resilient to Cr(VI) and can eliminate them from the environment (Upreti *et al.*, 2004). Not all of these bacteria can be proven without doubt that they are resistant to Cr(VI) as there may be other factors in play (Branco *et al.*, 2005; Upreti *et al.*, 2004). Cr(VI)-non-resistant strains have been shown to occur in environments together with Cr(VI)-resistant strains. A possible reason for this is that the Cr(VI) concentrations have been decreased to negligible amounts as a result of the reduction of Cr(VI) by Cr(VI)-reducers. This allows the stabilization of the bacteria in the environment relative to their Cr(VI)-resistance (Branco *et al.*, 2005). These reasons may indicate that *Bacteroidetes* species occur in the water samples.

The sequences of bands 5 and 6 had the most correspondence to uncultured beta-proteobacterium 16S rRNA genes (5-DGGE band). β -proteobacteria are a Gram-negative class of Proteobacteria. There are aerobic and facultative-aerobic bacteria as well as chemolithotrophic and phototrophic species (Schmeling & Fuchs, 2009; Wang *et al.*, 2007).

This class is important for plant growth and function, as it plays a role in nitrogen fixation. Various pathogenic strains exist such as the *Neisseriaceae* (Todar, 2012). The β -proteobacteria is a diverse group with a high diversity index. These organisms are prominent in biofilms and contribute in the hydrolysis or degradation of pollutants as they can oxidize ammonia. Most species are known to be resistant to Cr(VI) in contaminated areas (Branco *et al.*, 2005).

It is not specified in the BLAST results which particular β -proteobacteria occurred in the samples. It should be noted that many proteobacteria species in general have not been cultured, which makes identification of the particular species present in environmental samples nearly impossible (Muyzer *et al.*, 1993).

The sequence of band 7 had the most correspondence to an uncultured actinobacterium clone 16S rRNA gene. This group of bacteria is Gram-positive and their DNA contains high cytosine and guanine values. This bacterial phylum is one of the more dominant phyla, and generally occurs in soil and fresh- and marine water. They are a well-known active constituent of freshwater systems (Newton *et al.*, 2007). These organisms have the ability to degrade organic materials, like chitin and cellulose. For this reason they have a very important role in the carbon cycle (Servin *et al.*, 2008; Ventura *et al.*, 2007).

These organisms are not only resistant to Cr(VI), but have been proven to be Cr(VI)-reducers in various concentrations of Cr(VI) (Branco *et al.*, 2005). Therefore, these bacteria may very well be in the environmental samples of this study, but again, the particular species is not known.

The sequence of band 9 had the most correspondence to an uncultured *Rhodocyclaceae* clone 16S rRNA gene. These Gram-negative, rod-shaped bacteria have an order in the β -proteobacteria class (Imhoff, 2005). Most organisms are denitrifying and occur in aerobic environments and demonstrate a wide range of metabolic abilities. *Rhodocyclaceae* bacteria commonly occur in aqueous habitats, especially environments with oligotrophic conditions (Imhoff, 2005). For this reason many species can be used in bioremediation of waste water, such as *Azoarcus*. The genus *Rhodocyclus* is the only phototrophic species of the group. It is similar to the alpha-proteobacteria as it can perform anoxygenic photosynthesis by similar processes (Imhoff, 2005).

Species in the *Rhodocyclaceae* family have the ability to reduce uranium(VI). Many organisms that are capable of this, occasionally have the ability to reduce Cr(VI) (Martins *et al.*, 2010). The community structure, however, commonly differs. *Rhodocyclaceae* have been documented in Cr(VI) contaminated sources, which indicates that they are resistant

(Martins *et al.*, 2010). Members of this family have never been reported as Cr(VI)-removing, which may not be the case. These organisms may play a role in the bioremediation of metal contaminated waters (Martins *et al.*, 2010).

The sequences of bands 10 and 12 had the most correlation to an uncultured δ -proteobacterium clone 16S rRNA gene. Delta-proteobacteria is a gram-negative class of Proteobacteria. These organisms consist predominantly of aerobic genera and strictly anaerobic genera and occur in many diverse habitats in the environment, ranging from aqueous sources to sediment and soil. The aerobic genera are the *Myxobacteria* which form fruiting bodies. In unfavourable conditions they can release myxospores. The anaerobic genera consist of the majority of the sulphate- and sulphur-reducing bacteria, as well as some bacteria with different functioning, such as the iron-reducing *Geobacter* and many other metal-reducing organisms (Rodionov *et al.*, 2004; Thomas *et al.*, 2008).

Sulphate-reducers are of great importance environmentally and economically (Tebo & Obratzsova, 1998). δ -proteobacteria can be used in the bioremediation of metal-contaminated areas. Under anaerobic conditions they use sulphate as an external electron acceptor during the oxidation of organic compounds. H₂S is the final product of this reaction (dissimilatory sulphate reduction) and forms insoluble metal sulphides with heavy-metal ions. H₂S can also reduce soluble toxic metals to less toxic forms, such as reducing the toxic Cr(VI) to non-toxic Cr(III) (Fawcett, 1988; Tebo & Obratzsova, 1998). This is evidence that certain δ -proteobacteria members can survive in Cr(VI)-contaminated areas (Branco *et al.*, 2005; Tebo & Obratzsova, 1998).

The sequence of band 11 had the most correlation to an uncultured *Chloroflexi* bacterium clone 16S rRNA gene. This phylum comprises of extremely diverse phenotypes. Members from this phylum are mostly Gram-negative, but branch into very different functional groups. Certain members are aerobic thermophiles and flourish at high temperatures. Other members are anoxygenic phototrophs. The final group is anaerobic halo-respirers (Björnsson *et al.*, 2002). Members from the latter group use halogenated organic compounds as energy. An example of such a compound is toxic chlorinated ethenes (Sutcliffe, 2010). Filamentous *Chloroflexi* or green non-sulfur bacteria are used in waste water treatment in sewage plants (Björnsson *et al.*, 2002).

Chloroflexi species are not known to flourish in Cr(VI) contaminated environments. As chromium concentrations rise, the biomass of *Chloroflexi* tends to decline (Desai *et al.*, 2009). The reason for the presence of this phylum may be due to the reduction of the Cr(VI) in samples which is as a result of the metal reducers that occur in that same environment (Branco *et al.*, 2005)

Studies show that long-term chromium pollution leads to community shifts where the dominant organisms are the Proteobacteria (Desai *et al.*, 2009).

Studies have proven that resistance and resilience towards Cr(VI) may indirectly result in antibiotic resistance to several antibiotics (Branco *et al.*, 2005). The reason for this is that the plasmid with the Cr(VI)-resistant genetic sequence also contains the genetic sequence for antibiotic resistance, which poses additional health risks (Branco *et al.*, 2005).

All the BLAST results show that the sequences are from the 16S rRNA genes, which are correct as these were the genes used in this study, and indicative of some level of accuracy. All the samples were taken from water catchment areas on site, which is supported by the results which indicate that all the sequences analysed originated from organisms that often occur in water. It is difficult, however, to get precise results when such a small amount of the bacteria in the environment have been documented and characterized (Muyzer *et al.*, 1993). These are some of the limitations that come with the field and the technology.

A phylogenetic tree (Figure 5.10) was generated from the aligned genetic sequences to determine any evolutionary relationships between specific organisms. An UPGMA (Unweighted Pair Group Method with Arithmetic Mean) dendrogram (Figure 5.11) was generated from the bands on the DGGE profile to determine the microbial composition and biodiversity of the organisms within the samples. Correlations between samples were based on the similarities or dissimilarities of the DNA sequences and areas of band occurrence (Muyzer *et al.*, 1995; Baum, 2008).

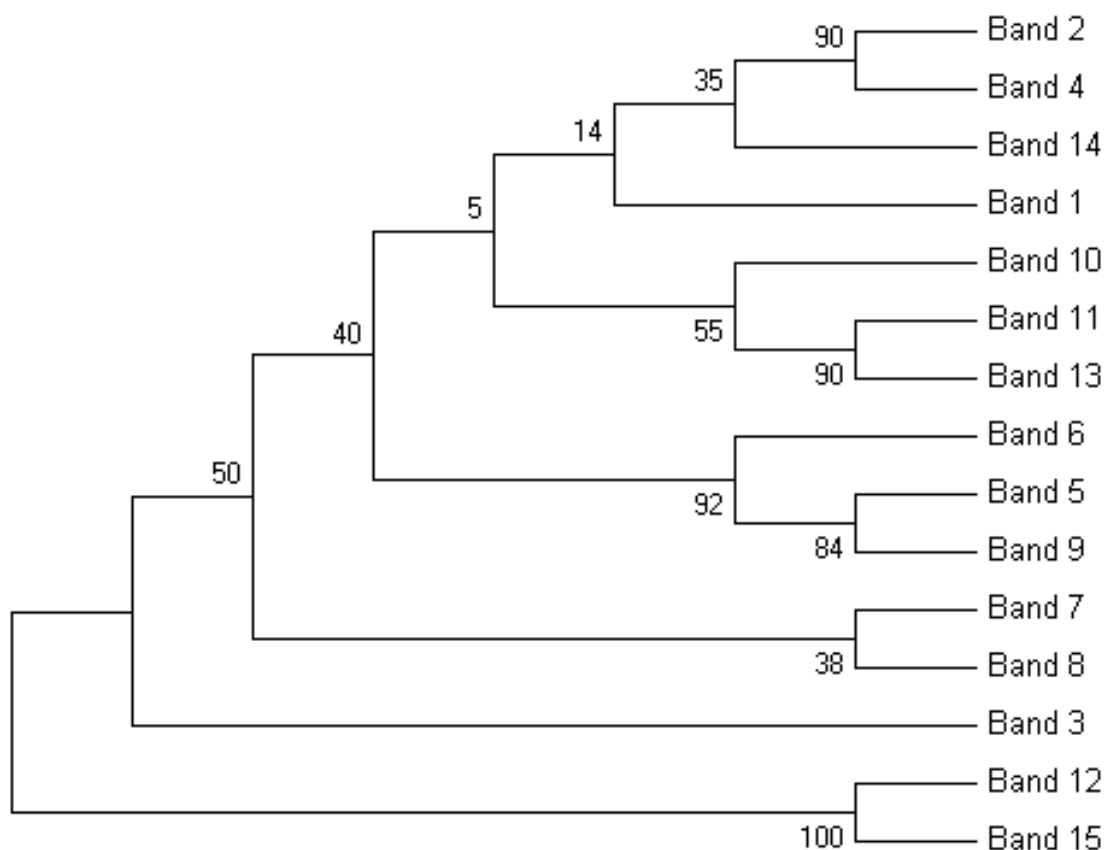


Figure 5.9: A neighbour-joining phylogenetic tree generated from the genetic sequences (bootstrap method)

The alignments of all the sequences were done from the 80-480 bp (400 bp in total).

The phylogenetic tree depicted in Figure 5.10 does not show many good correlations between the different bands. Bands 15 and 12 were depicted as having the exact same sequence, which is indicated by the subgroup they both fall under. Therefore, according to the tree they should be the same organism. This does not coincide with the results from the BLAST. This may be due to the fact that only certain areas of the sequence coincide with each other. However, it is obvious that these organisms are evolutionarily related to one another. Bands 2 and 4 had similar sequences, which is supported by the BLAST results. The organisms to which these sequences belong may be from the same phylum, but were different species (Ye, 2010). No further apparent correlations can be seen on this tree.

The UPGMA dendrogram is presented in Figure 5.11.

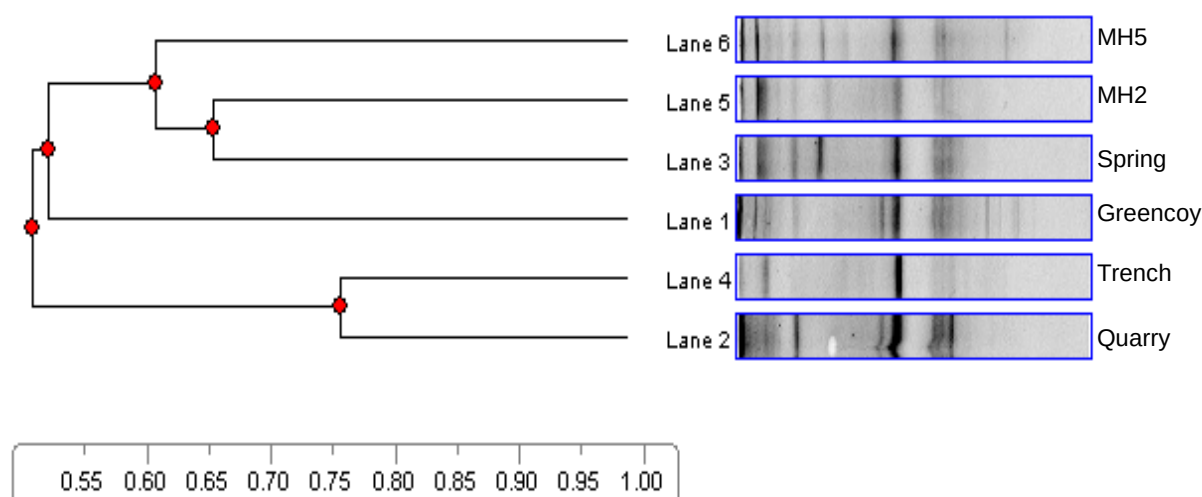


Figure 5.10: A UPGMA dendrogram generated from the DGGE profile

The Rf values of the bands are depicted in Appendix A. From the dendrogram in Figure 5.11 a few clusters can be viewed. They indicate the correspondence of the different bands in relation to each other. This indicates the similar organisms that were present within the different samples. From Figure 5.11 it can be noticed that a few bands occur at the same position (Rf) in each lane. This means that the sequences are similar, and therefore should belong to the same organism (Muyzer *et al.*, 1993; Janse *et al.*, 2004, Motta *et al.*, 2006). Lanes 1, 2 and 6 all have a band at the Rf value of approximately 0.55. The BLAST result of this band (band 1) was an uncultured cyanobacterium strain and is present in those five samples. Most of the lanes (excluding lanes 4 and 6) had a band at a Rf value of approximately 0.450. This value is the same as that of band 6 and was revealed to be an uncultured beta-proteobacterium strain by the BLAST results. All the samples had a band at the Rf value of 0.580, which was the same value as band 8. That means all the samples had the highly unidentified organism.

It is difficult to get exact results when so few microorganisms have been documented and their data added to the data source used for comparison and identification of the sampled organisms. However, a broad range of microorganisms could be identified and can aid in the determination of an environmental condition. They can be representative of the degree of rehabilitation of an area of interest, and in this way aid as a biomarker (Peters *et al.*, 2005; Ranjard *et al.*, 2000; Kölbel-Boelke *et al.*, 1988).

6 HYDROLOGICAL AND GEOHYDROLOGICAL ANALYSIS

6.1 MONITORING POSITIONS

The boundaries selected for the sub-catchment as shown in Figure 6.1 is discussed in Chapter 3. The monitoring sites shown in Figure 6.1 are a combination of data from the NGA (National Groundwater Archive) and the monitoring positions from the mine itself. The purple boundary within the sub-catchment is the delineation of the study area, with particular focus on the northern half.

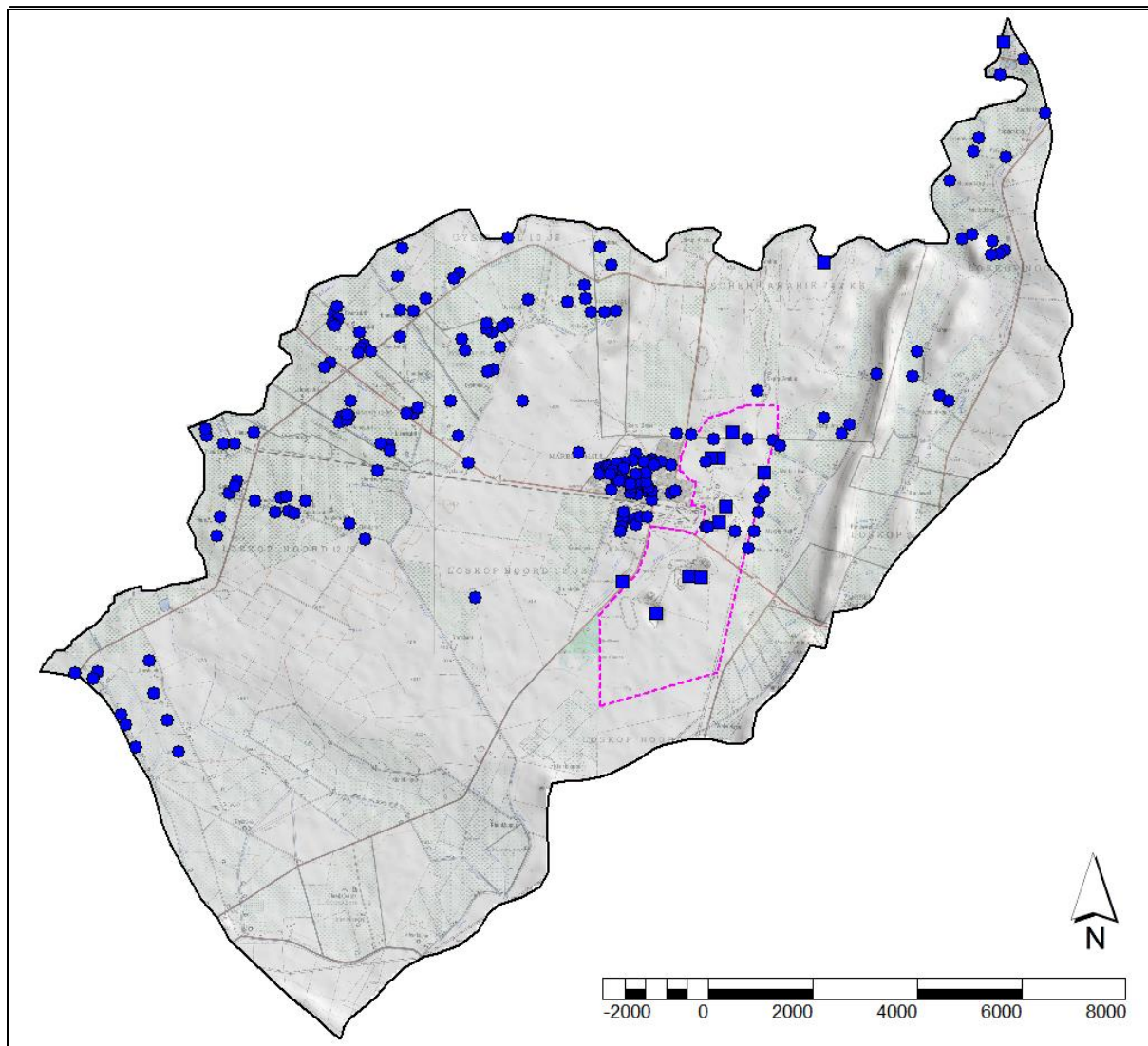


Figure 6.1: Sub-catchment boundary with surface and groundwater monitoring positions

Monitoring positions within and around the study area are shown in Figure 6.2. All sites depicted with a circle represent groundwater sites and all sites depicted with a square indicate surface water sites.

