Determining the effect of polluted mine water on the ecosystem health of a karstic cave environment in the Witwatersrand Basin

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“***I have been impressed with the urgency of doing. Knowing is not enough; we must apply. Being willing is not enough; we must do***.”

Leonardo da Vinci

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ABSTRACT

The Wonderfontein Cave is located within the Witwatersrand Basin (Gauteng province, South Africa) and is associated with the river banks of the Wonderfontein Spruit. This cave system has for many years been subjected to the influx of polluted mine water. Since subterranean environments remain poorly studied, it is unknown what the effect of this might be on the associated ecosystem. Furthermore, water that enters the Wonderfontein Cave poses a severe health threat as it drains into the underlying aquifer, which is abstracted for human and animal use. The general aim of this study was to determine the extent of metal pollution (enrichment), as well as to study the toxicity hazard potential of the soils and sediments associated with the Wonderfontein Cave. The objectives of this study were to (1) quantify the extent of anthropogenic metal pollution of water, soils and sediments associated with the Wonderfontein Cave (2) and assessing the toxicity hazard potential of these substrates; (3) determining whether nematode taxa and *C. gariepinus* individuals represent isolated communities and a population within the Wonderfontein Cave, respectively; (4) measuring the effect of mining-associated pollutants on the soil and sediment health of the Wonderfontein Cave by making use of nematodes to serve as bioindicators; (5) evaluating and comparing biomarker responses to metal bioaccumulation in *C. gariepinus* populations associated with the Wonderfontein Cave and epigean (surface) environments and lastly (6) comparing the results of the above identified assessments over both a temporal and spatial scale. Sampling was undertaken during April (1st sampling interval) and September (2nd sampling interval) 2013, which respectively represented the end of the high and low flow periods. Also, sampling of the subterranean (Wonderfontein Cave) and associated surface (Wonderfontein Spruit) environments were undertaken. The findings of this study suggested that especially the sediments associated with the Wonderfontein Cave have been subjected to severe nickel, copper, zinc, cobalt, aluminium, cadmium, lead and uranium enrichment. Also, the concentrations of many of the studied metals exceeded the respective water, soil and sediment environmental quality guidelines. Thus, also taking into consideration that most of the sediments were classified as being toxic, a severe threat is posed to the health of the associated biota. Although 60 nematode genera were identified from soil and sediments samples collected from the respective sampling sites associated with the Wonderfontein Cave and Spruit, it was concluded that most of these genera were likely only temporary residents of the subterranean environment. Even though plant-parasitic and non-parasitic nematodes were present, most of the collected soil and sediment samples were dominated by bacterivores (non-parasitic nematodes). Zero genetic divergence was recorded between the *C. gariepinus* populations associated with the Wonderfontein Cave and Stoffels Dam (Wonderfontein Spruit). However, significant temporal and spatial variation was observed in some bioaccumulated metals and biomarker responses within and between the respective *C. gariepinus* populations. Furthermore, the metal bioaccumulation levels present in both these fish populations pose a substantial threat to human health and are thus not fit for consumption. Also, no significant fish condition differences were observed between the *C. gariepinus* populations associated with the Wonderfontein Cave and Spruit. This study served as an initiative to create awareness and promote the conservation of Africa’s karst landscapes.

Keywords: Wonderfontein Cave; Witwatersrand Basin; metal pollution; plant-parasitic nematodes; non-parasitic nematodes

UITTREKSEL

The Wonderfonteingrot is in die Witwatersrandarea van die Gauteng Provinsie van Suid-Afrika geleë en word met die oewers van die Wonderfonteinspruit, wat vir baie jare al blootgestel word aan besoedelde mynwater, geassosieër. Aangesien ondergrondse ekosisteme swak bestudeer word en beperkte inligting daaromtrent beskikbaar is, is wetenskaplikes onseker oor die invloed van besoedeling daarop. Die omvang van voortgesette omgewingsbesoedeling is kommerwekkend aangesien gekontamineerde water, wat grootliks vanaf myne afkomstig is, deur die Wonderfonteingrot vloei en dan tot die onderliggende grondwaterbronne dreineer. Hierdie water word dan weer as drinkwater vir mens en dier gebruik. Die oorhoofse doel van hierdie studie was om die vlak van besoedeling (metaalverryking) sowel as die invloed daarvan op die welstand van ondergrondse grond- en sedimentekosisteme wat in die Wonderfonteingrot voorkom is, te bestudeer. Die doelwitte van hierdie studie was om: (1) die omvang van antropogeniese metaalverryking/-besoedeling in grond, water en sediment wat in die Wonderfonteingrot voorkom te kwantifisee asook (2) die potensiële gevaar wat laasgenoemde substrate inhou vir geassosieërde biota te evalueer; (3) te bepaal of die nematoodbevolkings (invertebrate) asook dié van die vis spesie *Clarias gariepinus* (vertebrate) wat in die grot voorkom, geïsoleerde bevolkings verteenwoordig; (4) vas te stel wat die invloed van myn-geassosieërde besoedeling op die grond- en sedimentekosisteme wat met die grot geassosieër is, behels; (5) die biomerker reaksie ten opsigte van metaalverryking in weefsel van *C. gariepinus* bevolkings wat in die grot sowel as buite die grot voorkom te evalueer en te vergelyk en laastens (6) om die data van bogenoemde parameters te vergelyk oor beide ‘n temporale en ruimtelike skaal. Monsternemings, wat water, grond asook sediment ingesluit het, is gedurende April (1ste monsternemingsinterval) en Septermber (2de monsternemingsinterval) 2013 onderneem en het onderskeidelik die einde van die laag- en hoogvloeiperiodes verteenwoordig. Verder is die bogenoemde substrate, sowel as *C. gariepinus* individue, versamel in die Wonderfonteingrot en –spruit, wat onderskeidelik die ondergrondse en bogrondse ekosisteme verteenwoordig. Toegepaste en wetenskaplik gefundeerde protokolle is geraadpleeg om die verskillende aspekte van hierdie studie uit te voer. Resultate wat verkry is, het aangetoon dat meeste van die sedimente wat met die Wonderfonteingrot geassosieër is, hoë vlakke van veral nikkel, coper, sink, kobalt, aluminium, kadmium, lood en uraan verryking bevat. Daar is ook bevind dat sommige metale in die water, grond en sediment maksimum waardes vir die beskerming van biota oorskry. As in ag geneem word dat meeste van dié sedimente ook as toksies geklassifiseer is, bestaan daar ‘n risiko dat biota in die grot negatief beïnvloed kan word. Alhoewel 60 nematoodgenera geïdentifiseer is uit grond- en sedimentmonsters wat in verskeie areas in die Wonderfonteingrot en –spruit geneem is, is dit bevind dat sogenaamde individue heel waarskynlik tydelike bewonders van die grot is. Plantparasitiese en nie-parasitiese nematode was in grond- en sedimentmonsters teenwoordig, met nie-parasitiese nematode wat in die meeste gevalle gedomineer het. Laasgenoemde is veral teenwoordig deur bakterievoedende nematode. Daar is ook bevind dat geen beduidende genetiese variase tussen die Wonderfonteingrot en Stoffelsdam (Wonderfonteinspruit) *C. gariepinus* populasies bestaan nie. Verskille ten opsigte van tyd en ruimtelike variasie in metaal bioakkumulasie en biomerkerreaksies in weefsels van laasgenoemde visspesiebevolkings wat in beide die Wonderfonteingrot en Stoffelsdam teenwoordig was, is aangeteken. Metaal bioakkumulasievlakke in beide vis populasies hou ‘n gesondheidsrisiko in vir die mens en is dus nie geskik om as ‘n voedselbron te dien nie. Daar is voorts geen beduidende verskille in die fisiologiese kondisie tussen die twee *C. gariepinus* populasies aangeteken nie. Hierdie studie as ‘n inisiatief het gedien om basislyninligting rakende die biota wat in kartsomgewings voorkom te verwerf asook om die bewaring van hierdie ondergrondse omgewings in Afrika te bevorder.

Sleutelwoorde: Wonderfonteingrot; Witwatersrandarea; metaalbesoedeling; plantparasitiese nematodes, nie-parasitiese nematodes

LIST OF ABBREVIATIONS

AEV	Acute effect values
AMD	Acid mine drainage
AMOVA	Analysis of molecular variance
ANOVA	Analysis of variance
ANZECC	Australia and New Zealand Environment and Conservation Council
ARC	Agricultural Research Council
ARD	Acid rock drainage
Ba	Bacterivores
BI	Basal index
BLAST	Basic local alignment search tool
BOD	Biochemical oxygen demand
Bp	Base pairs
Ca	Carnivores
CAT	Catalase
CCME	Canadian Council of Ministers of the Environment
CEQG	Canadian Environmental Quality Guidelines
CEV	Chronic effect values
CF	Condition factor
CI	Channel index
C-p	Colonizer-persister
CSQG	Canadian Soil Quality Guidelines
CWQG	Canadian Water Quality Guidelines
Cyt b	Cytochrome b
DEA	Department of Environmental Affairs

DNA	Deoxyribonucleic acid
DNPH	2, 4-Dinitrophenylhydrazine
DWAF	Department of Water Affairs and Forestry
DWAS	Department of Water Affairs and Sanitation
EDTA	Ethylenediaminetetraacetic acid
EF	Enrichment factors
EFSA	European Food Safety Authority
EI	Enrichment index
USEPA	United States Environment Protection Agency
Eu	Eukaryotic feeders
FPG	Formaldehyde propionic acid-water
FSANZ	Food Standards Australia New Zealand
Fu	Fungivores
GEL	Generally expected levels
Gu	Bat guano
HAI	Health assessment index
Her	Herbivores
HSI	Hepatosomatic index
ICP-MS	Inductively coupled plasma mass spectrometer
I_{geo}	Geoaccumulation index
MI	Maturity index
MT	Metallothioneins
mt DNA	Mitochondrial deoxyribonucleic acid
MUSCLE	Multiple sequence comparison by log-expectation
NINJA	Nematode indicator joint analysis
No.	Number

NPN	Non-parasitic nematodes
NWU	North-West University
Om	Omnivores
PC	Protein carbonyl
PCR	Polymerase chain reaction
PEC	Probable effect concentration
PPN	Plant-parasitic nematodes
PPRI	Plant Protection Research Institute
PTWI	Provisionally tolerable weekly intake
ROS	Reactive oxygen species
SD	Standard deviation
Sed	Sediment
SI	Structure index
SOD	Superoxide dismutase
SQG	Sediment quality guidelines
SQGQ	Sediment quality guideline quotients
SSV	Soil screen value
TCA	Trichloroacetic acid
TEC	Threshold effect concentration
TFM	Teflon lined vessel
TOC	Total organic carbon
UNESCO	United Nations Educational, Scientific and Cultural Organization
USA	United States of America
W	Water
ZAR	South African Rands

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Study overview

Subterranean environments, such as caves, are regarded as planet Earth's final frontier. These environments may host highly adapted species that are sometimes endemic to a single subterranean ecosystem (Romero, 2009; Culver & Pipan, 2010). Furthermore, groundwater serves as one of nature's most valuable sources of freshwater (Watson, 1997; Ford & Williams, 2007). Within caves scientists have also discovered what remains of ancient civilizations including burial sites, temples and hominid fossils (Watson, 1997; Bradley *et al.*, 2010). One of the most famous hominid discoveries that serves as a link in the evolution of the human species, was made in the Sterkfontein Cave, which today forms part of the greater Cradle of Humankind World Heritage Site (South Africa) (Bradley *et al.*, 2010; Durand *et al.*, 2010).

Unfortunately, these subterranean environments and especially the ecosystems they host, are threatened by anthropogenic activities such as groundwater abstraction, quarrying and pollution (Ford & Williams, 2007; Durand *et al.*, 2010). The risk of pollution is further intensified by the interconnected nature of karst landscapes that typically host cave and phreatic systems (Bonacci *et al.*, 2009). Although caves are well studied in some regions of the world, especially in parts of Europe and the Americas (Culver *et al.*, 2006), the ecosystems associated with many of Africa's karst landscapes remain understudied (Durand *et al.*, 2012).

One such karst landscape is located within the Witwatersrand Basin (South Africa), which is regarded as one of the biggest gold producing regions in the world (Osinski & Pierazzo, 2012). The Wonderfontein Cave, once utilized as South Africa's first touristic cave, is located within this landscape (Kent *et al.*, 1978). The Wonderfontein Cave is associated with the banks of Wonderfontein Spruit, which before the commencement of agricultural and large-scale mining activities, formed the centre point of a lush and species rich landscape (Swart *et al.*, 2003b). Regrettably, anthropogenic activities have led to the degradation of the landscape. One of the biggest threats that still remains is metal pollution in the Wonderfontein Spruit and the surrounding environment as the result of mineral processing plants, waste rock dumps and tailings runoff (Coetzee *et al.*, 2006; Hamman & Van Rensburg, 2012). The Wonderfontein Cave is subjected to the influx of polluted water from the Wonderfontein Spruit via a drainage area that feeds an underground river system. This river system runs through the Wonderfontein Cave and subsequently floods a vast extent of the 9 km (combined passage length) cave network. The water level within the Wonderfontein Cave is subject to seasonal climatic variations.

With the ultimate goal of promoting the conservation of Africa's subterranean environments and the ecosystems they host, this study focused on the effects of metal pollution on the ecosystem health of the Wonderfontein Cave and biota associated with it, as well as the toxic hazard potential of water, soil and sediment associated with the subterranean environment. Not only was the intention of this study to investigate the extent of anthropogenic pollution and the adverse effects it might present to biota associated with the subterranean environment, but also to consider the possible effect of the flow regime over a temporal scale. Subsequently, sampling was undertaken at the end of the high and low flow periods, which represented the 1st and 2nd sampling intervals, respectively. Nematodes (Phylum Nematoda) were selected as bioindicators of soil and sediment health, while *Clarias gariepinus* (African sharptooth catfish; Phylum Chordata) was used to evaluate biomarker responses to metal bioaccumulation compared to that of an epigean population. While nematode assemblage data was subjected to community and nematode specific indices (Ferris *et al.*, 2001; Ferris & Bongers, 2009; Ferris, 2010; Sieriebriennikov *et al.*, 2014), the bioaccumulation of selected metals, as well as biomarkers of oxidative stress and exposure was assessed in *C. gariepinus* (Van der Oost *et al.*, 2003; Farombi *et al.*, 2007; Wepener *et al.*, 2011; Van Dyk *et al.*, 2012). Also, the genetic distance between the *C. gariepinus* populations associated with the Wonderfontein Cave and Spruit, respectively, was measured in order to determine whether the population that was sampled in the cave has been isolated. If so, it would indicate that the cave population has only been subjected to the abiotic conditions associated with the subterranean environment.

An important factor that should be considered is the lack of a control cave system that has all the attributes required for this study. According to the knowledge of the author and the local caving community, the Wonderfontein Cave is the only known cave located within the Witwatersrand Basin that is subjected to the influx of surface river water. This problem has been overcome by using, with respect to the substrate (water, soil or sediment) in question, relevant guidelines and/or background values from appropriate studies. Furthermore, surface sites associated with the Wonderfontein Spruit were selected for investigations to serve as an indication of the extent of surface water pollution emanating from the mining activities.

The specific aims of this study were: (1) quantifying the extent of anthropogenic metal pollution; (2) assessing the toxicity hazard potential of water, soil and sediment associated with the Wonderfontein Cave; (3) determining whether nematode taxa and *C. gariepinus* individuals represent isolated communities and a population within the Wonderfontein Cave, respectively; (4) assessing the effect of mining-associated pollutants on the soil and sediment health of the Wonderfontein Cave by making use of nematodes to serve as bioindicators in both aquatic and terrestrial environments; (5) evaluating and comparing biomarker responses to metal bioaccumulation in *C. gariepinus* populations associated with the Wonderfontein Cave and epigean (surface) environments and (6) comparing the results of the above identified assessments over both a temporal and spatial scale. This was accomplished by conducting the following set objectives:

Part I (Chapter 3: Metal pollution and risk posed to water, soil and sediment quality of the Wonderfontein Cave):

- Collect representative substrate (water, soil and sediment) samples from the identified sampling areas in order to measure total metal concentrations as well as to extract, quantify and identify nematode assemblages.
- Determine particle size distribution, total organic carbon (TOC) and pH of soil and sediment samples.
- Identify and quantify the concentration of metals, as well as the degree of pollution in water, soils and sediments, across the extent of the Wonderfontein Cave and Spruit.
- Measure water quality parameters (pH and water hardness), as well as other physico-chemical characteristics in water samples from the selected sites to determine the source of pollution.

Part II (Chapter 4: Beneficial/non-parasitic nematodes as indicators of ecosystem health and their association with metals in soil and sediment from the Wonderfontein Cave):

- Extract nematodes from substrate samples using specific extraction methods and identify to highest possible taxonomic level.
- Use nematode assemblage data to assess the food web condition of each sampled substrate.
- Compare nematode assemblages associated with the Wonderfontein Cave to those associated with the surface environment.
- Study the relation between the nematode assemblage structures and metal enrichment.

Part III (Chapter 5: Metal bioaccumulation and biomarker responses in *Clarias gariepinus* of the Wonderfontein Cave and an epigean population):

- Determine the genetic distance between *C. gariepinus* populations that occur in the Wonderfontein Cave and Spruit, respectively, by using the cytochrome (cyt) b (mitochondrial DNA) molecular marker.
- Determine the current physiological state of *C. gariepinus* by applying health assessment and other gross body indices.

- Quantify the concentration levels of selected metals accumulated in muscle tissues of *C. gariepinus* collected from the Wonderfontein Cave and Spruit.
- Study specific biomarkers of oxidative stress and exposure that indicate the influence of toxic metal concentrations on *C. gariepinus*.

All data collected were statistically analyzed and compared for the all measured nematode and fish parameters. This has been accomplished by applying various univariate and multivariate approaches specific to each part of this study, which is further outlined in Chapter 2 (section 2.8). Ultimately, the concluding chapter (Chapter 6) provides a holistic view of the acquired results, as well as a final assessment on the ecosystem health of the Wonderfontein Cave, risk posed to biota associated with water, soils and sediments, as well as the effects of the flow regime.

Five hypotheses were formulated for this study. Firstly, it is hypothesized that elevated levels of anthropogenically sourced metals are present within the Wonderfontein Cave. Secondly, it is expected that nematode taxa and the *C. gariepinus* population associated with the Wonderfontein Cave are isolated communities and populations of the Wonderfontein Cave. Thirdly, it is foreseen that the pollutants present within the Wonderfontein Cave may have an effect on the nematode assemblages, subsequently affecting food web structures and threatening the soil and sediment ecosystem health. Fourthly, it is expected that metal bioaccumulation and biomarker responses in *C. gariepinus* specimens associated with the Wonderfontein Cave population are similar to that in the surface (Stoffels Dam) population. Lastly, it is anticipated that the flow regime, observed as differences between the high and low flow periods, has a substantial impact on the studied parameters.

1.2 General introduction to karst landscapes

1.2.1 What karst is

The term 'karst' originated from the Pre-Indo-European word "karra/gara", meaning stone (Williams, 2008) and is associated with any landscape created by the dissolution of water-soluble bedrock (Stokes *et al.*, 2010). Karst regions represent 7-12 % of the Earth's continental surface area and are an essential resource of freshwater, stored as groundwater, for a great part of the world's population (Ford & Williams, 2007; Hartmann *et al.*, 2014). Everyday 25 % of the global population makes use of either abstracted groundwater or spring water to meet personal requirements (Watson, 1997; Ford & Williams, 2007).

The main bedrocks in which karst can be found are limestone, dolomite and marble; these are generally referred to as carbonate rocks and constitute 15 million square kilometres (11 %) of Earth's continental ice-free surface area (Ford & Williams, 2007; Williams, 2008). However, karst features may also be found in evaporate rocks such as gypsum, rock salt and other rock types (Williams, 2008). It is important to note that karst principally defines a landscape partially eroded and thus an area of bedrock that has undergone the process of karstification.

1.2.2 The evolution of karst landscapes

Karst landscapes are typically characterized by depressions, dry valleys, sinking streams, fluted rock outcrops and caves; these characteristics are referred to as karst topography (Williams, 2008; Pankratz, 2010; Van Beynen, 2011). Significant karst features take many years to form and the rate of development is influenced by the interaction between climatic, topographic, geological, hydrological, as well as biological factors (Stokes *et al.*, 2010). In order to study these features and the factors affecting their formation, prior knowledge about the composition and dissolution of karst bedrock is essential. According to Stokes *et al.* (2010) carbonate rocks consists mainly out of two substances, namely calcite ($CaCO_3$) and dolomite ($CaMg[CO_3]_2$). Calcite is the primary mineral found in limestone and marble, while dolomite forms dolotone. The process of limestone formation starts by the deposition of plant and animal skeletal material as well as calcite from sea water on shallow marine seabeds (Hubert *et al.*, 2006). The deposited material undergoes diagenesis, which includes compaction, dissolution, calcite concentration, microbial micritization, dolomitization and crystal replacement. Over time sediments with an original porosity of between 40 and 80 % transform into limestone with a porosity of between 5 and 15 % (Ford & Williams, 2007). Limestone is thus a sedimentary rock and forms the basis of other carbonate rocks. The processes involved in the formation of dolomite are still today widely debated and various models exist to explain the process of dolomitization. The basic principle of dolomitization is the exchange of calcium (Ca^{2+}) ions, present in calcite, with magnesium (Mg^{2+}) ions sourced from the surrounding environment. If the stoichiometric relationship between the Ca^{2+} and Mg^{2+} ions in the newly formed compound is 1:1, it is referred to as dolomite and if not, as dolomitic limestone (Baker & Kastner, 1981; Morrow, 1982; Ford & Williams, 2007).

Over an extended period of time the sedimentary carbonate layers thicken and may even form bedrock hundreds of meters thick (Flügel, 2004). If the ocean level lowers and the water recedes, the bedrock may become exposed to physical and chemical weathering, subsequently subjecting the landscape to karstification. As a result karst evolution, roughly defined as the fashion in which both subterranean and surface karst features develop, is facilitated (Williams, 2004).

Chemical weathering is the main process involved in karstification and obtains energy from the hydrological cycle of which water is the main solvent and transport medium for dissolved carbonate ions (Williams, 2004). As water passes through the atmosphere with an average carbon dioxide (CO_2) concentration of 0.03 %, it interacts with CO_2 forming carbonic acid (H_2CO_3). Since soil has a CO_2 concentration of between 2 and 10 %, rainwater that enters and filtrates through the soil becomes even more acidic (Williams, 2004; Stokes *et al.*, 2010). Once the acidified water reaches the bedrock, it runs through pre-existing fractures and fissures initially created by physical forces. The H_2CO_3 partially dissolves the carbonate rock and transports the solution away from the site. The following equations illustrate the process of dissolution of (a) limestone and (b) dolomite with the addition of H_2CO_3 as reported by Leyland (2008):

(a) $CaCO_3 + H_2CO_3 \leftrightarrow Ca^{2+} + 2HCO_3^-$

$(K = 10^{-5.8})$

(b) $CaMg(CO_3)_2 + 2H_2CO_3 \leftrightarrow Ca^{2+} + Mg^{2+} + 4HCO_3^-$

$(K = 10^{-16.9})$

Over a great number of years this process results in the formation of conduits and subterranean voids, even extensive drainage and cave systems (Stokes *et al.*, 2010). Thus, karst landscapes are generally highly interconnected with substantial flow between surface water and groundwater systems (Bonacci *et al.*, 2009). The latter authors further stated that karst aquifers are characterized by a triple porosity system consisting of matrix permeability, fracture permeability and conduit permeability. Within this integrated network of spaces there exists a great variety of habitats (aquatic and terrestrial) for subterranean fauna. In fact, the importance of biological processes in the formation of karst landscapes is severely understated. According to Tabaroši (2002) many karst areas are the result of the complex relationship that exists between biotic and abiotic processes. It has even been suggested that karstification occurs as a result of biogenic processes (Bonacci *et al.*, 2009).

1.2.3 The importance of karst

Karst landscapes can host extensive cave systems of which there are globally a great number. The Mammoth Cave System in Kentucky (USA) is the world's longest cave system with more than 590 kilometres of surveyed passages (Culver & Pipan, 2010). And yet, size isn't the only characteristic that brings values to these subterranean voids.

In 1997 the International Union for Conservation of Nature (IUCN) Protected Area Program published a report titled the *Guidelines for Cave and Karst Protection* which provides reasoning and management strategies for the conservation of karst features. Following is a summary on the importance of caves and karst as outlined by Watson (1997):

1.2.3.1 Economic

Karst features are globally utilized for agriculture, forestry, water management and tourism purposes as well as limestone mining. Many karst regions provide highly productive soils and in some regions of the world, for example in Southeast Asia, caves are utilized to produce food sources and specialized products such as mushrooms and cheese. Also, one quarter of the globe's population is dependent on groundwater associated with karst landscapes for survival, while some other uses include irrigation, fisheries and hydro-electricity. Worldwide more than 20 million tourists visit caves annually with the Mammoth Cave System receiving over two million visitors each year.

1.2.3.2 Scientific

From a scientific perspective there exists great potential for research in karst landscapes. Geologists can study lithological units, geological structures, as well as the associated minerals. In addition, the bedrock in which karst mostly develops allows for good fossilization of various biotic groups and thus creates the opportunity for palaeontologists to study ancient species. One of the best examples may be in the Cradle of Humankind World Heritage Site, located in the Gauteng Province of South Africa, where hominid fossils have been excavated and intensely studied. Discoveries made here led to the greater understanding of the evolutionary path of the human race (Bradley *et al.*, 2010). Karst landscapes also host a vast array of endemic, highly adapted and possibly even singular plant and animal species (Romero, 2009; Culver & Pipan, 2010). The ecological study of subterranean environments is an intriguing and rewarding field of science.

1.2.3.3 Human

Many societies regard karst features, especially certain caves, as religious and spiritual places (Brockman, 2011). Today many Hindu and Buddhist groups utilize caves as temples; they even build temples that resemble caves. One of the most important human values must be the cultural value that caves hold. Excavations have provided insight into the culture of ancient communities and civilizations (Chase *et al.*, 2009; Ford, 2011). In addition, rock art has been studied and documented in depth and enable scientist to understand the mindset, social systems and behaviour of former cave inhabitants.