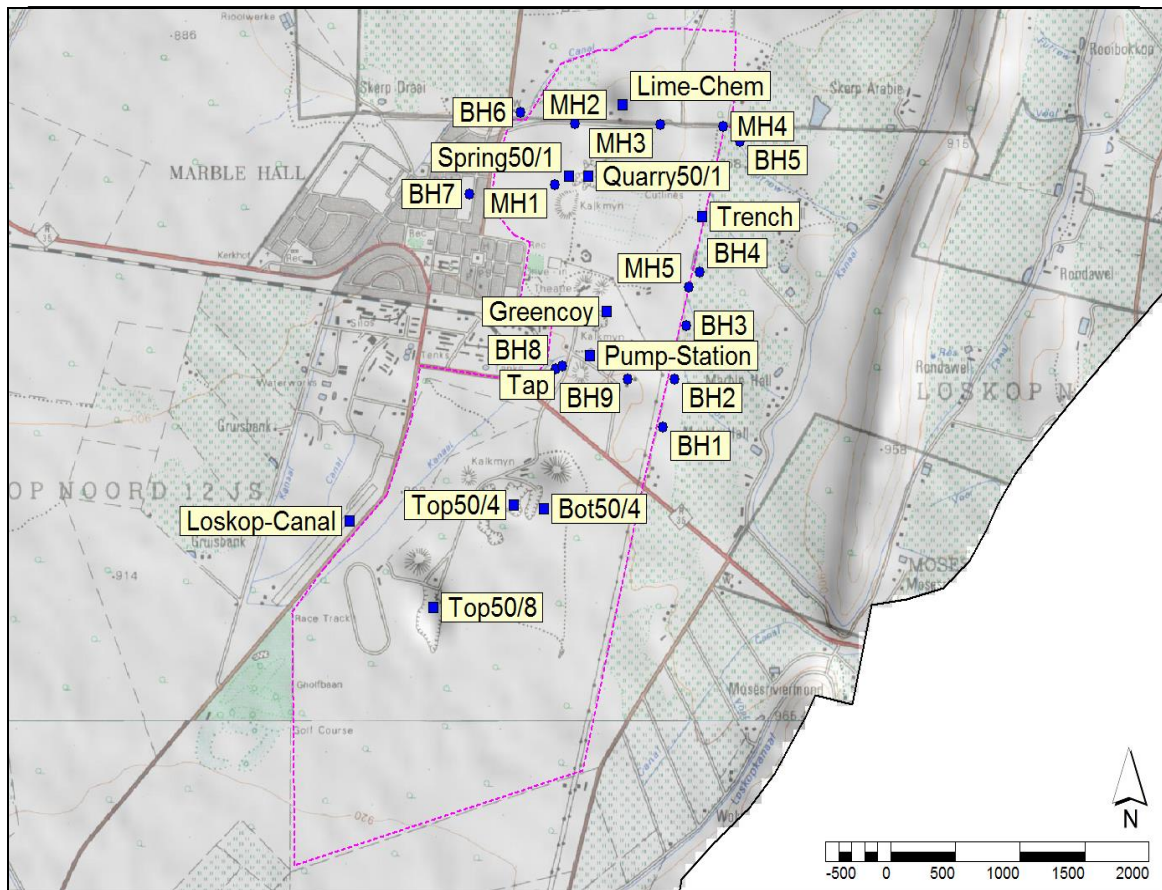


Figure 6.2: Study area monitoring positions

6.2 GROUNDWATER LEVELS

All available groundwater levels within the sub-catchment are shown in Figure 6.3. The water level data obtained from the NGA is considered a “historic” data set, as it has no recent water level measurements. This is indicated in the age analysis of the water levels shown in Figure 6.4.

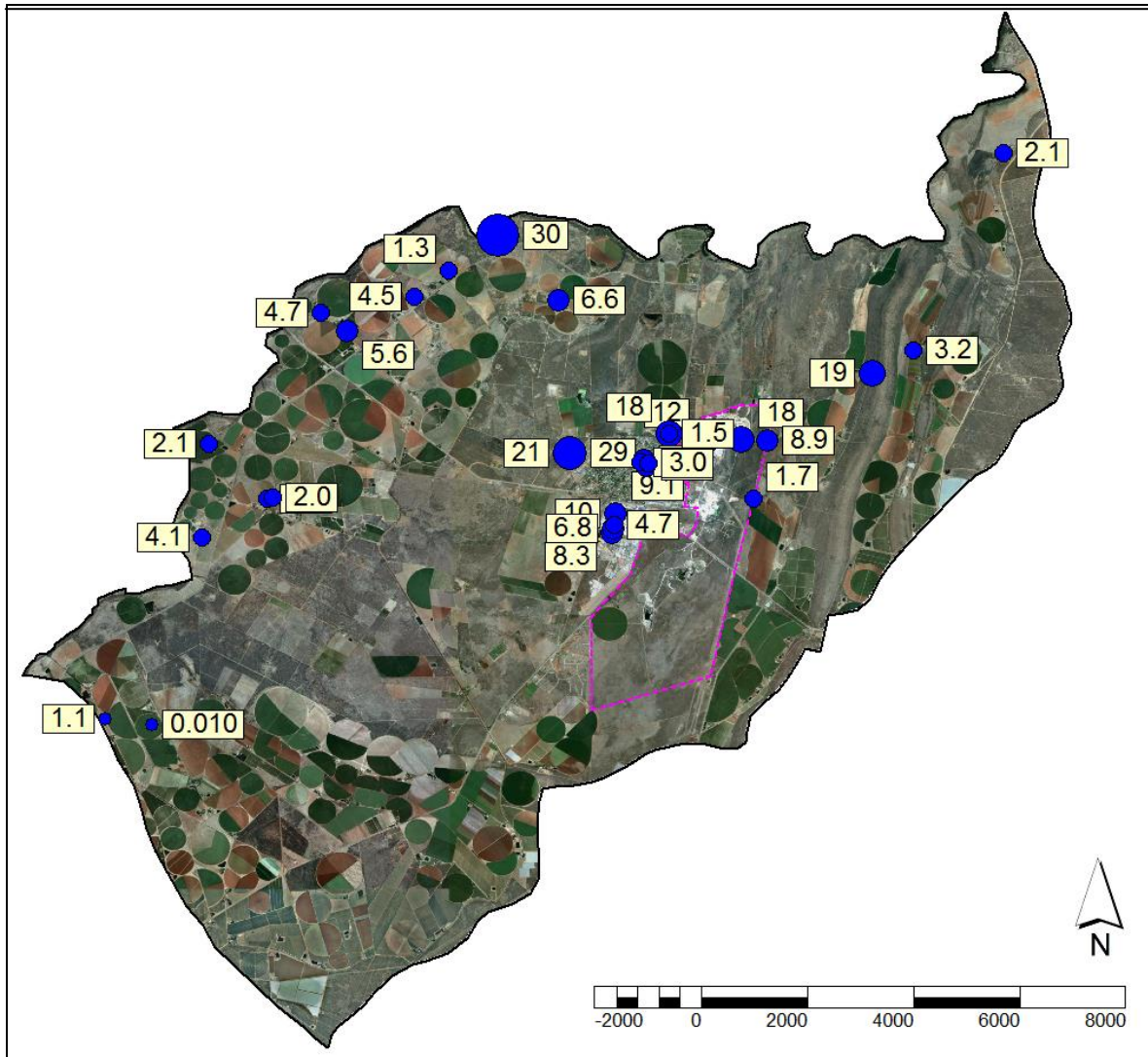


Figure 6.3: Groundwater levels within the sub-catchment (historic and current)

The highest groundwater level in close proximity to the study area, as depicted by Figure 6.3, is 1.5 mbgl. The lowest groundwater level is 29 mbgl. These values vary greatly and may be due to the different geological types in the area.

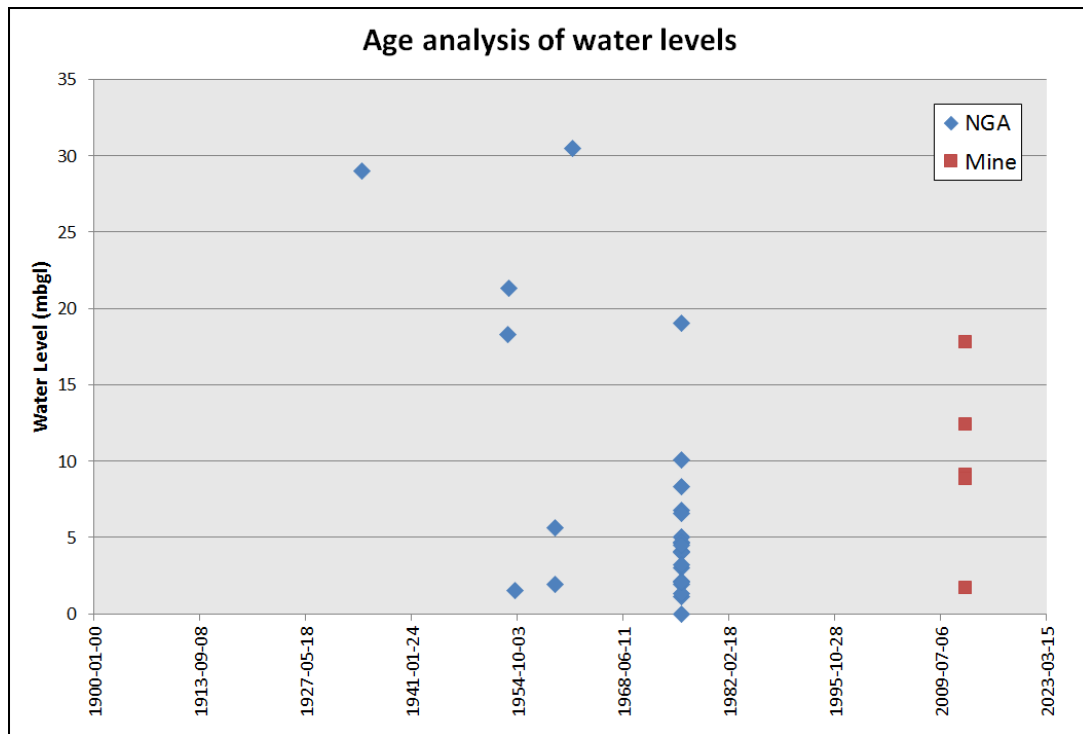


Figure 6.4: Age analysis of water levels

It is difficult to use the NGA data for current water levels due to large time gaps. From Figure 6.4, however, it can be seen that the NGA data from between 1968 and 1982 and the current data from the mine do not show many differences. This may indicate that water levels have not changed much in the area in recent years.

The positions of the recently acquired water samples and their corresponding water levels are shown in Figure 6.5 and Figure 6.6, respectively.

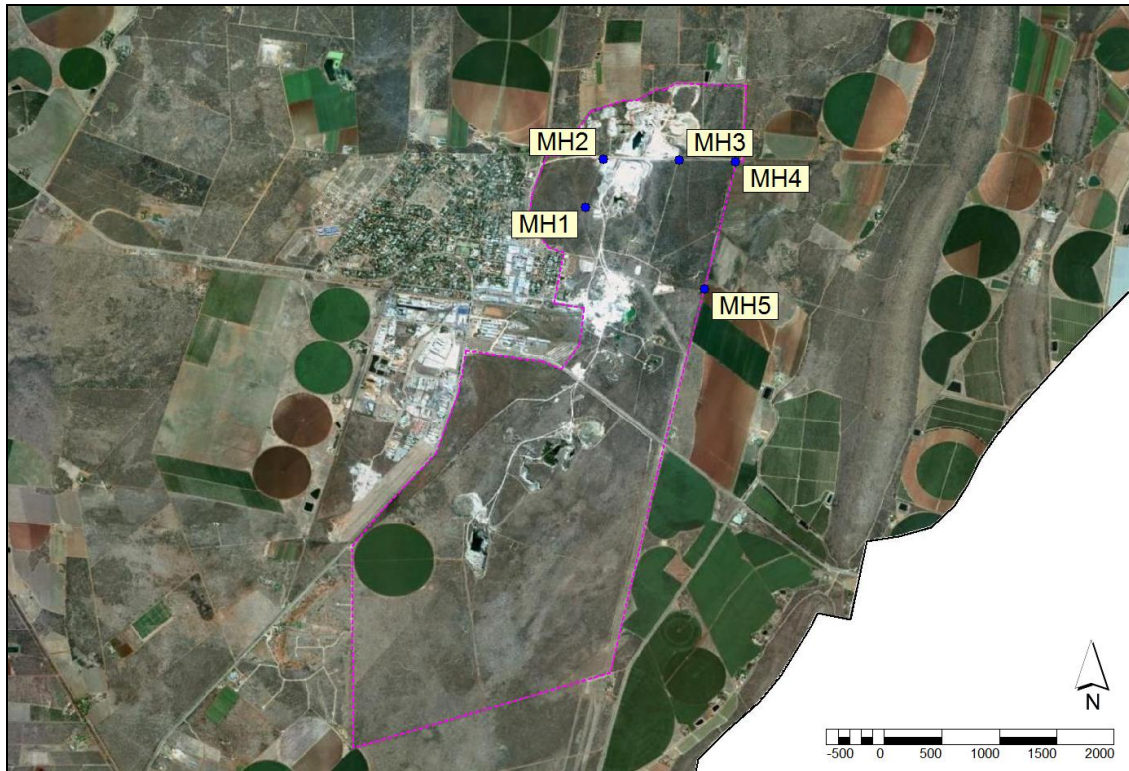


Figure 6.5: Positions of water level measurements

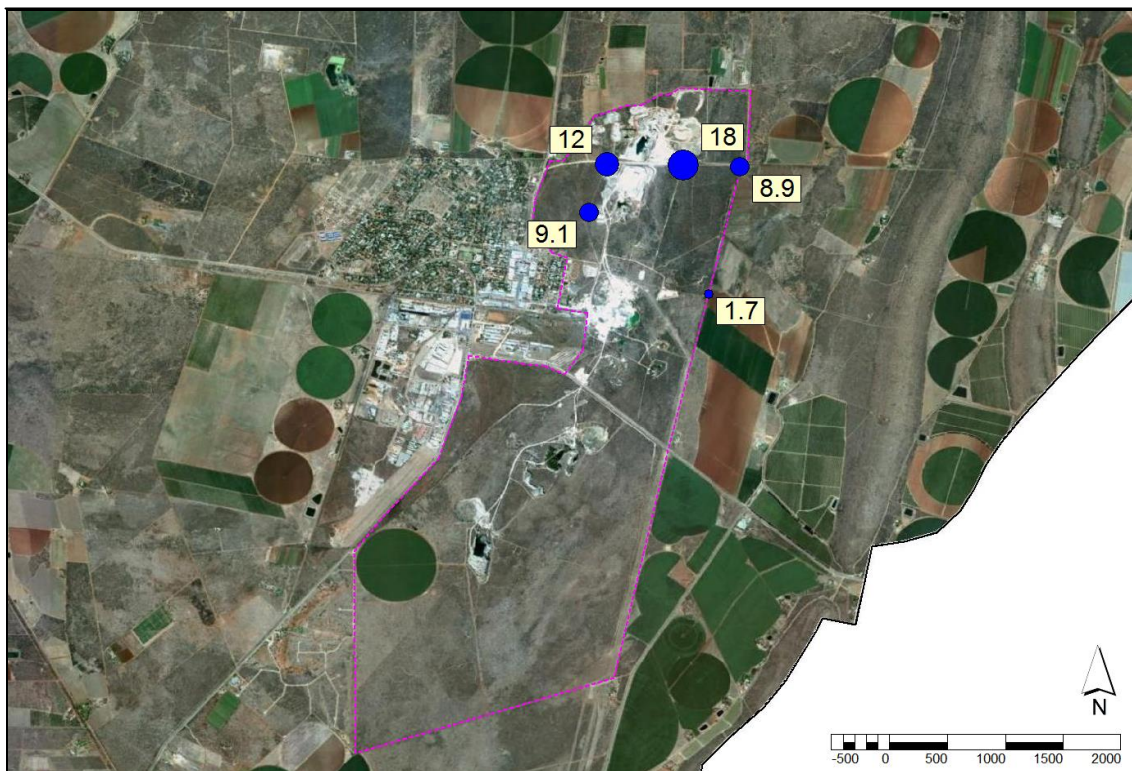


Figure 6.6: Spatial distribution of water level measurements (mbgl)

From Figure 6.6 it can be seen that MH3 has the lowest water levels and MH5 the highest.

The correlation plot between the water levels and elevation of the various monitoring sites is shown in Figure 6.7. It is clear that a strong correlation exists between the elevation and historic NGA water levels. Only four of the monitoring boreholes are used in the calculation of the water level correlation, due to the fact that one monitoring borehole (MH5) has got a very shallow water level. This shallow water level is due to seepage into the borehole which does not reflect a natural water level.

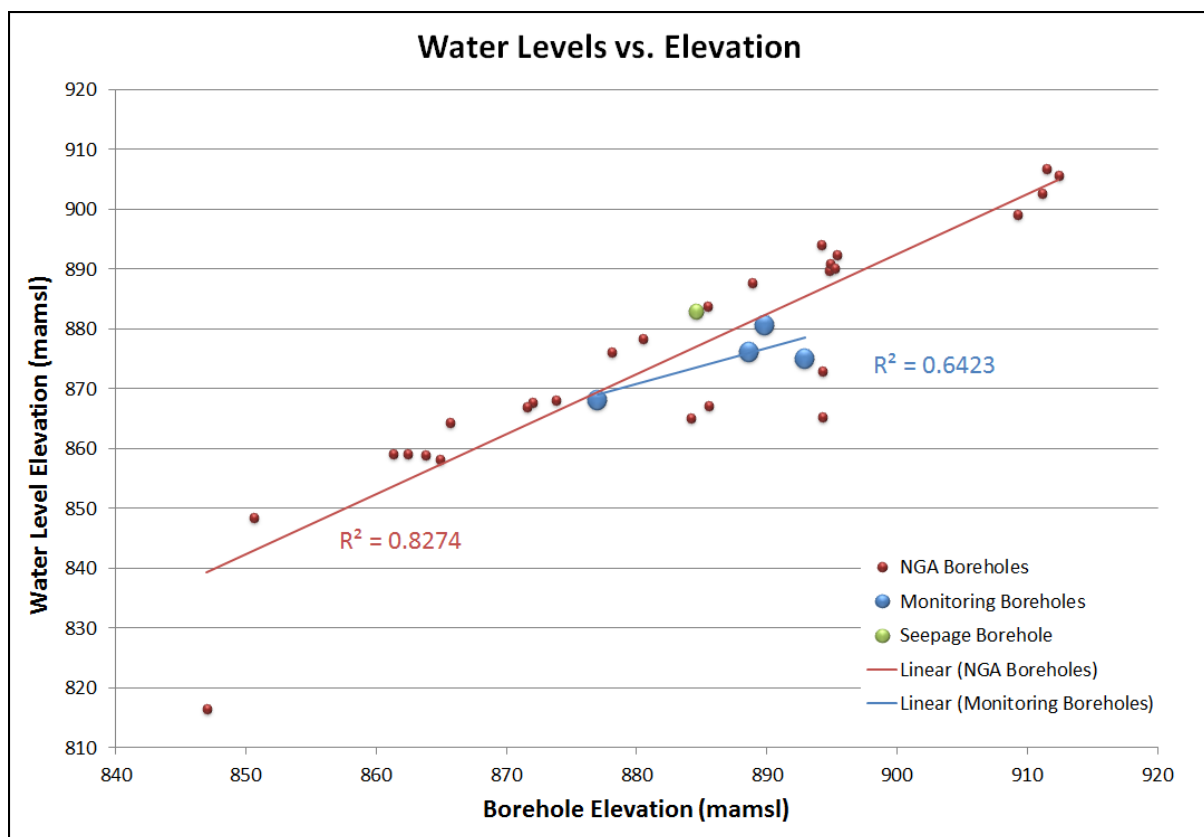


Figure 6.7: Water level vs. elevation

The correlation of the monitoring boreholes compared to the NGA boreholes are considered somewhat weaker, mainly due to the difference in scale. It is a well-known fact that the bigger the aerial extent is, the higher the correlation between water level and elevation and vice versa. A random selection of 60,000 NGA boreholes resulted in a correlation of 99.7% as shown in Figure 7.8, to support the above statement. In the light of this a 64% correlation of water levels in a 500m radius is considered good enough to warrant a Bayesian approach according to the Tripol help file (Dennis & Wentzel, 2007) to generate water levels for the whole area.

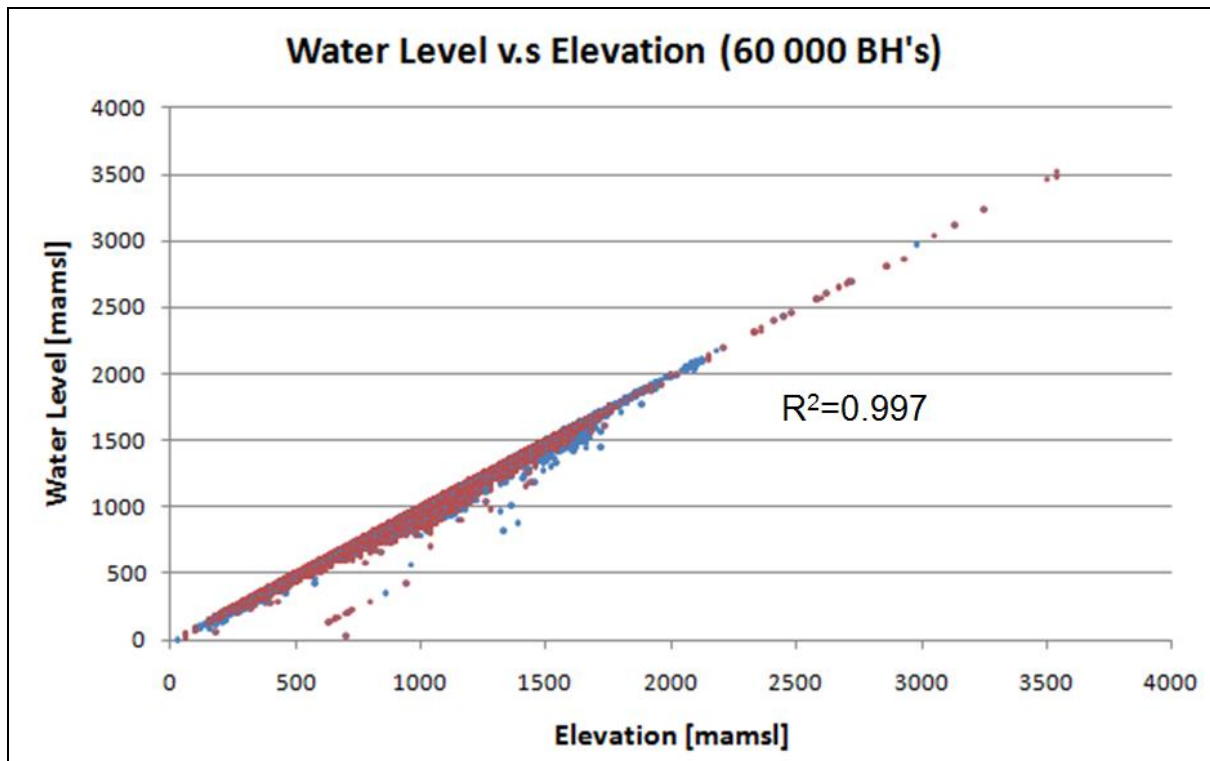


Figure 6.8: Water level vs. elevation over a large area

The resultant flow directions superimposed on the elevation map of the study area is shown in Figure 6.9, from which it is clear that the flow vectors follow topography (elevation in mamsl).

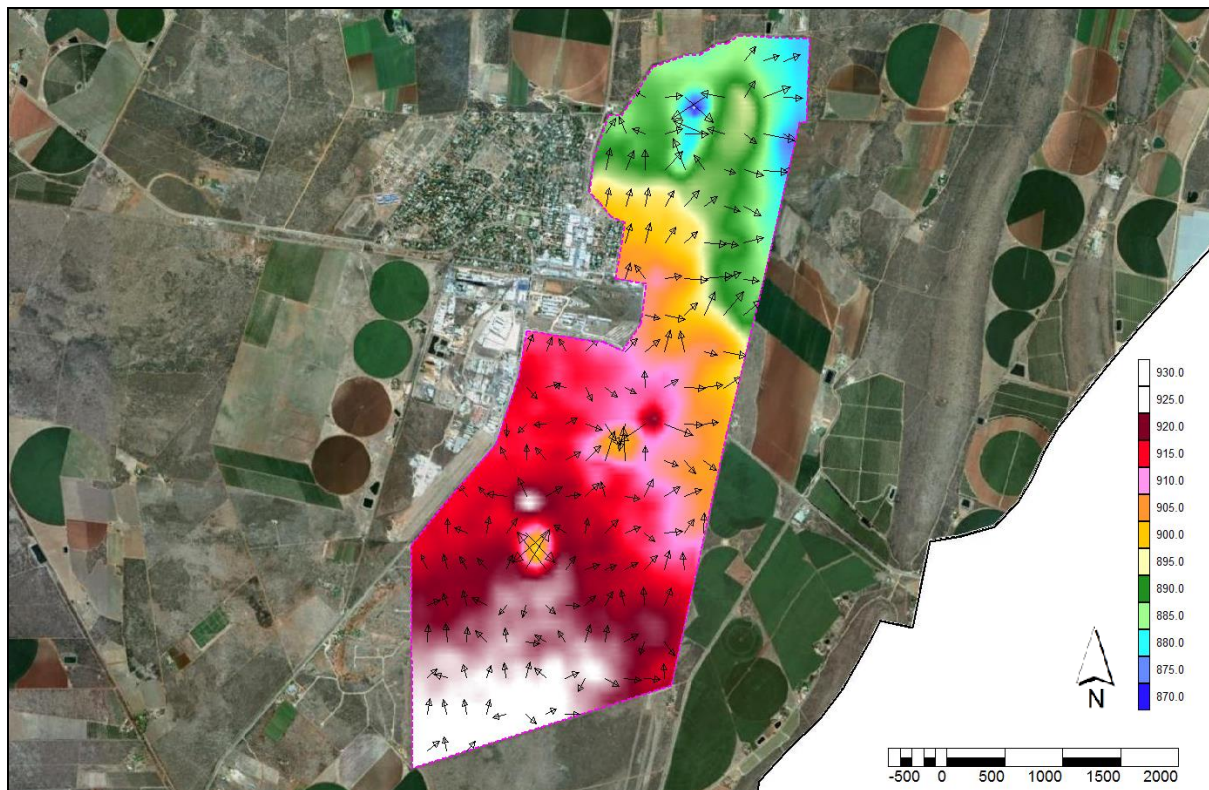


Figure 6.9: Flow directions superimposed on elevation map

Figure 6.10 indicates that there is a predominate flow towards the eastern boundary of the study area, which explains why the farmers along this boundary have dug a cut-off trench to try and minimize possible pollution. MH5 is also situated next to the trench explaining how seepage has caused a very shallow water level. Figure 6.10 also clearly illustrates the flow directions towards the open pits and away from elevated sites.

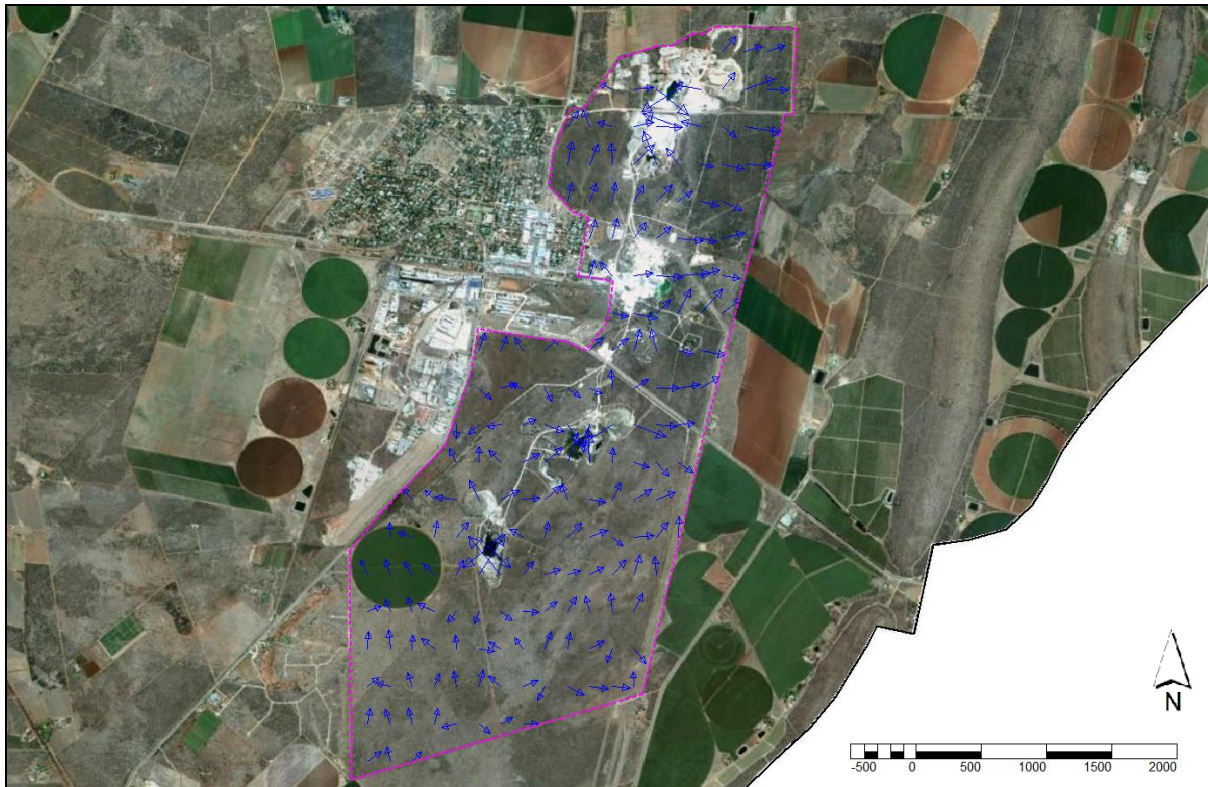


Figure 6.10: Flow directions based on Bayesian interpolation of water levels

6.3 WATER QUALITY

The sites where water samples were taken from for a screening analysis are shown in Figure 6.11.

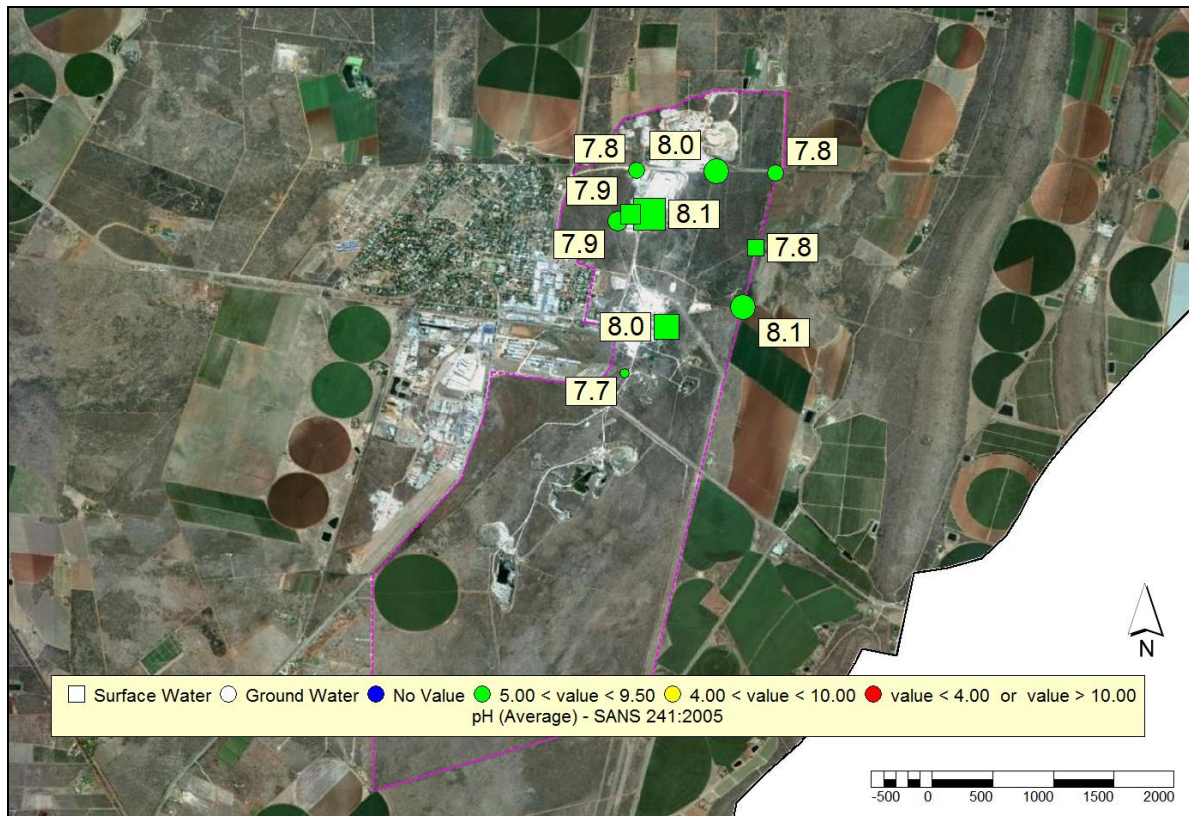


Figure 6.11: Sites used for screening analysis

Electrical conductivity (EC) is a good global indicator to identify possible exceedances in most water quality analysis (APEC, 2013; WHO, 1996; Hayashi, 2003). The average EC (mS/m) results of the screening analysis are shown in Figure 6.12. All surface water sites show elevated EC values together with monitoring boreholes MH3, MH4 and MH5. The Tap water sample is well below the SANS drinking water guideline as was expected (SABS, 2011:241).

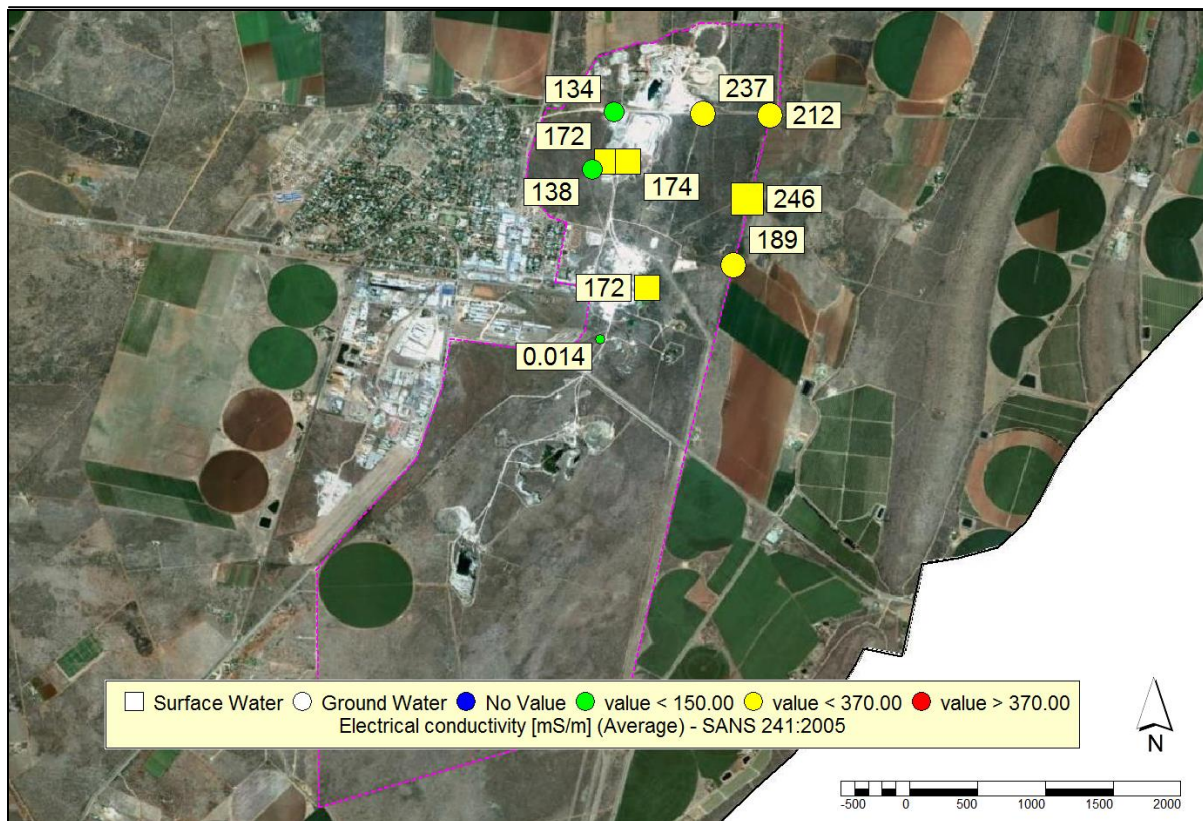


Figure 6.12: Average EC results of screening sites

EC profiles were conducted for all the monitoring boreholes (MH1 – MH5) to identify possible fracture zones. The profiling results are shown in Figure 6.13. Note that no information on casing lengths were available at the time of the investigation and the EC values displayed in Figure 6.13 are field measured values and differ from the laboratory measured EC values presented in Figure 6.12. This difference between EC values is not problematic as the field measured values are interpreted based on relative differences.

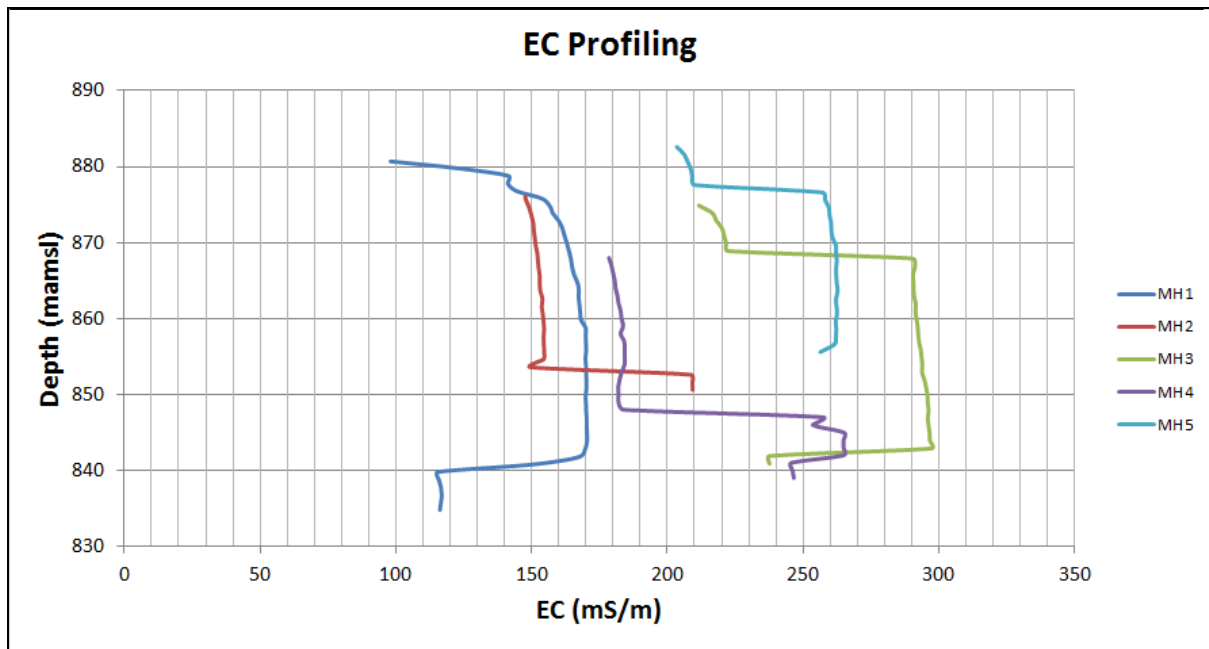


Figure 6.13: EC profiling results

The EC profiles show a fairly constant EC once the depth is greater than the casing length, or possibly a confining layer. Once the bottom of the boreholes are reached another EC jump occurs, which is either related to a geological layer that was drilled into or possibly mud at the bottom of the borehole. The fact that there is no sudden increase or decrease in EC values across the “constant” sections of the profile indicates a uniform geological formation with no major fractures. This observation is also consistent with all the available boreholes logs presented in Appendix A with the exception of one, which consists of two geological types.

The positions of the boreholes logs presented in Appendix A are shown in Figure 6.14.

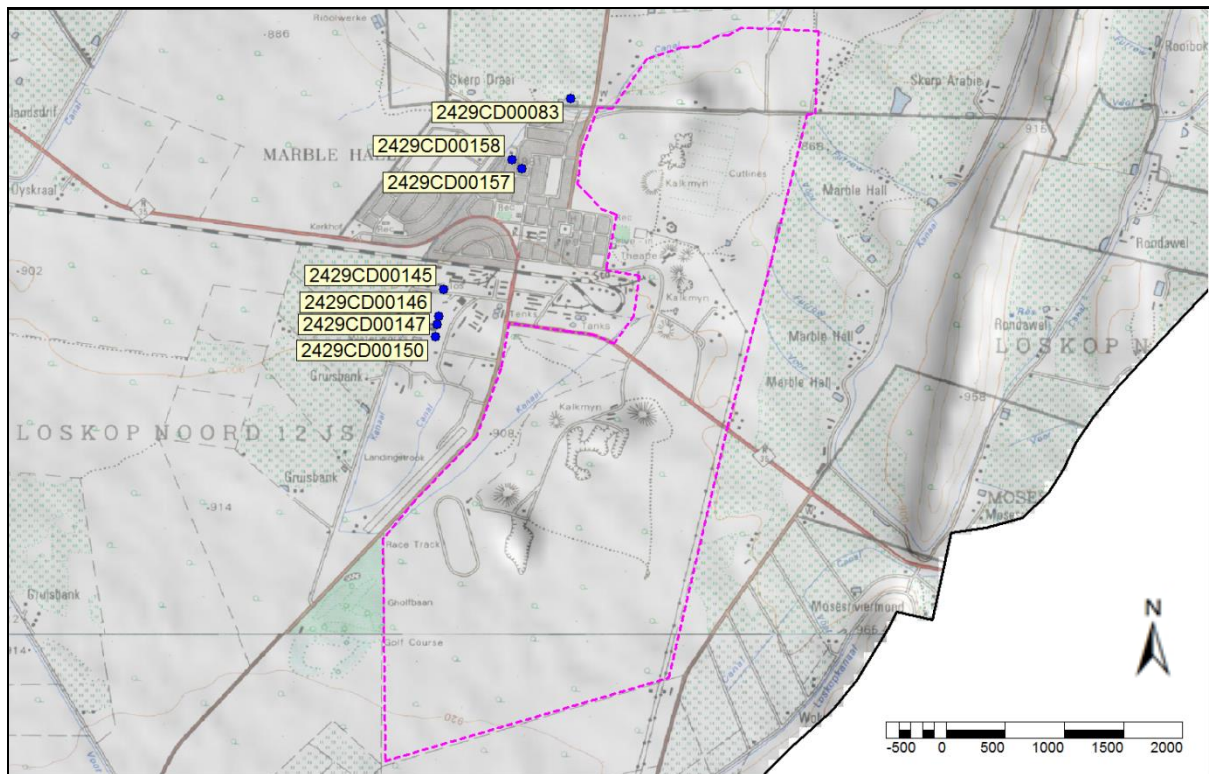


Figure 6.14: Borehole log positions

The pH values for the monitoring sites where the screening was conducted, is shown in Figure 6.15. All sites comply with the specified standard, which is to be expected due to the dolomitic subsurface which has a buffering ability.