1.2.4 Potential threats to the integrity of karst landscapes

It is evident that karst landscapes have great scientific, social and economic value. It would thus be expected that these landscapes are adequately protected and conserved. Unfortunately, various karst landscapes are at risk of degradation as a result of anthropogenic activities (Van Beynen & Townsend, 2005; De Waele *et al.*, 2011).

In order to conserve the functionality and integrity of karst landscapes, it is necessary to identify, appreciate and study the activities that threaten it. Karst landscapes are especially prone to pollution as a result of its interconnected nature (Ford & Williams, 2007). The latter authors explain that karst hydrological systems effectively drain surface water through sinking streams, joints and conduits that represent a pathway for pollutants to the underlying aquifer. Watson (1997) related to this by stating that functional relationships exist between the land, water and biota associated with the greater catchment (water drainage) area. Any alteration to one of these could have a significant impact on a karst system and may occur as a result of physical, social and/or economic activities (Van Beynen & Townsend, 2005).

1.2.4.1 Anthropogenic impacts on karst landscapes

Ford and Williams (2007) provided a comprehensive summary (Table 1.1) of human activities that pose a threat to karst systems. Major anthropogenic activities resulting in the degradation of karst landscapes include agriculture, deforestation, industry and urbanization, mining and water exploitation. Other activities include recreation and tourism as well as military activities. The agricultural sector produces waste water that may contain elevated levels of suspended solids. This can result in the increase of the biochemical oxygen demand (BOD), which subsequently causes water quality to deteriorate (Liu, 2008). A study conducted by Boyer and Pasquarell (1996) revealed that the quality of karstic water associated with the Appalachian Region (USA) was adversely affected by cattle raising activities. Readings of up to 70 % increase in nitrogen load were recorded from an underlying cave system.

Table 1.1: A summary of adverse impacts inflicted on karst systems as a result of the effects created by human activities (Ford & Williams, 2007).

HUMAN ACTIVITIES	EFFECTS	IMPACT ON KARST SYSTEM
Agriculture	1) Increased runoff and erosion 2) Discharge of waste water	1) Degradation and loss of soil 2) Water quality deterioration
Deforestation	1) Loss of biota 2) Decreased evapotranspiration	1) Deteriorating ecological function 2) Increased runoff and erosion
Industry and urbanization	1) Increased runoff and erosion 2) Discharge of waste water 3) Rock and mineral remotion 4) Acidification of meteoric water	1) Degradation and loss of soil 2) Water quality deterioration 3) Landform destruction 4) Water quality deterioration
Mining	1) Increased sediment discharge 2) Discharge of waste water 3) Rock and mineral remotion 4) Production of chemical wastes	1) Sedimentation of caves 2) Water quality deterioration 3) Landform destruction 4) Water quality deterioration
Upstream dams	1) Allogenic recharge reduction	1) Lowering of water table
Downstream dams	1) Flooding	1) Flooded karst systems
Groundwater abstraction	1) Lowering of water table	1) Marine water intrusion; springs dry up; soil and structure collapse

Simsek *et al.* (2011) revealed that sewage and waste water, which was dumped into marble pits in the Iznik area in Turkey, polluted the underlying groundwater system and subsequently discharged pollutants into the alluvial unit. Urbanization is responsible for substantial habitat destruction that results in the extinction of species endemic to a single location. Also, mining can cause significant damage to the geomorphological setting of a karst landscape (Romero, 2009). The latter author further stated that mining activities alter the hydrological equilibrium and structural integrity of karst landscapes, resulting in sinkhole formation and cave system destruction. One such activity is groundwater abstraction, which is necessary for lowering the water table (Ford & Williams, 2007). The latter authors reported that eight sinkholes, each 50 m in diameter and deeper than 30 m, were formed after the compartments of the Far West Rand (located in South Africa) were dewatered. As a result, a total of 29 people lost their lives in 1962 at West Driefontein Mine when a three-storey crusher disappeared into a sinkhole (Ngcobo, 2006).

Mining activities may also lead to groundwater pollution as mine effluent is sometimes released into karst landscapes (Culver & Pipan, 2010). The latter is well documented in the Witwatersrand Basin (South Africa) and poses a threat to the United Nations Educational, Scientific and Cultural Organization (UNESCO) status of the Cradle of Humankind World Heritage Site (Durand *et al.*, 2010; Durand, 2012). It is evident that anthropogenic activities pose a serious threat to the hydrological and geomorphological integrity of a karst landscape, but what about the integrity of the associated ecosystem?

1.2.4.2 The conservation of karst ecosystems

A study conducted by Petit *et al.* (2006) revealed that the survival of several bat species on the island of Curacao, an island in the southern Caribbean Sea off the Venezuelan coast, were being threatened by urbanization and other anthropogenic activities. Also, Graening *et al.* (2006) documented that *Cambarus aculabrum*, an endangered species of cave crayfish, is globally limited to four locations (three in Benton County and one in Washington County, Arkansas, United States) and being threatened by habitat degradation. It was discovered that septic system discharges and animal feeding operations alter the water quality of a system that hosts a *C. aculabrum* population. Both Graening *et al.* (2006) and Petit *et al.* (2006) provided management strategies that can ultimately prevent the extinction of threatened species.

Conservationists and decision makers should act diligently in order to prevent damage to karst landscapes and the associated ecosystems. In an attempt to accomplish this, 86 % of the total karst area in Belize (a country on the north-eastern coast of Central America) has been incorporated into karst protection and development programs (Day, 1996). Also, interdisciplinary research teams are studying, documenting and continuously surveying the biodiversity associated with selected karst landscapes. In 2004 the Nature Conservancy and Indonesian Institute of Science orchestrated a study on the biodiversity of the Sangkulirang’s karst mountains that are situated in the east of Indonesia. Preliminary results revealed that the mountains host a great diversity of animal and plant species, however, some are threatened by fires, logging and hunting (Salas *et al.*, 2005).

1.2.5 South Africa’s karst landscapes

Within South Africa there are three major karst landscapes (Kuykendall, 2012) as illustrated by Figure 1.1 in blue. One landscape is located along the southern and eastern coastline of the Eastern and Western Cape provinces. This area forms part of the Cape Supergroup that consists of limestone, sandstone, quartzite and shale. The remaining two landscapes form part of the Transvaal Supergroup, which consists of limestone, dolomite, iron formation, quartzite and shale.

The first stretches from Prieska in the Northern Cape upwards past Kuruman until Vryburg in the North-West province. The second forms an elliptical sphere covering parts of the North-West, Gauteng and Mpumalanga provinces. It is within the latter that the Cradle of Humankind World Heritage Site is located, while it also hosts the well-known Sterkfontein and Wonderfontein Caves (Durand *et al.*, 2010).

A fourth substantially smaller karst landscape extends a few hundred kilometres North and South of Durban (South Africa) along the coastline and forms part of the Natal Group. The latter consists of the same major rock types as the Transvaal Supergroup (Kuykendall, 2012).

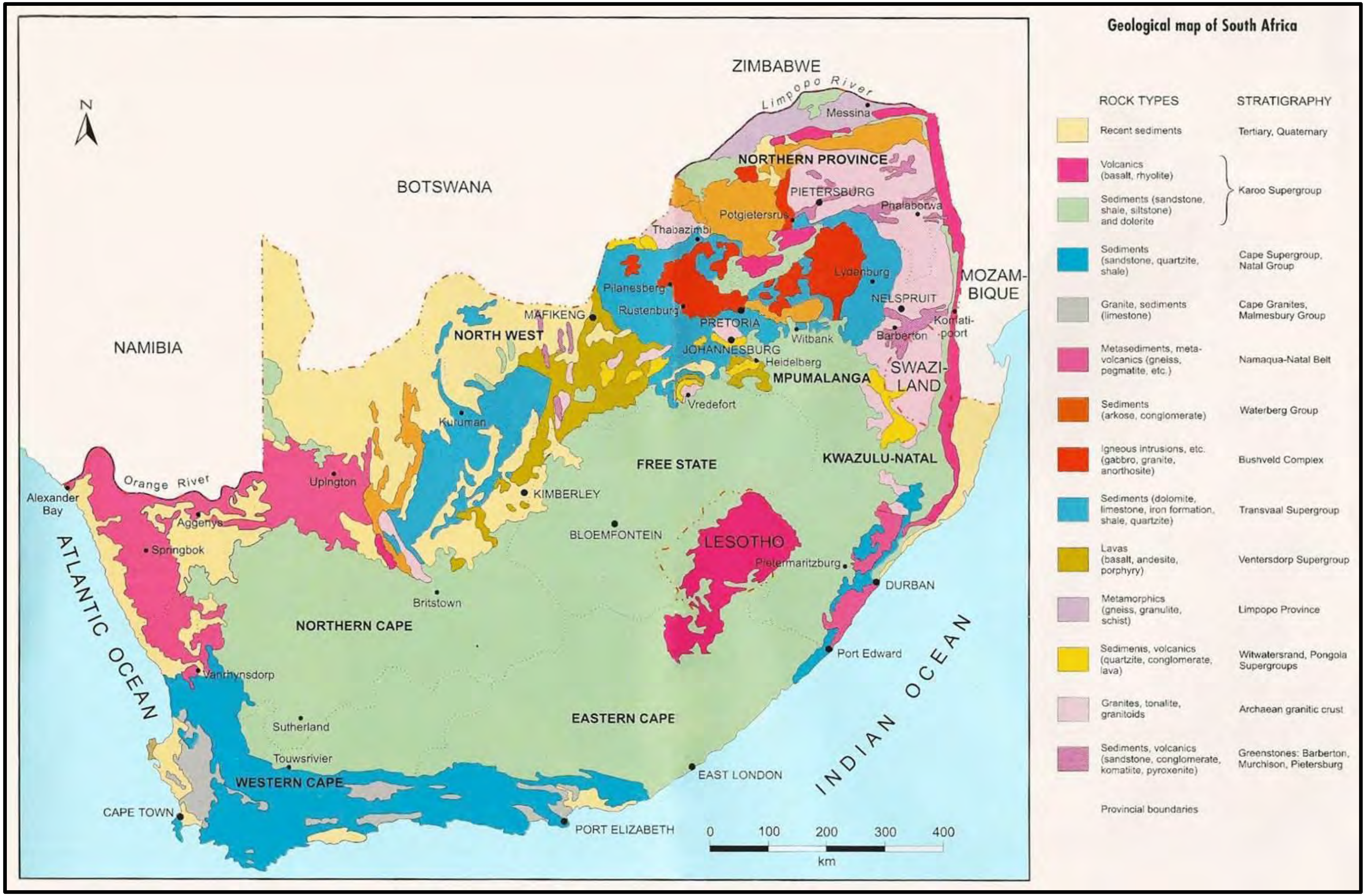

Figure 1.1: A geological map of South Africa with karst landscapes illustrated in blue (Kuykendall, 2012).

1.3 The subterranean milieu

1.3.1 An ecological definition of a cave

Caves are important scientific landscapes that deserve the attention of researchers from various disciplines. In order to study caves, a clear and concise definition of the boundaries of such as system is required. However, the ideal definition applicable to all fields of science does not exist. Different definitions (Table 1.2) are applied to delineate the subterranean environment with respect to a specific application (Culver & Pipan, 2010).

Table 1.2: Different definitions of caves.

Num.	Definition	Reference
A	'*A natural cavity, recess, chamber, or series of chambers and galleries beneath the surface of the earth, large enough for a person to enter*.'	Bates and Jackson (1980)
B	'*A natural opening that is large enough to admit a human being*.'	Ford and Williams (2007)
C	'*A natural opening in solid rock with areas of complete darkness, and larger than a few millimetres in diameter.*'	Culver and Pipan (2010)
D	'*A natural or artificial cavity in rock in which large-scale scalar phenomena are actually or potentially ecologically significant. These phenomena include the presence of surfaces (which may be at rock-air, rock-water and or water-air interfaces) available for utilization by mesofauna and/or macrofauna. Usually, though not invariably, they also include the presence and effects of fluid flow (air currents, streams, springs or tidal flow) and they also commonly have accumulations of bulk substrates such as guano, vegetable debris, talus and sediments. There is also potential for access and utilization by flying animals (bats, birds and insects) and by terrestrial and aquatic animals that are unable, because of their size, to utilize mesocaverns or smaller voids*.'	(Moseley, 2009)

The first definition (A) (Table 1.2) is generally used by geologists, while a simpler version (B) is recognized by the International Union of Speleology. However, Moseley (2009) reasons that none of the definitions meet the requirements of an biological/ecological study. The latter author further stated that if a cave is perceived as an ecological unit it should be defined with relation to the spatial scale. This is necessary since macrocaverns (large cavities) present special characteristics that have an influence on the ecology of cave fauna. As a result, Moseley (2009) proposed a more encompassing definition (D), which is accepted by the author of this thesis.

1.3.2 The cave environment

Although the boundaries of a cave system can be ecologically defined, it should be noted that the cave environment is further subdivided into different zones including the entrance, twilight, transitional and deep zones (Gillieson, 1996; Humphreys, 2012). Gillieson (1996) depicted the typical characteristics of each zone as follow:

The entrance zone is evidently very similar to the surface environment, however, it is sheltered and the flow of energy and mass focused. In addition, this zone may be littered with cave development debris such as boulders and sediment. Since food resources in this area are abundant, it serves as a habitat for a diversity of animal and even some plant species, though not obligatory cave-dwelling organisms.

The twilight zone is characterized by more stable atmospheric conditions with less fluctuating temperatures and higher humidity levels. Although some light still reaches this zone, it is not uncommon for cave-dwelling organisms (cave fauna) to show some form of adaptations to cave life. It should be noted that cave-dwelling organisms typically refer to animals (fauna) that reside within cave environments and not plants as cave systems are not able to sustain plant growth due to the lack of sunlight. The term ‘cave-dwelling organisms’ will be further applied in the remainder of this chapter as it refers to all organisms residing within a cave (facultative and obligatory) and are widely used in literature sources.

The dark or deep zone is a hostile place for most species that live on the surface, thus, only adapted species can truly survive and reproduce within this zone. No light would ever reach this section of the cave and temperatures are even more constant than that of the twilight zone. However, according to Humphreys (2012) research conducted by Howarth and Stone (1990) in an Undara lava tube located in North Queensland in Australia, led to the classification of a fifth zone named the stagnant-air zone. The latter authors noted that elevated CO_2 and lowered oxygen (O_2) levels were recorded and that true cave adapted species were restricted to this zone.

1.3.3 Sources and availability of energy

Although caves may provide suitable physical environments for the survival of various animal species, the availability of food resources play an important role in the functioning of subterranean ecosystems. As caves are typically energy-deprived environments, they are not able to sustain extensive populations (Poulson, 2012).

As part of nature, energy is transformed through various processes of which the single most important is the process of photosynthesis. Light energy is transformed into chemical bonds and stored in plants as carbohydrates, which represents a bioavailable-food source to other forms of life (Hall & Rao, 1999). If light is permitted to enter the entrance and twilight zones of a cave, residing organisms are capable of harnessing it. However, organisms residing in the deeper sections of a system generally have to make use of alternative energy sources for survival (Gillieson, 1996). These alternative energy sources include organic matter that is transported from the surface ecosystem (Culver & Pipan, 2010; Poulson, 2012), thus, its presence subterraneanly is the indirect result of photosynthesis. Culver and Pipan (2010) also reported that energy sources are able to enter a cave system in various ways, *viz*:

(1) as percolating water that originates as meteoric water. After precipitation, water drains through the soil allowing organic matter to become dissolved (or suspended), which serves as a source of carbon (C). Also, microbes and small invertebrates may become trapped and subsequently transported by percolating water. Ultimately, energy in the form of organic matter reaches the cave system, which may even serve as the main energy source.

(2) as flowing water that may enter a cave system as a sinking stream and functions as an energy source similar to that of percolating water. However, the size of the organic particles, including plant material and animal carcasses, transported by flowing water can be significantly bigger than that contained in percolating water.

(3) as animals, for example bat colonies that actively migrate out of a cave and feed on food sources that is present on the surface. After foraging they return to the safety of the cave where food is digested and bat guano is excreted. The latter not only provides habitat for specialised species, but also serve as an energy source for other cave-dwelling organisms.

(4) in the form of wind and gravity that causes organic matter, such as plant material and animals, to either blow or fall into caves. If an organism enters a cave system and dies, it subsequently serves as an energy source for cave-dwelling organisms.

(5) as roots of plants that penetrate subterranean voids to acquire water from either underground lakes/rivers or by absorbing moisture from the humid cave atmosphere. However, roots of plants may also serve as a habitat for a variety of specialized cave-dwelling organisms.

(6) by means of chemoautotrophy. Sarbu *et al.* (1996) documented that the latter process provides Moville Cave, located in Romania, with sufficient energy to sustain a community of 48 obligatory cave-dwelling terrestrial and aquatic invertebrate species. Chemoautotrophic bacteria convert hydrogen sulphide (H_2S) to organic C, which is then utilized by the cave-dwelling organisms as an energy source (Sarbu *et al.*, 1996; Engel, 2012). Sarbu *et al.* (1996) also mentioned that Moville Cave represents the first cave ecosystem known to be sustained by autotrophic production.

1.4 Life “down under”

1.4.1 Classification

The classification of cave-dwelling organisms is based on their morphology, distribution, ecology and behaviour (Gillieson, 1996; Romero, 2009; Trajano, 2012). Schiner (1854) proposed that cave-dwelling species, including temporarily visiting species, should be classified as either trogloxenes, troglophiles or troglobites. Romero (2009) and Trajano (2012) further explains that trogloxenes are organisms that occasionally make use of a cave environment for shelter and/or to utilize food resources. Trogloxenes are thus regarded as surface species such as moths and mosquitoes that only temporarily visit caves. It would thus be impossible for them to complete a full life cycle confined to a typical cave environment (Sket, 2008). On the contrary, troglophiles are capable of completing a full life cycle without leaving the cave. However, it would also be possible for the same individuals to reproduce in the surface environment and include many terrestrial vertebrates (Sket, 2008) and even fish species (*Forbesichthys agassizi*) (Christiansen, 2012). Finally, troglobites are typically highly adapted species that reside beyond the twilight zone and present specific characteristics such as depigmentation, elongated appendages, loss of eyesight, extended life cycles and chemosensory enhancement as traits of adaptation. Also, troglobites are sensitive to sudden environmental changes and will most likely die if removed from their subterranean habitat (Hunt & Millar, 2001; Trajano, 2012). Globally, there are many known troglobitic species, however, some of the highly adapted fish species such as *Typhlichthys subterraneus* remain peculiar (Christiansen, 2012). Groundwater-inhabiting organisms are generally referred to as stygobionts and/or stygofauna, while all cave fauna can be classified as either obligatory/compulsory or facultative/temporarily cave-dwelling/cavernicolous species (Romero, 2009).

1.4.2 Global perspective on the colonization, biodiversity and distribution of subterranean animal species

According to Trajano and Cobolli (2012) there are various ways in which surface species can enter and subsequently colonize subterranean habitats. Marine populations may enter both freshwater and marine environments through pathways that include interstitial spaces, submarine karst springs and anchihaline systems. Epigean aquatic species typically enter subterranean environments by means of sinking streams and resurgences. Regarding terrestrial species, the colonizer’s physical size plays a definitive role in its mode of entry. Smaller species may gain entry into a subterranean habitat by migrating through interstitial spaces in soils, but also by utilizing other pathways such as interconnected cracks and crevices. Larger species can enter a cave system by means of bigger natural entrances.

The diversity of species found in cave environments compared to that of the surface, is substantially lower (Sket, 1999a). The latter author explained that this is the result of the lack of habitat diversity and available food resources within caves as well as the fragmented nature of cave environments. Yet, according to Romero (2009) all major taxa are represented as either visitors or permanent residents of caves. Crustaceans, for example, account for 43 % of all known stygobiont species, which represent 9 % of all known aquatic species (Gibert & Culver, 2009). In subterranean environments, insects are represented by 700 families of 30 orders and springtails (Collembolans) by more than 400 species (Romero, 2009). Scientists are constantly discovering new species and since the year 2000, more than 5 000 obligatory cave-dwelling species have been described from Europe alone (Deharveng *et al.*, 2012). However, this is not the case for all cave-rich regions. According to Gibert and Culver (2009) there have been only 1 200 species described from Asia, 1 000 from North America, and 500 from Africa. Deharveng *et al.* (2012) explained that there are two main factors responsible for this phenomenon. Firstly, epikarst and subterranean environments other than caves are extensively studied in Europe, while vast areas in Africa and Asia remain isolated and undiscovered in terms of its residing cave-dwelling organisms. Secondly, the diversity of subterranean species is known to be the highest in Europe. Culver *et al.* (2006) identified seven well-studied karst regions within America and Europe in search of subterranean species; the two most diverse regions are both located in Europe and represent Ariège, a region located in South West France and the Slovenian Dinarides, a section of a mountain chain located in Slovenia.

Although subterranean life is well documented in many regions of the world, others regions and especially those in Africa, remain poorly studied. As a result there is a definitive lack of baseline data for the ecological study of Africa’s subterranean environments (Durand *et al.*, 2012).

1.4.3 A general report on cave taxa with emphasis on African species

Although most of Africa’s subterranean realms remain unstudied, research has produced some baseline data of especially invertebrate taxa (Lawrence, 1964; Sharratt *et al.*, 2000; Dippenaar-Schoeman & Myburgh, 2009; Durand *et al.*, 2012). In this section a brief report on taxa recorded from caves that are located in southern Arica, as well as their general characteristics, will be provided.

Mites and spiders constitute a diverse class (Arachnida) of organisms that occur in cave systems worldwide. Various species have been recorded from local caves, e.g. mites such as *Chiropturopoda coprohila*, *Laelaps* sp., *Sancassania* sp. as well as 41 spider species of which only 10 were classified as obligatory cave-dwellers (Dippenaar-Schoeman & Myburgh, 2009). Pseudoscorpions (Arachnida) are also found in southern African caves, with a peculiar troglobitic genus having been recently described from Botswana (Harvey & Du Preez, 2014). Arachnid-cave representatives commonly present troglobitic characteristics and their feeding habits include predation, parasitism and consumption of detritus (Romero, 2009). Another group of organisms found in cave environments are the worm-like arthropods of which cave-dwelling populations have been reported from such subterranean ecosystems in Botswana (Du Preez *et al.*, 2013). They are classified as Myriapoda and are generally associated with decaying, organic material (Romero, 2009). According to the latter author Hexapoda are the greatest representative of cave fauna and include insects, diplurans and springtails (Collembola). The lepidopteran species *Monopis transeans* occur in great numbers in the Bakwena Cave (South Africa) (Durand *et al.*, 2012). From the same cave bat flies (family Nycteribidae) have also been identified. Crustaceans, which includes amphipods and copepods, are also commonly associated with aquifers, but can also be found in cave pools and rivers. Amphipods sampled from the Bakwena Cave belong to the genus *Sternophysinx* and most likely the species *S. filaris*. Another species of the same genus, *S. calceola*, has been reported from the Matlapitse (Limpopo Province, South Africa) and Koelenhof (Cradle of Humankind World Heritage Site, South Africa) caves (Holsinger, 1992; Durand *et al.*, 2012). Eight fungi genera and bacteria taxa have also been identified from the Bakwena Cave (Durand *et al.*, 2012). Such microorganisms also play an important role in the food web of cave systems not only as nutrient cyclers (Northup & Lavoie, 2004), but also as a food source for other cave-dwelling organisms such as some non-parasitic (NPN), terrestrial and aquatic nematode and collembolan taxa (Romero, 2009).

A study by Sharratt *et al.* (2000) revealed that the sandstone caves of the Cape Peninsula (South Africa) host 85 cave-dwelling invertebrate species. Of these, Hexapoda represent 38 % and Chelicerata 34 % of the total invertebrate assemblage. Furthermore, most of the chelicerans were classified as either spiders (Araneae) or harvestmen (Opiliones). The authors also reported that six new species were discovered, with some presenting troglobitic characteristics. According to the latter author, a total of 21 invertebrate species are endemic to the Peninsula region. Furthermore, a number of species are critically endangered such as the crustacean species *Spelaeogriphus lepidops*. Conservation efforts to ensure the survival of such invertebrates has thus been strongly motivated (Sharratt *et al.*, 2000). Other groups of organisms also known to inhabit cave environments include nematodes and fishes. These two invertebrate groups are relevant to this study since ecological and/or biochemical data obtained from studying these taxa was used to evaluate the ecosystem health of the Wonderfontein Cave, as well as the potential risk of anthropogenic metal pollution (Gauteng Province, South Africa).

1.4.4 Cave-dwelling nematodes: A general perspective

Nematodes, generally known as round- and/or eelworms, play an important role as part of the food web in cave ecosystems. Some NPN/beneficial species feed on bacteria that is present in bat guano and in turn serve as a food source for mites and other arthropods (Romero, 2009). Studies by Van Rensburg (2010) and Durand *et al.* (2012) were most likely the first to survey a South African cave in search of nematodes. Both studies were conducted in the Bakwena Cave (Irene area, Gauteng Province) and results indicated that bacterivorous nematodes dominated the nematode communities in the various substrates sampled. Also, a range of other trophic groups that included herbivores was reported from the same cave's ecosystem (Table 1.3).

Most nematodes, however, are believed to be accidental inhabitants of caves that gain entry by means of substrates that are deposited and/or water that flushes through cave openings (Poinar & Sarbu, 1994; Hodda *et al.*, 2006; Romero, 2009; Durand *et al.*, 2012). Hodda *et al.* (2006) created a list (Table 1.3) of 28 cave-dwelling nematode genera, which was updated by the thesis author. Especially relevant to this study is the applied update of cave-dwelling nematode genera reported from the above mentioned Bakwena Cave (Durand *et al.*, 2012), as well as those reported by Du Preez *et al.* (2013). While 27 nematode genera were found within the Bakwena Cave, the latter study collectively reported 12 genera from two South African and two Botswana caves. The remainder of the list was compiled from existing literature sources and includes individuals of beneficial, bacterivore and predatory nematode genera such as *Monhystera*, *Plectus* and *Dorylaimus* as well as those of plant-parasitic nematode (PPN) genera such as *Criconema* and *Rotylenchus*.