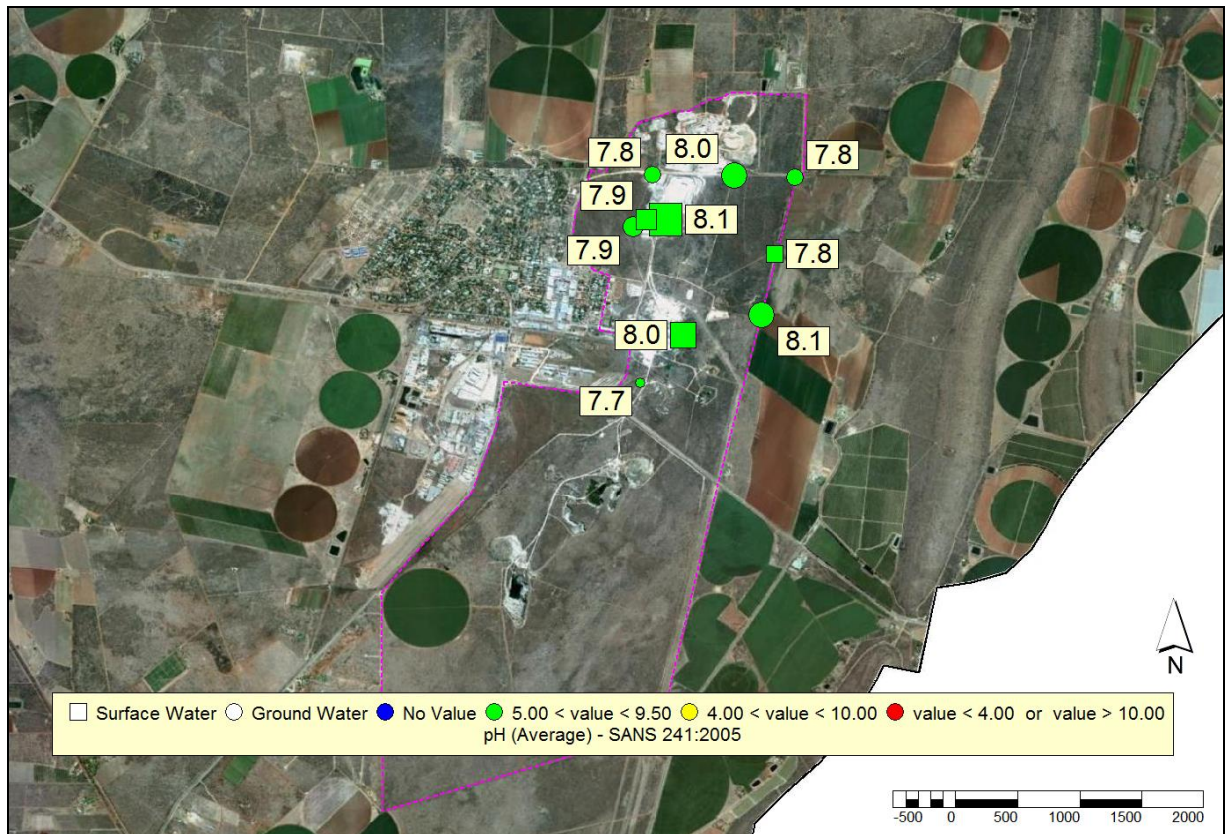


Figure 6.15: pH values for screening sites

The nitrate values at the screening sites are shown in Figure 6.16, which are in all probability a result of blasting in the mining operations.

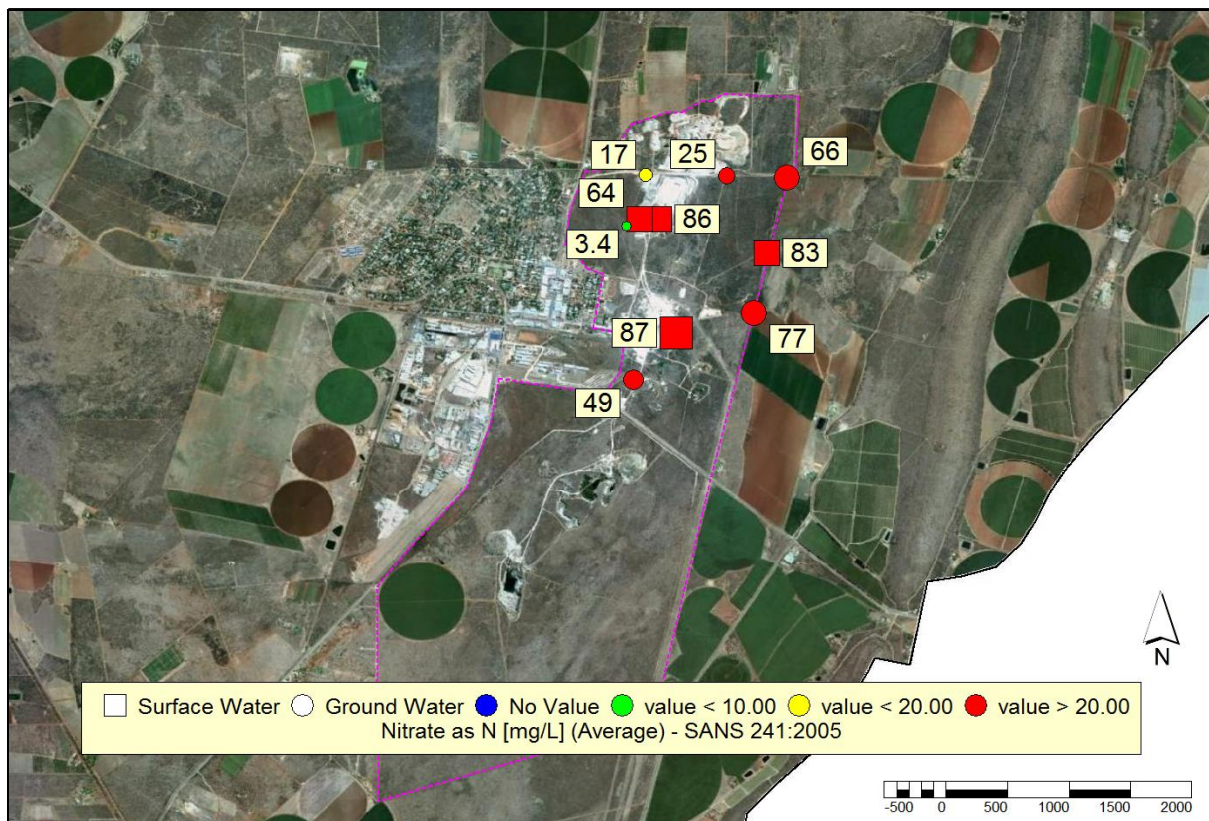


Figure 6.16: Nitrate values at screening sites

6.4 SOURCES OF POLLUTION

The main source of pollution on the site is buried Cr(VI) waste that originated from World War II where leather products were made for the war effort. It was felt at the time that the Cr(VI) would be harmless when capped and placed in marble settings, hence the storage of this waste at this site. The main challenge lies in identifying all of the positions of the buried deposits.

All monitoring sites where Cr(VI) are monitored are shown in Figure 6.17 and the average Cr(VI) values for these sites are shown in Figure 6.18. It is clear from Figure 6.18 that all the Cr(VI) concentrations of the surface and groundwater monitoring points exceed the drinking water guidelines.

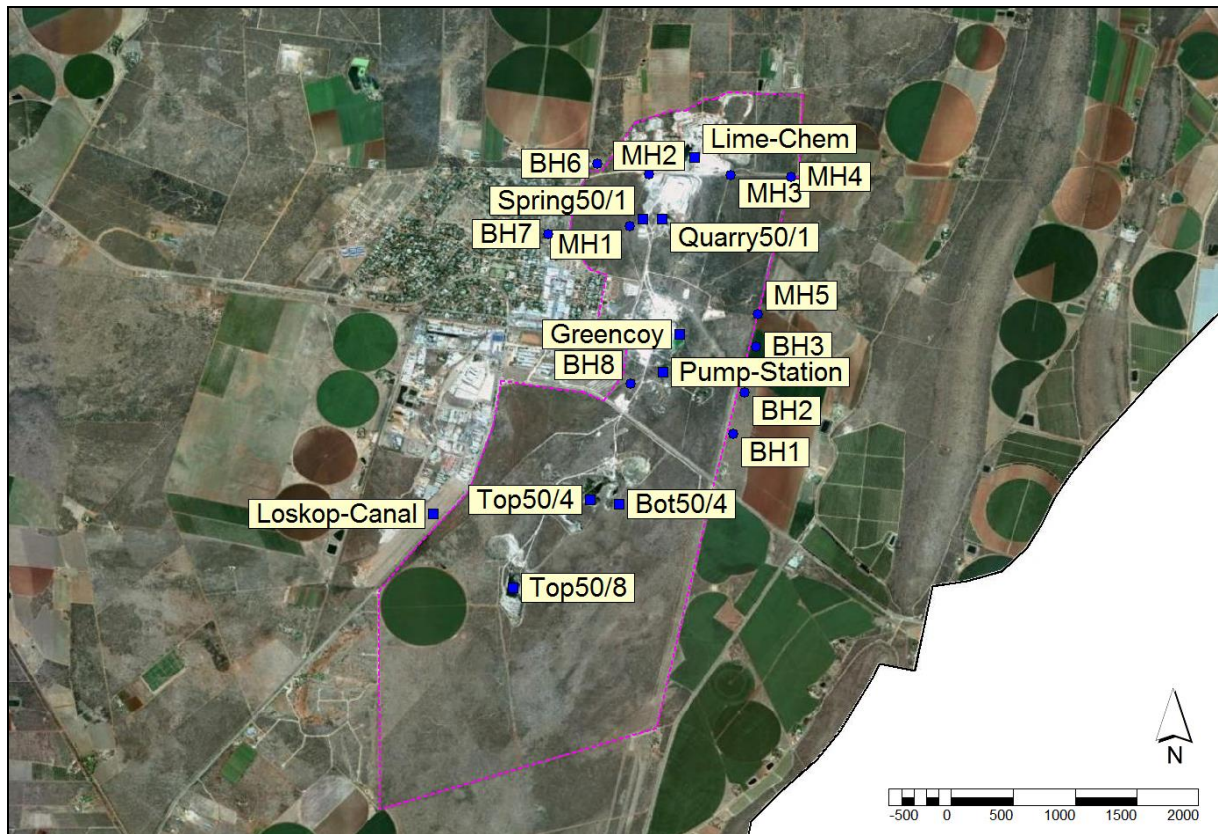


Figure 6.17: Cr(VI) monitoring sites

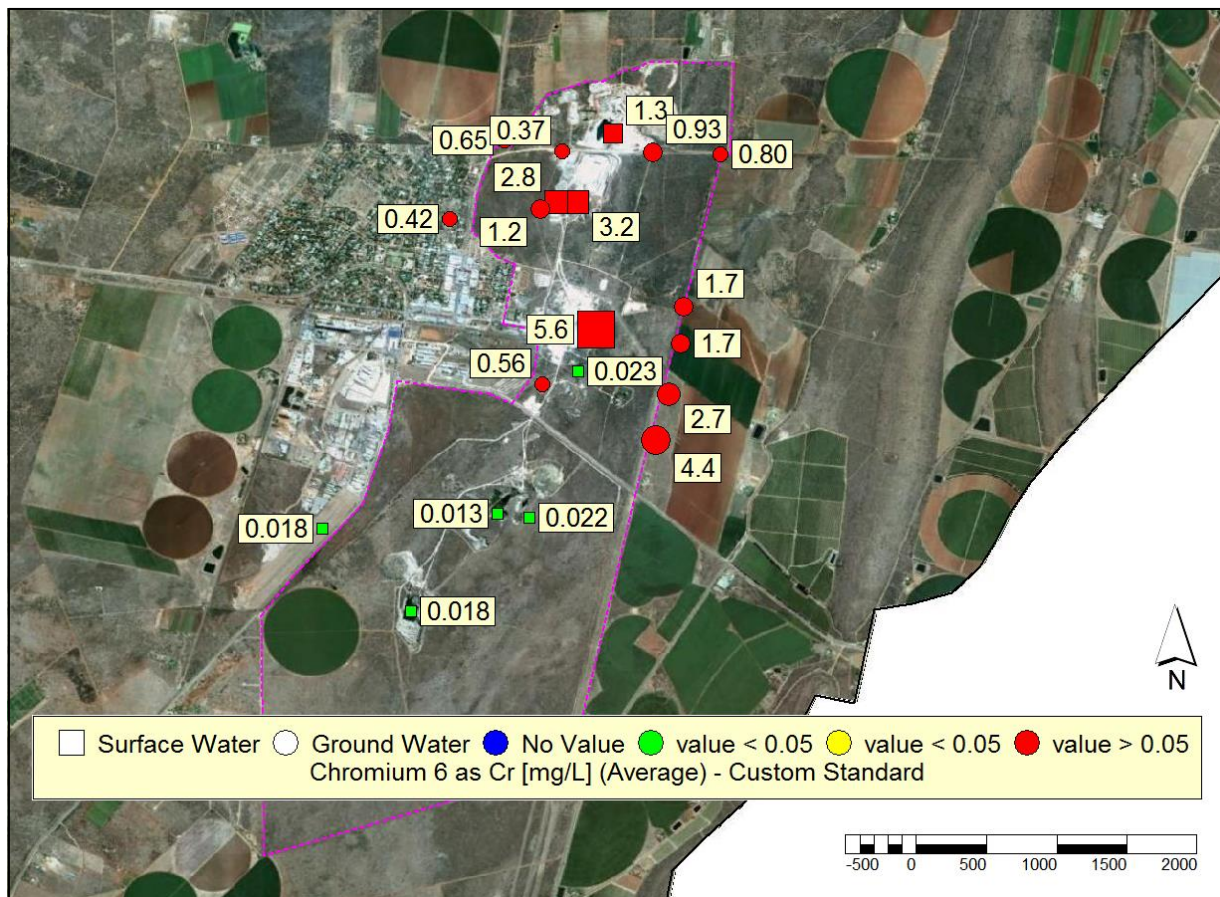


Figure 6.18: Cr(VI) average values

The Cr(VI) time series values for some surface and groundwater sites are presented in Figure 6.19 and Figure 6.20, respectively.

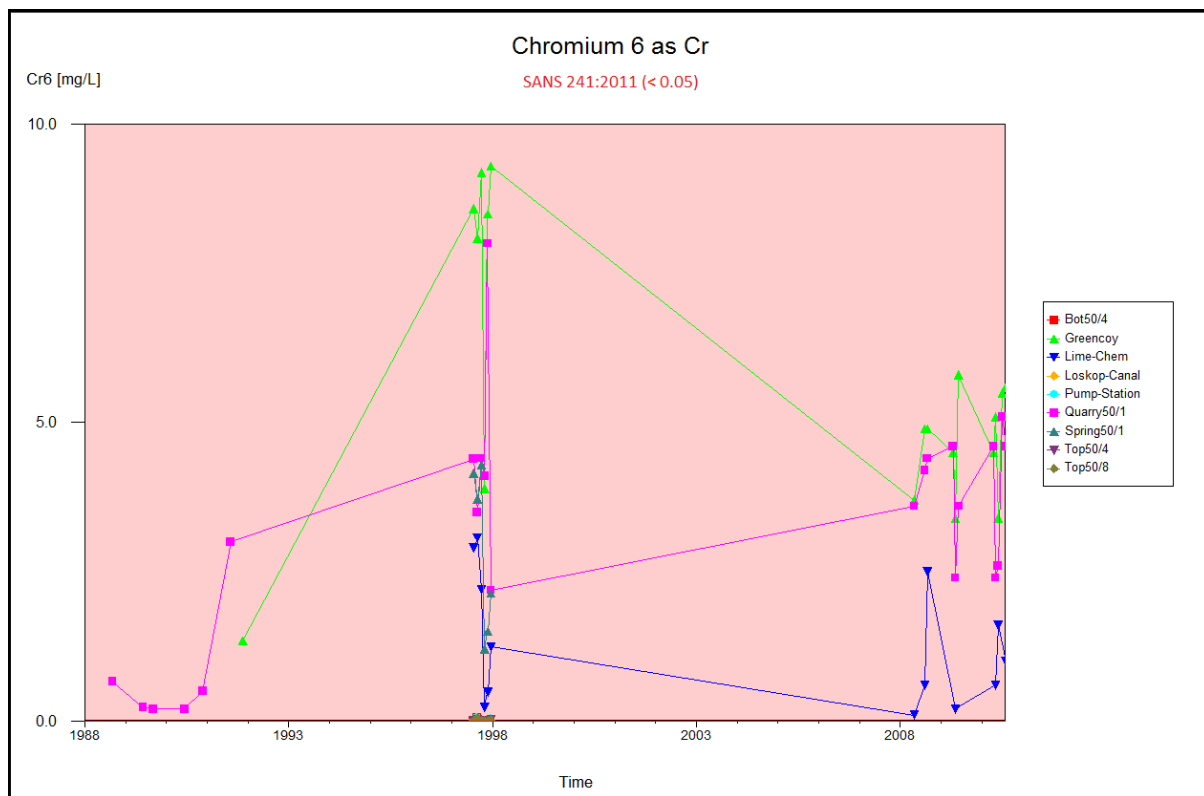


Figure 6.19: Time series data for Cr(VI) levels at surface water sites

A peak is seen during 1998. The reason for this is unknown. More recent data indicate that the Cr(VI) levels remain above the TWQR.

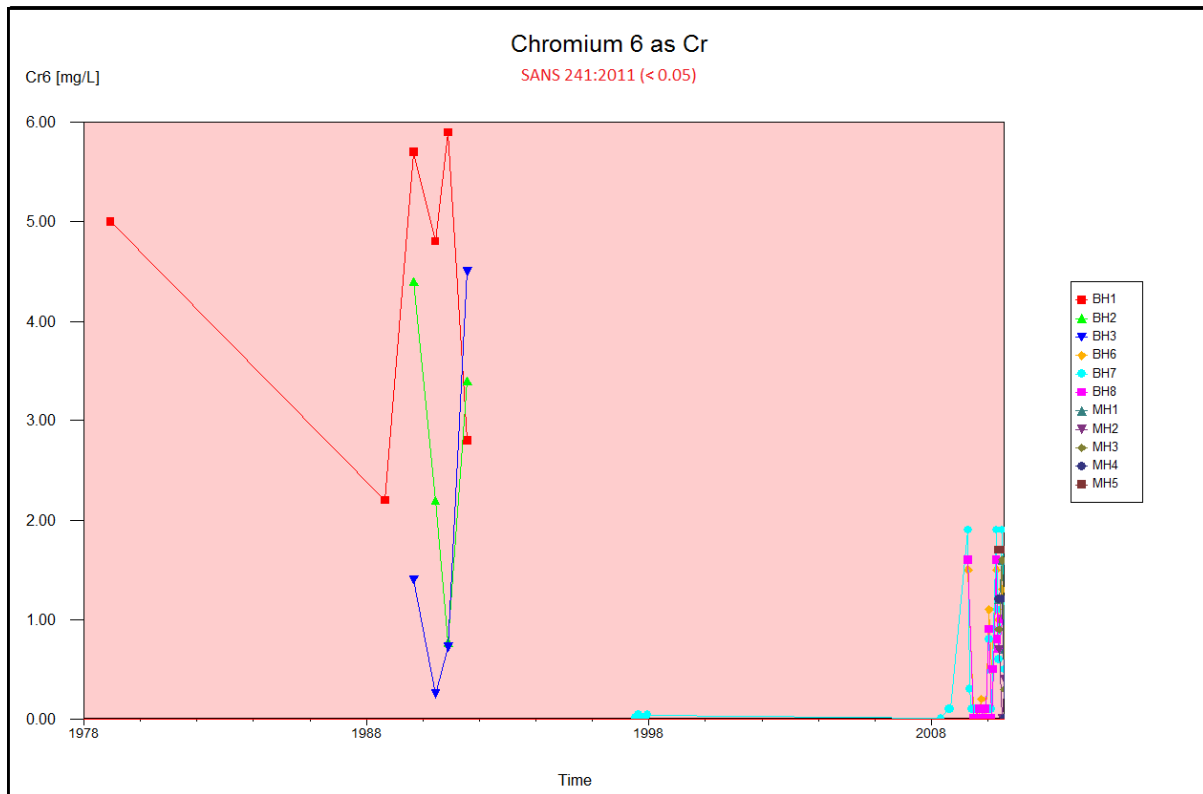


Figure 6.20: Time series data for Cr(VI) levels at groundwater sites

Again a peak is seen during 1998, which correlates with the peak values in Figure 6.19. The reason for this peak is unknown and could possibly be contributed to the analysis technique used in the past. There is no evidence that currently suggest that flushing is taking place which could explain the drop in Cr(VI) levels. More recent data indicate that the Cr(VI) levels remain above the TWQR.

Excavations within the study area have revealed some of the buried deposits, indicated in Figure 6.21 as Dig1 – Dig11. The digout site details are listed in Table 6.1.

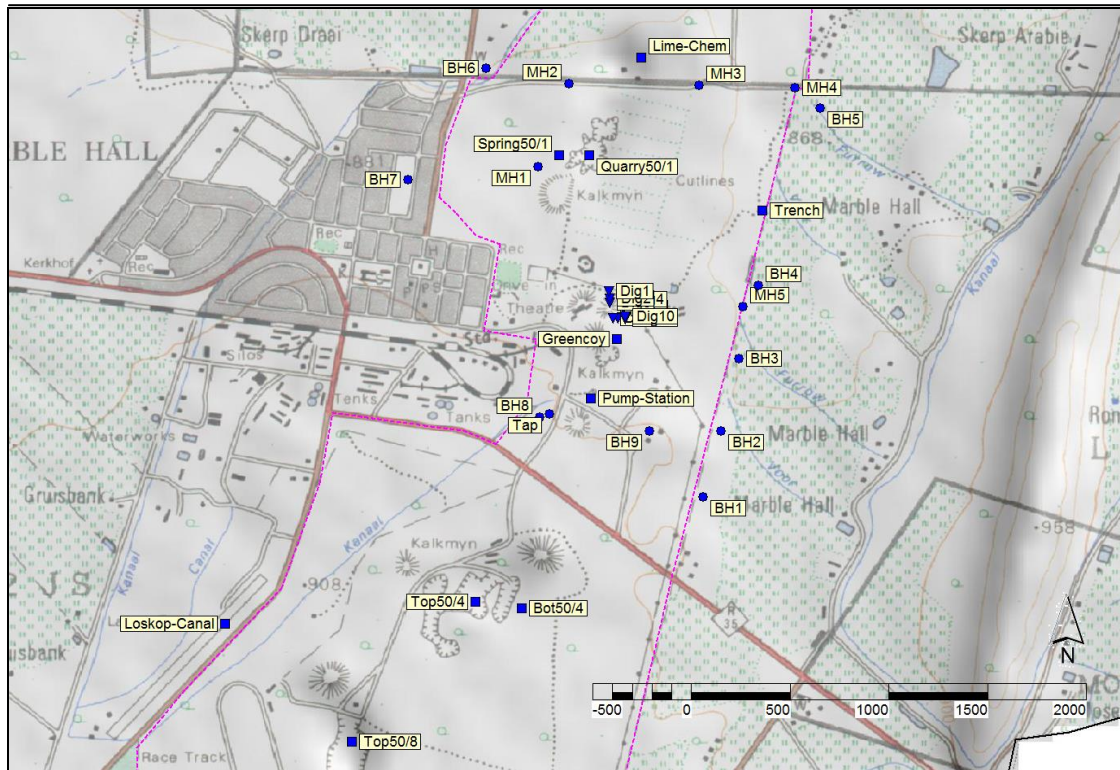


Figure 6.21: Positions of digout sites

Table 6.1: Digout site positions and deposit thickness

Longitude	Latitude	Elevation (mamsl)	Soil Thickness (m)	Deposit Thickness (m)
29.304583	-24.970294	899	0.5	0.025
29.304622	-24.970658	900	1.5	1.5
29.304628	-24.970850	900	1.3	1
29.305258	-24.970692	898	2.2	1
29.305289	-24.970886	898	2	1
29.305331	-24.971156	898	1.5	1
29.304781	-24.971661	900	0.5	0.1
29.305031	-24.971631	899	0.8	0.2
29.305231	-24.971594	898	1.3	0.5

29.305397	-24.971542	897	0.75	0.5
29.305406	-24.971669	897	0.3	0.15

At this stage the position of other buried deposits remains pure speculation. One of the aims of this study is to assist in the establishment of future methodologies to help identify possible deposit positions through the use of biomarkers.

6.5 CONCEPTUAL MODEL

A simple conceptual model was created to illustrate the cross section from MH2 to Greencoy. This area is depicted in Figure 6.22.

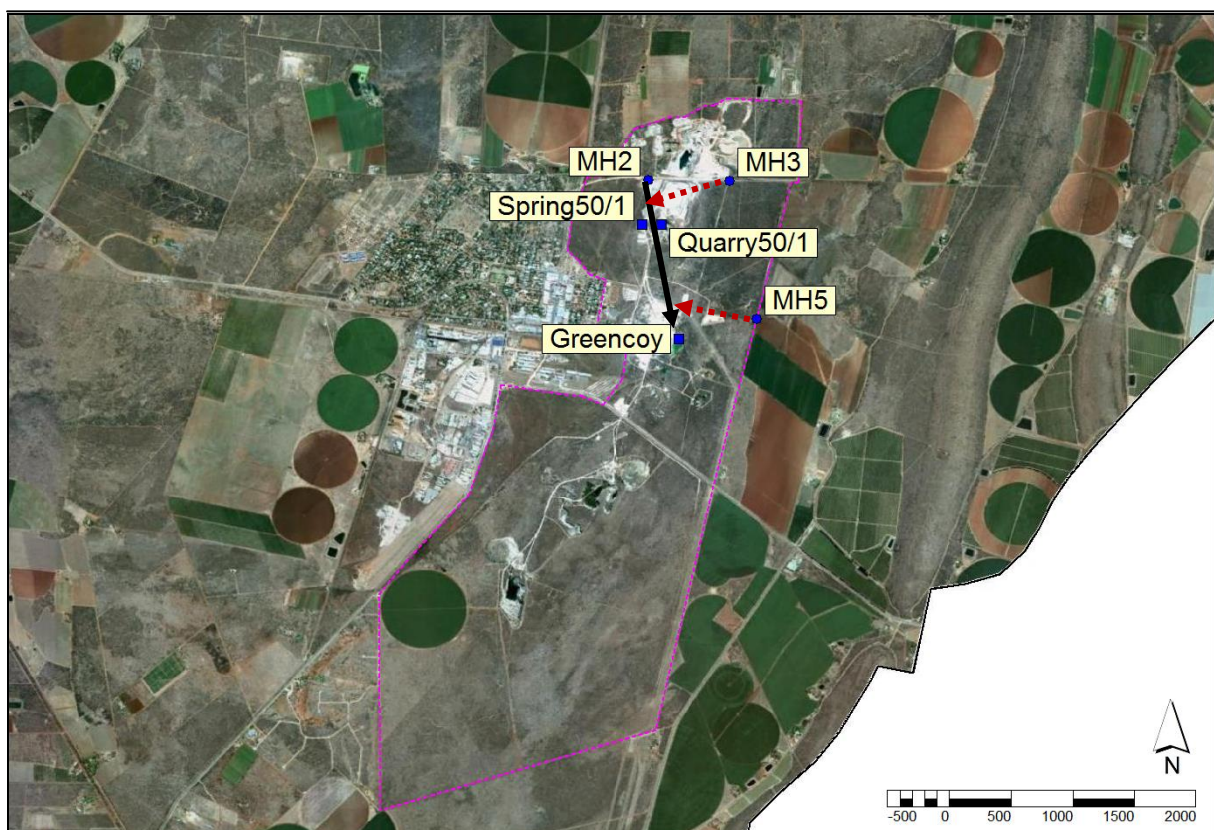


Figure 6.22: The cross section area for the conceptual model

Note that MH3 and MH5 are projected onto the cross section line, although in reality they are some distance away. The flow directions shown in Figure 6.23 indicate the possibility that these two sites could be reached from the digout points, hence their inclusion in the conceptual model.

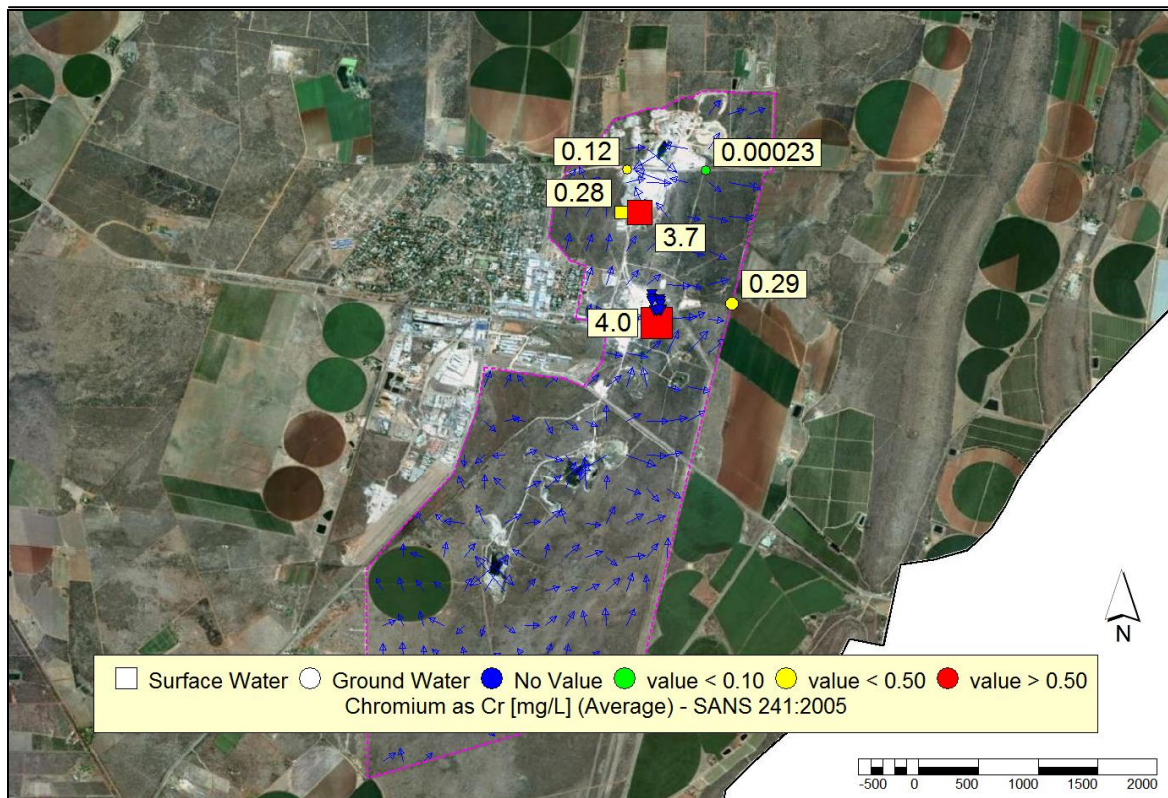


Figure 6.23: Flow directions with Cr levels at screening sites

The conceptual model created from the cross section is shown in Figure 6.24.

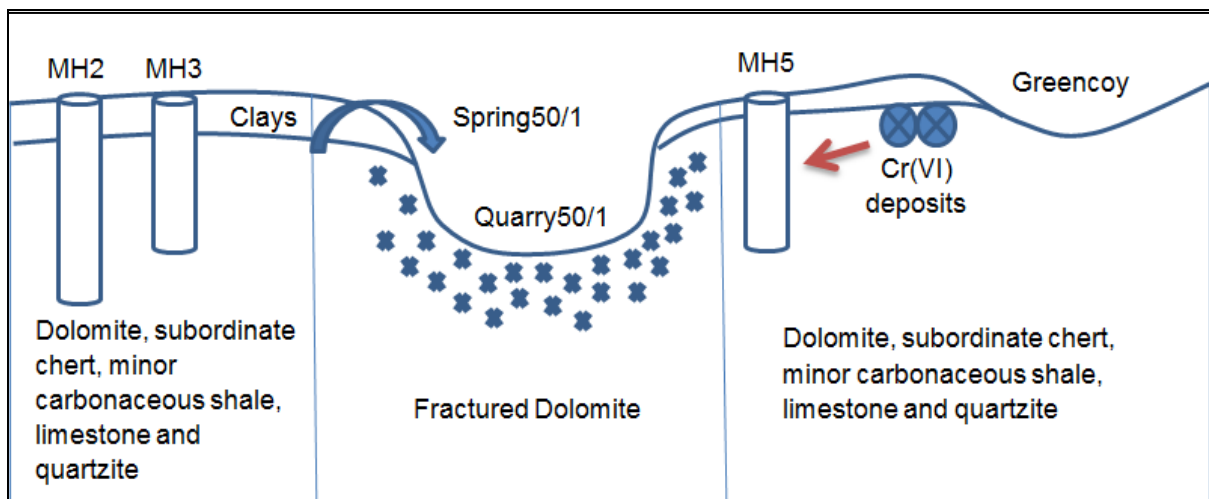


Figure 6.24: The conceptual model

From the conceptual model it is clear that the known deposits could seep to MH5 and Quarry50/1 due to the elevation differences. Evidence of fracturing around the quarry is visible, as well as the seepage of Cr(VI) pollution into the pit as shown in Figure 6.25. At this stage there is, however, no confirmation that the seepage into the pit originated from the

known deposits. Furthermore, the conceptual model indicates fractured dolomite which could be due to the blasting of the quarry wall.



Figure 6.25: Fractured quarry wall with Cr(VI) polluted seepage

Figure 6.26 indicates the down gradient nature of MH5 with respect to the deposit; these are actual elevations with the modelled Bayesian water level.

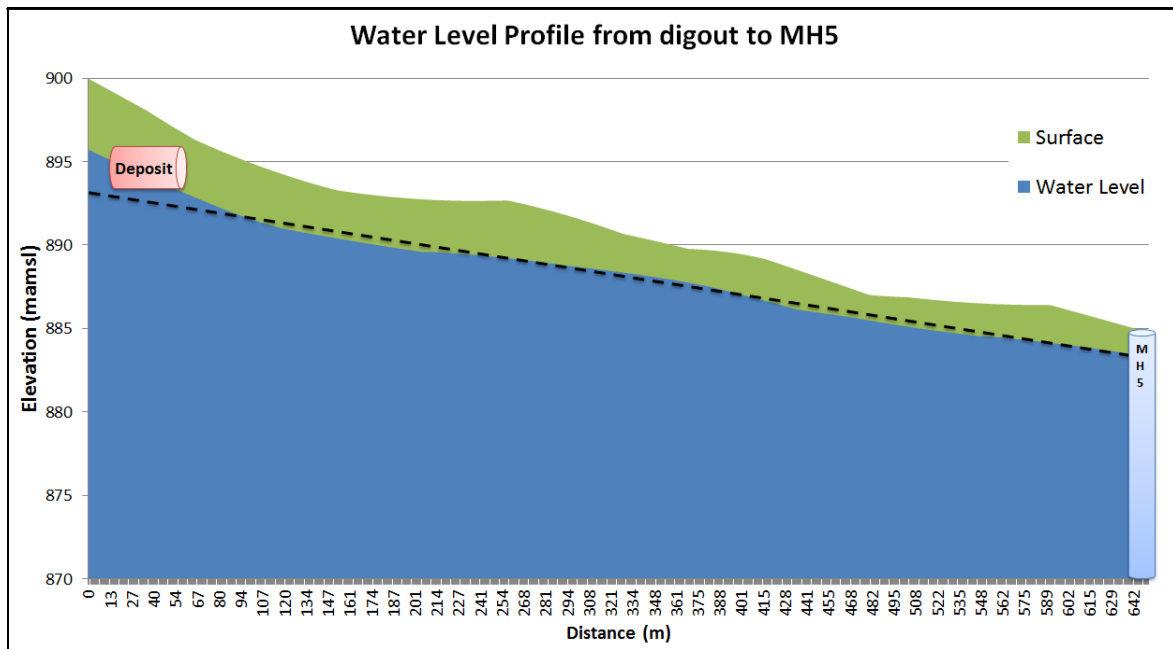


Figure 6.26: Down gradient nature of MH5 with respect to the deposit

Figure 6.26 illustrates that there is an elevation difference of approximately 8 mamsl. The water, therefore, moves along the gradient from the mine pit towards MH5.

7 DISCUSSION OF RESULTS

7.1 INTRODUCTION

In order to relate the biomarkers to individual sites associated with a dominant chemistry, Principal Component Analysis (PCA) was employed. PCA allows the display of data correlation in 2 dimensions for multi variables. More detail about PCA is given in Appendix A.

The sites used in the biomarker analysis are shown in Figure 7.1.



Figure 7.1: Sites used in the biomarker analysis

MH2 and MH5 are groundwater sites. Greencov and Quarry50/1 are open water bodies. Quarry50/1, however, is a smaller body of water that is driven by seepage. The Greencov and Quarry50/1 sites are shown in Figure 7.2 and Figure 7.3, respectively. Spring50/1 and Trench are related to seepage, although Trench intersects the subsurface seepage whereas Spring50/1 is visible on the surface as shown in Figure 7.4.

7.2 SITE CLASSIFICATION

The dominant chemistry for each site, as well as the biomarkers identified, will be used to setup a site classification for future use. For the purpose of the classification, the following definitions are assumed:

- *Surface Water* – Water from a surface water body exposed to sunlight
- *Groundwater* – Water from the subsurface not exposed to sunlight
- *Seepage* – Water originating from groundwater seeping into a surface water body e.g. a trench and spring

Note that seepage, as per definition, is linked to groundwater. However, a distinction is drawn to accommodate a site like a trench which is an open body of water driven by groundwater, specifically seepage. Figure 7.2, Figure 7.3 and Figure 7.4 are photographs of three sample sites. Figure 7.2 illustrates the surface body Greencoy. Figure 7.3 and Figure 7.4 illustrate the different rock layers through which the Cr(VI) polluted water seeps.



Figure 7.2: Greencoy site



Figure 7.3: Quarry50/1 site

The light green areas in the rock layer are where the Cr(VI) polluted water seeps through.



Figure 7.4: Spring50/1 site

7.2.1 SITE CHEMISTRY

The Piper diagram for the sites in Figure 7.1 is shown in Figure 7.6. It is visible from Figure 7.6 that the two surface sites (Greencoy and Quarry) group together, as is the case with the two seepage sites (Spring50/1 and Trench). The two groundwater sites (MH2 and MH5), however, exhibit different a character based on the major anions and cations. The fact that MH5 is close to the trench and intersects some of the seepage moving toward the trench can also be seen from Figure 7.6, as these sites are related with respect to their hydrochemistry. A more detailed discussion of the Piper Diagram is documented in Appendix A.