Although many of the reported taxa might be classified as accidental inhabitants, true cavernicolous nematodes such as *Chronogaster troglodytes* have also been reported. The latter species was discovered in fungal mats from a Romanian cave that is subjected to an autochthonous primary production system as a result of the interaction between microorganisms and sulphur-enriched thermal spring water (Poinar & Sarbu, 1994; Hodda *et al.*, 2006). Poinar and Sarbu (1994) further stated that *C. troglodytes* might even be the only true obligatory cave-dwelling nematode as it has adapted and resided within the cave for millions of years. Nematodes are also known to inhabit groundwater systems deep (greater than 3 km) below the surface of the earth (Borgonie *et al.*, 2011). The latter authors sampled and described a new nematode species, *Halicephalobus mephisto*, from filtered fracture water. It was also reported that *H. mephisto* (bacterivore) are tolerant to high temperatures and reproduce asexually.

Table 1.3: An updated list of reported cave-dwelling (cavernicolous) nematodes as modified from Hodda *et al.* (2006).

Genus*	**Country**	**Cp - Group**	**Reference**
Achromadora	South Africa	Eu (3)	Durand *et al.* (2012)
Acrobeles	South Africa	Ba (2)	Durand *et al.* (2012)
Acrobeloides	France, South Africa	Ba (2)	Cayrol (1973); Durand *et al.* (2012)
Alaimus	South Africa	Ba (4)	Durand *et al.* (2012)
Amphidelus	Mexico, Guatemala	Ba (4)	Zullini (1977)
Anaplactus	France, South Africa	Ba (2)	Cayrol (1973); Durand *et al.* (2012)
Aphelenchus	South Africa	Fu (2); Her	Durand *et al.* (2012)
Aphelenchoides	South Africa, Botswana	Fu; Her	Durand *et al.* (2012); Du Preez *et al.* (2013)
Aporcelaimellus	Mexico, Guatemala	Om (5)	Zullini (1977)
Aporcelaimus	South Africa	Om (5)	Durand *et al.* (2012)
Boleodorus	South Africa	Her	Durand *et al.* (2012)
Cephalobus	France, Botswana	Ba (2)	Andrássy (1967); Cayrol (1973); Du Preez *et al.* (2013)
Cervidellus	France	Ba (2)	Cayrol (1973)
Chiloplectus	South Africa	Ba (2)	Durand *et al.* (2012)
Chronogaster	Romania	Ba (3)	Poinar and Sarbu (1994)
Criconema	Austria, Botswana	Her	Schneider (1940); Du Preez *et al.* (2013)
Criconemoides	Austria, France	Her	Schneider (1940); Cayrol (1973)
Cylindrolaimus	Hungary, South Africa	Ba (3)	Andrássy (1959); Durand *et al.* (2012)
Desmoscolex	Slovenia	Ba (3)	Stammer (1935)
Diplogasteroides	South Africa	Ba (1)	Durand *et al.* (2012)
Diploscapter	South Africa	Ba (1)	Durand *et al.* (2012)
Discolaimus	South Africa	Ca (5)	Durand *et al.* (2012)
Ditylenchus	South Africa	Fu/Her (2)	Durand *et al.* (2012)
Dorylaimus	Yucatan (Mexico)	Om (4)	Chitwood (1938)
Eumonhystera	South Africa	Ba (1)	Durand *et al.* (2012)
Halalaimus	Austria	Unknown	Schneider (1940)
Helicotylenchus	South Africa, Botswana	Her	Durand *et al.* (2012); Du Preez *et al.* (2013)
Meloidogyne	Botswana	Her	Du Preez *et al.* (2013)
Mermis	Romania	EPN	Schuurmans-Stekhoven (1950)
Mesodorylaimus	France, Mexico, Guatemala, Romania	Om (5)	Cayrol (1973); Zullini (1977); Poinar and Sarbu (1994)
Mesorhabditis	South Africa	Ba (1)	Durand *et al.* (2012)
Miconchus	France	Ca (4)	Cayrol (1973)
Monhystrella	France, South Africa	Ba (1)	Cayrol (1973); Durand *et al.* (2012)
Monochromadora	France	Unknown	Cayrol (1973)
Mononchus	France, Mexico, Guatemala, South Africa	Ca (4)	Cayrol (1973); Zullini (1977); Durand *et al.* (2012)
Mylonchulus	Austria, Mexico, Guatemala, South Africa	Ca (4)	Schneider (1940); Zullini (1977); Durand *et al.* (2012)

*For each genus entry, the location (country) of the cave, cp-group classification and literature reference is provided. Trophic group and cp-value classification of nematode genera was based on entries listed in the Nemaplex online database (Available at http://plpnemweb.ucdavis.edu/nemaplex) and respectively represent Eukaryote feeders (Eu), Bacterivores (Ba), Fungivores (Fu), Omnivores (Om), Carnivores/Predators (Ca), Herbivores (Her) and Entomopathogenic (EPN) nematodes.

Table 1.3: Continued.

Genus	Country	Cp - Group	Reference
Myolaimus	Hungary	Ba (2)	Andrássy (1959)
Neoactinolaimus	Botswana	Om (4)	Du Preez *et al.* (2013)
Panagrolaimus	Romania, South Africa, Botswana	Ba (1)	Muschiol and Traunspurger (2007); Durand *et al.* (2012); Du Preez *et al.* (2013)
Paracrobeles	Botswana	Ba (2)	Du Preez *et al.* (2013)
Plectus	France, South Africa	Ba (2)	Cayrol (1973); Durand *et al.* (2012)
Poikilolaimus	Romania	Ba	Muschiol and Traunspurger (2007)
Pratylenchus	South Africa	Her	Durand *et al.* (2012)
Prismatolaimus	South Africa, Botswana	Ba (3)	Durand *et al.* (2012); Du Preez *et al.* (2013)
Rhabditis	France, South Africa, Botswana	Ba (1)	Cayrol (1973); Du Preez *et al.* (2013)
Rotylenchus	France	Her	Cayrol (1973)
Stegelletina	Spain	Ba?	Abolafia and Pena-Santiago (2006)
Stenochulus	Austria	Ca (3)	Schneider (1940)
Thallassoalaimus	Austria	Ba?	Schneider (1940)
Tobrilus	France	Ca (3)	Cayrol (1973)
Trichodorus	South Africa	Her	Durand *et al.* (2012)
Tylenchus	France	Her/Fu	Cayrol (1973)
Tylenchorhynchus	South Africa	Her	Durand *et al.* (2012)
Xiphinema	Botswana	Her	Du Preez *et al.* (2013)
Zeldia	Botswana	Ba (2)	Du Preez *et al.* (2013)

1.4.5 Nematodes as environmental indicators

1.4.5.1 Classification and ecosystem function of nematodes

Nematodes inhabit a diverse range of environments and represents 80 % of the world's metazoans (Platt, 1994; Bongers & Ferris, 1999; Ferris & Bongers, 2009). According to Bongers and Ferris (1999) nematodes can inhabit any marine, aquatic and terrestrial environment, provided there is an adequate source of organic C. Also, nematodes are primarily regarded as aquatic organisms as even in terrestrial environments they occupy thin water films that surrounds soil particles (Bongers & Ferris, 1999). When considering soil ecosystems, nematodes can broadly be classified as either being PPN or NPN. A more in depth classification according to feeding types of this phylum would include 1) herbivores (PPN), 2) bacterial feeders (bacterivores), 3) hyphal/fungal feeders (fungivores), 4) eukaryote feeders, 5) substrate ingesters, 6) predators and 7) omnivores (Yeates *et al.*, 1993). Herbivores only represent PPN and the rest NPN nematodes.

Although PPN are known to cause devastation to various crops, NPN/beneficial nematodes help maintain soil health by fulfilling vital ecosystem functions (Neher, 2010). The latter author explains that nematodes play an important role in regulating 1) the community structure of decomposing microflora, 2) element cycles and 3) litter decomposition. According to Neher (2001) nematodes alter the metabolic activity of microbes as well as the size of the microbial community and thus partially controls the overall rate of decomposition that occur in soil substrates. Nematodes also serve as nutrient cyclers by directly and indirectly altering the availability of nitrogen; directly by excreting ammonia and indirectly by metabolizing, excreting and facilitating the dispersal of microbes (Ingham *et al.*, 1985; Neher, 2010). Another vital ecosystem function is the control of plant diseases as nematode predators also feed on PPN (Kerry, 2000; Neher, 2010).

1.4.5.2 Nematode as indicators of disturbance: Available ecological indices

As a result of their diversity, abundance and importance in maintaining soil health, nematodes are considered to be one of the most significant bioindicators among soil fauna (Neher, 2001; Ekschmitt & Korthals, 2006). The use of nematodes as bioindicators of pollution and especially indicators of toxic-metal contamination has been extensively studied (Ekschmitt & Korthals, 2006; Pen-Mouratov *et al.*, 2008; Šalamún *et al.*, 2012). The latter authors agree that metal contamination has a severe effect on the community structure of nematodes. Furthermore, various general community and nematode specific indices can be applied as an alternative to chemical approaches in determining the pollution effect.

Community indices are widely applied in order to analyse and interpret nematode species data with the aim of providing information on either a past or future ecological process (Neher & Darby, 2006; Neher & Darby, 2009). According to the latter authors community indices include both univariate ones such as Margalef’s richness and Shannon’s diversity indices as well as multivariate techniques such as cluster analysis and data transformation. Consideration should always be given to selecting the appropriate indices necessary to fulfill the goals of a specific study. Also, to derive the meaning of especially identity-independent indices without the support of sufficient evidence should be avoided. For further reading on general community indices available for the ecological study of nematode assemblages, the chapter of Neher and Darby (2009) could be recommended.

While community indices can be very useful, nematode-specific indices provides us with more information on the ecosystem functioning and includes the Maturity index (MI) family, Enrichment index (EI), Structure index (SI), Channel index (CI) and Basal index (BI) as well as metabolic footprints (Ferris *et al.*, 2001; Ferris & Bongers, 2009; Ferris, 2010; Sieriebriennikov *et al.*, 2014). The MI, originally developed by Bongers (1990), is a very useful method for evaluating ecosystem disturbance using nematode assemblages. This index is based on the r/K strategists concept where r-selected individuals are favored for reproducing rapidly, while K-selected individuals are favored for their substantial contribution to a population where the size of the population nears the environment’s carrying capacity (Begon *et al.*, 2009). While the former (r-strategy) is typically associated with unpredictable environments and highly variable mortality rates, the latter (K-strategy) is associated with constant environments and minimal change in population size. Based on this concept, nematodes are categorized into a 1-5 colonizer-persister (cp) series (Table 1.4) (Bongers, 1999; Bongers & Ferris, 1999; Ferris *et al.*, 2001). According to the latter authors, nematode taxa with the same cp classification have similar responses to ecosystem disturbance. For example, a low MI represents an unbalanced predator (low biomass) - prey (high biomass) relationship and may indicate environmental disruption of higher cp class nematodes (Ferris & Bongers, 2009). Thus, if applied to the study of the ecological effect of metal pollution, Bongers and Ferris (1999) stated that stress induced as a result of metal pollution will be reflected in a lowered MI value as the ecosystem becomes dominated by opportunistic (cp-1), NPN species less sensitive to pollution.

Table 1.4: Colonizer-persister (cp) series classification of non-parasitic nematodes (NPN) as modified from Ferris *et al.* (2001).

	Generation Time	**Fecundity**	**Trophic groups**	**Sensitivity**	**Other**
CP-1	Short	High	Ba	Tolerant	Feed on enriched media
CP-2	Longer	Lower	Ba, Fu	Tolerant	Constant feeders
CP-3	Longer	Lower	Ba, Fu, Ca	More sensitivity	
CP-4	Longer	Lower	Om	Greater sensitivity	
CP-5	Long	Low	Om, Ca	Great sensitivity	

Pen-Mouratov *et al.* (2008) also concluded that the MI was the most sensitive index for determining the influence of metal contamination on NPN populations in soils. There are, however, a series of MI collectively referred to as the MI family. It is thus crucial that attention should be given to select the appropriate nematode indices most suitable for each ecological study (Ferris & Bongers, 2009). According to the latter authors the MI, that excludes cp-1 nematodes (MI_{2-5}), is more suitable for the assessment of ecosystem disturbance induced by metal pollution than other MI.

Since cp-2 nematodes are found in most food webs and are tolerant to environmental change, they are regarded as basal to the food web (Ferris *et al.*, 2001; Ferris & Bongers, 2009). From this the EI and SI are conceptualized. The EI is calculated by considering the weighted abundance of cp-1 bacteriovore and cp-2 fungivore nematodes proportional to all cp-1-2 nematodes. Conversely, the SI is calculated by considering the weighted abundance of cp-3-5 nematodes proportional to all cp-2-5 nematodes (Ferris & Bongers, 2009). According to the latter authors the BI can also be calculated by considering cp-2 nematodes proportional to all nematodes present in a sample obtained from a specific ecosystem.

1.4.5.3 Nematode as indicators of disturbance: Food web structure and metabolic footprint analyses

The continuous research and model validation associated with the MI family has resulted in the development of a functional guild classification of nematodes (Ferris *et al.*, 2001; Ferris & Bongers, 2009). The guild classification plays a fundamental role in the analysis and comparison of ecosystem processes (Bongers & Bongers, 1998). By applying this classification, a nematode-faunal profile (Figure 1.2) with a quadrat structure ordination can be created, providing information on the community structure and resource availability. Furthermore, the food web condition (structured, enriched and basal) are conjunctively used with the quadrat structure to plot the SI and EI (Bongers & Bongers, 1998; Ferris *et al.*, 2001). Ideally, equilibrium should exist between the enrichment and structure of a food web that is representative of an ecosystem where nematodes occur. If one trajectory outweighs the other, it might be indicative of a disturbed environment in terms of nematode communities present.

It is evident that community- and nematode-specific indices can provide ecologists with an accurate assessment of ecosystem health. However, little can be derived about the magnitude of ecosystem functioning and services provided by the associated nematode assemblage. According to Ferris (2010) calculating the metabolic footprint of an assemblage can provide additional insight into the extent that a specific nematode trophic group contributes towards ecosystem functioning. While a functional footprint can be calculated for the entire nematode assemblage, separate enrichment and structure footprints provide insight into the role that nematodes associated with the enrichment and structure of food webs play, respectively (Ferris, 2010).

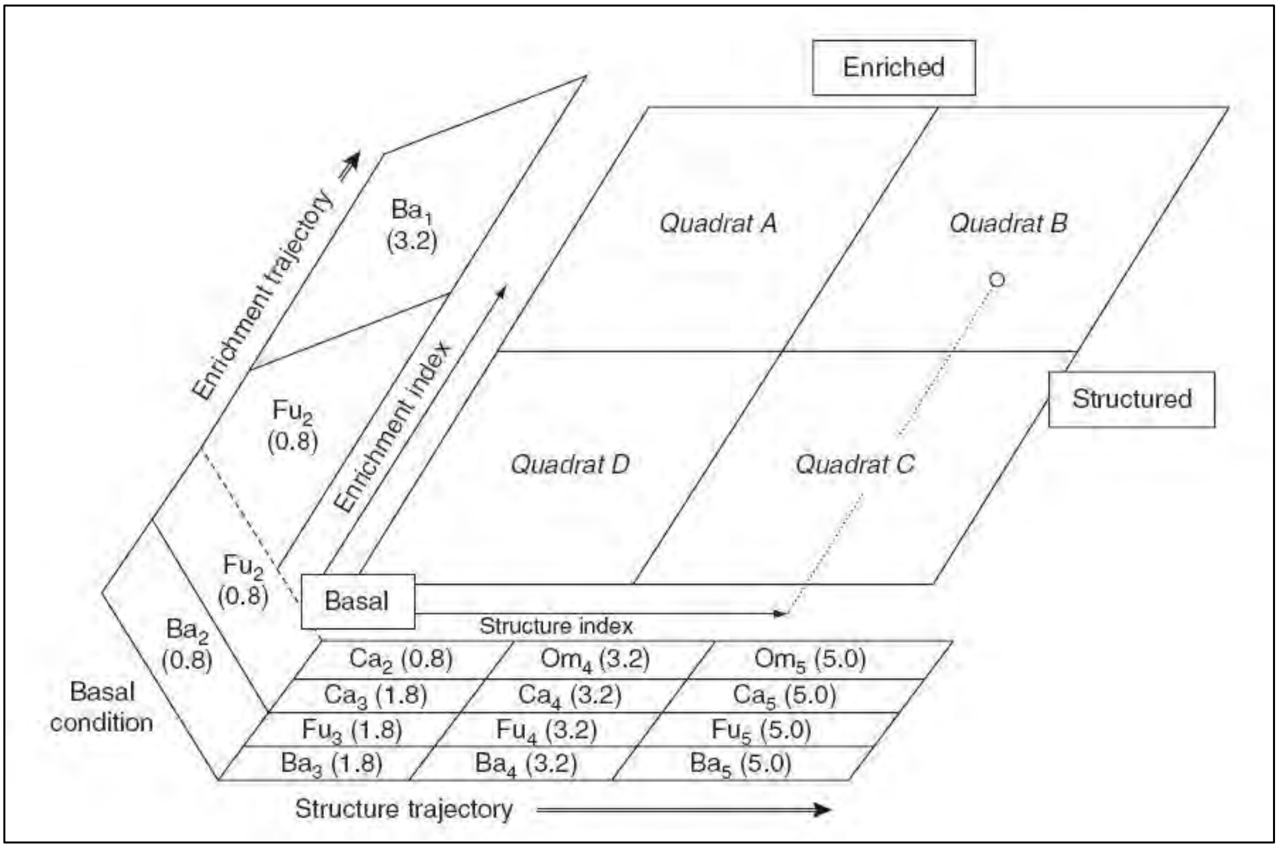

Figure 1.2: Illustration of the nematode-faunal profile with enrichment (y-axis) and structure (x-axis) trajectories indicated. Each quadrat represents a different faunal profile: A is enriched and unstructured; B is enriched and structured; C is resource-limited and structured; D is resource-depleted with minimal structure. Trophic groups of non-parasitic nematodes (NPN) are identified and each guild weighted for calculating the respective trajectories (Ferris & Bongers, 2009).

1.4.5.4 Nematode response to metal-induced stress

When considering the physical effects of metal-induced stress on nematode assemblages, Ekschmitt and Korthals (2006) studied published data from six fields investigated and reported that nearly 50 % of the identified nematode taxa presented significant correlation with at least one metal (toxicant) substance. Although the results indicated that a broad range of PPN and NPN were similarly affected by toxicants, some genera showed specific tolerances to particular contaminants. Generally, nematodes presented three major response types to metal contamination (Ekschmitt & Korthals, 2006), namely:

1. Copper (Cu)/Zink (Zn) sensitivity,
2. Nickel (Ni) sensitivity and
3. Genera specific tolerances to Cu, Zn, Chromium (Cr) and Ni

Most importantly, however, these authors concluded that individuals from the genera *Pratylenchus* and *Chiloplacus* are effective bioindicators of Cu, *Tylenchus* and *Cephalobus* of Zn and

Paratylenchus and *Criconemoides* of Cr. All of these nematode genera presented a single and direct positive correlation with the respective metals.

1.4.6 Subterranean fish

1.4.6.1 Diversity of subterranean fish with emphasis on African species

Subterranean fish species have globally been extensively studied from various regions of the world. According to Proudlove (2010) there are more than 2 500 publications that relate to the biology of subterranean fishes. The latter author also stated that there are more than 150 freshwater fish species reported from subterranean environments. On the contrary, Romero (2009) reported 299 fish species from subterranean environments, however, also included marine species. Although the number of known subterranean fish species is increasing fast, it is unlikely to exceed 1 000 species (Proudlove, 2010). Currently, subterranean fish species are known from 34 countries with China, Brazil and Mexico accounting for more than half of the known species, while many of the remaining countries are only represented by a single species (Proudlove, 2010). According to the latter author a number of factors might account for this phenomenon, but the lack of focus on caving and the studying of subterranean environments in some regions play an important role. As would be expected, many of these fish species present typical adaptive traits. Romero and Green (2005) stated that troglomorphic adaptations are common in subterranean fish species and include depigmentation, loss of eyesight, reduced gas bladder size and reduced number and size of scales. Although many species are reported from cave habitats, some are also found in groundwater (phreatic zones) and anchialine systems (Romero, 2009).

The first African cave-dwelling fish was discovered in 1917 by amateur cave explorers and named the Congo blind barb (*Caecobarbus geertsii,* 1921) (Berti & Messana, 2010). Berti and Messana (2010) further stated that only eight valid African cave fish species have been described mostly as a result of civil unrest. Of the remaining seven species, three were discovered in Somalia, three in Madagascar and one in Namibia. The latter fish species, *Clarias cavernicola*, is the only one known from southern Africa. Two of the species, *Uegitglanis zammaranoi* from Somalia and *C. cavernicola* from Namibia, belong to the family Clariidae, which is the same family that the fish species relevant to this study, *C. gariepinus*, is classified under (Berti & Messana, 2010). According to the latter authors *C. cavernicola* was first described from a lake in Aigamas Cave, which is located a short distance north of Otavi (Namibia). However, it is possible that this fish species inhabits also other caves in the region.

1.4.6.2 Threats posed to the survival of the *Clarias cavernicola* population

Unfortunately, many subterranean fish populations are threatened by anthropogenic activities such as habitat degradation, hydrological manipulations and the introduction of invasive species (Proudlove, 2001; Bichuette & Trajano, 2010). Another threat posed to subterranean fish species is environmental pollution, including exposure to toxic metals (Proudlove, 2001). Many subterranean fish populations that are threatened by metals have, however, not yet been documented (Bichuette & Trajano, 2010).

Proudlove (2001) lists a number of cave-dwelling fish species that are threatened by especially hydrological manipulations, one of which is *C. cavernicola.* Water is abstracted from the same groundwater system that is inhabited by the latter species. Subsequently, Proudlove (2001) reported a 20 m drop in the water level, exposing the feeding area of this fish species. It was noted by several authors, including Skelton and Teugels (1991) and Bruton (1995), that the continued abstraction of groundwater might severely inhibit the feeding ability of *C. cavernicola* populations. Populations of the latter fish species is also threatened by the introduction of *C. gariepinus* specimens that outcompete the native *C. cavernicola* population (Bruton, 1995; Proudlove, 2001). According to the latter author *C. cavernicola* has been classified as a critically endangered species since 1996.

1.4.7 Fish as environmental indicators

1.4.7.1 Bioindicators and metal bioaccumulation as an assessment of ecosystem disturbance

Fish occur in almost all aquatic environments and play a major role in the ecology of food webs in such ecosystems. As a result fish are considered as one of the most important aquatic bioindicators used in environmental risk analyses (Van der Oost *et al.*, 2003; Di Giulio & Hinton, 2008). Although decreasing populations and physiological alterations may indicate the presence of toxicants, its manifestation often resembles the point of no return (Van der Oost *et al.*, 2003). Therefore, it is necessary to identify and manage the detrimental effects of toxicants before the state of an ecosystem is severely affected.

Serving as an early-warning system, biomarkers are utilized and consists of measured indicators of tissues, cells, and/or fluids that are altered as a result of toxification or response by the host (Henderson *et al.*, 1987; Van der Oost *et al.*, 2003). According to the latter authors oxidative stress parameters are one group of biomarkers used in fish ecotoxicological studies. Oxidative stress that occur in organisms, such as fish, is defined by Betteridge (2000) as: “A disturbance in the balance between the production of reactive oxygen species (free radicals) and antioxidant defenses, which may lead to tissue injury." Various pollutants are related to the induction of reactive oxygen species (ROS), which can cause enzyme deactivation, DNA impairment, lipid peroxidation and even cellular death. Some oxidative stress parameters measured in organisms include antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) (Van der Oost *et al.*, 2003; Di Giulio & Meyer, 2008). Superoxide dismutase is present, within vertebrates, in three forms including CuZnSOD, Mn(manganese)SOD and extracellular SOD and serve as a response mechanism to ROS by reducing superoxide (O_2^-) concentrations. As a result of the latter, however, hydrogen peroxide (H_2O_2) is produced which may in itself be damaging to cells, especially in combination with of Cu and Fe (Di Giulio & Meyer, 2008). Fortunately, vertebrate species utilize other enzyme systems such as CAT, which metabolizes H_2O_2 to O_2 and water (H_2O) (Van der Oost *et al.*, 2003; Di Giulio & Meyer, 2008). Another measurable biomarker induced by oxidative stress is protein carbonyl (PC) content. According to Parvez and Raisuddin (2005) PC may be used as a biomarker of exposure in fish since carbonyl groups are formed as a result of the presence of the highly reactive hydroxyl radical (OH^-). Also regarded as a biomarker of exposure are the cysteine-rich proteins called metallothioneins (MT) which are known to regulate essential metals such as Cu and Zn (Van der Oost *et al.*, 2003). According to the latter authors, MT also intercept and bind to metal ions, as well as remove metals from non-thionein ligands. Metallothioneins thus also present a detoxification function, which is induced by exposure to especially cadmium (Cd), Cu, Zn, mercury (Hg), cobalt (Co), Ni, bismuth (Bi), and silver (Ag).

Bioaccumulation is defined by Jorgensen (2010) as the: "… accumulation and enrichment of contaminants in organisms, relative to that in the environment." It is thus the concentration of substances that prevail in muscle tissues/organs of live organisms after all uptake and loss processes. The bioaccumulation of contaminants in fish can provide insight into the type and extent of exposure to pollutants as well as the possible adverse effects that the aquatic ecosystem might face (Franke *et al.*, 1994). According to Van der Oost *et al.* (2003) uptake of contaminants by fish may occur through ingestion of suspended particles and/or intake of contaminated food, as well as directly from water via the gills. Bioaccumulation is especially relevant when metal contamination is considered as various studies reported the concentration of metal toxins present in the muscle tissue/organs of fish (Avenant-Oldewage & Marx, 2000; Murtala *et al.*, 2012).

1.4.7.2 The fish health assessment index and other gross body indices as an assessment of ecosystem disturbance

A useful tool that allows the rapid assessment of the physiological health of a fish specimen is the Health Assessment Index (HAI) (Adams *et al.*, 1993). According to the latter authors environmental stressors can reflect on the physiological condition of fish as reduced growth, reproduction impairment and increased susceptibility to diseases, only to name a few. The HAI, which uses the fish necropsy protocol provided by Goede and Barton (1990), has been widely applied in the physiological health assessment of *C. gariepinus* populations (Crafford & Avenant-Oldewage, 2009; Marchand *et al.*, 2009; McHugh *et al.*, 2013). Gross body indices, such as the condition factor (CF) and hepatosomatic index (HSI), are also useful tools for assessing the health of a fish specimen (Bervoets & Blust, 2003; McHugh *et al.*, 2013). While the former is based on the body weight and length relationship of a fish (Bervoets & Blust, 2003), the latter considers the relative size of the liver to that of the total body weight, which relates to the energetic state and metabolic activity of a fish (Pyle *et al.*, 2005).

1.4.7.3 African sharptooth catfish (*Clarias gariepinus*) as an environmental indicator

The African sharptooth catfish (*C. gariepinus*) has been extensively used as a bioindicator of pollution in various African countries including South Africa, Nigeria and Egypt (Crafford & Avenant-Oldewage, 2010; Osman & Kloas, 2010; Murtala *et al.*, 2012). This fish species has a wide distribution and is found from the Afrotropical region of the Nile to its most southern natural habitats, namely the Orange and Umtamvuna river systems in South Africa. *Clarias gariepinus* has been translocated to the Eastern and Western Cape provinces and is also found in Israel, Turkey and Lebanon (Skelton, 2001). Furthermore, *C. gariepinus* is cultivated in fish farms in Europe, Asia and Latin America (Vitule *et al.*, 2006). According to the latter authors *C. gariepinus* has also been introduced into the Guaraguaçu river basin in Parana´ State (Brazil), which poses a threat to local endemic species.

A preliminary study conducted by Van der Merwe *et al.* (1993) reported lethal Cu concentrations (LC50) for *C. gariepinus* adults at 1.29 mg/l during the summer and 1.38 mg/l during the winter. However, Cu and other mining-associated pollutants could present adverse effects at much lower concentrations. Farombi *et al.* (2007) reported a significant increase in the activity of oxidative stress related enzymes in *C. gariepinus* populations sampled from the polluted Ogun river (Nigeria) as a result of metal induced stress.

1.5 Metal pollution of water, sediment and soil

As mentioned previously, pollution poses a severe threat to the integrity of karst landscapes and the associated ecosystems. Furthermore, the interconnected nature of these landscapes facilitate the dispersion of pollutants to neighboring systems (Watson, 1997; Ford & Williams, 2007). According to Hill (2010) pollutants, such as metals, are transported by running water and may precipitate as a result of change in water flow and/or chemistry. Deposited pollutants associated with sediments also pose a threat to the ecosystem as it may be ingested by benthic organisms. It also serves as a reservoir since pollutants can become resuspended/redissolved in the water column with a change in environmental conditions (Horowitz, 1991). Furthermore, percolating water poses a threat as it may transport pollutants, especially pesticides, from the soil layers to an underlying aquifer (Hill, 2010).

The sampling and subsequent chemical analyses of cave substrates as part of an ecological study has been well documented (Simon & Buikema Jr, 1997; Graening & Brown, 2003; Wood *et al.*, 2008). However, most of these studies were focused on the identification and quantification of organic pollutants present in the collected substrates. Nevertheless, Vesper (2012) stated that a number of karst regions in the United States, southern China and other parts of the world are exposed to acid mine drainage (AMD) and subsequently metal pollution. For the purposes of this study the focus will be on metal concentrations in water, sediments and soils collected from the Wonderfontein Cave and Spruit. The latter includes the Cave Inflow Area, Wonderfontein Canal and Stoffels Dam sampling sites. While the Cave Inflow Area resembles the surface drainage area where water from the Wonderfontein Spruit drains into the Wonderfontein Cave, the Wonderfontein Canal resembles a man-made structure that runs past the Wonderfontein Cave. This canal was previously used to provide water for the irrigation of agricultural fields and to dispose of excess mine water. The third surface sampling site, Stoffels Dam, is located downstream of the Wonderfontein Cave and is subjected to water inflow from the Wonderfontein Spruit. For a full description on the respective sampling sites, please refer to Chapter 2 (section 2.3).

1.5.1 Water quality assessment

Water quality refers to the chemical, physical and biological state of water, which determines its suitability for human consumption as well as the impact it might have on the health of an ecosystem (Li & Migliaccio, 2010). When considering water quality assessments, there are various parameters (characteristics) that are typically measured. According to Li and Migliaccio (2010) chemical characteristics include the concentration of dissolved substances such as gases, nutrients and pesticides, while physical characteristics represent the colour, smell, temperature, taste and turbidity of the water. Biological characteristics represent all living organisms such as bacteria, protozoans, insects, plants and fish. Kumar (2004) stated that other physico-chemical parameters such as the pH, conductivity, dissolved O_2 and total alkalinity should also be considered. Especially relevant to this study is the dissolved concentration of metals such as lead (Pb), arsenic (As), Ni, Cu, Cr, Cd, Zn and Hg since exposure to these metals can have a toxic effect on most living organisms including humans (Sarkar, 2002; Kumar, 2004).

However, the chemical, physical and biological characteristics of water provide little information if not compared to baseline data or specific water quality guidelines. The South African Water Quality Guidelines, obtainable from the Department of Water Affairs and Forestry (http://www.dwaf.gov.za), consist of a series of eight volumes with each providing water quality standards for a specific sector (DWAF, 1996). Water quality guidelines produced by other institutions, such as the Canadian Council of Ministers of the Environment (CCME), the United States Environment Protection Agency (USEPA) and the Australia and New Zealand Environment and Conservation Council (ANZECC), can also be used. These guidelines typically provide pre-determined threshold concentrations of all the important pollutants as well as limits for various other water quality parameters. Also, aquatic ecosystem guidelines typically provide a target quality range, a chronic effect value, as well as an acute effect value for each pollutant.

It might also be necessary to determine the water type and possible source of a water sample for which Piper diagrams may be used (Piper, 1944; Hounslow, 1995; Kresic, 2006). Piper diagrams (Figure 1.3) incorporate the percentage concentrations of selected anions and cations plotted on two separate trilinear diagrams that collectively project a third quadrilateral (diamond shaped) plot (Hounslow, 1995). For determining the water type of any plotted water sample, the diamond plot is considered. According to Hounslow (1995) each corner of the diamond represents a water type: Samples plotted at the top is considered to have a permanent hardness as it is associated with high Ca^{2+} + Mg^{2+} and chloride (Cl^-) + sulphate (SO_4^{2-}) concentrations. Water samples that plot in the left corner represent temporary hardness with Ca^{2+} + Mg^{2+} and bicarbonate (HCO_3^-) concentrations being high, while those in the lower corner is characterized as alkali carbonates with high sodium (Na^+) + potassium (K^+) and HCO_3^- + carbonate (CO_3^{2-}) concentrations and those in the right corner representing saline water with high Na^+ + K^+ and Cl^- + SO_4^{2--} concentrations. Another useful diagram for characterizing water samples is the Durov plot and involves a similar plotting approach as that of the Piper diagrams (Zaporozec, 1972).

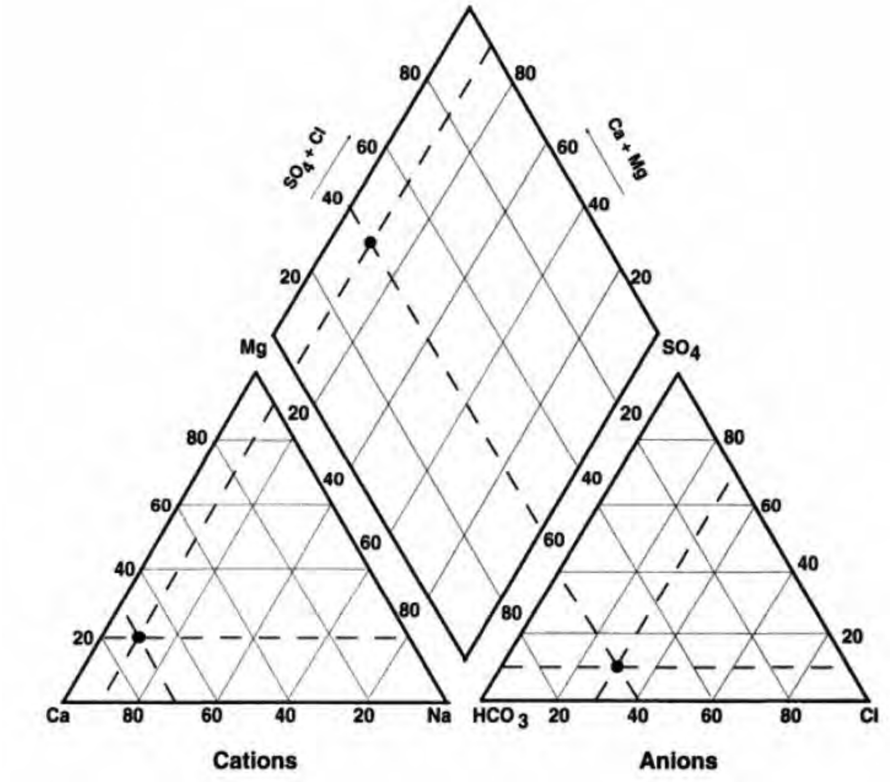

Figure 1.3: Piper diagram used to classify water samples according to the percentage concentrations of selection ions (Hounslow, 1995).

1.5.2 Sediment and soil pollution

Metals such as Co, Cu and Zn naturally occur in nature and in small quantities serve as nutrients necessary for the survival of many living organisms (Singh *et al.*, 2011; Vesper, 2012). However, anthropogenic activities sometimes result in the enrichment of some metals toxic at higher concentrations (Vesper, 2012). Karst environments are especially vulnerable to metal pollution as the higher alkalinity of karstic water, due to the nature of the bedrock, result in the precipitation of metals (Vesper, 2012).

An important factor to consider in metal pollution is the bioavailability of it that is roughly defined by Horowitz (1991) as: "…that portion of dissolved, biologically-, or sediment-associated chemical constituents that are readily accessible to biota either through physical contact or by ingestion". According to Horowitz (1991) physico-chemical parameters such as pH, dissolved O_2 and bacterial activity play a significant role in the bioavailability of metals. The particle size distribution and TOC should also be considered especially since high concentrations of the latter is capable of immobilizing metals (ANZECC, 2000; Skłodowski *et al.*, 2006; Agarwal, 2009). Unfortunately, metal chemistry remains complex and challenging to interpret (Vesper, 2012). As a result of this and constraints by limited funding, many researchers would rather analyse the total metal concentration, which can then be compared to sediment and soil quality guidelines in order to assess the possible threats posed by pollution.

As with water quality guidelines, many environmental ministries provide sediment quality guidelines (SQG) that aid researchers in determining the ecological threat posed by pollutants. MacDonald *et al.* (2000) integrated research and produced consensus-based SQG for aquatic ecosystems, with various threshold values for pollutants such as metals and pesticides included. This consensus-based SQG is very useful as it provides both a threshold effect concentration (TEC) and probable effect concentration (PEC) for each chemical of concern. Also, mean sediment quality guideline quotients (mean SQGQ) have been developed with the purpose of providing a single, easily interpretable numerical index that highlights the relative degree of pollution (Fairey *et al.*, 2001; Long *et al.*, 2006). According to Long *et al.* (2006) SQGQ have been accurately used to classify sediment samples as either toxic or non-toxic. However, users should be aware of certain limitations which are discussed in depth by Long *et al.* (2006).

Another approach to consider, especially if baseline data is not available, is the use of enrichment factors (EI) and the geoaccumulation index (I_{geo}), both of which have been extensively applied in various studies (Miko *et al.*, 1999; Loska *et al.*, 2004; Buccolieri *et al.*, 2006; Zhuang & Gao, 2014). According to Loska *et al.* (2004) the I_{geo} assesses the extent of contamination by comparing current to preindustrial metal concentrations. Similarly, EF compare current to background data and produce a standardized assessment as a reference element with low variability is incorporated; Mn, aluminum (Al), iron (Fe) and titanium (Ti) are commonly used reference elements (Sutherland, 2000; Loska *et al.*, 2004).

1.6 The Wonderfontein Spruit catchment

1.6.1 A historical perspective on its degradation

The Wonderfontein Spruit has an unprecedented history of which the earliest records depicts a landscape characterized by exuberant gardens, overgrown river banks and a diversity of wildlife (Swart *et al.*, 2003b). These are the accounts of Thomas Baines, William Cornwallis, Karl Munch and others who from 1836 till 1877 explored the catchment while documenting their findings (Baines, 1964; Mauch, 1969; Swart *et al.*, 2003b). Unfortunately, it was shortly after this that gold was discovered and large-scale mining commenced, which resulted in the progressive degradation of the landscape (Swart *et al.*, 2003a; Stoch *et al.*, 2008; Van Eeden *et al.*, 2008).

The first settlers arrived in 1838 near Potchefstroom (North-West Province). The fertile soil and a sufficient supply of water provided potential for agricultural practices. Subsequently, agricultural lands developed all along the Mooi River and Wonderfontein Spruit (Stoch *et al.*, 2008). However, the discovery of gold in the 1890's near Venterspost marked the beginning of new era. In the 1930's the first successful shaft was sunk through the dolomitic bedrock by the Ventersdorp Gold Mining Company (Van Niekerk & Van der Walt, 2006). The dewatering of the compartments commenced in the 1940's and resulted in some of the local springs to dry up. According to Swart *et al.* (2003b) the Venterspost Compartment was dewatered by the mid-1940's, the Oberholzer Compartment by the mid-1950's, the Bank Compartment by 1969 and the Gemsbokfontein West Compartment by 1986. The effect of the dewatering of these compartments was devastating. Due to the decrease in geomorphological stability, many sinkholes were formed, which led to the death of 38 people and infrastructure damage accounting for millions of South African Rands (ZAR) (Ford & Williams, 2007).

1.6.2 The polluted Wonderfontein Spruit

The Wonderfontein Spruit originates from the Tudor Dam in Krugersdorp. From here it flows through a series of water systems including the Lancaster, Cooke Attenuation and Donaldson Dams. From the Donaldson Dam the water of the Wonderfontein Spruit is diverted into a 1 m diameter pipeline with a total length of 32.5 km, which spills directly north of Carletonville (Opperman, 2008). Along the extent of the Wonderfontein Spruit, polluted water is dumped into the river system by decanting shafts, runoff from tailings dams as well as runoff from informal settlements. For more than a hundred years, the Wonderfontein Spruit has been exposed to metals and other mining associated pollutants (Hamman & Van Rensburg, 2012). These pollutants are generally sourced from tailings, waste rock dumps, underground mines and open pits (Ritcey, 2005). It is considered that AMD, also known as acid rock drainage (ARD), is the main contributor to the deterioration of the water quality and ecological state of the Wonderfontein Spruit and associated environment, respectively (Opperman, 2008).

1.6.3 The Wonderfontein Cave: A karst system exposed to the influx of polluted water

Since the initial exploration of the Wonderfontein Cave it has been exposed to the influx of water from the Wonderfontein Spruit. Early records dating back to 1909 indicate that within the Wonderfontein Cave water flowed down the "River Passage" and drained in the most northern section (Kent *et al.*, 1978). According to the latter authors the eastern passages, which branched from the main river passage, were also flooded. In addition, the north-eastern section of the cave also contained water of which levels would fluctuate during flooding events. In 1969 the mining company Goldfields South Africa Ltd. built a weir that diverged water from a concrete canal into the cave. This was undertaken in an effort to dispose of water pumped from the Carletonville mines. The mine water left a residual line on the walls and speleothems of the cave. This line was used as a datum to calculate the elevations of the system (Kent *et al.*, 1978). Today, the function of the weir has been revoked as it is overflowing. Also, initial investigations revealed that no sinking stream was associated with the Wonderfontein Cave, but rather water that drained through the dolomitic bedrock.

Recent exploration of the Wonderfontein Cave system has revealed that it remains flooded. Although dependant on the season, it is estimated that 90 % of the cave system is underwater while some areas of the cave remain inaccessible to humans. The probable cause for the flooding is likely the change in the morphology of the surface water body as well as the lack of water management. Some areas within the cave remain dry as they are elevated above the cave development level. Although subterranean rivers are common in karst landscapes and may even host endemic animal species, an influx of polluted water such as that from the Wonderfontein Spruit could result in severe ecosystem degradation.

1.7 References

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CHAPTER 2

SITE DESCRIPTION

The Wonderfontein Cave is located north of central Carletonville (Gauteng Province, South Africa) and is associated with the river banks of the Wonderfontein Spruit. The latter extends through the Far West Rand, which forms part of the Witwatersrand Basin (Figure 2.1) (Coetzee *et al.*, 2006). This site represented the main study area where nematode (Phylum Nematoda; representing invertebrates) and fish (Phylum Chordata; representing vertebrates) were sampled and investigated.

2.1 A general description of the Wonderfontein Spruit

According to Coetzee *et al.* (2006) the Wonderfontein catchment, which hosts the Wonderfontein Cave and Spruit, extends over both the West Rand (upper region) and the Far West Rand (lower region) (Figure 2.1) goldfields of the Witwatersrand Basin. According to Swart *et al.* (2003a) the rock formations of the Far West Rand belong to the Transvaal Supergroup. Dolomitic bedrock associated with the latter was formed early during the Proterozoic eon and belongs to the Malmani Subgroup that forms part of the greater Chuniespoort Group. The Malmani Subgroup is further divided into different dolomitic formations (Table 2.1) that vary in thickness and mineral composition. Although the dolomitic bedrock is in total 1 500 m thick, only the upper layers (100 – 200 m section) have been exposed to intense physical and chemical weathering (Coetzee *et al.*, 2006). It is thus not unusual for this karst landscape to host a great number of cave systems. Also, the five most extensive South African systems (Table 2.2), measured by combining the length of all the surveyed passages, are all found within this landscape (Swart *et al.*, 2003b).

The total passage length of Apocalypse and Wonderfontein Caves exceeds more than 12 and nine kilometres, respectively. It is thus evident that the Malmani dolomites have been subjected to some considerable weathering and are likely to be highly interconnected. The Wonderfontein catchment has great potential for storing substantial amounts of groundwater and is divided into different groundwater compartments by impermeable dolerite dykes (Van Eeden *et al.*, 2008). Although many springs were associated with this landscape, most dried up as mining activities dewatered the Oberholzer, Bank and Venterspost compartments (Figure 2.1).

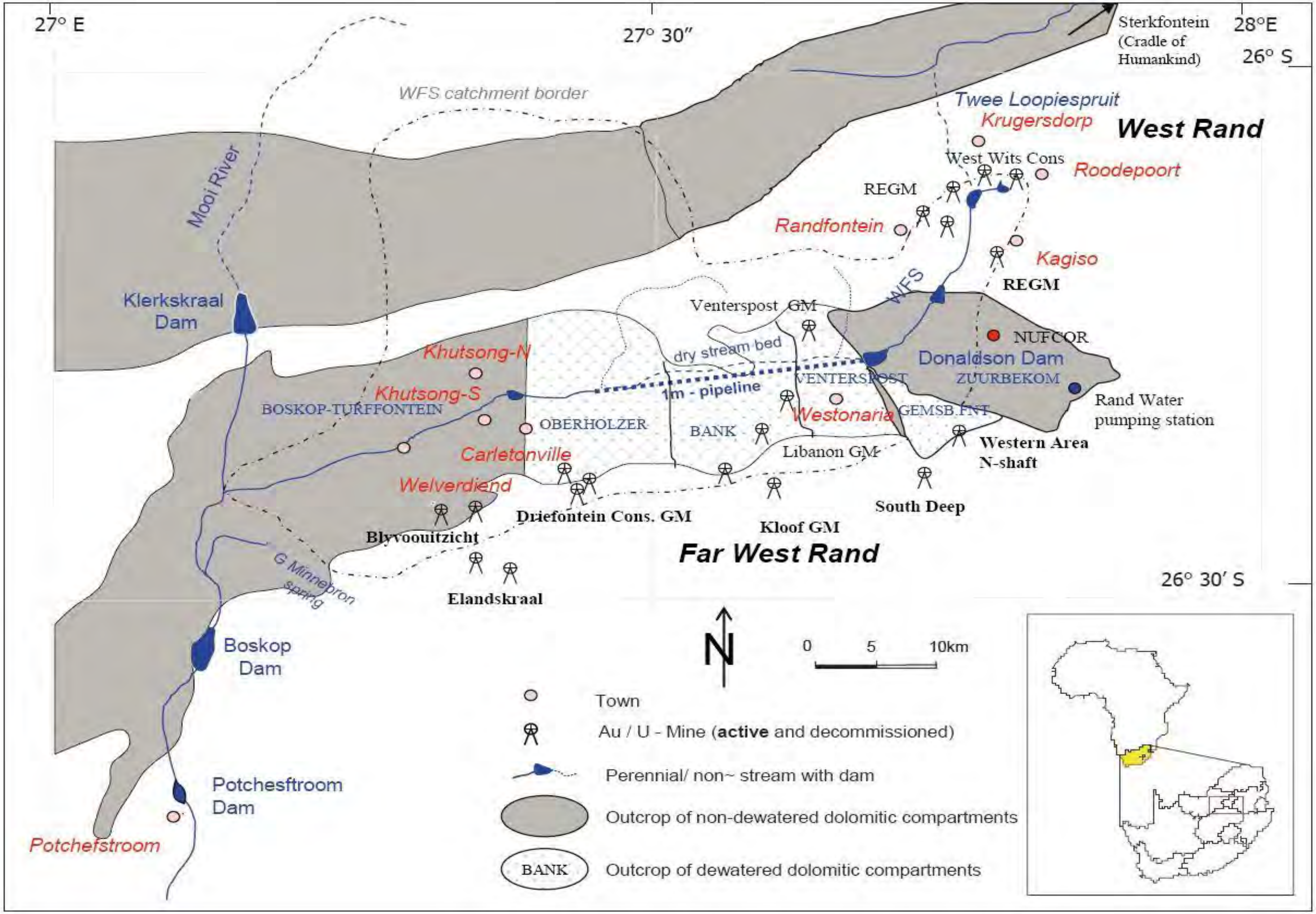

Figure 2.1: Map of the Far West Rand (Witwatersrand Basin) with associated water compartments, mines and the Wonderfontein Spruit indicated (Winde & Stoch, 2010).

Table 2.1: Lithostratigraphic composition of the Malmani dolomites (Transvaal Supergroup) with general characteristics listed (Brink, 1979; Wolmarans, 1986).

			Formation	Thickness (m)	Description
Transvaal Supergroup	Chuniespoort Group	Malmani* Subgroup	Eccles	380	Dark-coloured dolomite, chert-rich
			Lyttleton	150	Dark-coloured dolomite, chert-free
			Upper Monte Christo	258	Chert-rich dolomite
			Middle Monte Christo	162	Chert-poor dolomite
			Lower Monte Christo	275	Chert-rich dolomite
			Oaktree	200	Chert-poor dolomite with carbon-rich shales

*Total dolomite bedrock thickness is between 1200 and 1450 m

Table 2.2: Location, length and depth of the five most extensive South African dolomitic cave systems; modified from Swart *et al.* (2003b).

Cave	Location	Combined passage length (km)	Maximum depth (m)
Apocalypse	13 km north-west of Carletonville (Wonderfontein catchment)	12.4	85
Wonderfontein	6 km north-north-west of Carletonville (Wonderfontein catchment)	9.4	15
Crystal	25 km west-south-west of Carletonville (Wonderfontein catchment)	8.05	85
Chaos	25 km west-south-west of Carletonville (Wonderfontein catchment)	6.1	112
West Driefontein	7 km south-west of Carletonville (Wonderfontein catchment)	5.7	185

2.2 A geological description of the Wonderfontein Cave

The Wonderfontein Cave is located 4.5 km downstream of the pipeline, 3.5 km west of Khutsong (an informal settlement located on the banks of the Wonderfontein Spruit) and 6 km north of central Carletonville. Figure 2.2 illustrates that the cave inflow area (26°19'1.78"S; 27°21'44.57"E), where water from the Wonderfontein Spruit drains into the cave system, is located on the northern bank of the Spruit. From this point the cave extends in a north-westerly direction at a general development level of 20 m below surface.

Kent *et al.* (1978) provided a comprehensive description on the geology and general structure of the cave system. The latter authors stated that the cave developed in a 25 m dolomitic sequence that forms part of the uppermost section of the Monte Christo Formation (Table 2.1). This sequence consists of the following layers (from top to bottom):

- 10 m chert-free dolomite,
- 1-1,5 m dolomite and chert breccia in matrix with black shale,
- 5 m dolomite with spongy chert,
- 0,3 m massive chert,
- 10 m chert-free dolomite.

The cave passage network presents an interesting maze structure unique to the landscape (Figure 2.2). The two principal directions of development, respectively 5° and 105°, is thought to be the result of a regional tensional fracture system, which caused the cave system to develop with a grid-like structure. The cave system thus consists of a series of interconnected passages that vary in size (Kavalieris & Martini, 1976; Kent *et al.*, 1978). According to the latter authors the cave system has four shaft entrances, many chimneys, as well as a slope entrance and extends below 8.9 hectares of surface area.

As was stated in Chapter 1, the flow of water entering the cave from the Wonderfontein Spruit was previously controlled by a weir built in the 1969 near the Cave Inflow Area. However, Figure 2.3 illustrates that this weir is non-functional. Satellite imagery reveals that while the weir reserved some function in 2004, it was overflowing by 2013. Also visible in Figure 2.3 is the canal that once provided agricultural lands with water and even this is overgrown and overflowing. Due to the lack of adequate management the water from the Wonderfontein Spruit thus freely drains into and floods the Wonderfontein Cave system. Preliminary investigations revealed that some areas within the cave remain dry, which have likely never before exposed to the influx of water from the Wonderfontein Spruit.

Figure 2.2: A survey map of the Wonderfontein Cave system plotted over a satellite image of the area where it is situated, illustrating the extent of the cave system and associated Wonderfontein Cave inflow area (Photo: Google Earth, available at https://www.google.com/earth).