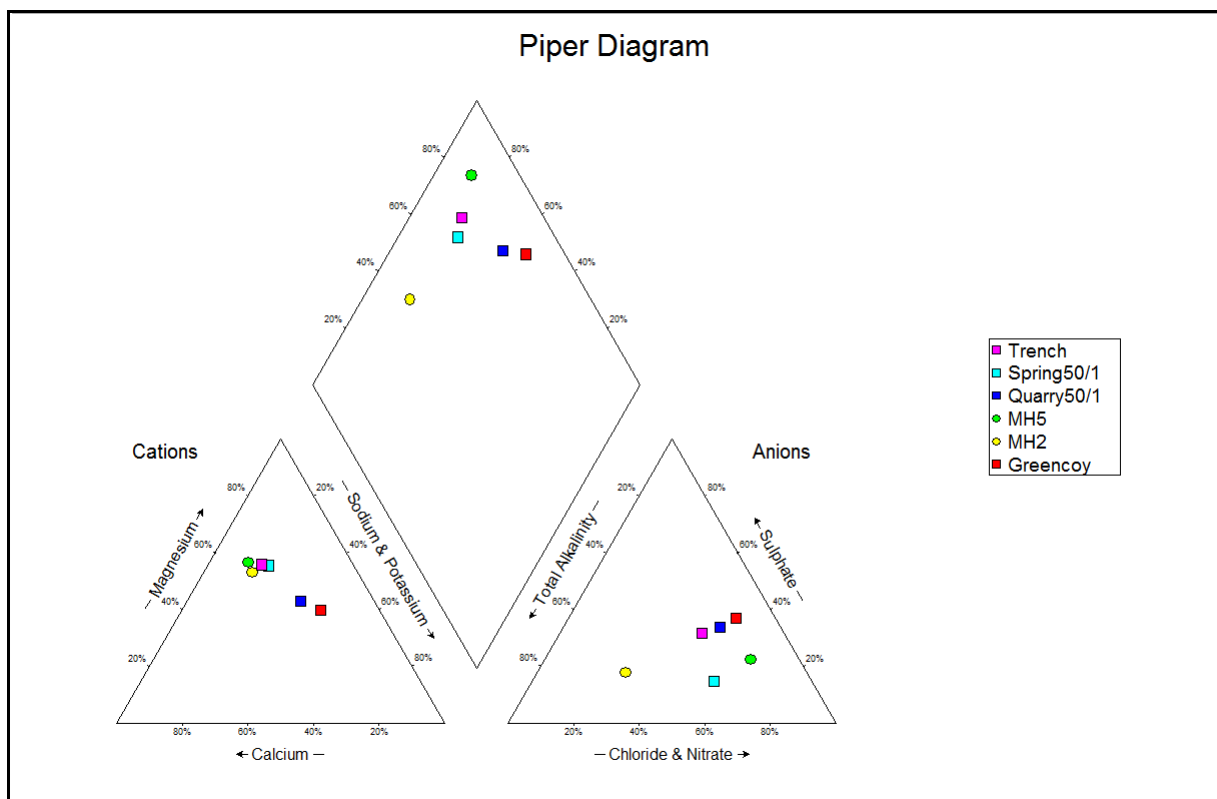


Figure 7.5: A Piper Diagram for biomarker sites

From the Piper diagram it is clear that a logical site grouping can be made based on the major anions and cations. Each of the sites were analysed for 40 chemical elements, excluding parameters like EC, TDS and pH. The ten most dominant anions and cations were per site as shown in Figure 7.6 and Figure 7.7, respectively.

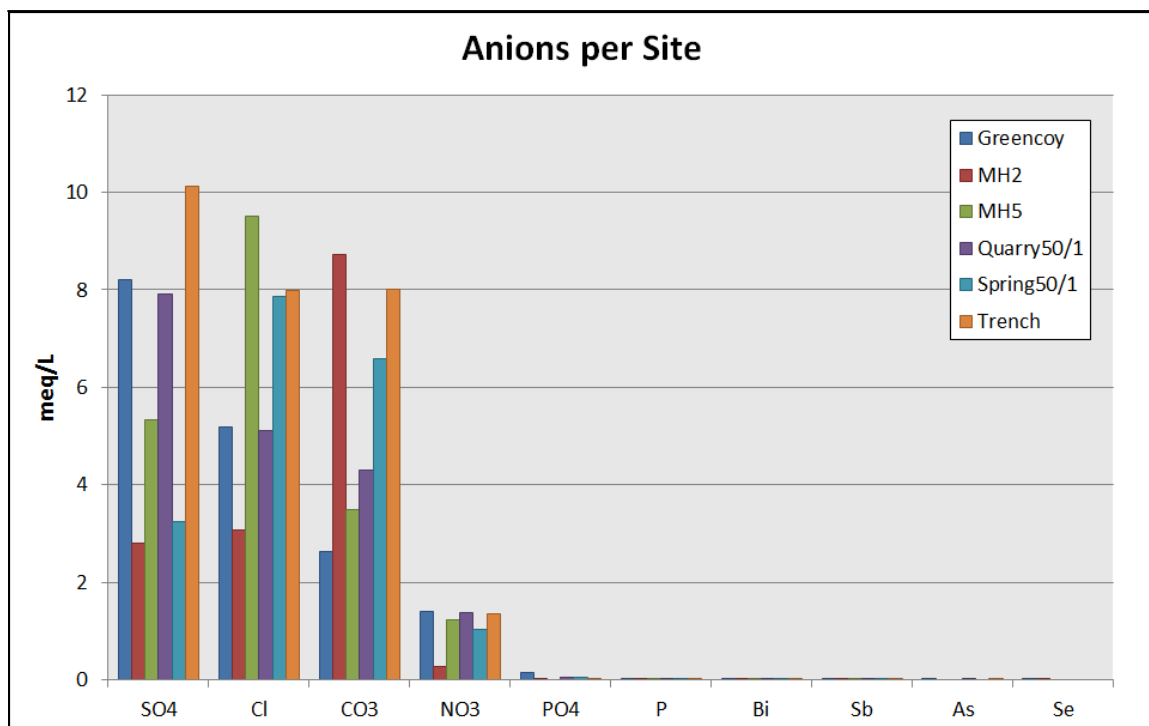


Figure 7.6: Anions per site

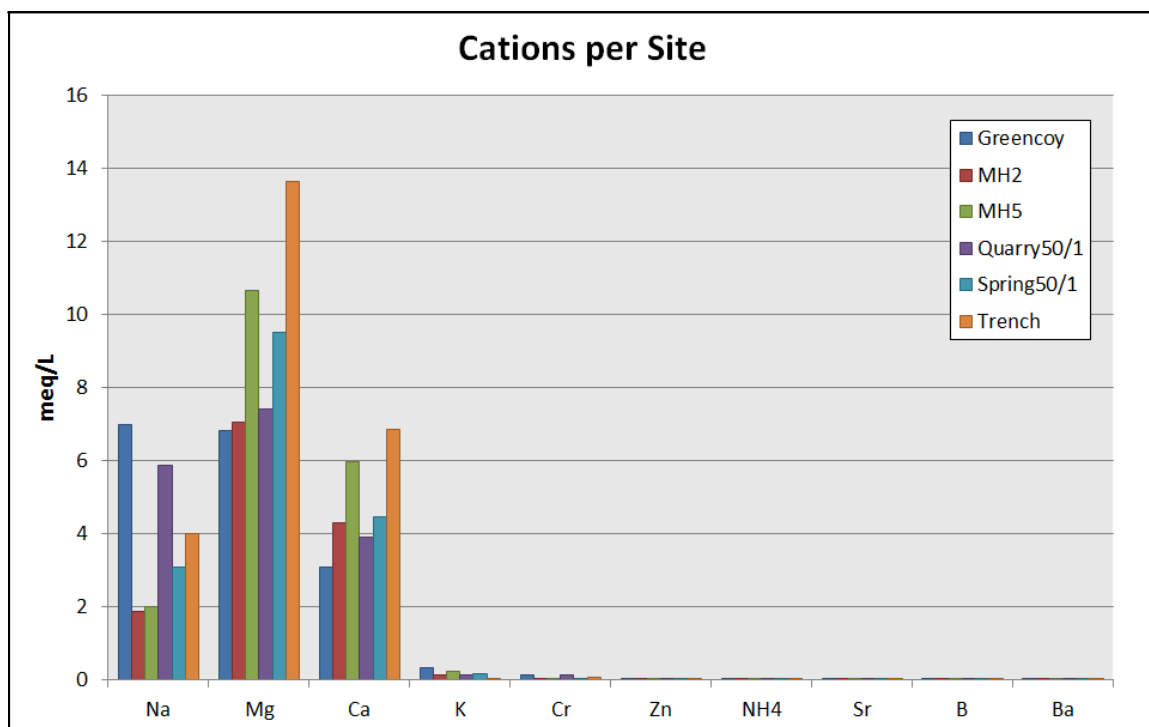


Figure 7.7: Cations per site

PCA is used when the data set has a large number of original variables; this method takes the observed variables as inputs and yields composite variables, or principal components,

thereby reducing the dimensionality. Through this PCA determines which variables play a larger role in the total sample variance (Jolliffe, 2002).

Principle components can therefore be used to describe almost all the variance in a sample, but by using a smaller number of principle components than there are observed variables, and in doing so achieves parsimony. The sum of the principle component's variances is equal to the total sample variance and it should be noted that the correlation between any two principle components is zero (Jolliffe, 2002).

Principle components are obtained by performing an eigenvalue decomposition of either the correlation or covariance matrix. Each principle component is fully characterised by its eigenvalue-eigenvector pair as follows: a principle component is a linear combination of the original variables, where the weights assigned to each are contained in the eigenvector; the variance of a principle component is equal to its eigenvalue. The proportion of the total sample variance that is accounted for by the principle component, measures the importance of each component (Jolliffe, 2002).

The five most dominant anions and cations were selected per site. The resultant data used in the PCA is listed in Table 7.1, where cations are presented in blue and anions in red.

Table 7.1: Anion and cation percentages

	Greencoy	MH2	MH5	Quarry50/1	Spring50/1	Trench
Na	6.97	1.87	2.01	5.88	3.07	4.00
Mg	6.82	7.06	10.66	7.42	9.50	13.64
Ca	3.09	4.29	5.97	3.89	4.47	6.85
K	0.34	0.13	0.24	0.14	0.16	0.05
Cr	0.15	0.00	0.01	0.14	0.01	0.07
SO4	8.20	2.81	5.34	7.92	3.25	10.14
Cl	5.19	3.09	9.51	5.12	7.86	7.99
CO3	2.64	8.72	3.49	4.30	6.59	8.00
NO3	1.40	0.27	1.24	1.38	1.03	1.34
PO4	0.16	0.03	0.03	0.05	0.05	0.03

The PCA eigenvalues of the correlation matrix of Figure 7.8 are listed in Table 7.2. The eigenvalues are used to determine how many principal components need to be considered. Nearly 80% of the dataset is covered using two principal components.

Table 7.2: Eigenvalues of correlation matrix for analysis of ions

	Eigenvalue	Variance %	Cumulative %
1	4.87369	48.74%	48.74%
2	3.09468	30.95%	79.68%
3	1.47202	14.72%	94.40%
4	0.36937	3.69%	98.10%
5	0.19024	1.90%	100.00%

The resultant biplot of the PCA is shown in Figure 7.8 together with a basic site classification. It is also clear from Figure 7.8 that the groundwater and seepage have a dolomitic character ($\text{CaMg}(\text{CO}_3)_2$), which is to be expected from a dolomitic area. The chloride does not occur naturally and can be an indication of possible pollution.

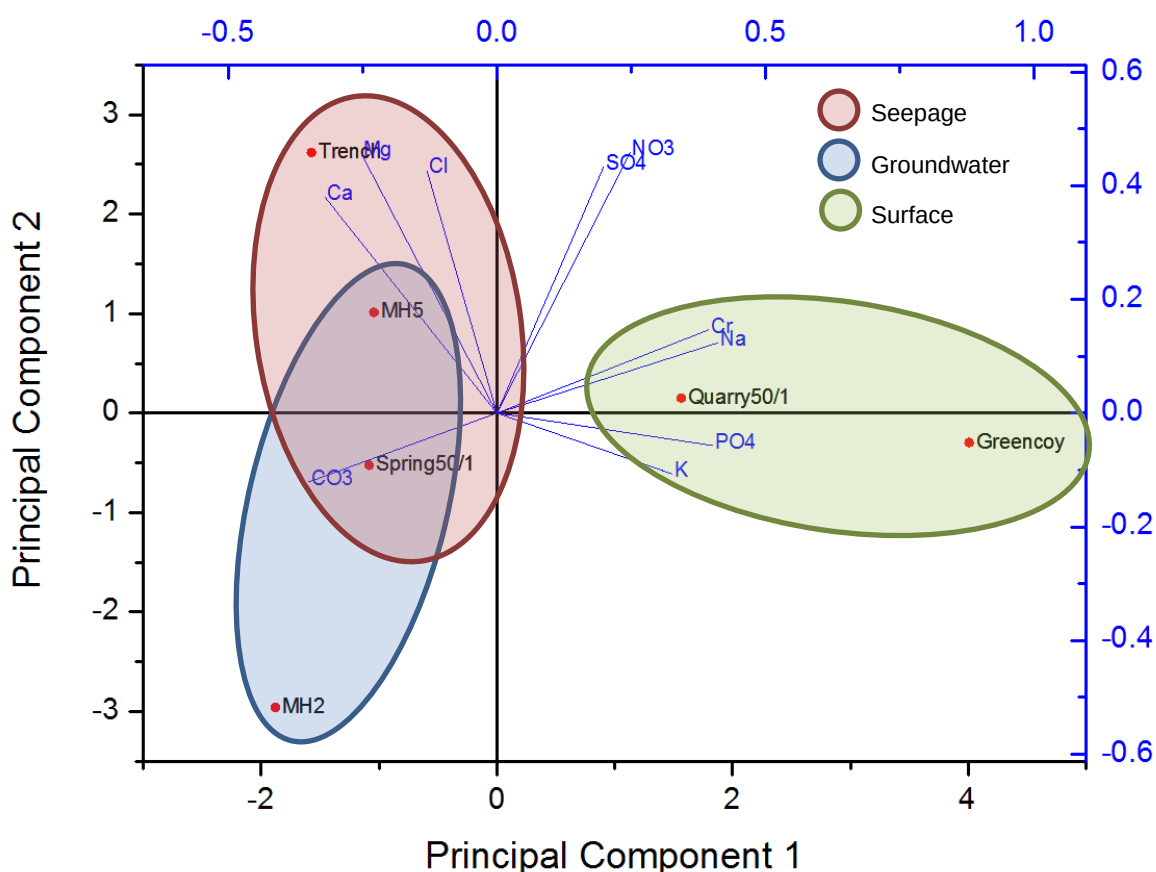


Figure 7.8: Biplot of the chemical PCA

7.2.2 SITE BIOMARKERS

The results from the microbial analysis related to the various sites are listed in Table 7.3, where a zero represents no trace and a 1 confirms detection.

Table 7.3: Biomarker results

	Greencoys	Quarry50/1	Spring50/1	Trench	MH2	MH5
Band1	1	1	0	0	0	1
Band2	1	1	0	0	0	0
Band4	0	0	1	0	0	1
Band5	1	1	0	0	0	0
Band6	1	1	1	0	1	0
Band7	0	1	1	1	0	1
Band9	0	1	0	1	0	0
Band10	1	0	0	0	0	0
Band11	1	0	0	0	0	0
Band12	1	0	0	0	0	0
Band13	0	0	0	0	0	1
Band14	0	0	0	1	0	0
Band15	0	0	0	0	1	0

The PCA eigenvalues of the correlation matrix for Figure 7.9 are listed in Table 7.4.

Table 7.4: Eigenvalues of correlation matrix for analysis of biomarkers

	Eigenvalue	Variance %	Cumulative %
1	5.52938	42.53%	42.53%
2	2.62427	20.19%	62.72%
3	2.51823	19.37%	82.09%
4	1.47838	11.37%	93.46%
5	0.84974	6.54%	100.00%

The resultant biplot of the PCA is shown in Figure 7.9 together with a basic site classification.

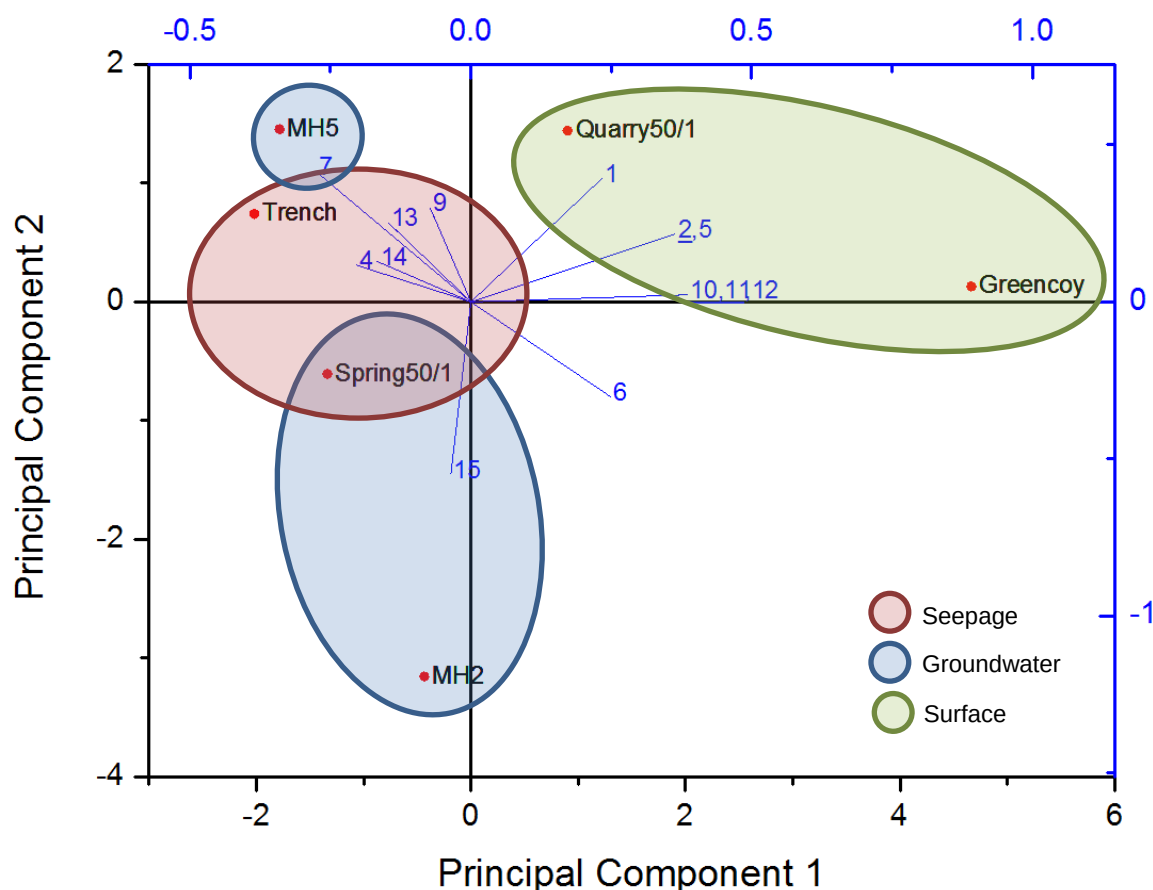


Figure 7.9: Biplot of the biomarker PCA

The surface samples (Greencoys and Quarry 50/1) are exposed to oxygen and sunlight. Therefore, organisms that occur within these samples are aerobic and possibly phototrophs (depending at the depth in which they occur). Bands 5 and 6 are from beta-proteobacteria species. These organisms are either aerobic or facultative aerobes, which mean they require a certain amount of oxygen (Schmeling & Fuchs, 2009; Wang *et al.*, 2007). Certain species are also phototrophs, which mean they form chlorophyll from sunlight, water and carbon dioxide. It is for this reason that these organisms may be present in the surface water bodies. Bands 10 and 12 are potentially sulphate reducing, anaerobic delta-proteobacteria. This can be seen as they occur in areas of elevated SO₄ levels. This ability also allows them to reduce Cr(VI) (Rodionov *et al.*, 2004; Thomas *et al.*, 2008).

Band 7 originated from an actinobacterium species and are well known Cr(VI)-reducers (Branco *et al.*, 2005). Therefore, they may occur in the sample Spring50/1 and commonly are a portion of the freshwater bacterioplankton (Hahn, 2009).

Band 9 originated from *Rhodocyclaceae*. Most bacteria from this family are aerobic; one strain, however, is anaerobic and can perform anaerobic photosynthesis. They predominantly occur in oligotrophic water bodies processes (Imhoff, 2005). As water seeps into MH2 it may contain elevated concentrations of nutrients and therefore contain this organism. These bacteria are known to be resistant to Cr(VI) and possibly able to reduce Cr(VI) (Martins *et al.*, 2010).

Bands 4 and 14 are from the non-Cr(IV)-resistant, anaerobic *Bacteroidetes* species. They occur in areas without oxygen. These organisms are able to survive in Cr(VI) polluted areas when together with Cr(VI)-reducers (Branco *et al.*, 2005). This is the case in samples MH5 (Cyanobacterium) and MH2 (Cyanobacterium and *Rhodocyclaceae*). As the water seeps underground to these sources, there may be active cell growth in this anoxygenic water source. This may finally result in the presence of these organisms in the water samples. *Chloroflexi* (band 11) is not resistant to Cr(VI), either. It occurs in the water due to all the Cr(VI)-reducers that are with it in sample Greencoy. These organisms lower the Cr(VI) concentration in certain environments (Branco *et al.*, 2005).