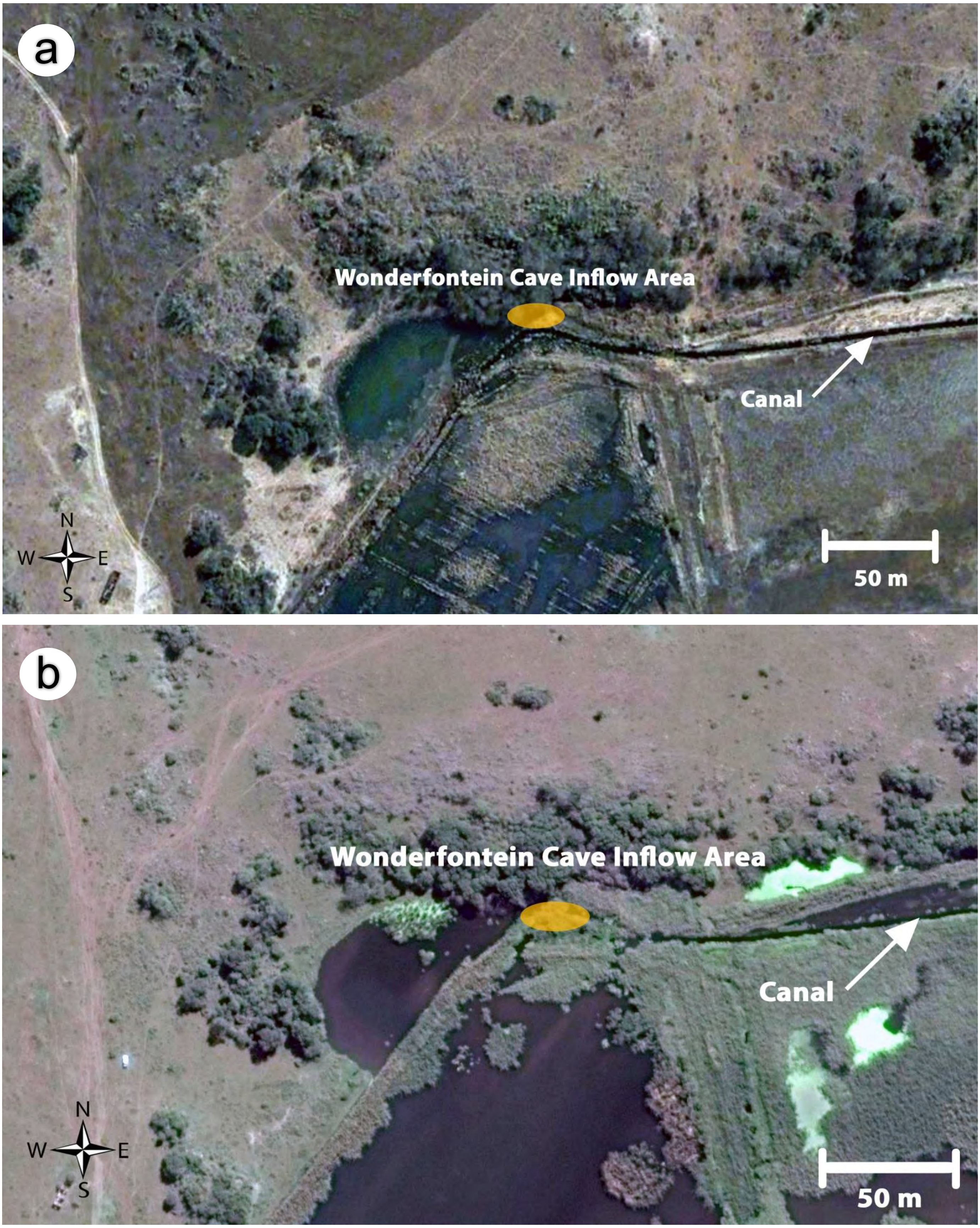

Figure 2.3: Satellite images of the canal associated with the Wonderfontein Spruit (Cave Inflow Area area) in (a) 2004 when it reserved some function and in (b) 2013 when it was overgrown and water freely flowed into the adjacent swampland. Furthermore, water drains into the karst landscape at an unknown rate, subsequently flooding the Wonderfontein Cave system (Photos: Google Earth, available at https://www.google.com/earth).

2.3 Sampling intervals, site locations and substrates collected

Subterranean and surface sites where selected for this study. The former were located throughout the extent of the Wonderfontein Cave, while the latter included three sampling areas namely the Cave Inflow Area, Wonderfontein Canal and Stoffels Dam, all of which are directly/indirectly associated with the Wonderfontein Spruit. Not all of the sampling methods and analyses performed in this study were applied at each site as the objectives of this study, in some cases, remained site specific.

Sampling was undertaken in 2013 during the months of April and September (Table 2.3), which represented the end of the high and low flow periods, respectively. During the 1st (April) and 2nd (September) sampling intervals, both the cave and surface environments were sampled. Substances sampled for nematode and chemical analyses included soil (So), sediment (Sed), bat guano (Gu) and water (W). *Clarias gariepinus* specimens were collected from the cave environment during both sampling intervals, but from the surface environment (Stoffels Dam), only during the 2nd sampling interval.

2.3.1 Description of subterranean sites associated with the Wonderfontein Cave

This section will focus on the description of the features associated with the individual sampling sites. Throughout the extent of the Wonderfontein Cave system (Figure 2.4) a total of seven sampling sites were selected for investigations. The selection of sites represent most, if not all of the habitat types inhabited by nematodes and/or fish found within the Wonderfontein Cave and include both anthropogenically influenced and natural, pristine environments.

2.3.1.1 Kent’s Entrance

Kent’s Entrance (Figure 2.4) is located nearest to the inflow area and within the main river passage that transports water into the cave. Furthermore, this site is exposed to rainfall/runoff water, invertebrates and organic matter that enter from the surface through a vertical shaft. A heap of soil mixed with organic matter is located directly below the shaft entrance. Sediment can also be found in the vicinity of the heap. Water and sediment was sampled in the vicinity of the heap and soil directly from it.

Table 2.3: List of site names and coordinate positions associated with the Wonderfontein Cave (overlying surface position) and Spruit environments as well as the type of substrates collected from each site.

Site	Overlying surface point	1st Interval (04/2013)	2nd Interval (09/2013)
Wonderfontein Cave (subterranean) environment			
Kent's Entrance	-26.316426° 27.361925°	1, 2, 3*	1, 2, 3
Keyhole	-26.316026° 27.360364°	2, 3	2, 3
Main Entrance	-26.315052° 27.361677°	1, 2, 3, 4	1, 2, 3
Fault Passage	-26.314678° 27.361076°	-	1, 2, 3
Pristine Chamber	-26.313904° 27.361758°	1, 2, 3, 4	1, 2, 3, 4
Elevation Pit	-26.314468° 27.362032°	1, 2, 3, 4	1, 2, 3, 4
North-Eastern Section	-26.314146° 27.362458	1, 2, 3	1, 2, 3
Derek's Exit	-26.316280° 27.360232°	1, 2, 3	1
Surface environment			
Cave Inflow Area	-26.317124° 27.362366°	1, 2, 3	1, 2, 3
Stoffels Dam	-26.368762 27.257331°	-	1, 2, 3
Wonderfontein Canal	-26.314514° 27.368811°	-	1, 2, 3

*1 = Soil; 2 = Sediment; 3 = Water; 4 = Guano

2.3.1.2 Keyhole

The Keyhole site (Figure 2.4) is found further down the main river passage towards the most eastern section of the cave and is characterized by an open area with boulders underlying a keyhole entrance. The latter is a vertical shaft that, as with Kent's Entrance, permits rainfall/runoff water, invertebrates and organic matter to enter from the surface. Water and sediment were collected in the vicinity of the boulders.

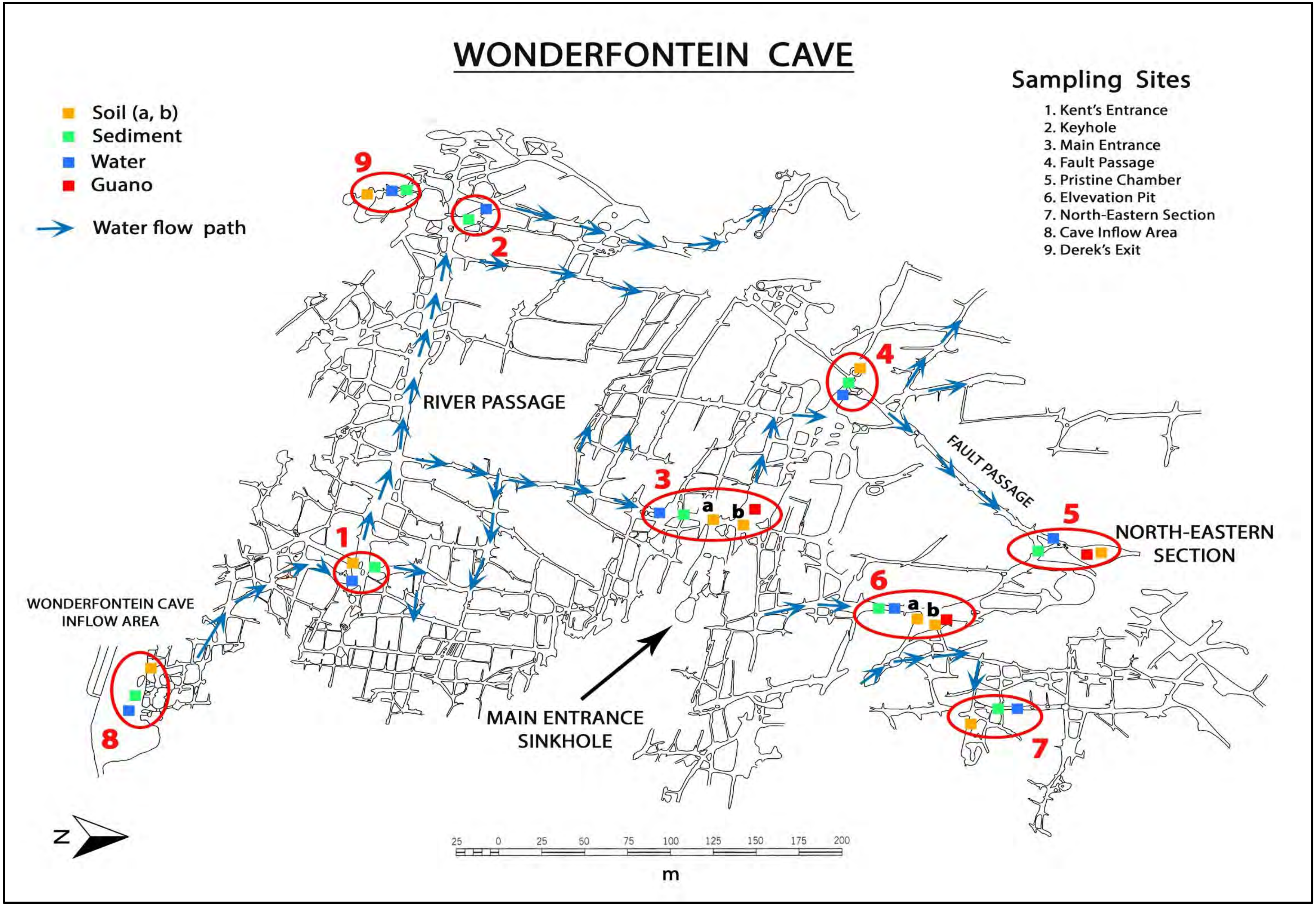

Figure 2.4: Surveyed map of the Wonderfontein Cave system with subterranean and Cave Inflow Area (surface) sampling sites indicted. Also, the general water flow path is illustrated as blue arrows. Main cave features, as well as the sampling location of each substrate type collected per site, are also indicated; modified by from Kent *et al.* (1978).

2.3.1.3 Main Entrance

The Main Entrance (Figure 2.4) is an extensive site reached when entering via the main entrance sinkhole. Typically, surface runoff water can reach this area by flowing down the sinkhole and into the main entrance area. At the southern end of this site water and sediment samples were collected, while a soil sample was collected from the main entrance opening. An extension of this site is a side chamber located directly north of the main entrance area. It is linked to the latter via a horizontal slot that requires a caver to crawl in order to pass it. Within the side chamber a heap of dry red soil and minimal amounts of guano can be found. Samples of both the latter substances were collected.

2.3.1.4 Fault Passage

A fault line, extending through the dolomitic bedrock, allowed the development of a 100 m passage (Figure 2.4). The latter is joined at its south-eastern end with the central section of the cave. This joint is an open area associated with boulders, a pool of water and infiltrating red soil. The site is referred to as Fault Passage from which soil, sediment and water samples were collected.

2.3.1.5 Pristine Chamber

This chamber is located (Figure 2.4) at the end of the fault line passage that extends in a north-easterly direction. Although preliminary observations revealed that this site is well elevated above the water level, water and sediment samples were collected at the entrance of the chamber, which remains at a lower level. Furthermore, this chamber hosts a characteristically different environment from the rest of the cave system as red soil with beds of fresh guano and spider webs are associated with the floor. Both soil and guano samples were collected.

2.3.1.6 Elevation Pit

This site (Figure 2.4) is an extensive area that features a soil heap with guano deposits, water and sediment most likely associated with the main river passage and soil from the cave floor in which seedlings, transported by runoff water from the surface, germinate. Thus, some areas such as the cave floor are exposed to surface runoff, which transports organic matter into the cave system, most likely via a sinkhole entrance located not more than 20 m away. Also, since the soil and guano found on the heap were dry and elevated above the water level, it is unlikely that water from the Wonderfontein Spruit ever reaches this area. Soil and guano samples were collected from the heap as well as a soil sample containing seedlings, a water sample and a sediment sample.

2.3.1.7 North-Eastern Section

The North-Eastern Section (Figure 2.4) is a small network of passages connected to the rest of the system by only a single known passage. However, there is the possibility that other undiscovered connections exist. Although this section is isolated to an unknown degree, organic matter flushed in from the surface can be found in this section, while most of it remains flooded. Accounts from Kent *et al.* (1978) revealed that water levels within the cave system may fluctuate as a result of either an increased influx of water from the Wonderfontein Spruit or heavy rainfall. As no guano could be found in this section, only water, sediment and soil samples were collected. It should be noted that many of the flooded passages are associated with a thick layer of sludge, which is anticipated to be the result of decomposing organic matter.

2.3.1.8 Derek’s Exit

Derek’s Exit (Figure 2.4) sampling site is located towards the western end of the cave system and directly south of the Keyhole site. However, according to the survey map created by Kent *et al.* (1978), Derek’s Exit is elevated 10 m above the general cave development level of 20 m below surface. Since the passages associated with this section is very small, entry was gained via another surface entrance. However, the connection indicated on the survey map could not be confirmed and neither could the source of water associated with this sampling site be established. During the 1st sampling interval water, sediment and soil samples were collected, however, during the 2nd sampling interval all the water had dried up, thus, only soil samples were collected.

2.3.2 Description of surface sites associated with the Wonderfontein Spruit

Surface sites, both of natural and anthropogenic origins, were also selected for investigations. This allowed the comparison of the extent of pollution as well as ecosystem health and toxicity hazard potential of water, soils and sediments between the subterranean and surface environments. Also, the degree of divergence between the *C. gariepinus* populations associated the Wonderfontein Cave and Spruit was determined.

In total three surface sites (Table 2.3) were selected. All of these, as illustrated by Figures 2.5 and 2.6, are directly associated with the Wonderfontein Spruit and include the Cave Inflow Area (located 5.4 km downstream of the Pipeline Spilling Point), Stoffels Dam (located 13.5 km downstream of the Cave Inflow Area) as well as the Wonderfontein Canal (located less than one kilometre upstream of the Cave Inflow Point).

2.3.2.1 Cave Inflow Area

Water reaches the Wonderfontein Cave from the Wonderfontein Spruit through a drainage area referred to as the Cave Inflow Area (Figures 2.5, 2.6 and 7 a). Although there is no visible sinking stream, it was observed that the surface water drains slowly towards the development direction of the cave. Associated with this sampling site are dolomitic rocks covered by thick reeds and other vegetation. Sediment and water samples were collected by climbing down between the rocks in order to reach the water level, while soil was collected from the nearest river bank.

2.3.2.2 Stoffels Dam

Directly north of Welverdiend (Carletonville-area, North-West Province) the Wonderfontein Spruit flows into Stoffels Dam (Figures 2.5 and 2.7 b). Warning signs under the authority of the Department of Water Affairs and Sanitation (DWAS) indicate that the water is polluted and not suitable for human and/or livestock consumption. This was the only surface site where *C. gariepinus* specimens were found. Although attempts were made to sample specimens closer to the Cave Inflow Area from both the canal and associated wetlands, it was without success. The banks of Stoffels Dam are overgrown with many thick reeds, nonetheless, an open section on the southern bank allowed entry. Sampling of soil, sediment and water, as well as *C. gariepinus* specimens representing the surface population, was undertaken at this sampling site.

Figure 2.5: The location of the Pipeline Spilling Point in relation to the Cave Inflow Area and Stoffels Dam sampling sites (Photo: Google Earth, available at https://www.google.com/earth).

Figure 2.6: The Cave Inflow Area and Wonderfontein Canal surface sampling sites with the sampling location of each substrate type indicated (Photo: Google Earth, available at https://www.google.com/earth).

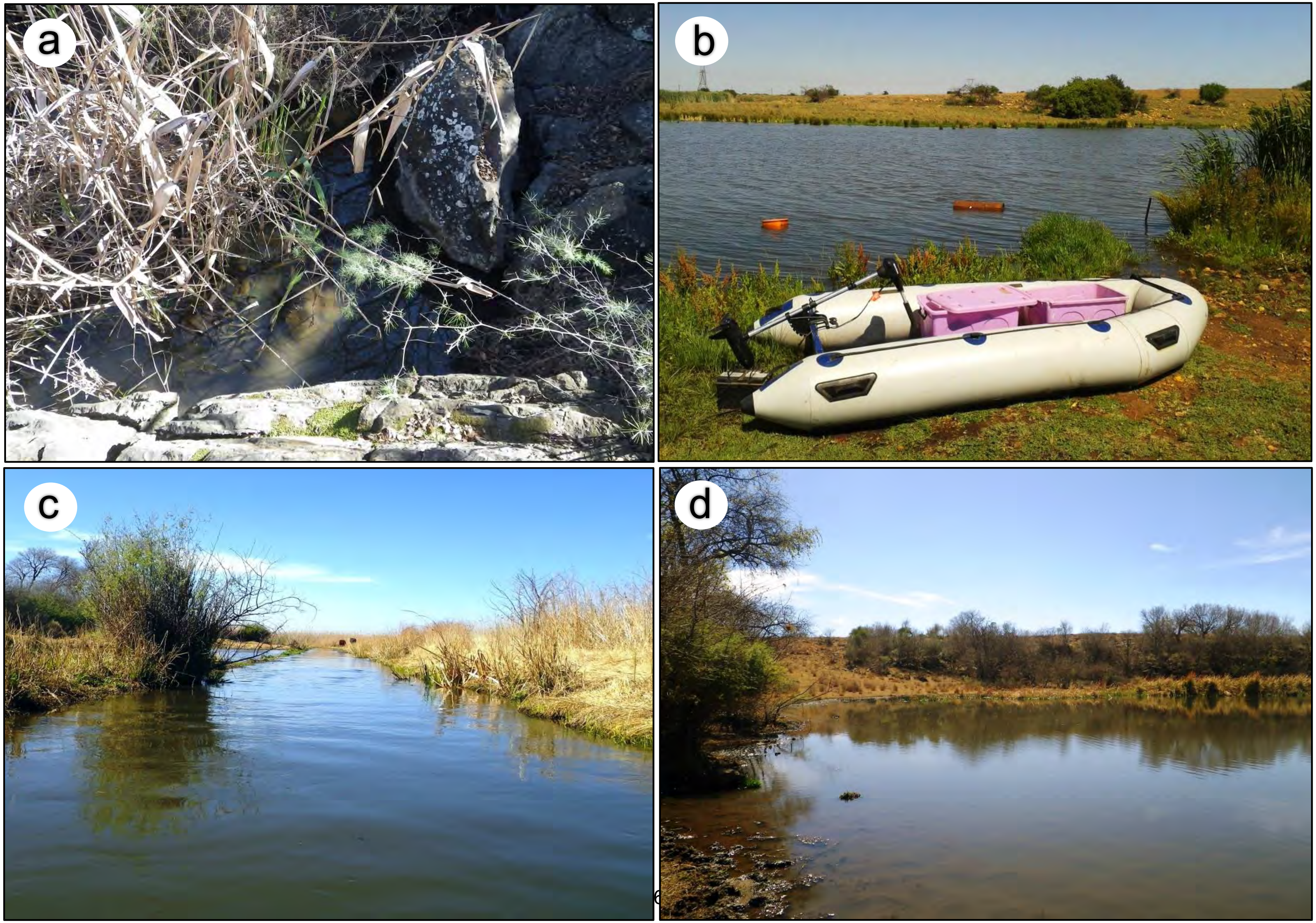

Figure 2.7: Picture plate of the surface sampling sites: (a) Cave Inflow Area; (b) Stoffels Dam; (c) Wonderfontein Canal; (d) water pool adjacent to Wonderfontein Canal (Photos: Gerhard du Preez, NWU).

2.3.2.3 Wonderfontein Canal

Although the canal (Figures 2.6 and 2.7 c) was originally created to provide water for agricultural activities, all management of it has ceased. The canal is mostly underwater (Figure 2.7d) and only visible due to the outline created by the reeds. As a result, the overflowing water creates water pools (Figures 2.7 d) that likely resemble the original landscape before the water was canalised. Water was sampled directly from the canal, while soil and sediment samples were collected from the river bank and adjacent pool, respectively.

MATERIAL AND METHODS

2.4 Collection of substrate samples

From both the subterranean and surface sites substrate samples were collected that were used for nematode extractions and physico-chemical analyses. Water samples were collected according to the protocols provided by the USEPA (Forrest, 2000). A sterilized 200 ml plastic container was rinsed in the respective site's water and sealed with a lid. The container was submerged 30 cm below surface, the lid removed and while the opening faced upstream it was allowed to fill until all the air was replaced. While still being submerged, the lid was replaced and the container sealed. Contamination by re-suspended sediments was avoided by sampling upstream of the sampler's wading path.

Due to the inaccessibility of especially the subterranean sites as well as the quantity of materials that was necessary, composite (soil, sediment and guano) rather than discrete samples were collected. Soil and guano samples were collected by making use of a soil plastic probe (25 x 3.5 cm) (Figure 2.8 a). The probe was drilled into the substrate by means of mechanical action after which it was removed and the substrate scraped into a labelled, plastic zip-lock bag (18 x 32 cm). After each sample was collected, the probe was rinsed in order to avoid the contamination of other samples. From each site, 10 sub-samples were collected in order to create one composite sample. Sediments were also collected using a sterilized glass jar (Figure 2.8 b). Ten sediment sub-samples that were probed 7 cm deep were obtained at each site where sediment occurred. Again, the sub-samples were combined in a plastic zip-lock bag creating a single composite sample. During the 1st sampling interval, one composite sample of each substrate was collected per site. However, during the 2nd sampling interval three composite samples were collected of each substrate per site to allow for statistical analyses. All the samples were clearly marked, stored in their respective containers and transported to the EcoRehab facility at the North-West University (NWU) for nematode and chemical analyses.

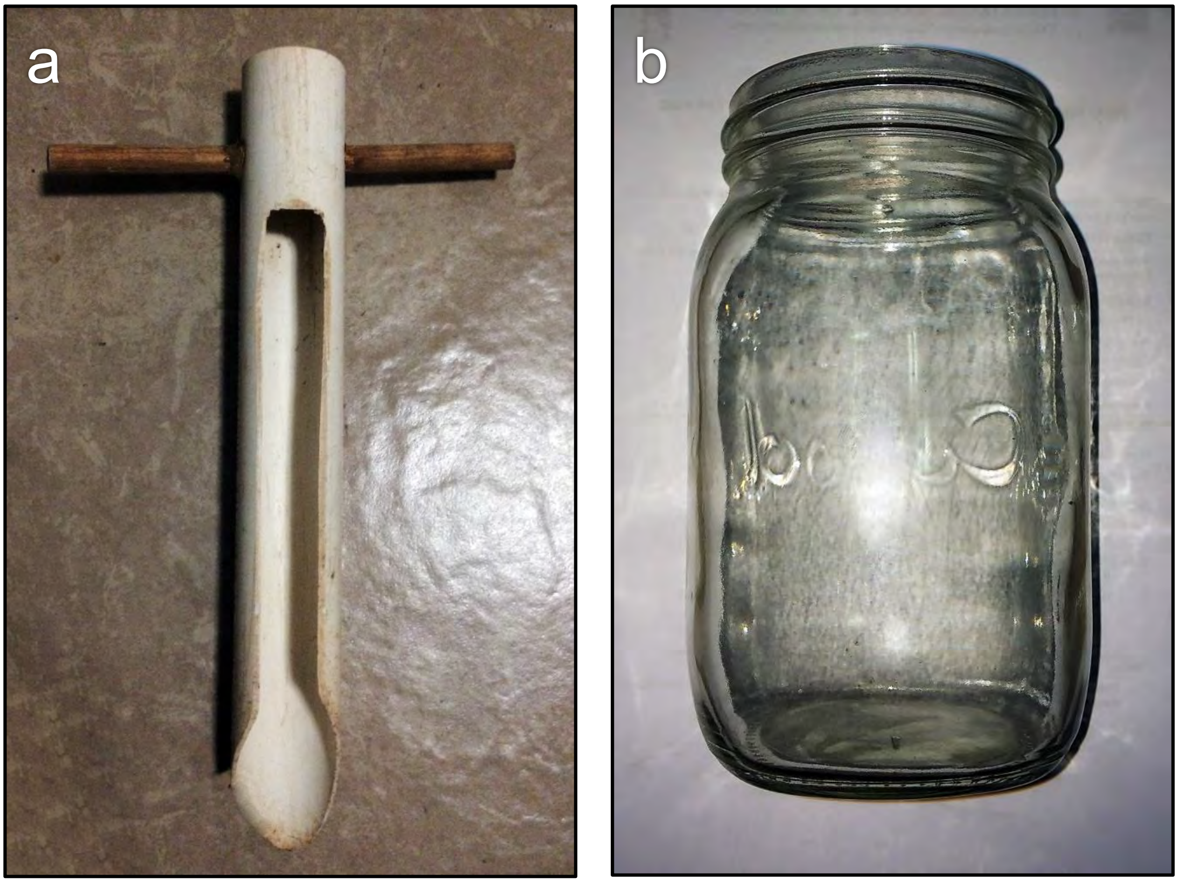

Figure 2.8: Substrate sampling equipment that includes a (a) soil probe and (b) sediment jar that were sterilized before use.

2.5 Extraction, counting and fixating of nematodes

Nematodes were extracted by using different methods according to the type of substrate sampled (Table 2.4). Preliminary investigations revealed that nematodes are best extracted from water by using the sieve-rinsing method (Hodda & Eyualem-Abebe, 2006) and from soil by using the decanting and sieving followed by sugar centrifugal flotation method (Hooper *et al.*, 2005). From guano samples, nematodes were extracted using the modified Baermann funnel method (Hooper *et al.*, 2005) and from sediments using the decanting and sieving followed by sugar centrifugal flotation method (Hooper *et al.*, 2005). After the nematodes were extracted from each of the water and composite soil, guano and sediment samples the organisms were kept in tap water in a cold room (5 ºC) until identification, counting and fixation could continue. It should be noted that in order to avoid the disintegration of the nematodes, they were fixated in formalin (4 %) if processing extended more than three weeks after sampling (Bridge & Starr, 2007).

Table 2.4: Nematode extraction methods applied for the different substrate samples collected.

METHODS APPLIED	Water	Guano	Soil	Sediment
Sieve-rinsing	√			
Modified Baermann		√		
Decanting and sieving followed by sugar centrifugal flotation			√	√

2.5.1 Water samples: Sieve-rinsing method

Water was the only substrate from which the nematodes were extracted *in situ*. Ten litres of water (5 x 2 l buckets) were poured through a set of two sieves: A 75 µm mesh sieve was placed on top in order to trap unwanted particles and a 20 µm mesh sieve below to collect the nematodes. A squeeze bottle that contained tap water was used to flush the nematodes into a 50 ml Falcon tube. Care was taken to scoop water upstream of the wading path of the sampler in order to avoid sampling nematodes re-suspended from sediments. The Falcon tubes containing the nematodes were transported on ice in a cool box to the EcoRehab facility at the NWU for further extractions. At the laboratories the same set of sieves was used to remove any remaining unwanted particles.

2.5.2 Guano samples: Modified Baermann method

Since guano has a high organic content, it proved difficult to extract nematodes from it by using the decanting and sieving followed by sugar centrifugal flotation method. For this reason the modified Baermann method (Hooper *et al.*, 2005) was applied in order to acquire maximum extraction (Swart, 2010a). The latter method is based on the mobility of nematodes and as a result only mobile nematodes can be extracted this way. The procedure of the modified Baermann method was applied as follows:

a) Using a decimal scale, a 100 g representative portion of guano was weighed and placed in a labelled container.

b) A sieve with a 750 µm mesh size was placed inside the labelled tray with an approximate dimension of 60 x 30 x 15 cm.

c) The sieve was carefully lined with a single layer of paper towel and the guano sample gently poured onto the paper towel. Care was taken to ensure that none of the guano came in direct contact with the mesh.

d) Tap water (2 l) was slowly poured into the tray while avoiding direct water contact with the guano.

e) The setup was left undisturbed for 72 h. During this time nematodes present in the guano migrated through the paper towel into the water.

f) After the 72 h waiting period expired, the sieve was carefully removed with only the water containing the nematodes remaining in the tray.

a) The water containing the nematodes was poured through a 75 and an underlying 25 µm mesh sieves. The first sieve served as a barrier for impurities, while the second sieve trapped the nematodes. The nematodes were flushed into a 100 ml labelled bottle and stored at 5 °C in a cold room until counting and identifications were completed.

g) Nematodes were extracted four times from each of the guano samples to allow for statistically-related data to be obtained.

2.5.3 Soil and sediment samples: Decanting and sieving followed by sugar centrifugal flotation method

All soil and sediment samples were extracted by applying the decanting and sieving followed by sugar centrifugal flotation method (Hooper *et al.*, 2005). According to the latter authors flotation methods are regarded as some of the most efficient methods to extract nematodes from various substrates. Flotation methods don’t depend on the mobility of the nematodes, but rather on their specific gravity. Furthermore, flotation methods also allow for immobile, dead and fixated nematodes to be extracted. Principally, flotation methods extract nematodes from soil and organic matter by suspending them in a solution with a greater specific gravity than their own. The procedure of the decanting and sieving followed by sugar centrifugal flotation method was applied as follows:

a) Using a decimal scale, a 100 g representative portion of substrate (soil and sediment) was weighed and washed through a 750 µm mesh sieve into a 5 l bucket. The contents of the sieve were discarded.

b) The bucket was filled to the brim with tap water, the mixture mechanically stirred and left for 30 s. During the waiting period, unwanted sediments precipitated out of the mixture while the nematodes stayed in suspension.

c) The supernatant containing the nematodes was decanted through a 25 µm mesh sieve, trapping the nematodes on the sieve. This process was repeated to ensure that most of the nematodes were extracted.

d) The retained materials were collected in 50 ml centrifuge tubes, filled with tap water and thoroughly mixed using a spoon, which was rinsed between samples to avoid cross contamination. The tubes were subsequently spun at 1800 g for 5 min using a Hettich Rotofix 32 A (Beverly, United States) centrifuge. Since nematodes have a greater specific gravity than water, they accumulated at the bottom of the tubes after the 5 min centrifuge procedure.

e) The supernatant was decanted and replaced by a sucrose solution (specific gravity of 1.15 g/cm^3), mixed and spun at 1 800 g for 1 min using the same centrifuge. The sucrose solution has a greater specific gravity than nematodes, thus the nematodes remained in the supernatant during the latter procedure.

b) The supernatant was poured through a 75 µm mesh sieve that was nested on a 25 µm mesh sieve. The top sieve served to trap unwanted, debris particles and the bottom sieve the nematodes. The nematodes were flushed from the bottom sieve into a 100 ml labelled bottle and stored at 5 ºC in a cold room until counting and identification proceeded.

f) Nematodes were extracted four times from each of the substrate samples to allow for statistically-related data to be obtained.

2.5.4 Counting, fixation and mounting of nematodes on permanent slides

Nematodes that were extracted from the samples obtained during the two sampling intervals were counted using a De Grisse counting dish (Figure 2.9 a) (De Grisse, 1963) under a stereo microscope with up to 100 x magnification. Initially, morpho species were identified and counted after which the nematodes were fixated and mounted on permanent slides for morphological identification.

Nematodes were fixated by making use of an adapted method that consists of two main procedures (Swart, 2010b), namely: (1) the initial fixation and (2) dehydration processes. The following solutions were required for nematode-fixation procedures:

FPG fixative: 100 ml of a 40 % formalin solution

10 ml propionic acid

890 ml distilled water

Picric acid (Only enough to colour the solution citrus yellow)

Solution 1: 200 ml of a 95 % ethanol solution

10 ml glycerine

790 ml distilled water

Solution 2: 950 ml of a 95 % ethanol solution

50 ml glycerine

Initial fixation process:

a) Nematodes were caught using a small, modified dermatologist's needle (Figure 2.9 b) and placed in a Syracuse dish. Next, most of the water in the dish was drawn off using a glass pipette.

b) Formaldehyde propionic acid-water (FPG) fixative was poured into a test tube and the latter placed in a glass beaker. Hot water was added to the glass beaker and a thermostat inserted into the fixative solution.

c) Once the temperature reading on the thermostat that was immersed in the fixative solution reached 65 ºC, the test tube was removed and the FPG fixative added to the Syracuse dish with a glass pipette.

d) The Syracuse dish was then placed in a glass Petri dish, which was covered and placed in a desiccator that contained approximately 100 ml of the FPG fixative solution at the bottom instead of silica.

e) The desiccator containing the Petri dish with nematodes was then placed in an incubator (38-40 ºC) for a minimum of 3 days, but on average for 2 weeks.

Dehydration process:

f) The Syracuse dish that contained the nematodes was removed from the desiccator, half of the FPG fixative solution drawn off and enough of Solution 1 added to suspend the nematodes in it.

g) The nematodes that were contained in the Syracuse dish was then returned to the desiccator with an atmosphere of 95 % ethanol and placed back in the incubator for 24 h. The Syracuse dish was, however, not covered with a lid during this step of the procedure.

h) After 24 h, half of the Solution 1-mixture was drawn off from the nematodes in the Syracuse dish and enough of the Solution 2 added to suspend the nematodes in it.

i) The Syracuse dish with the nematodes was this time partially covered with a lid to allow slow evaporation and placed back in the incubator, however, not inside a desiccator.

j) After most of the ethanol has evaporated from the Syracuse dish that contained the nematodes, 2-3 drops of glycerine was added and the Syracuse dish placed in a desiccator containing silica at the bottom.

k) After another 24 h passed for the dehydration process to finish, the mounting of the nematodes was undertaken.

Regarding the fixation and dehydration of the nematodes, the following were considered throughout this study:

- When the different fixation solutions were drawn off from the nematodes that were contained in the Syracuse dish, it was done using a microscope to ensure that no nematodes were lost.
- It was ensured that the oven did not overheat, especially when sealing the lid of the desiccator with wax/petroleum jelly since the nematodes would have been distorted.
- Special care was taken not to spill the FPG fixative solution during the processes it was used in since it contains formalin, which is a carcinogenic substance, as well as a toxic staining agent, namely Picric acid.

Permanent slides (Figure 2.9 c) were prepared by using a copper tube, paraffin wax and clean microscope slides. The open end of the copper tube was preheated with a paraffin lamp, briefly inserted into the wax and then firmly pressed at a 90º angle against the slide until a wax ring was created. A drop of glycerine was placed in the centre of the wax ring, the nematodes fished from the Syracuse dish and carefully placed in the centre of the glycerine drop by using the modified fishing needle. A cover glass was placed over the glycerine drop and the slide heated on a hot plate over a lower heat setting. As soon as the wax melted, the slide was removed and the edges of the glass cover sealed with colourless acrylic nail hardener (cutex) to enclose and preserve the nematode specimens to be identified.

This method ensured that the nematodes were kept hydrated, sealed and fixated. The slides were sent to a nematode taxonomist, Dr. Antoinette Swart from the Agricultural Research Council (ARC) - Plant Protection Research Institute (PPRI) (Roodeplaat) for identifications to genus and where possible species level.

It should be noted that in order to avoid any confusion, all terrestrial, beneficial/NPN assemblages will for the remainder of this dissertation be referred to as NPN and not free-living nematodes.

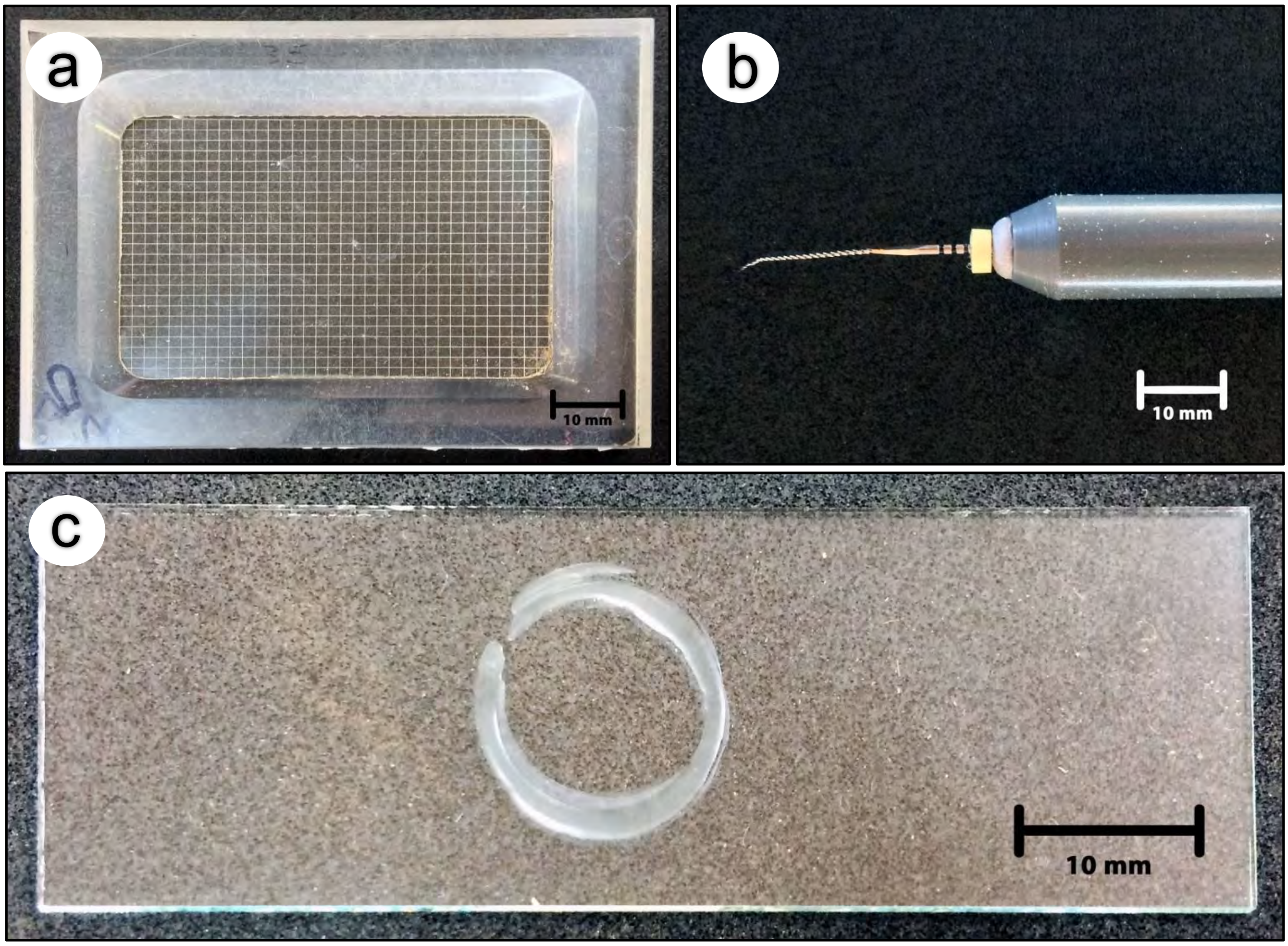

Figure 2.9: Equipment used during nematode counting and fixation included a (a) De Grisse dish used for counting nematodes, (b) modified dermatologists needle used for collecting nematodes and (c) permanent slide for mounting of nematodes.

2.6 Measurement of water quality parameters and physico-chemical characteristics of substrates

The abiotic conditions of each sampling site were quantified by means of various analyses in order to determine the extent of anthropogenic pollution, as well as its effect on the biotic components namely, nematodes and *C. gariepinus*. All of the collected substrate (water, soil and sediment) samples were subjected to various physico-chemical analyses at the EcoRehab laboratories (NWU). Water samples were analysed for (1) total metal concentration, (2) anion (including Cl^- and SO_4^{2-}) and nutrient [including nitrate (NO_3^-), ammonium (NH_4^+) and phosphate (PO_4^{3-})] concentrations and (3) pH. Soil and sediment samples were analysed for (1) total metal concentration, (2) anion (including Cl^- and SO_4^{2-}) and nutrient (including NO_3^-, NH_4^+ and PO_4^{3-}) concentrations, (3) pH, (4) TOC and (5) particle size distribution.

2.6.1 Total metal concentration

The total metal concentration analysis was performed according to the protocol provided by EPA-Method-3050b (1996). Soil, sediment and guano samples were dried, homogenised and sieved. A representative 1 g sample was weighed (0.01 g accuracy) and 10 ml of a 1:1 nitric acid (HNO_3) solution added under a fume hood. The mixture was covered with a watch glass, heated to 95 ºC and refluxed for 15 min. In order to prevent losses, care was taken to prevent the mixture from boiling. After the sample cooled, 5 ml of a concentrated HNO_3 mixture was added and refluxed for 30 min. The latter step was repeated until no more brown fumes, indicating oxidation, were released. The solution was allowed to evaporate until 5 ml of volume remained. After the sample cooled, 2 ml of distilled water and 2 ml of a 30 % hydrogen peroxide (H_2O_2) solution was added. The mixture was covered and heated until effervescence lessened. Extra portions (1 ml) of a 30 % H_2O_2 solution were added when necessary. The sample was allowed to evaporate until a volume of 5 ml remained. After cooling, the solution was diluted (total volume of 100 ml) with distilled water and filtered through a Whatman Grade No. 41 filter paper. An Agilent 7500 CE series Inductively Coupled Plasma Mass Spectrometer (ICP-MS) was used to measure the metal concentrations. Collected water samples did not require any preparation and were directly analysed by using the latter apparatus.

2.6.2 Soil and sediment character analysis by means of a 1:2 volume extract

In order to quantify the concentration of selected anions and nutrients as well as the pH of the substrates, it was necessary to create a water dissolved solution by means of a 1:2 volume extract (Sonneveld & Van Den Ende, 1971). A plastic bottle with a sealable lid was used and 200 ml deionized water with 3 drops of super flocculent (1 % solution) added. A 100 ml representative substrate sample was added to the deionised water after which it was shaken for 30 min. The water column was decanted and centrifuged at 2 000 rpm for 30 min after which the supernatant was decanted. The process was continued by centrifuging the supernatant at 16 500 rpm for 12 min after which it was filtered. Subsequently, after all the particles were removed, the pH was measured with a Radiometer PHM 80 Portable Meter, NH_4^+ concentration with a Radiometer TTT85 Titrator, selected anion concentrations (Cl^-, NO_3^-, SO_4^{2-} and PO_4^{3-}) with a Metrohm 761 Compact IC and HCO_3^- concentrations (pH < 8.2; alternatively total alkalinity) by means of titration.

As with the total metal concentration analysis (under section 2.6.1), collected water samples did not require any preparation. However, if some particles were suspended in a sample, the latter was filtered using Whatman Grade No. 41 before the commencement of analyses.

2.6.3 Percentage of total organic carbon in solid substrates

The loss-of-ignition method (Donkin, 1991) is widely used to determine the TOC content of substrates. A 15 g representative soil/sediment sample (oven-dried at 60 ºC) was weighed (0.0001 g accuracy) and placed in a ceramic crucible. The crucible was inserted into a pre-heated furnace at 600 ºC and left undisturbed for 6 h to ensure that all organic carbon had been ignited. After cooling at 25 ºC, it was weighed (0.0001 g accuracy) again and the percentage of organic content was calculated based on the differences in weight.

2.6.4 Particle size distribution and soil classification of substrates

The particle size distribution protocol was based on Laker and Du Preez (1982) and Gee and Or (2002). A 100 g representative oven-dried sample was sieved (2 mm mesh size), the > 2 mm fraction weighed and 50 g of the sieved substrate added to a 500 ml glass beaker. The content of the beaker was moisturized with distilled water after which 10 ml of H_2O_2 was carefully added under a fume hood. After 10 min the mixture was placed on a heating plate covered with sand. The beaker was covered with a watch glass and boiled for 4 h. The beaker was allowed to cool to 25 ºC after which 125 ml of Calogen was added and mixed well. A 53 µm mesh sieve was placed over a sedimentation cylinder (1 000 ml capacity) and the mixture washed through the sieve with tap water and a small brush. The fraction that remained in the sieve was then oven-dried. The content of the sedimentation cylinder was well mixed, left undisturbed and the first measurement made after 40 s by using a hydrometer. The second measurement was recorded after 7 h. In both cases the temperature was also noted. The oven-dried fraction was sieved (53 µm mesh size) for 3 min into a pan and the content of the latter weighed. The cumulative percentage for each hydrometer reading and subsequently the particle sizes was calculated with formulas provided by the Standards Association of Australia (Standard No.: AS 1289.C6.2-1976):

- Clay = < 0.002 mm
- Silt = 0.002 – 0.02 mm
- Fine sand = 0.02 – 0.2 mm
- Coarse sand = 0.20 – 2 mm
- Gravel = > 2 mm

The percentage of particles (classified as sand, silt and clay) was calculated and used to determine the soil classification (Figure 2.10) of each substrate sample as defined by the Soil Classification Working Group (1991). It should be noted that since fractions larger than 2 mm were only calculated as a percentage per sample, sediment samples were also subjected to the same classification system.

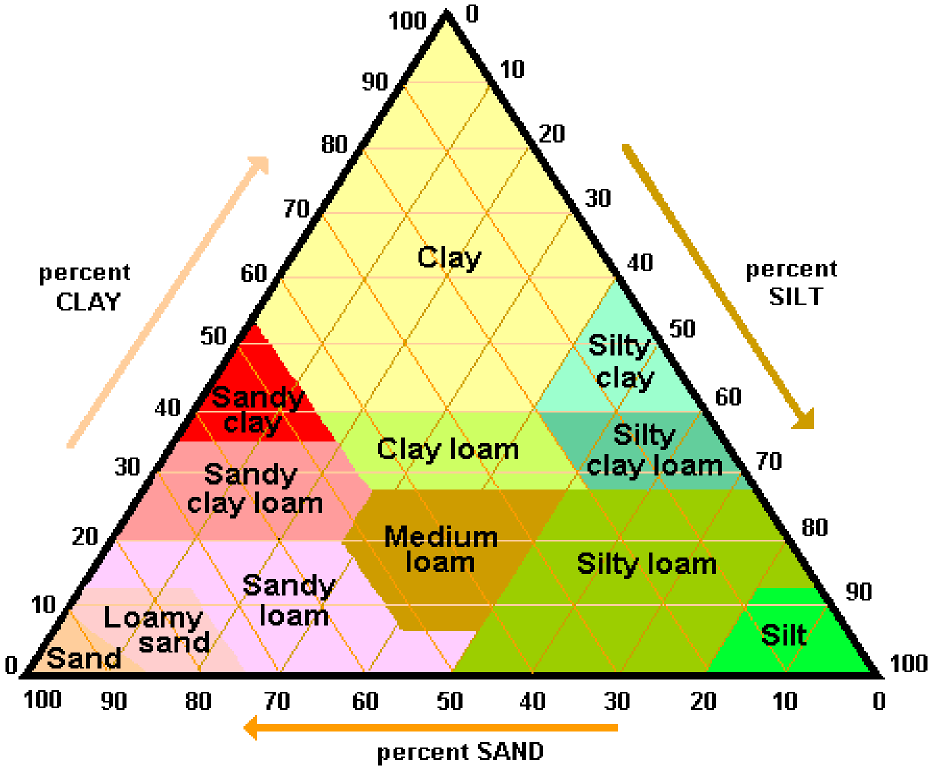

Figure 2.10: Soil texture classification triangle (Soil Classification Working Group, 1991).

2.7 Sampling and health assessment of *Clarias gariepinus* specimens

All sampling efforts to obtain *C. gariepinus* specimens during this study were permitted by Gauteng Nature Conservation under permit number: CPF6 0181. All experiments were approved by the NWU Ethical Committee under approval no: NWU-00095-12-A4 before onset of this research.

2.7.1 Sampling of *Clarias gariepinus* specimens

During the 1st sampling interval, *C. gariepinus* specimens were only collected from the Wonderfontein Cave, representing the subterranean environment. However, during the 2nd interval specimens were obtained from both the cave environment and Stoffels Dam (representing the surface environment). It should be noted that due to permit limitations, only 19 specimens were collectively sampled from the cave (n = 9) and surface (n = 10) environments. Sampling of *C. gariepinus* in the Wonderfontein Cave was undertaken by making use of large hand nets and sufficient lighting. It was discovered that the most effective approach for sampling the cave specimens was to first trap them against a wall after which a quick swooping action would force them into the net.

Sampling in Stoffels Dam was undertaken by using a small inflatable boat with a battery operated on-board motor (Figure 2.7 b). Gill nets (3 x 20 m lengths combined; respective mesh sizes of 93, 120 and 150 mm) were placed in *C. gariepinus* feeding areas alongside reeds, anchored and left undisturbed for 3 h. After this period the anchors were lifted, the nets hauled in and the fish removed. The live specimens were placed in plastic containers with sufficient water. All other fish species caught together with *C. gariepinus* specimens were immediately released.

Coleman cooler boxes filled with site water and a battery-operated air pump installed to ensure adequate aeration, was used to transport the *C. gariepinus* specimens to the NWU's School for Biological Sciences. After being transferred to larger aerated aquariums, further analyses were continued the same day of sampling.

2.7.2 Techniques applied for determining the health, exposure level and degree of isolation of *Clarias gariepinus* specimens

2.7.2.1 A quantitative health assessment index (Fish health index)

Adams *et al.* (1993) described a quantitative HAI that allows a rapid field evaluation of the observable physical health of fish specimens. This technique was applied at the NWU on all *C. gariepinus* specimens sampled from both the Wonderfontein Cave and surface environments during this study. The fish necropsy was carried out as proposed by Goede and Barton (1990). Each specimen was weighed and its length measured before being euthanized by severing the spinal cord (approval no: NWU-00095-12-A4). The physical outer appearance was evaluated by considering the condition of the eyes, skin, fins, opercula as well as the presence of parasites. Without delay, the specimen was dissected, sexed and the liver, spleen and gonads removed. These organs were weighed and in the case of a male, the length of the testes measured. Also, the percentage mesenteric fat, colour of the bile and the condition of the liver, spleen, hindgut and kidneys were evaluated. All the remaining intestines were removed after which the gutted specimen was weighed. All the variables were assigned weighted values and applied in the HAI as described under section 2.8.3.2 (Adams *et al.*, 1993). Also, liver and muscle tissue samples were collected, placed in labelled cryogenic vials and stored in an ultra-low temperature freezer at -80 °C, until further analyses could be continued.

2.7.2.2 Oxidative stress biomarkers

The liver tissue samples of the *C. gariepinus* specimens were used in the analyses of the following oxidative stress biomarkers: (1) PC, (2) SOD and (3) CAT activity, as well as the biomarker for metal exposure: (4) MT. The protocols for the respective biomarker assays are described below. It should be noted that the protein content was determined according to the Bradford (1976) method.

(1) Protein carbonyl content: This protocol was performed as described by Parvez and Raisuddin (2005), as assayed by Levine *et al.* (1990) and modified by Floor and Wetzel (1998). Liver tissue obtained from each fish specimen was homogenized, filtered with a muslin cloth and centrifuged at 10 500 g for 30 min. The proteins in solution were allowed to react with 2, 4-Dinitrophenylhydrazine (DNPH) in a 2 M HCl solution for 1 h, precipitated with a 6 % trichloroacetic acid (TCA) solution and centrifuged at 10 000 g for 3 min. The pelleted protein was re-suspended and washed three times with a 1 ml ethanol/ethyl ether (1:1) solution. Following, proteins were solubilized in 400 µl Guanidine hydrochloride, centrifuged at 16 000 g for 5 min and the carbonyl content read in triplicate at 366 nm with a spectrophotometer. A blank, which contained all the reagents except the PC, was used to calibrate the spectrophotometer. The results were expressed as nanomoles of DNPH incorporated/mg protein when considering the molar extinction coefficient as 21 000 $M^{-1}cm^{-1}$.