Cyanobacteria (bands 1, 13 and 15) are extremely diverse and adapt rapidly to their surroundings (Ñancucheo & Johnson, 2012; Whitton & Potts, 2000). It is possible that they occur in samples MH5 and MH2 as they have the ability to reduce Cr(VI) (Gupta & Rastogi, 2008). This may also indicate that the water in these samples derive from the same sources. Although these organisms are Cr(VI)-reducers, they do not necessarily indicate any other environmental characteristics.

7.2.3 CONCLUSIONS OF THIS CHAPTER

A good correlation exists between Figure 7.8 and Figure 7.9, which enables site classification based on both the biomarkers and predominate ions. A logical grouping of the correlation can be depicted as listed in Table 7.5, which can give a good first indication of the hydrological nature of a sample based on either the dominant ion in a sample or a biomarker identified.

Table 7.5: Logical grouping of PCA correlations

	Na	K	Cr	PO4	Mg	Ca	Cl	CO3
Band 1	Surface	Surface	Surface	Surface				
Band 2	Surface	Surface	Surface	Surface				
Band 5	Surface	Surface	Surface	Surface				
Band 10	Surface	Surface	Surface	Surface				
Band 11	Surface	Surface	Surface	Surface				
Band 12	Surface	Surface	Surface	Surface				
Band 4					Seepage	Seepage	Seepage	
Band 7					Seepage	Seepage	Seepage	
Band 9					Seepage	Seepage	Seepage	
Band 13					Seepage	Seepage	Seepage	
Band 14					Seepage	Seepage	Seepage	
Band 15								

8 CONCLUSION AND RECOMMENDATIONS

8.1 CONCLUSION

Water sources on the mining site are polluted with chromium, which naturally occurs as the non-mobile Cr(III) and/or the mobile toxic Cr(VI). Varying concentrations of Cr(VI) were found in the selected water samples, six of which were above the stipulated Target Water Quality Range of 0.05 mg/l. Factors that influence the pollution pathways of Cr(VI) include geology, topography and drainage. The mine is surrounded by agricultural and urban land use and the water resources of that area is mainly used for agricultural and domestic supply. A great problem is presented if high concentration of Cr(VI) are absorbed by plants or ingested by humans or animals. Cr(VI) creates immense adverse effects on human and environmental health when exposed to elevated concentrations (>0.05 mg/l or TWQR) for extended periods of time. As Cr(VI) is mobile and the soils permeable in this area, this causes a great risk for Cr(VI) polluted leachate from the mine to seep into the water table. From this study, a possible mechanism to determine the pathway of Cr(VI) polluted groundwater sources was tested. The water moves along the hydraulic gradient into neighbouring water sources. The areas affected by the Cr(VI) polluted water are within 1200 m from the known pollution source.

The area analysed during this study was quite small, and was chosen to be representative of the entire mining area. Samples analysed had high chromium concentrations compared to other water sources in the area. In order to complete a full investigation of the mine, a greater area must be analysed and more intrusive work would be required.

This study indicates that microorganisms may be reliable biomarkers in areas of Cr(VI) pollution. Certain microorganisms identified in this study may indicate a certain level of Cr(VI) pollution. These organisms may be applied in the determination of pollutant pathways of Cr(VI). It is difficult, however, to prove without a doubt that the identified microorganisms are appropriate biomarkers for Cr(VI) pollution in water. The reason for this is that a few of these organisms may also occur in non-polluted water sources and may not be resistant to Cr(VI) pollution. Further studies should be conducted to determine exactly which microorganisms can be used as biomarkers for Cr(VI) polluted water sources.

8.2 RECOMMENDATIONS

More documentation on bacterial or microbial strains is required as to narrow down to the specific bacterial strains present in certain habitats. From this data their characteristics can be determined, which would aid in the determination of specific parameters in the environment. This means more than one variable may be determined at a time and results would be more definite.

The biomarkers can be applied in reverse-engineering processes as to locate deposits that have not yet been detected. As each organism inhabits specific environments, they can be used to determine pollution sources in the rest of the mine.

A complete study of the site should be conducted to ensure accurate results of the entire mining area. This will determine if the results obtained during this study, with the small amount of samples, can be implemented correctly during deposit location and mitigation applications.

Appropriate mitigation and continuous monitoring methods are required on site to prevent seepage of the Cr(VI) contaminated water to the surrounding water sources. An eco-friendly and cost effective alternative is the implementation of bioremediation. This implies the use of biological elements, which makes this alternative a 'green' and environmentally friendly method. One such method is the implementation of bioreactors. These use specific bacteria with the ability to utilize, and therefore remove, the pollutant. Many bacteria are Cr(VI)-reducers and can be used in such a bioreactor on site.

A complete risk assessment should be conducted to determine the full environmental and health implications that may be caused by the seepage and distribution of Cr(VI) throughout the water sources. Although this study gives an understanding of this aspect, explicit values may aid in the prevention of the distribution of pollution, appropriate mitigation implementation and ultimate rehabilitation of this site.

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APPENDIX A: RF VALUES

Table A.1: Rf values for the bands in the DGGE gel

Ref Band	Number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Lane 1	Band No	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Lane 1	Rf	0.055	0.086	0.097		0.169						0.414		0.456	0.561	0.577	0.605		0.638	0.707	0.743		0.782	0.796	0.837
Lane 1	Peak Height	68.86	24.07	19.23		20.62						45.70		132.80	48.92	52.10	25.72		5.27	35.26	13.47		12.69	20.66	5.30
Lane 2	Band No	1	2		3	4	5	6	7	8		4	5	6	7	8									
Lane 2	Rf	0.058	0.083			0.171						0.414		0.450	0.564	0.586		0.613							
Lane 2	Peak Height	91.44	84.07			121.31						50.46		172.24	79.34	73.26		111.10							
Lane 3	Band No	1				2	3							4	5	6	7								
Lane 3	Rf	0.064				0.160	0.235							0.453	0.564	0.580	0.605								
Lane 3	Peak Height	183.74				47.36	101.00							155.93	46.20	50.56	37.90								
Lane 4	Band No		1											2	3	4	5								
Lane 4	Rf		0.080											0.461	0.558	0.580		0.610							
Lane 4	Peak Height		72.11											184.62	7.11	6.19		11.67							
Lane 5	Band No	1	2		3	4		5		6	7			8	9	10	11								
Lane 5	Rf	0.055	0.074		0.110	0.160		0.253		0.347	0.386			0.449	0.559	0.581	0.598								
Lane 5	Peak Height	189.17	84.08		6.35	17.72		18.33		3.59	4.99			58.36	19.72	29.55	15.44								
Lane 6	Band No	1	2		3	4	5		6				7		8	9	10		11			12			
Lane 6	Rf	0.055	0.085		0.115	0.162	0.242		0.308				0.442		0.566	0.582	0.599		0.637			0.761			
Lane 6	Peak Height	146.43	10.95		6.41	28.37	44.67		12.10				100.47		32.84	39.38	12.76		4.74			19.78			