(2) Superoxide dismutase content: The protocol used to determine the SOD content was adapted from Greenwald (1989). Four microliter of each SOD sample was added to the wells of a microtitre plate. Following, a solution of 242 µl Tris/DTPA buffer and 4 µl of pyrogallol was also added to the wells. Firstly, a blank measurement was made after which the absorbance of each sample was read kinetically over 5.5 min in 30 s intervals (first measurement at 0 s).

(3) Catalase: The CAT protocol was performed as described by Cohen *et al.* (1970). Liver tissue obtained from each fish specimen was homogenized in PO_4^{3-} buffer (pH 7.4) and centrifuged at 10 000 g for 10 min. The supernatant was used and 0.06 ml H_2O_2 per 100 ml PO_4^{3-} buffer added to it. The solution was thoroughly mixed and left for three min after which 5 ml of a 98 % H_2O_2 solution mixed with 50 ml deionised water was added. Following, 0.0316 g of $KMnO_4$ dissolved in 100 ml deionised water was added to the solution and the CAT content read at 492 nm for 30-60 s.

(4) Metallothioneins: This protocol was performed as described by Viarengo *et al.* (1997) and as modified by Atli and Canli (2008), while sample preparation was performed as by Fernandes *et al.* (2008). Liver tissue obtained from each fish specimen was homogenised in homogenising buffer (1:3) and centrifuged at 30 000 g at 4 ºC for 20 minutes. The pellet was discarded and 500 µl of the supernatant measured after which 500 µl of cold absolute ethanol (-20 ºC) and 40 µl of chloroform was added. The solution was vortexed and centrifuged at 7 000 g for 10 min. Another three volumes of cold absolute ethanol (-20 ºC) was added, vortexed and incubated at -20 ºC for 2 h. The supernatant was discarded and the pellet washed twice with 1 ml of washing buffer. After the pellet was dried, it was re-suspended in 300 µl Tris (5 mM)-EDTA (1 mM). Next, 210 µl Ellman's reagent and 15 µl of the blank, standards and samples were added in triplicate to a microtitre plate. The plate was incubated at 25 ºC for 15 min after which the content was read at 412 nm with a spectrophotometer.

2.7.2.3 Total metal concentration

The protocol performed for determining the total metal concentration in muscle tissue samples of *C. gariepinus* samples obtained for this study was based on the EPA-Method-3052 (1995). All muscle tissue samples were oven-dried (60 ºC) for 24 h to ensure that no moisture remained. A representative 0.5 g sample was weighed and added to a Teflon (TFM) lined vessel after which it was inserted into a HTC safety shield and 10 ml of a 65 % Suprapur HNO_3 concentration added. The vessel was closed, tightened with a torque wrench and inserted into a Mars 5 microwave digester. Before commencement of the microwave program, the temperature and pressure sensors were connected and tested. The microwave program, which consisted of two steps of 10 min at 200 ºC (1 000 Watt) as well as 15 min at 200 ºC (1 000 Watt), was initiated. After completion, the vessel was allowed to cool and the pressure released under a fume hood. The content was transferred to a volumetric flask and diluted HNO_3 (1 % solution) added to a total volume of 50 ml. The solution was transferred to a 50 ml capacity Falcon tube and stored in a dark, room temperature environment until the metal concentrations could be measured by using the Agilent 7500 CE series ICP-MS.

2.7.2.4 Deoxyribonucleic acid (DNA) extraction, polymerase chain reaction (PCR) amplification and further analyses of the cytochrome b gene (mitochondrial DNA) in *Clarias gariepinus* muscle tissue

Extraction of highly pure deoxyribonucleic acid (DNA) by using a NucleoSpin Tissue DNA extraction kit

The DNA (deoxyribonucleic acid) was extracted from the respective samples according to the standard protocol for human or animal tissue and cultured cells as detailed in the instruction manual of the NucleoSpin Tissue DNA extraction kit (Macherey-Nagel, Düren, Germany). A ground representative 25 mg portion of muscle tissue was placed in a microcentrifuge tube, 180 µl of Buffer T1 and 25 µl of Proteinase K added after which each tube was briefly vortexed. The mixture was incubated at 56 °C for 3 h until complete lysis was obtained. Following, 200 µl of Buffer B3 was added, the mixture vortexed and the solution incubated 70 °C for 10 min. After the incubation period expired, 200 µl of 96 % ethanol was added and vortexed. Each sample was applied to a NucleoSpin Tissue Column placed in a collection tube and centrifuged at 11 000 g for 1 min. The contents of the collection tube were discarded and the Column re-inserted into the collection tube. This washing step was repeated twice with 500 µl of Buffer BW and 600 µl of Buffer B5, respectively. In order to elute the highly pure DNA, the column containing the DNA was placed into a 1.5 ml microcentrifuge tube, 100 µl of Buffer BE (70 °C) added and incubated at room temperature for 1 min. Lastly, the Column was centrifuged at 11 000 g for 1 min. The contents collected in the microcentrifuge tubes were stored at -20 °C until PCR amplification could continue.

Polymerase chain reaction (PCR) amplification of cytochrome b gene

In order to amplify the *C. gariepinus* cytochrome (cyt) b gene (found in the mitochondrial DNA), the primer pair L15267 and H15891 (Briolay *et al.*, 1998) was used. A solution consisting of 1 µl mM of L15267 primer, 1 µl mM of H15891 primer, 12.5 µl of DreamTaq DNA polymerase (Inqaba Biotec) and 9 µl of nuclease-free water was added to 25 µl microcentrifuge tubes. Lastly, 1 µl of 20 ng/µl pure DNA was added to the microcentrifuge tube. Using a Bio-Rad C1000 Touch^{TM} Thermal Cycler (Bio-Rad, Hemel Hempstead, UK), the PCR sequence runs were performed with the following conditions: Initial denaturing step at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing temperature at 55 °C for 30 s and extension at 72 °C for 45 s. The final extension step was performed at 72 °C for 5 min after which the samples were kept at 4 °C until retrieved from the thermo cycler (Roodt-Wilding *et al.*, 2010).

The amplicons were visualized in ultraviolet light using a 1 % agarose gel (GelRed) on a ultra-violet Bio-Rad GelDoc Imaging System (Bio-Rad). Successfully amplified PCR products were sent to Inqaba Biotec (South African Genomics Company) for sequencing in both directions.

Alignment and analyses of *Clarias gariepinus* cytochrome b sequences

All sequences were edited and aligned using Geneious (v7.1) bioinformatics software package (created by Biomatters. Available at http://www.geneious.com). In order to create high quality sequences, chromatogram based contigs were generated in the above named software package. Following, sequences were manually checked for any undefined base pairs (bp), corrected accordingly and aligned with the MUSCLE (Edgar, 2004) algorithm implemented within Geneious bioinformatics software package. All the specimen samples were identified by using the Basic Local Alignment Search Tool (BLAST) (Available at http://www.ncbi.nlm.nih.gov/blast/Blast).

All of the sequences were imported into DnaSP (v5.10.1) software package (Available at http://www.ub.edu/dnasp), which was used to identify and group haplotypes as well as calculate haplotype and nucleotide diversity. The nucleotide data was exported in the relevant file formats and further analysed in Arlequin (v3.5.1.3) (Available at http://cmpg.unibe.ch/software/arlequin35) and Network (v4.612) (Available at http://www.fluxus-engineering.com) software packages. While the former software package was used to analyse the genetic distance [Pairwise F_{ST} and analysis of molecular variance (AMOVA)] between the respective populations, the latter software package was used to create haplotype (median-joining) networks.

2.8 Statistical analyses of data

2.8.1 Statistical calculation of water, soil and sediment physico-chemical parameters

Specific water, soil and sediment parameters were calculated by making use of different statistical analyses. The percentage concentration of specific ions [Ca^{2+}, Mg^{2+}, sodium (Na^+), potassium (K^+), SO_4^{2-}, CO_3^{2-}, HCO_3^-, Cl^-] in water samples were plotted on Piper diagrams (Piper, 1944) using AquaChem (v2012.1) Water Quality Analysis and Reporting Software (Available at http://www.swstechnology.com/). This analysis grouped the respective water samples collected from the Wonderfontein Cave and Spruit according to their ionic character. Furthermore, water hardness (mg/l $CaCO_3$) was calculated using Lenntech online water hardness calculator (Available at http://www.lenntech.com/ro/water-hardness.htm).

Enrichment factors and the I_{geo} were used for determining the extent of anthropogenic metal enrichment and contamination, respectively, in soil and sediment samples collected from the Wonderfontein Cave and Spruit. It should be noted that enrichment and contamination classes of the EF and I_{geo}, respectively, are provided in Chapter 3 (section 3.2.2).

Enrichment factors were calculated as follows (Miko *et al.*, 1999; Loska *et al.*, 2004; Zhuang & Gao, 2014):

$$EF = \frac{Cn\ (sample)}{Cref\ (sample)} / \frac{Bn\ (backgrounf)}{Bref\ (background)}$$

where $\mathrm{Cn\ (sample)}$ represents the concentration of the examined element, $\mathrm{Cref\ (sample)}$ = concentration of reference element, $\mathrm{Bn\ (background)}$ = concentration of the examined element in reference environment and $\mathrm{Bref\ (background)}$ = concentration of reference element in reference environment. Titanium was used as a reference element for the calculation of EF (Loska *et al.*, 2004).

The **I_{geo}** was calculated as follows (Muller, 1969; Loska *et al.*, 2004; Ji *et al.*, 2008):

$$Igeo = \log 2 \frac{Cn}{1.5Bn}$$

where $\mathrm{C_n}$ represents the measured concentration of n (element) and $\mathrm{B_n}$ the geochemical background value.

Box-and-whisker plots were created by using GraphPad Prism 6 Scientific software package (Available at http://www.graphpad.com/) with the calculated mean I_{geo} values of the soil and sediment samples collected from the Wonderfontein Cave and the Cave Inflow Area sampling site.

2.8.2 The application of community and nematode specific indices in the analysis of nematode assemblages

General community identity-independent indices, as well as nematode-specific indices of ecosystem function, were applied. While the former included the Shannon's diversity, Margalef's richness and Pielou's evenness indices, the latter comprised of the MI, faunal profile (EI and SI) and metabolic footprint analysis (Ferris & Bongers, 2009; Neher & Darby, 2009; Ferris, 2010). These indices were applied in order to interpret the nematode data obtained during this study, assess the health of the associated ecosystem and evaluate the possible influences of metal pollution. It should be noted that the NINJA: Nematode Indicator Joint Analysis (available at http://spark.rstudio.com/bsierieb/ninja) online system was used as a control for the calculation of all the nematode specific indices (Sieriebriennikov *et al.*, 2014).

2.8.2.1 The calculation of general community indices

Shannon's diversity index (Shannon, 1948; Neher & Darby, 2009):

$$H' = -\sum P(lnPi)$$

where P_i represents the portion of the i-th genus in a sample. Shannon's diversity index was applied in order to quantitatively measure the diversity of genera in a nematode assemblage associated with a site and substrate type.

Margalef's richness index (Margalef, 1958; Neher & Darby, 2009):

$$D = \frac{(S-1)}{ln(N)}$$

where S represents the total genera and N the number of individuals. Margalef's richness index measures the total number of taxa (genera) in a community and thus has no limiting range, which allows for easy comparison between sites.

Pielou's evenness index (Pielou, 1966):

$$J' = \frac{H'}{H'max}$$

where $H'\mathrm{max}$ is calculated as follows:

$$H'\,\mathrm{max} = -\sum \frac{1}{S} \ln \frac{1}{S} = lnS$$

Pielou's evenness index measures how evenly individuals are distributed among different taxa (genera) and has a range of between zero and one. While values nearing zero represents uneven distribution, values nearing one represents high evenness.

2.8.2.2 The calculation of nematode specific indices

The **MI** is calculated as follows (Ferris & Bongers, 2009):

$$\mathrm{XI} = \sum_{i=1,f} vi\, ni \,/ \sum_{i=1,f} ni$$

where XI is the index in question, vi represents the cp-value of taxon i and ni the total number of individuals in each f taxa.

While the MI includes all NPN taxa, $MI_{2\text{-}5}$ is similarly calculated with the exception that cp-1 nematodes are excluded. Lower MI values are indicative of a disturbed/enriched environment, while higher MI values reflect stability (Bongers, 1990).

The EI and SI, necessary to plot the faunal profile, are calculated by incorporating the weighted faunal components basal (b), enrichment (e) and structure (s) (Ferris & Bongers, 2009):

$b = (Ba_2+Fu_2) * W_2$, where $W_2 = 0.8$

$e = (Ba_1*W_1) + (Fu_2*W_2)$, where $W_1 = 3.2$ and $W_2 = 0.8$

$s = (Ba_n*W_n) + (Ca_n*W_n) + (Fu_n*W_n) + (Om_n*W_n)$, where $n = 3\text{-}5$, $W_3 = 1.8$, $W_4 = 3.2$ and $W_5 = 5$

> where b represents the basal index, e = enrichment index and s = structure index. Also, Ba represents bacterivores, Ca = carnivores, Fu = fungivores, Om = omnivores and W = the respective weight class values.

The **EI and SI** indices are calculated as follows (Ferris & Bongers, 2009):

$El = (100*e) / (e+b)$

$SI = (100*s) / (s+b)$

The percentage values of the EI and SI obtained as a result of such calculations are plotted on the y-axis and x-axis of the nematode faunal profile, respectively, which is described and provided in Chapter 1 (section 1.4.5.3).

The **metabolic footprint analysis** was solely calculated and plotted by using the NINJA platform in order to ensure accuracy. However, the expanded equation for the metabolic footprint of nematodes (F) as provided by Ferris (2010) is as follows:

$F = \sum (N_t(0.1(W_t/m_t) + 0.273(W_t^{0.75})))$

> where N_t represents the number of individuals, W_t = body weight and M_t the cp classification of taxon t.

This equation represents the sum of the production and respiration components ($F = P + R$) and thus carbon utilization of component taxa.

2.8.3 Statistical analyses performed on metal bioaccumulation, gross body indices and biomarkers of oxidative stress and exposure population datasets

2.8.3.1 Statistical significance comparison between populations

Several gross body indices, including the CF and HSI, were calculated as a means of assessing and comparing the health of the respective *C. gariepinus* populations. Using GraphPad Prism 6 Scientific software package (Available at http://www.graphpad.com/), population data of metal bioaccumulation, biomarkers of oxidative stress and exposure, as well as the above named indices, were subjected to the D'Agostino & Pearson omnibus normality test (D'agostino, 1986). This test was performed in order to determine if the respective data sets represented a normal (Gaussian) or non-parametric distribution. If a normal distribution was presented, a one-way analysis of variance (ANOVA) was performed together with Tukey's multiple comparison test (Tukey, 1991), which allowed a multiple comparison test between the respective populations. However, if a dataset presented a non-parametric distribution, the Kruskal-Wallis test (Kruskal & Wallis, 1952) and Dunn's multiple comparison test (Dunn, 1964) was performed. Significance was regarded at $p < 0.05$.

2.8.3.2 Calculation of health assessment index and gross body indices

In order to calculate the HAI, numerical values were assigned to variables listed in section 2.7.2.1 according to Adams *et al.* (1993). Following, the HAI value for each population was calculated by summing the variable values and dividing that by the total number of individuals in that population. Furthermore, a standard deviation (SD) was calculated as follows (Adams *et al.*, 1993):

$$SD = \frac{\sum_{i=1}^{N}(Vi - X)^2}{N - 1}$$

where N represents the number of individuals in the fish population, $x =$ average index and Vi the index value for fish i.

The fish CF and HSI was calculated as follows (Bagenal & Tesh, 1978; Goede & Barton, 1990; Bervoets & Blust, 2003; McHugh *et al.*, 2013):

$$CF = W/L^{b}\ x\ 100$$

where W represents the weight of the individual, L = fork length (mm) and b the exponent derived from the length-mass relationship, which is described as $W = aL^{b}$.

$$HSI = \frac{Liver\ weight}{Total\ body\ weight}\ x\ 100$$

2.8.4 Multivariate analysis of species and environmental data

The ordination diagrams reported in Chapters 3, 4 and 5 were created using Canoco 5 software package (Available at http://www.canoco5.com). The response (species) data was log transformed and a principal component analysis (PCA) (Wold *et al.*, 1987) performed. A PCA represents an unconstrained analysis and illustrates the variation in species composition, which could also be interpreted with the help of environmental variables (Ter Braak & Smilauer, 2012).

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CHAPTER 3

METAL POLLUTION AND RISK POSED TO WATER, SOIL AND SEDIMENT QUALITY OF THE WONDERFONTEIN CAVE

3.1 Introduction

The Wonderfontein Spruit has been extensively subjected to the deleterious activities associated with predominantly gold mining in the Witwatersrand Basin, which have resulted in pollutants entering the surrounding environment (Swart *et al.*, 2003; Coetzee *et al.*, 2006). Mining-associated pollutants, emanating from mineral processing plants, waste rock dumps and tailings runoff, enter the Wonderfontein Spruit, which subsequently interlinks with other surface as well as groundwater systems. Many people and their livestock, as well as the town of Potchefstroom, are located downstream of the Wonderfontein Spruit (Coetzee *et al.*, 2006). Also, the geology consists mainly of karst (dolomitic) landscapes and as a result of the dewatering of the compartment, sinkholes have formed claiming the lives of many people (De Bruyn & Bell, 2001). Associated with the river banks of the Wonderfontein Spruit is the Wonderfontein Cave, which developed at a general level of 25 m below surface (Kent *et al.*, 1978). The Wonderfontein Cave has been subjected (at least since 1969 when a weir was built in order to dispose of mine water) to the influx of polluted water from the Wonderfontein Spruit.

Caves present unique environments that may host singular species, many of which remain unknown to science (Romero, 2009). As a result of the interconnected nature of karst landscapes, caves are at threat as pollution can easily enter and disperse in systems such as the Wonderfontein Cave (Ford & Williams, 2007). The aims of this part of the study were to 1) determine the extent of metal enrichment and contamination as a result of the influx of polluted water and to 2) evaluate the pollution threat posed to the ecosystem hosted by the Wonderfontein Cave by considering the toxicity hazard potential of the respective substrates. Both these aims were considered over a temporal scale as the sampling of cave water, soils and sediments were undertaken during the months of April (1st sampling interval) and September (2nd sampling interval) 2013, representing the end of the high and low flow periods, respectively. During the 1st sampling interval, a composite sample, consisting of 10 sub-samples, were collected for each substrate from seven subterranean and three surface sites. In order to allow statistical analyses, three replicate samples were collected of each substrate during the 2nd sampling interval.

Since there exists no known cave that can serve as a control for the Wonderfontein Cave, EF and the I_{geo} were applied in order to identify anthropogenically enriched metals and determine the degree of contamination, respectively. Background values for sediments was acquired from a study by Coetzee *et al.* (2006) who reported metal concentration values from the Klerkskraal Dam (see Appendix A), a pristine system with a similar geological setting. Soil collected from an unpolluted site (Pristine Chamber) located within the Wonderfontein Cave also provided background values. Metal concentrations were compared to environmental quality guidelines in order to assess the toxicity hazard potential of the respective substrates. Also, water quality parameters, as well as the physical and chemical characteristics of the soil and sediment samples, were determined.

It is hypothesized that the Wonderfontein Cave has been subjected to the influx of polluted water from the Wonderfontein Spruit, which subsequently influences the toxicity hazard potential of the subterranean abiotic environment.

3.2 Results

3.2.1 Classification of water, sediment and soil samples

There are a number of important factors to take note of when considering the ecological threat posed by metal pollution. Water quality parameters such as pH and hardness, as well as soil and sediment characteristics such as the pH, particle size distribution and TOC, influence the concentration and bioavailability of metals (Violante *et al.*, 2010).

The water quality parameters (Table 3.1) indicate that all of the collected water samples were alkaline and classified as very hard. In order to further analyse the character and determine the possible source of the water, the concentrations of selected ions (see appendix A) were used to plot the respective samples on Piper diagrams. This included water samples collected during both the 1st (Figure 3.1 a) and 2nd (Figure 3.1 b) sampling intervals. The results indicated that the samples were associated with relatively high concentrations of Ca^{2+} and Mg^{2+} cations, which is indicative of hard water as reported above, as well as Cl^- and SO_4^{2-} anions. Minimal seasonal variation was observed with slightly lower SO_4^{2-} and Cl^- concentrations recorded during the 2nd sampling interval. Furthermore, no variation was evident between the surface and subterranean sites.

Soil and sediment characteristics (particle size distribution, TOC and pH) of all the collected samples are presented in Table 3.2. Since fractions larger than 2 mm were only calculated as a percentage per sample, sediment samples were also subjected to the soil classification triangle provided in Chapter 2 (section 2.6.4). This classification method revealed that most soil samples were classified as either 'loamy sand' or 'sandy loam', while sediment samples were mostly classified as 'sandy loam' or 'loam'. The percentage fraction larger than 2 mm only exceeded 50 % in soil collected at Kent's Entrance and sediments collected at Kent's Entrance and Elevation Pit. All of these samples were collected during the 1st sampling interval.

During both sampling intervals the TOC (Table 3.2) in the soils ranged from 0.11 to 7.08 % and in the sediments from 0.73 to 5.88 %, while the soil pH values ranged from 4.50 to 8.44 and the sediment pH values from 4.87 to 8.42. Low pH values where observed in soil samples collected during both sampling intervals at Elevation Pit (soil type B), as well as sediment samples collected from the same site during the 2nd sampling interval.

Table 3.1: Water quality parameters (pH and hardness) of water samples collected from the Wonderfontein Cave and Spruit during the 1st (April 2013) and 2nd (September 2013) sampling intervals.

	pH	**Hardness (mg/l $CaCO_3$)**	**pH**	**Hardness (mg/l $CaCO_3$)**
	1st Sampling Interval		**2nd Sampling Interval**	
Kent's Entrance	9.15	281.5 (Very hard)	8.7	381.9 (Very hard)
Keyhole Entrance	8.51	276.1 (Very hard)	8.64	384.4 (Very hard)
Main Entrance	8.63	266.8 (Very hard)	8.54	366.7 (Very hard)
Fault Passage	-	-	8.59	369.3 (Very hard)
Pristine Chamber	8.46	266.9 (Very hard)	8.45	355.4 (Very hard)
Elevation Pit	8.41	263.6 (Very hard)	8.45	363.7 (Very hard)
North-Eastern Section	8.39	258.6 (Very hard)	8.4	359.4 (Very hard)
Cave Inflow Area	8.7	277.2 (Very hard)	8.55	391.7 (Very hard)
Derek's Exit	8.92	270.1 (Very hard)	-	-
Wonderfontein Canal	-	-	8.39	380.8 (Very hard)
Stoffels Dam	-	-	8.59	384.1 (Very hard)

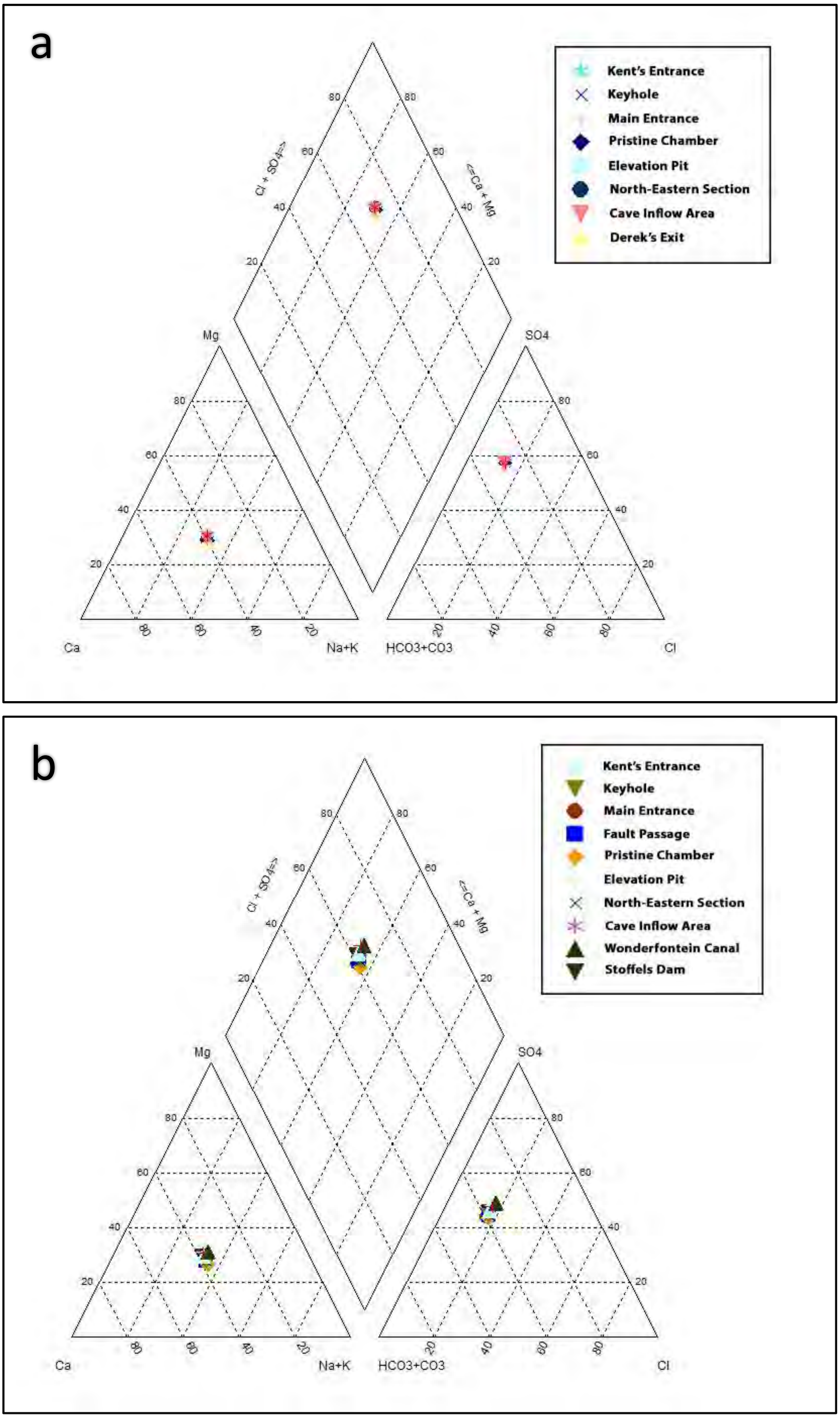

Figure 3.1: Concentrations of selected ions, associated with water samples collected from the Wonderfontein Cave and Spruit during the a) 1st (April 2013) and b) 2nd (September 2013) sampling intervals, were used to plot and characterize each water sample by means of a Piper diagram.