APPENDIX B: BOREHOLE LOGS

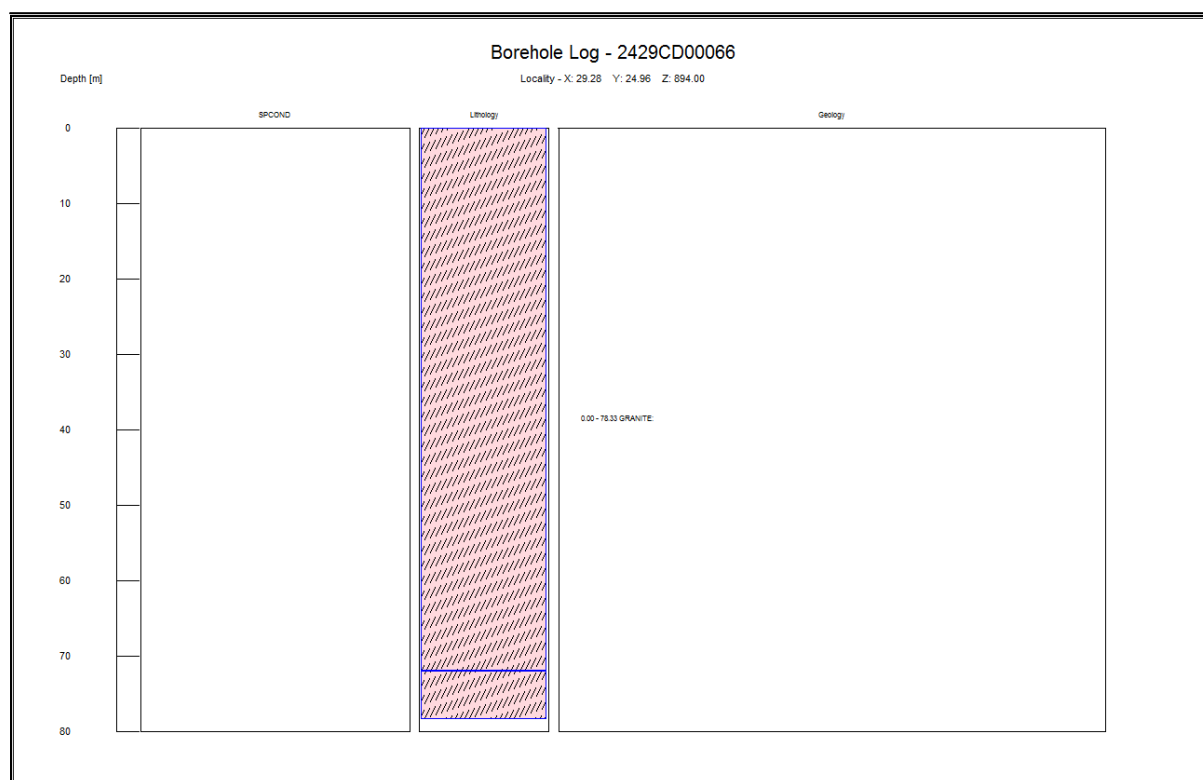


Figure B.1: Borehole log type 1

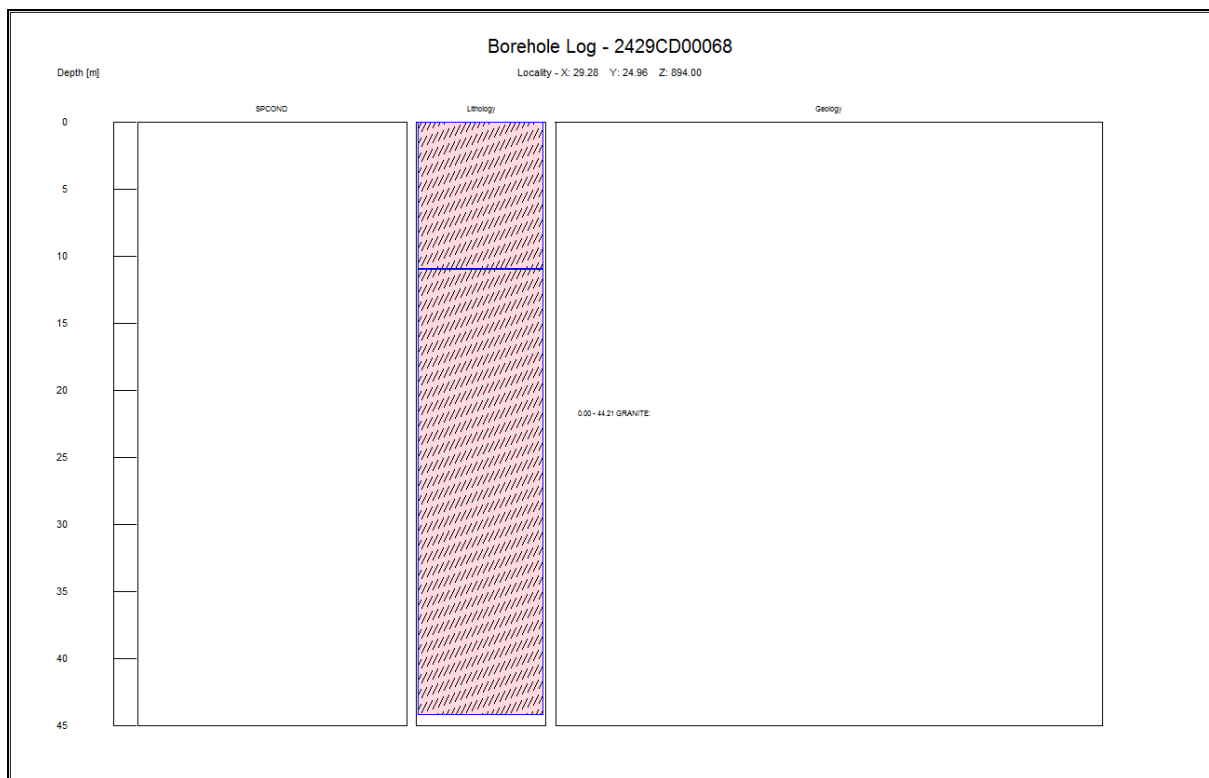


Figure B.2: Borehole log type 2

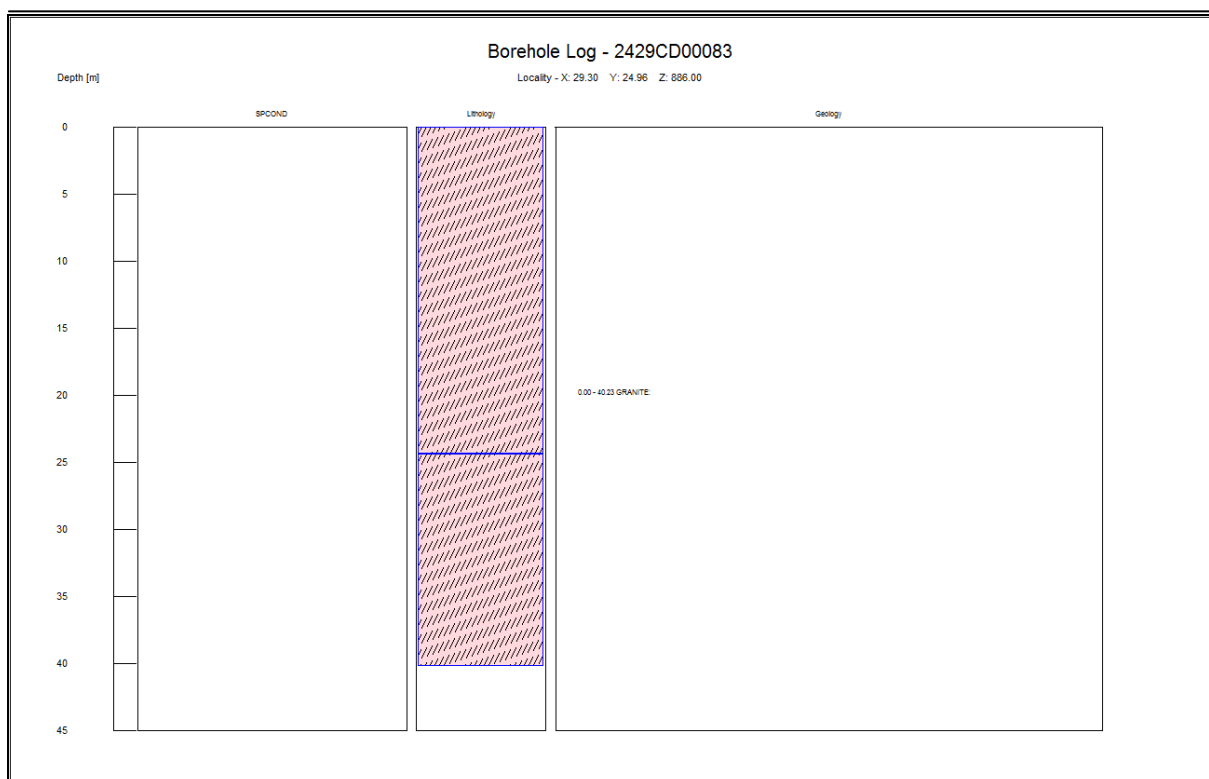


Figure B.3: Borehole log type 3

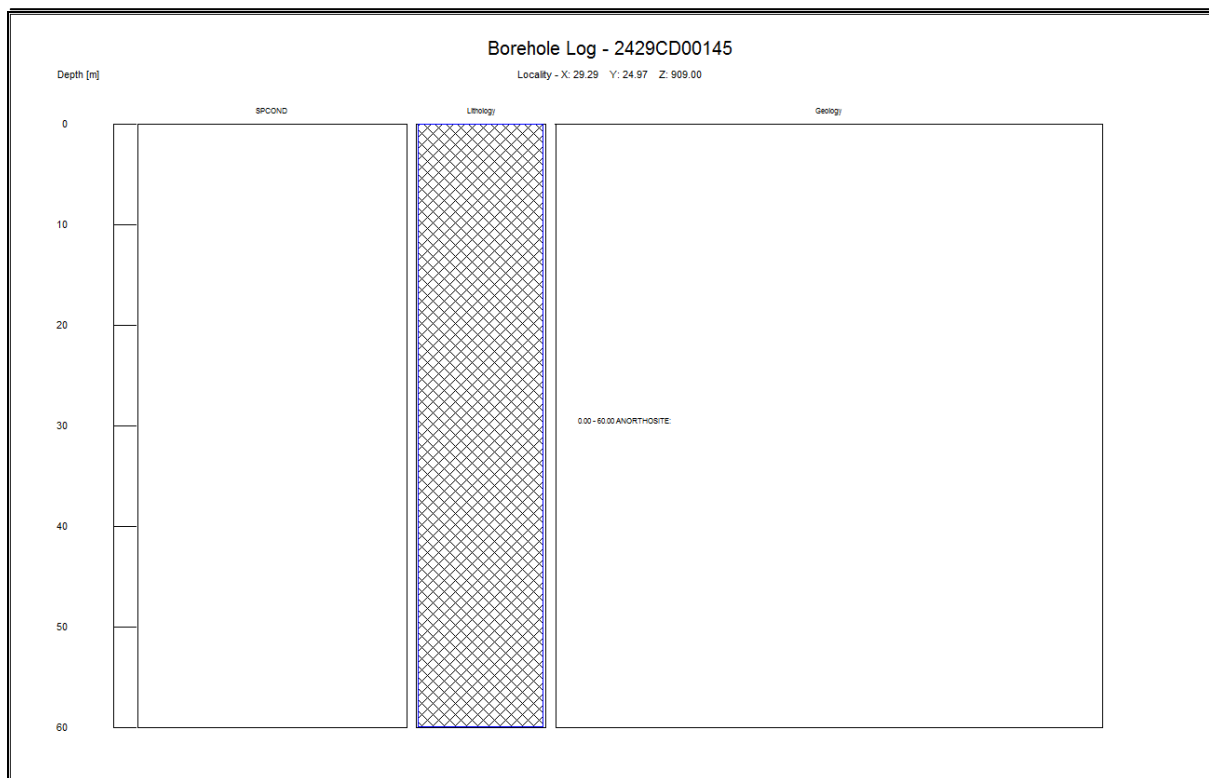


Figure B.4: Borehole log type 4

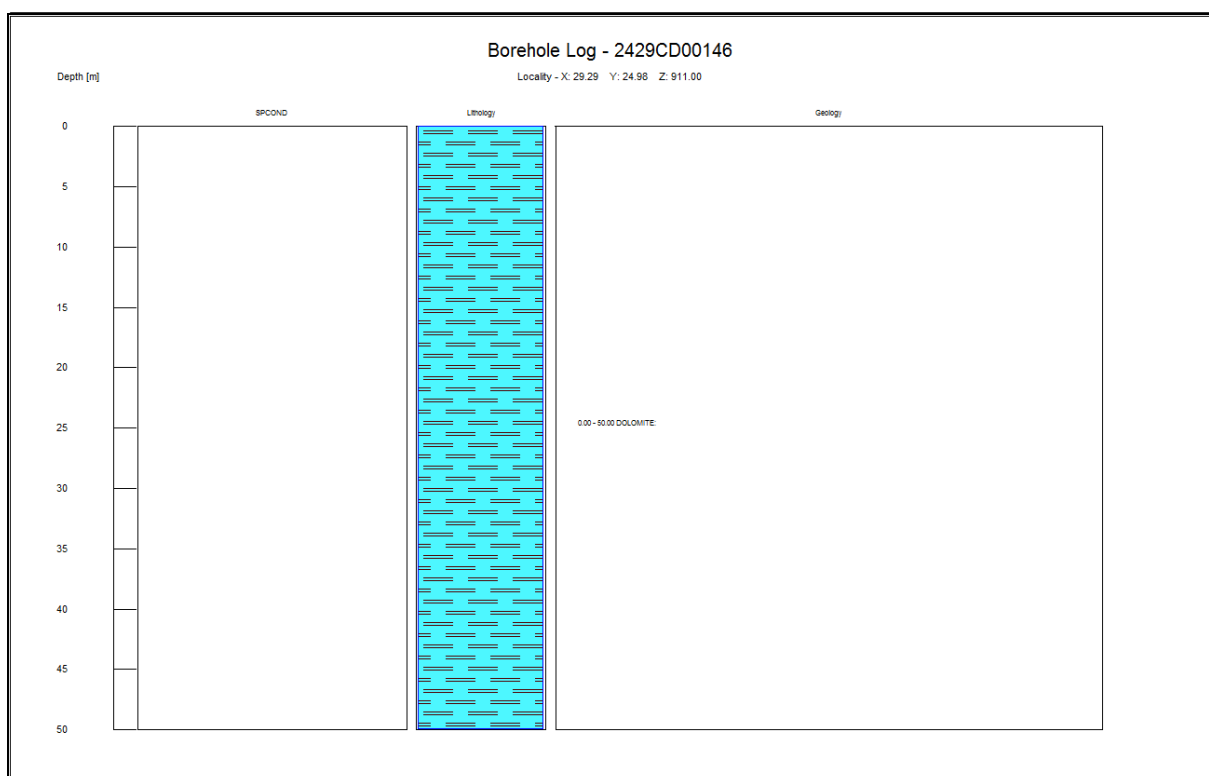


Figure B.5: Borehole log type 5

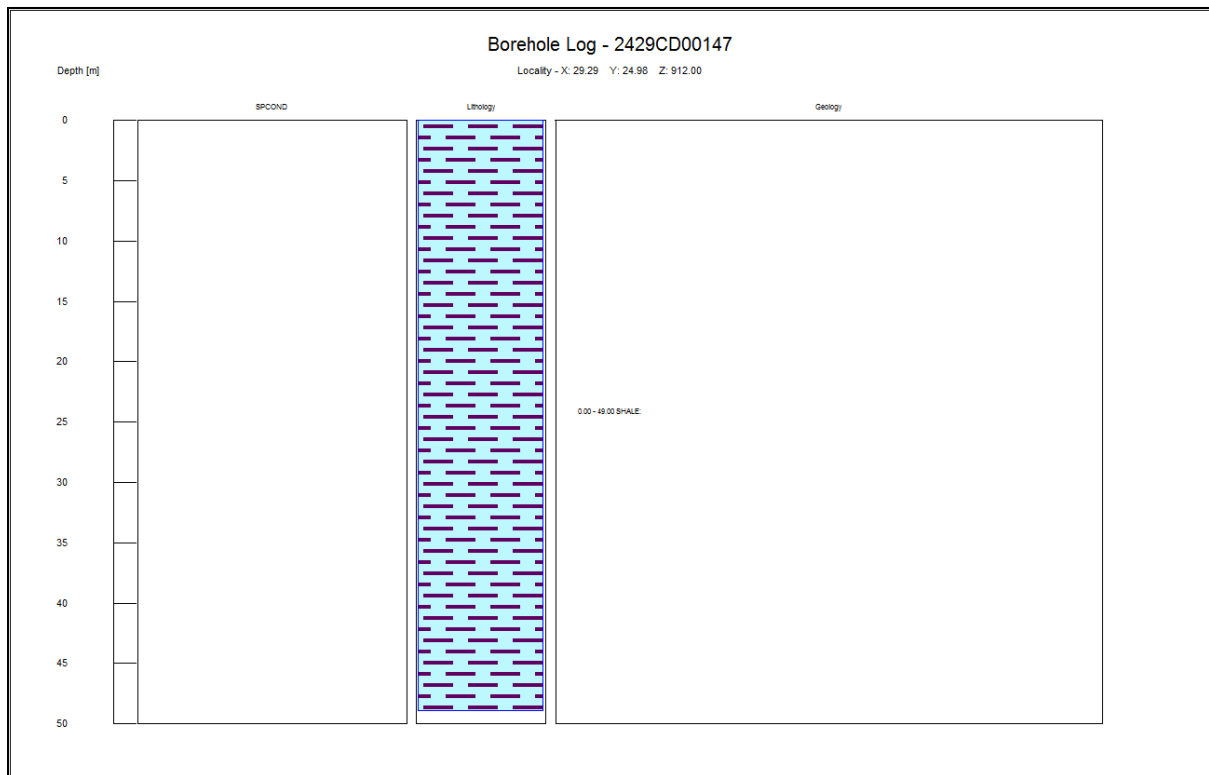


Figure B.6: Borehole log type 6

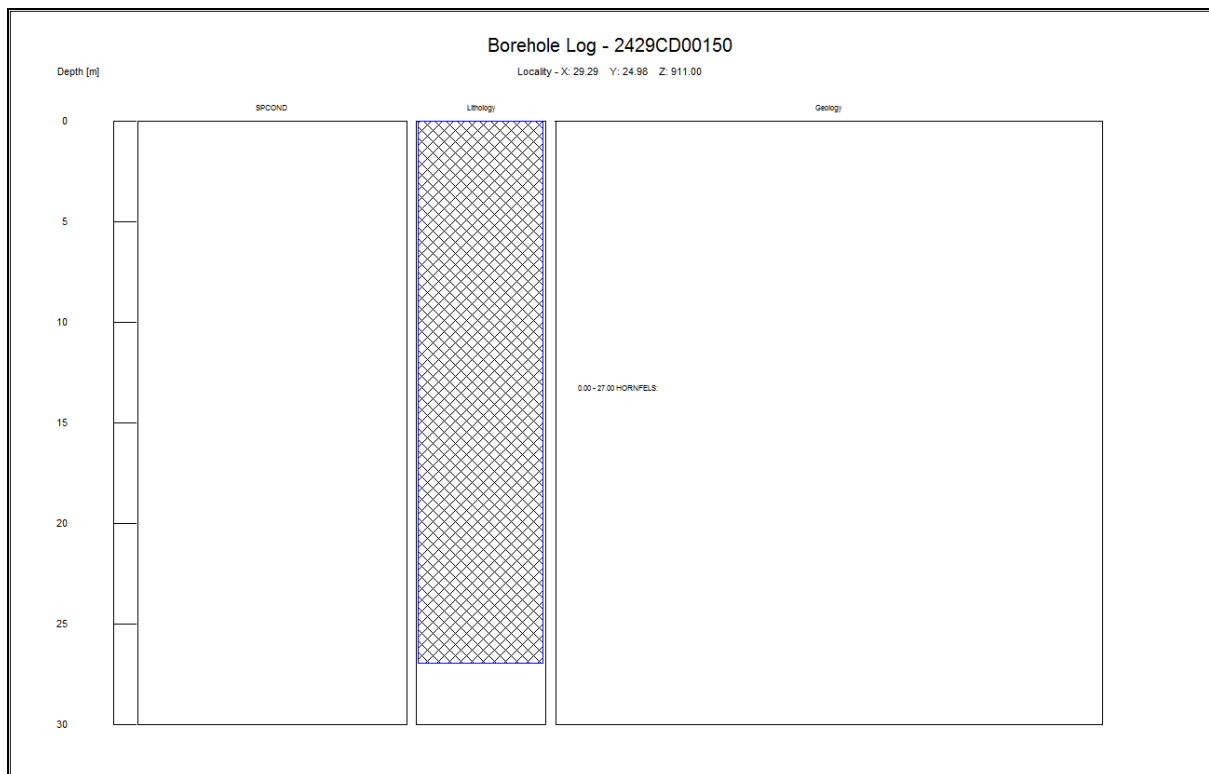


Figure B.7: Borehole log type 7

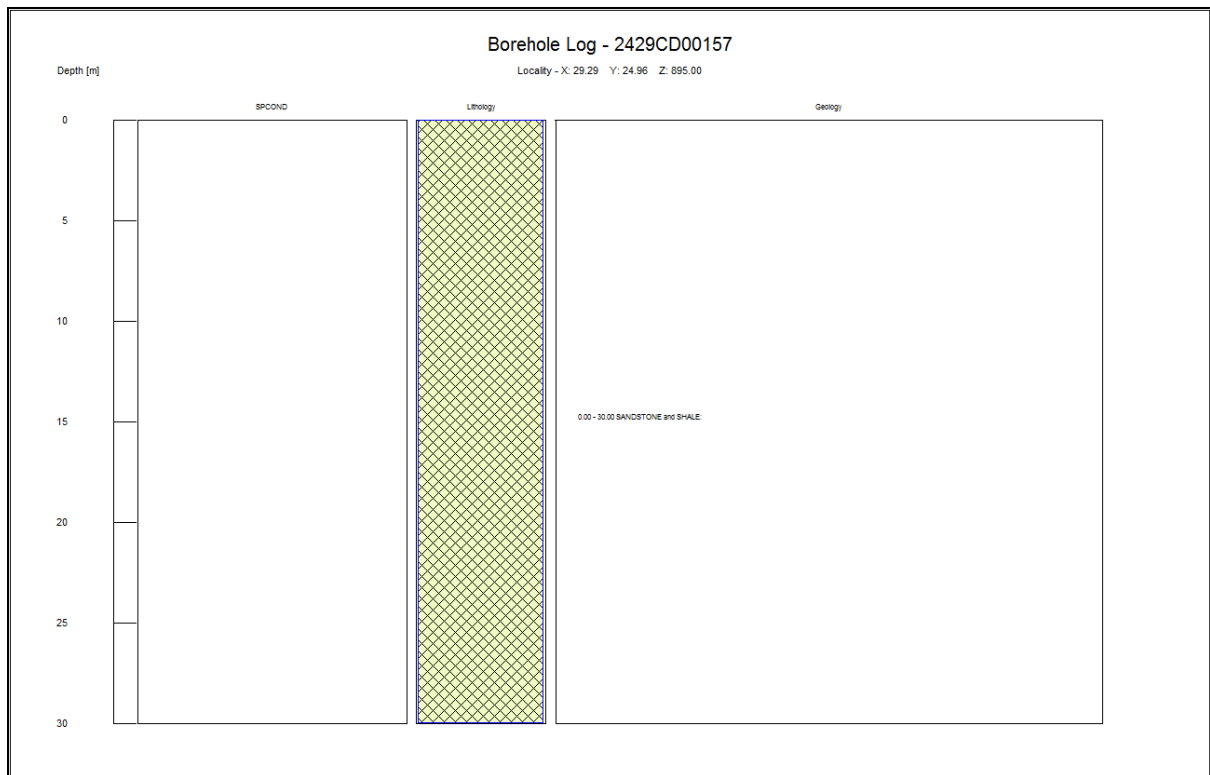


Figure B.8: Borehole log type 8

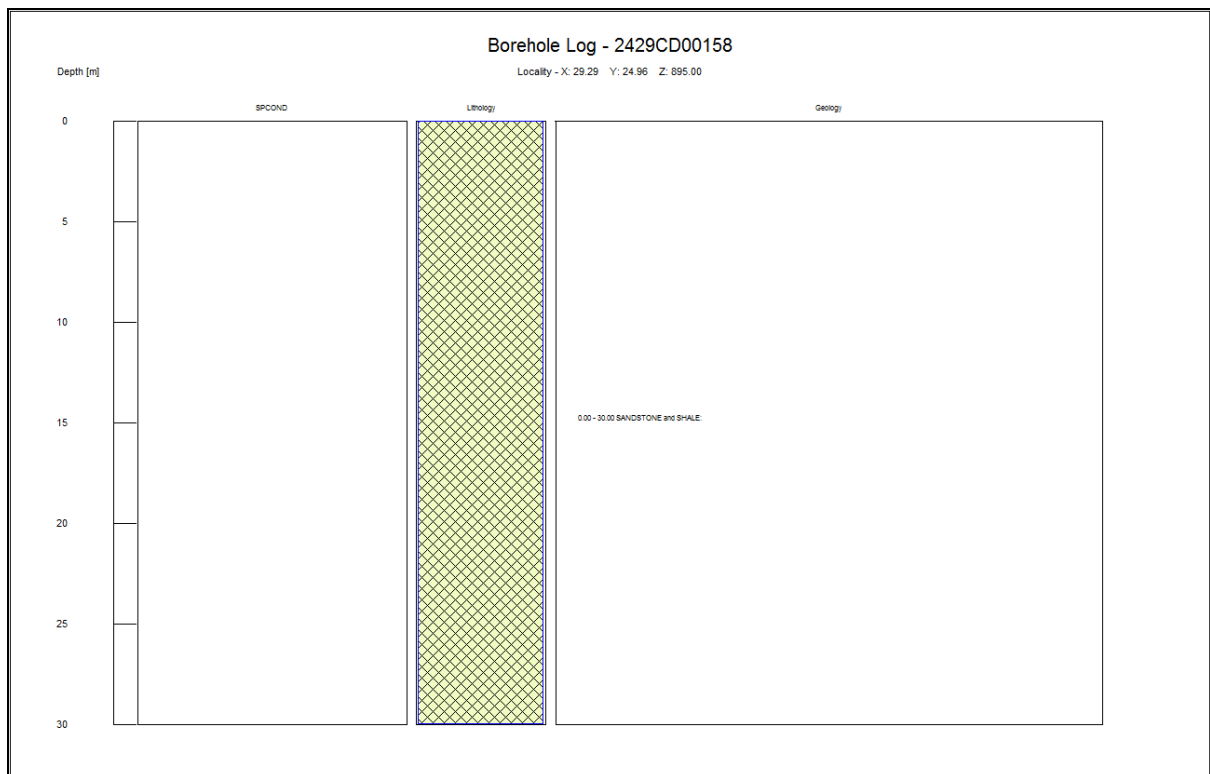


Figure B.9: Borehole log type 9

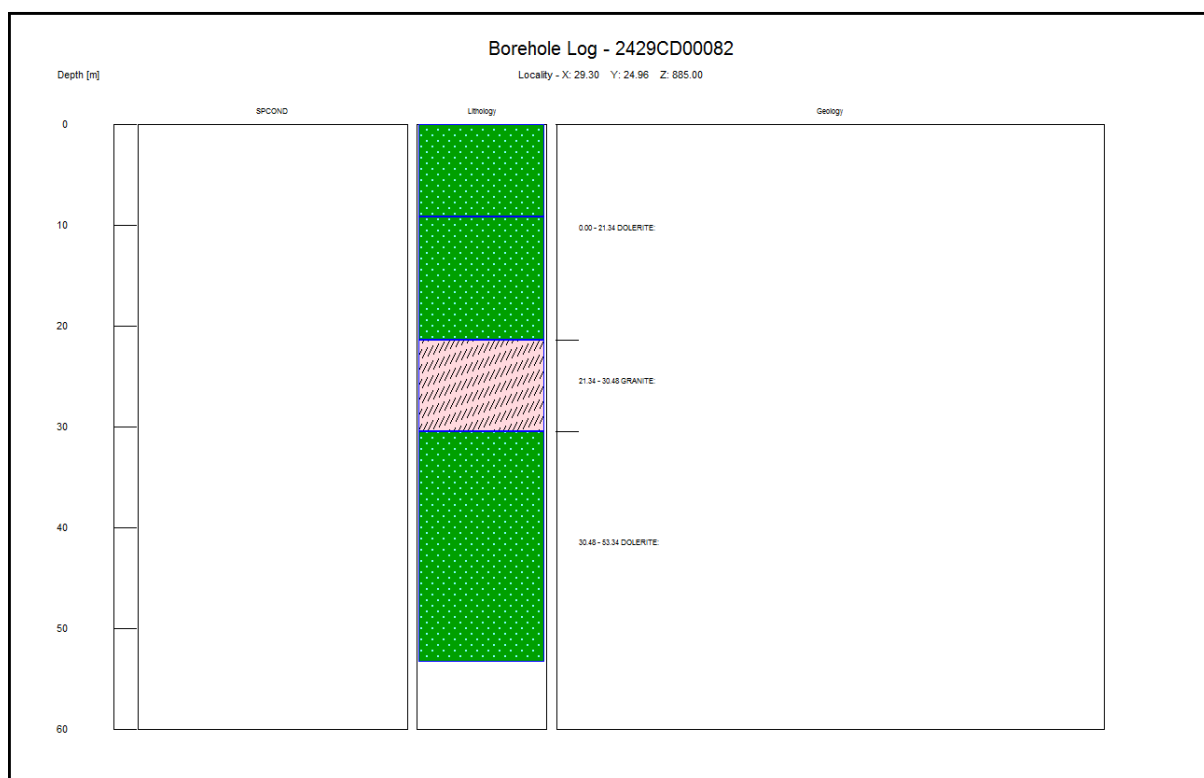


Figure B.10: Borehole log type 10

APPENDIX C: THE PIPER DIAGRAM

This diagram calculates the concentrations of the input parameters in milli-equivalents per litre. After calculation of the relative percentages for anions and cations, the Piper Diagram plots these percentages in the bottom right triangle and bottom left triangle, respectively. Finally the diagram plots the projection of the cross section (marks location) of the anions and cations to the central block. Trends are created through these diagrams, from which polluted samples can be identified (Piper, 1953). The plotting procedure is shown in Figure C.1.

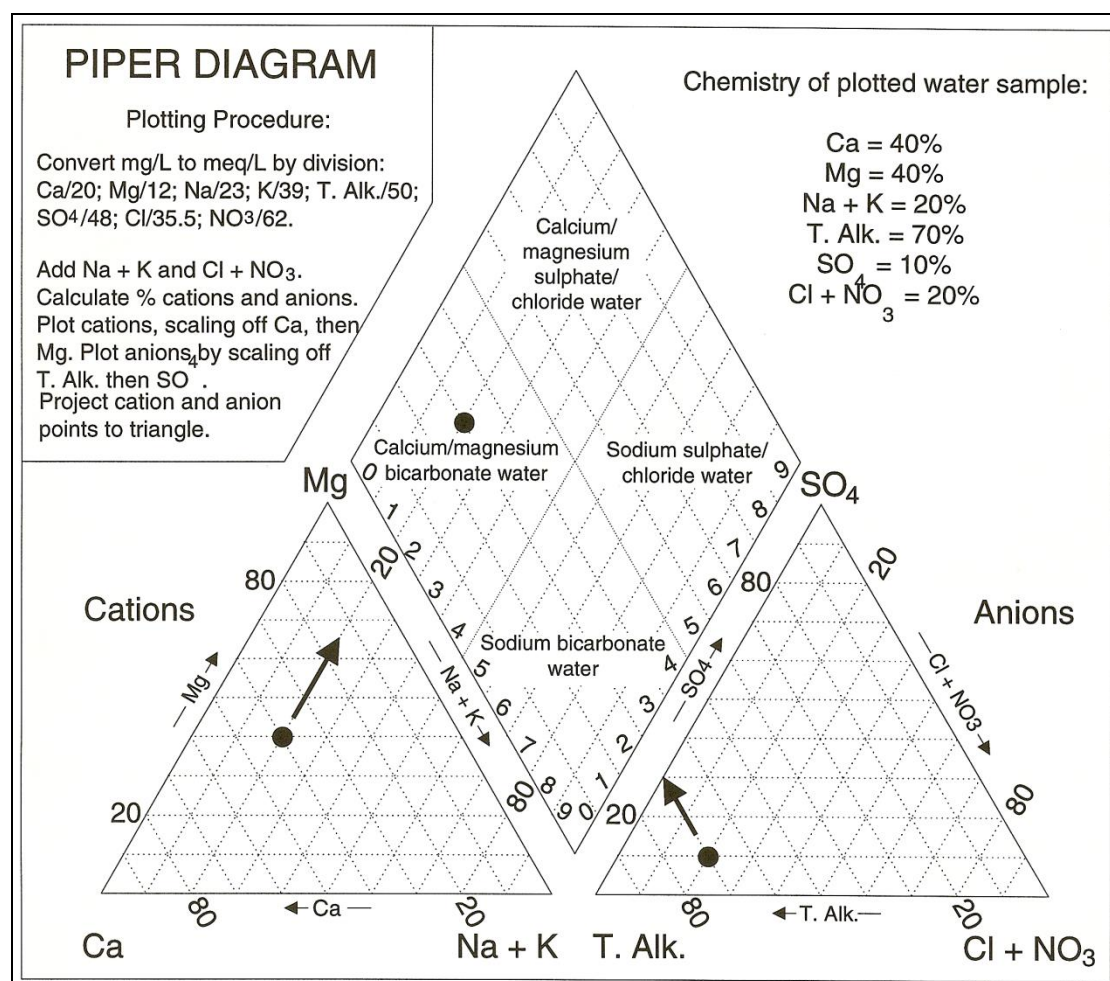


Figure C.1: A representation of the Piper Diagram

The total percentage of each chemical parameter forms in this diagram to determine the quality of the water source. The marks in the centre block are the corresponding water sources and can then further be interpreted.