Table 3.2: Classification, fraction percentage larger than 2 mm, total organic carbon (TOC) and pH values of soil and sediment samples collected from the Wonderfontein Cave and Spruit during the 1st (April 2013) and 2nd (September 2013) sampling intervals.

Sample	Class.	Frac > 2 mm (%)	TOC (%)	pH	Class.	Frac > 2 mm (%)	TOC (%)	pH
	1st Sampling Interval				**2nd Sampling Interval**			
SOIL								
Kent's Entrance	Loamy Sand	57.64	0.91	7.96	Sandy Loam	32.42 ± 6.07	1.47 ± 0.09	8.02 ± 0.16
Main Entrance A	Loamy Sand	21.19	0.41	4.55	Sandy Loam	33.22 ± 7.95	0.72 ± 0.03	7.87 ± 0.20
Main Entrance B	Sandy Loam	25.96	0.31	7.13	Loamy Sand	29.30 ± 13.47	1.92 ± 0.24	7.51 ± 0.19
Fault Passage	-	-	-	-	Sandy Loam	16.78 ± 7.32	0.57 ± 0.19	7.37 ± 0.06
Pristine Chamber	Sandy Loam	7.69	0.40	7.53	Sandy Loam	18.15 ± 1.95	2.67 ± 2.95	7.31 ± 0.41
Elevation Pit A	Sandy Loam	7.11	2.55	7.85	Sandy Loam	7.50 ± 1.85	1.40 ± 0.38	7.62 ± 0.13
Elevation Pit B	Sandy Loam	28.02	0.11	4.88	Sandy Clay Loam	22.86 ± 16.17	3.33 ± 1.53	4.50 ± 0.08
North-Eastern Section	Clay Loam	3.57	0.37	7.8	Clay	8.41 ± 11.15	2.26 ± 0.13	7.93 ± 0.19
Cave Inflow Area	Sandy Loam	1.38	4.08	6.69	Loam	2.57 ± 1.48	6.14 ± 2.74	6.93 ± 1.61
Derek's Exit	Loamy Sand	25.48	4.22	7.77	Sandy Loam	29.39 ± 6.56	3.57 ± 0.45	7.40 ± 0.15
Wonderfontein Canal	-	-	-	-	Loamy Sand	5.02 ± 2.40	0.97 ± 0.18	5.28 ± 0.61
Stoffels Dam	-	-	-	-	Sandy Loam	7.40 ± 2.38	7.08 ± 2.11	8.44 ± 0.06
SEDIMENT								
Kent's Entrance	Sandy Loam	59.51	2.21	7.54	Sandy Loam	32.62 ± 10.39	2.36 ± 0.14	8.19 ± 0.05
Keyhole Entrance	Sandy Loam	39.58	2.94	7.82	Sandy Clay Loam	21.55 ± 17.28	2.75 ± 1.37	7.96 ± 0.07
Main Entrance	Loam	4.15	5.88	8.05	Loam	4.20 ± 1.37	3.69 ± 0.50	8.28 ± 0.08
Fault Passage	-	-	-	-	Sandy Loam	9.57 ± 8.14	2.67 ± 1.67	7.93 ± 0.04
Pristine Chamber	Sandy Loam	34.65	0.73	7.77	Sandy Clay Loam	15.32 ± 2.89	1.36 ± 0.78	8.04 ± 0.04
Elevation Pit	Loam	52.05	4.86	8.05	Sandy Loam	24.21 ± 7.40	4.66 ± 1.17	4.87 ± 0.19
North-Eastern Section	Loam	13.82	0.65	7.99	Loam	20.91 ± 17.06	2.55 ± 1.18	8.09 ± 0.23
Cave Inflow Area	Sandy Loam	0.79	4.27	7.98	Sandy Loam	6.57 ± 4.51	3.61 ± 2.17	8.15 ± 0.15
Derek's Exit	Sandy Loam	12.18	3.30	8.03	-	-	-	-
Wonderfontein Canal	-	-	-	-	Sandy Loam	2.33 ± 2.12	2.45 ± 2.02	8.42 ± 0.14
Stoffels Dam	-	-	-	-	Loamy Sand	2.26 ± 1.12	1.17 ± 0.15	7.76 ± 0.23

3.2.2 Anthropogenic metal enrichment and contamination of soil and sediment samples

Several metals, including Cr, Ni, Cu, Zn, Co, Fe, Al, Mn, Cd, Pb and Uranium (U), as well as the metalloid As, were selected for investigations. For the purpose of simplicity, As will be referred to as a metal for the remainder of this dissertation. These metals represent those, as identified by Herselman *et al.* (2005), environmentally important to the South African landscape, as well as metals known to be released into the environment by gold mining activities in the Witwatersrand Basin (Coetzee *et al.*, 2006; Usher & Vermeulen, 2006; Durand *et al.*, 2010).

The concentrations of metals in soils and sediments collected from each site associated with the Wonderfontein Cave and Spruit are provided in Appendix A. In order to assess the extent of anthropogenic metal enrichment, as well as the degree of contamination, EF and the I_{geo} were applied, respectively. While the former served as a tool to identify anthropogenically enriched metals, the latter was used to numerically indicate the actual degree of contamination.

However, both indices require background values for calculation and although the world average shale and average Earth's crust values are commonly used, these values are very general and may produce inaccurate results (Ji *et al.*, 2008). As there exists no other known cave system that could've served as a control for the Wonderfontein Cave, background values from unpolluted systems associated with similar geology were used. Sediment background values were acquired from a study conducted by Coetzee *et al.* (2006), which produced the total concentrations of metals associated with sediments collected from the unpolluted Klerkskraal Dam. Furthermore, the total concentrations of metals associated with Pristine Chamber, a sampling site within the Wonderfontein Cave, provided baseline data for the soil samples. Pristine Chamber has not been exposed to the influx of polluted water as it is well elevated above the general cave development level and the cave datum as stated by Kent *et al.* (1978). It should be noted that Ti, commonly used in metal enrichment analyses, was applied as a reference element for the calculation of EF (Loska *et al.*, 2004).

The calculated EF and I_{geo} for soil and sediment samples collected from the Wonderfontein Cave and Spruit (both sampling intervals) are provided in Tables 3.4 and 3.5 and colour coded according to a classification scheme (Table 3.3) to allow for easy identification and comparison. When considering EF, 'significant' enrichment does not imply the application of a statistical test, however, only represent a class/degree of enrichment as defined by Loska *et al.* (2004) and provided in Table 3. For further details on the methodology and calculation of the respective indices, please refer to Chapter 2 (section 2.8.1).

Also as part of this section, variability in contamination levels of the selected metals was assessed by plotting mean metal I_{geo} values on box-and-whisker plots (Figures 3.2, 3.3). It should be noted that only the values of the sediments directly associated with the Wonderfontein Cave, which includes the Cave Inflow Area surface site, were included in the dataset.

Table 3.3: Classification system of enrichment factors (EF) and the geoaccumulation index (I_{geo}) as provided by (Loska *et al.*, 2004). Colour coding was applied for easy identification of different enrichment and contamination classes.

EF	Minimal	Moderate	Significant	Very high	Extreme	
Range	EF < 2	EF = 2 – 5	EF = 5 - 20	EF = 20 - 40	EF > 40	

I_{geo}	Uncontaminated	<= Moderate	Moderate	Mod. – Heavy	Heavy	Heavy - Extreme	Extreme
Range	$I_{geo} < 0$	$0 < I_{geo} < 1$	$1 < I_{geo} < 2$	$2 < I_{geo} < 3$	$3 < I_{geo} < 4$	$4 < I_{geo} < 5$	$5 < I_{geo}$

3.2.2.1 Anthropogenic metal enrichment and contamination of soils

The calculated EF for selected metals present in soil samples collected from the Wonderfontein Cave and Spruit are provided in Table 3.4. Although most of the soil samples presented moderate enrichment of some metals, other soil samples presented significant to extremely high enrichment of specific metals. Soils collected during the 1st sampling interval, especially from North-Eastern Section and Cave Inflow Area sampling sites were highly enriched. While U presented very high enrichment at the latter sampling site, Cr, Zn, Fe, Al and U were recorded as significantly enriched in soils collected at North-Eastern Section. Significant U and Al enrichment was also recorded at Kent's Entrance and Cave Inflow Area, respectively. During the 2nd sampling interval higher enrichment levels were observed. While soils collected at North-Eastern Section and Cave Inflow Area sampling sites again presented significant enrichment, soil samples collected at Elevation Pit sampling site presented significant, very high and even extremely high enrichment for selected metals. Soil sample A (Elevation Pit) presented significant enrichment for Zn and extremely high enrichment for U, while soil sample B (Elevation Pit) presented significant enrichment for Cr, Cd and U and very high enrichment for Zn. The two surface sampling sites associated with the Wonderfontein Spruit (Wonderfontein Canal and Stoffels Dam) both presented extremely high enrichment of U, while the Wonderfontein Canal also presented significant enrichment of Cr and Zn.

The degree of contamination of these metals was considered by means of the I_{geo} (Table 3.4). During both sampling intervals only Cr, Zn, Al and U were identified as occurring in levels 'moderately contaminated' and above. Moderate contamination of Cr was only recorded at North-Eastern Section (2nd sampling interval), Zn at Main Entrance (soil sample

A) and Derek's Exit during the 1st sampling interval and at Kent's Entrance, North-Eastern Section, Cave Inflow Area and Derek's Exit during the 2nd sampling interval. Although Al presented moderate contamination at Main Entrance (soil samples A and B), North-Eastern Section and Cave Inflow Area during the 1st sampling interval, moderate contamination was only observed at North-Eastern Section during the 2nd sampling interval. Uranium contamination in the Wonderfontein Cave soils during the 2nd sampling interval ranged from being uncontaminated (Main Entrance soil sample A; Pristine Chamber) to heavily contaminated (Elevation Pit soil sample A; Cave Inflow Area). Also, soils collected from Kent's Entrance were subjected to moderate to heavy U contamination during both sampling intervals. The Wonderfontein Canal and Stoffels Dam were subjected to moderate to heavy and heavy to extreme U contamination, respectively.

The mean I_{geo} values of sampled cave soils ranged from – 1.27 (Mn) to 1.01 (U) during the 1st sampling interval (Figure 3.2 a) and from -1.65 (Mn) to 1.2 (U) during the 2nd sampling interval (Figure 3.2 b). Although the mean I_{geo} values do not reflect a severe degree of contamination, maximum (max) values for U (both sampling intervals) neared 4, which represents heavy contamination. Furthermore, during the 2nd sampling interval the max value for Zn neared 3 representing moderate to heavy contamination. Figures 3.2 a and b clearly illustrate that although most metals associated with the soils of the Wonderfontein Cave presented relatively low variability, Mn and U contamination levels were highly variable. Ranges of 4.55 and 4.42 (1st sampling interval) and 4.55 and 4.36 (2nd sampling interval) were recorded for Mn and U, respectively. Also, skewness was observed at most of the I_{geo} values and especially from those associated with the 2nd sampling interval.

Table 3.4: Enrichment factors (EF) and geoaccumulation index (I_{geo}) values for soil samples collected during the 1st (April 2013) and 2nd (September 2013) sampling intervals. Colour coding, as presented in Table 3.3, were used to identify specific enrichment and contamination classes as defined by Loska *et al.* (2004).

	Cr	Ni	Cu	Zn	Co	Fe	Al	As	Mn	Cd	Pb	U	Cr	Ni	Cu	Zn	Co	Fe	Al	As	Mn	Cd	Pb	U
	EF												I_{geo}											
1st Sampling Interval																								
Kent's Entrance	1.02	2.69	2.30	1.18	2.19	0.88	2.51	1.31	1.71	1.25	1.59	9.58	-0.73	0.66	0.44	-0.52	0.36	-0.94	0.56	-0.38	0.01	-0.44	-0.10	2.49
Main Entrance A	2.01	1.62	1.26	3.08	1.62	0.88	3.04	0.77	0.64	0.81	1.12	1.13	0.66	0.35	-0.02	1.27	0.35	-0.53	1.25	-0.72	-1.01	-0.66	-0.19	-0.18
Main Entrance B	1.25	0.53	0.88	1.43	0.48	1.10	2.48	1.12	0.53	0.52	1.09	2.63	0.07	-1.16	-0.43	0.27	-1.32	-0.11	1.06	-0.08	-1.17	-1.19	-0.13	1.15
Pristine Chamber	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-0.58	-0.58	-0.58	-0.58	-0.58	-0.58	-0.58	-0.58	-0.58	-0.58	-0.58	-0.58
Elevation Pit A	1.28	1.70	1.93	2.16	1.85	0.98	1.54	0.87	1.52	1.74	1.75	3.55	-0.50	-0.08	0.10	0.26	0.04	-0.87	-0.23	-1.05	-0.25	-0.05	-0.04	0.98
Elevation Pit B	1.59	0.93	1.60	3.59	1.50	1.21	2.40	0.96	0.82	1.10	1.33	1.79	-0.27	-1.05	-0.27	0.90	-0.37	-0.67	0.32	-1.00	-1.23	-0.81	-0.54	-0.11
North-Eastern Section	11.85	4.46	3.02	8.76	2.80	5.15	12.41	4.41	1.44	2.46	2.75	10.45	0.96	-0.45	-1.02	0.52	-1.12	-0.24	1.02	-0.47	-2.08	-1.31	-1.15	0.78
Cave Inflow Area	3.43	2.63	1.03	4.23	1.55	0.82	5.54	1.27	0.11	1.97	0.95	36.26	0.44	0.05	-1.30	0.74	-0.71	-1.62	1.13	-0.99	-4.48	-0.36	-1.41	3.84
Derek's Exit	1.99	2.01	1.42	4.86	1.18	1.07	3.34	1.29	1.26	1.83	1.43	3.78	-0.01	0.01	-0.50	1.28	-0.76	-0.91	0.74	-0.63	-0.67	-0.13	-0.49	0.92
2nd Sampling Interval																								
Kent's Entrance	1.52	4.21	2.50	4.80	2.65	1.05	1.84	1.83	1.35	2.44	1.14	14.66	-0.47	0.99	0.24	1.18	0.33	-1.01	-0.20	-0.21	-0.65	0.20	-0.89	2.79
Main Entrance A	1.43	1.29	1.14	1.29	1.10	1.06	1.11	0.63	1.15	1.24	1.25	1.62	-0.37	-0.53	-0.70	-0.52	-0.75	-0.81	-0.74	-1.56	-0.68	-0.58	-0.57	-0.20
Main Entrance B	1.52	1.51	1.29	3.19	1.41	0.99	2.04	0.71	1.10	1.42	1.61	4.20	-0.19	-0.20	-0.43	0.88	-0.30	-0.82	0.23	-1.29	-0.66	-0.29	-0.11	1.27
Fault Passage	0.99	1.11	0.78	1.25	0.85	0.99	1.37	0.88	0.73	0.84	0.80	2.18	-0.96	-0.80	-1.30	-0.63	-1.19	-0.97	-0.49	-1.14	-1.40	-1.21	-1.27	0.18
Pristine Chamber	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-0.58	-0.58	-0.58	-0.58	-0.58	-0.58	-0.58	-0.58	-0.58	-0.58	-0.58	-0.58
Elevation Pit A	1.32	4.19	0.94	6.04	1.75	1.25	1.93	1.30	0.66	2.63	0.83	51.53	-1.50	0.16	-1.99	0.69	-1.10	-1.59	-0.96	-1.53	-2.50	-0.51	-2.17	3.78
Elevation Pit B	5.37	1.25	2.52	24.42	1.07	2.38	3.61	2.48	0.87	7.76	1.61	6.11	0.43	-1.67	-0.66	2.62	-1.90	-0.74	-0.14	-0.68	-2.19	0.96	-1.31	0.62
North-Eastern Section	9.21	2.42	1.69	16.08	1.28	4.17	8.81	3.20	0.88	2.51	1.21	4.57	1.14	-0.79	-1.31	1.94	-1.70	0.00	1.08	-0.38	-2.25	-0.74	-1.79	0.13
Cave Inflow Area	3.17	1.58	0.98	9.23	0.66	1.04	4.50	1.07	0.08	2.65	0.85	25.07	0.28	-0.71	-1.40	1.83	-1.98	-1.32	0.79	-1.28	-5.10	0.03	-1.61	3.27
Derek's Exit	1.57	2.29	1.56	6.93	1.29	1.30	2.32	1.27	2.00	3.83	1.48	4.54	-0.89	-0.35	-0.90	1.25	-1.18	-1.17	-0.33	-1.20	-0.55	0.39	-0.98	0.64
Wonderfontein Canal	6.52	4.97	0.95	6.01	1.91	0.51	4.15	1.88	0.03	3.38	0.56	51.57	-0.53	-0.92	-3.30	-0.64	-2.30	-4.19	-1.18	-2.32	-8.28	-1.47	-4.07	2.46
Stoffels Dam	3.13	0.88	1.13	4.34	0.42	1.47	3.16	2.88	0.02	3.34	0.81	78.57	-0.06	-1.89	-1.53	0.41	-2.95	-1.15	-0.05	-0.18	-7.28	0.03	-2.00	4.59

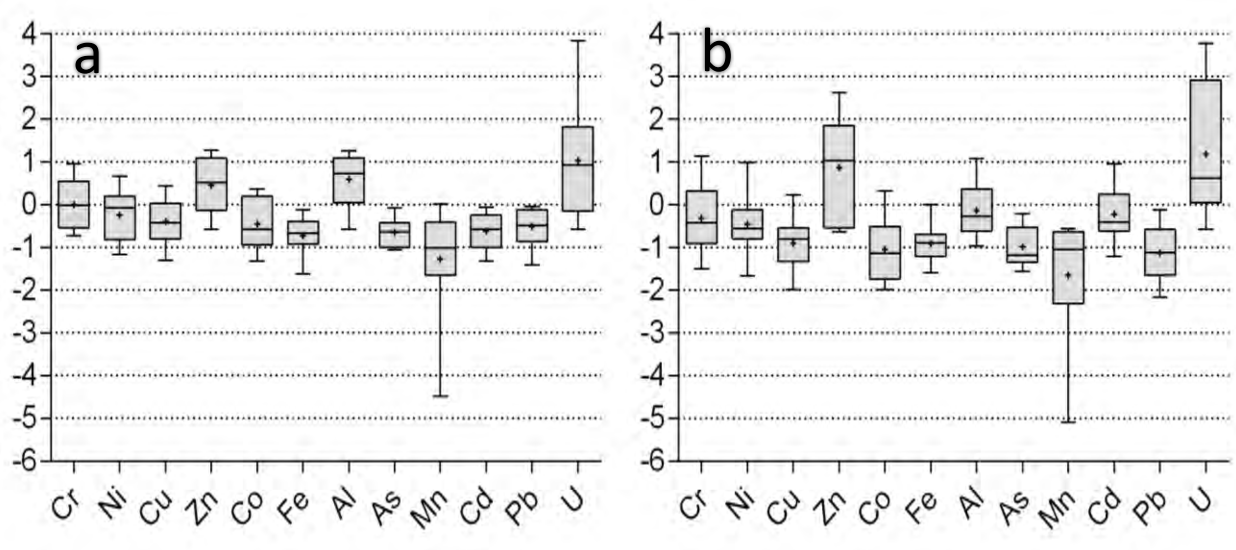

Figure 3.2: Box-and-whisker plots illustrate the variability and skewness of mean I_{geo} values calculated for soil samples collected during the a) 1st (April 2013) and b) 2nd (September 2013) sampling intervals from the Wonderfontein Cave.

3.2.2.2 Anthropogenic metal enrichment and contamination of sediments

As reported in Table 3.5, sediment samples collected from the Wonderfontein Cave during both sampling intervals presented moderate to extremely high enrichment of metals, with only Cr, Fe, As and Mn presenting minimal enrichment. Sediments collected from Kent's Entrance, Main Entrance, Elevation Pit and North-Eastern Section presented significant enrichment of Ni, Cu, Zn and Co during the 1st sampling interval. Also, Co and Al was recorded as significantly enriched in sediment sampled from Keyhole and North-Eastern Section, respectively. Furthermore, significant Zn and Al enrichment was recorded at Derek's Exit. During the 2nd sampling interval significant Cu and Zn enrichment was recorded at all of the subterranean sites as well as the Wonderfontein Canal surface site. However, sediments collected at Kent's Entrance and Pristine Chamber were highly enriched with Cu. Furthermore, significant Ni enrichment was recorded at Kent's Entrance, Fault Passage and Cave Inflow Area and very high enrichment at Pristine Chamber. Cobalt was recorded as being significantly enriched in all of the collected sediments, except for Elevation Pit and Stoffels Dam, which presented minimal enrichment and Pristine Chamber and Cave Inflow Area which presented extreme and very high enrichment, respectively. Significant Al enrichment was recorded at Pristine Chamber, Elevation Pit, North-Eastern Section, Cave Inflow Area and Wonderfontein Canal, while significant Pb enrichment was recorded from all the sampling sites except Elevation Pit and Stoffels Dam. Very high enrichment of Pb was recorded at Pristine Chamber. During both sampling intervals extremely high enrichment of Cd, but especially U, was recorded from sediments collected from all of the sampling sites.

Although Cr, Fe, As and Mn presented minimal contamination in the sediment samples collected during both sampling intervals, the contamination levels of the remaining metals ranged from moderate to extreme. Moderate to heavy contamination was recorded for Ni, Cu, Zn, Co, Al and Pb in sediments collected from Kent's Entrance, Keyhole, Main Entrance (with the exception of Pb), Elevation Pit (with the exception of Co) and Derek's Exit (with the exception of Pb) during the 1st sampling interval and in sediments collected from Kent's Entrance (with the exception of Al), Keyhole, Main Entrance, Fault Passage, Pristine Chamber and the Cave Inflow Area during the 2nd sampling interval. Furthermore, moderate contamination of Cu, Co and Al was recorded at Pristine Chamber, while moderate contamination of Zn, Co and Al was recorded at North-Eastern Section during the 1st sampling interval. Yet, heavy to extreme Co contamination was recorded at Elevation Pit during the same sampling interval. During the 2nd sampling interval, moderate Al contamination was recorded at Elevation Pit and North-Eastern Section. Extreme contamination of Cd and U was recorded at all of the sites, except at Stoffels Dam (2nd sampling interval) where Cd contamination was classified as heavy.

The mean I_{geo} values of the sediments (Figures 3.3 a, b) presented substantially higher contamination levels than the soils (Figures 3.2 a, b). During the 1st sampling interval the mean I_{geo} values for Ni, Cu, Zn and Al were recorded as moderately contaminated, while the mean I_{geo} value for Co represented moderate to heavy contamination. Also, higher variability was observed in Ni, Cu, Zn and Co. During the 2nd sampling interval mean I_{geo} values for Cu, Zn, Co and Pb were classified as moderately to heavily contaminated with higher variability observed in Co and Pb contamination levels. Furthermore, the mean I_{geo} value for Al was again classified as moderately contaminated, however, presented minimal variability with a range of only 0.83. The biggest range, when considering only the above named metals, was recorded for Co (4.86) during the 1st sampling interval and both Co (4.48) and Pb (4.48) during the 2nd sampling interval. However, the mean I_{geo} values for Cd and U were considerably higher, respectively calculated as 7.97 and 13.14 during the 1st sampling interval and 7.68 and 13.84 during the 2nd sampling interval, all of which represent extreme contamination. Substantial variability was recorded for U especially during the 2nd sampling interval with a range of 8.01. Also, skewness was observed with most of the metal I_{geo} values.

Table 3.5: Enrichment factors (EF) and geoaccumulation index (I_{geo}) values for sediment samples collected during the 1st (April 2013) and 2nd (September 2013) sampling intervals. Colour coding, as presented in Table 3.3, were used to identify specific enrichment and contamination classes as defined by Loska *et al.* (2004).

	Cr	Ni	Cu	Zn	Co	Fe	Al	As	Mn	Cd	Pb	U	Cr	Ni	Cu	Zn	Co	Fe	Al	As	Mn	Cd	Pb	U
	EF												I_{geo}											
1st Sampling Interval																								
Kent’s Entrance	0.32	5.93	6.11	7.11	14.51	1.15	3.33	0.64	0.94	481.70	3.43	14723.69	-1.99	2.23	2.27	2.49	3.52	-0.14	1.39	-0.98	-0.43	8.57	1.43	13.50
Keyhole	0.20	2.65	3.74	3.32	6.62	0.84	2.27	0.26	1.20	272.90	2.43	6880.41	-1.99	1.71	2.21	2.03	3.03	0.05	1.48	-1.64	0.57	8.40	1.59	13.05
Main Entrance	0.48	7.33	7.22	7.82	12.76	1.01	4.84	0.55	0.09	453.22	1.52	23672.07	-0.96	2.96	2.94	3.06	3.76	0.10	2.36	-0.79	-3.32	8.91	0.69	14.62
Pristine Chamber	0.20	1.56	2.43	1.33	2.57	0.93	2.65	0.35	0.55	132.75	1.54	4392.73	-2.29	0.66	1.30	0.43	1.38	-0.09	1.42	-1.49	-0.84	7.07	0.64	12.12
Elevation Pit	0.55	6.34	10.36	12.83	15.96	1.25	4.59	0.81	0.13	649.71	2.44	23768.82	-0.59	2.94	3.65	3.95	4.27	0.59	2.47	-0.03	-2.70	9.62	1.56	14.81
NE Section	3.50	5.95	7.75	8.62	10.68	1.87	16.52	0.56	0.15	578.05	3.25	27184.49	-0.27	0.49	0.87	1.03	1.34	-1.17	1.97	-2.92	-4.85	7.10	-0.38	12.65
Cave Inflow Area	0.72	1.21	1.82	2.34	2.29	0.86	4.64	0.31	0.05	271.19	1.37	11405.55	-2.26	-1.51	-0.92	-0.56	-0.59	-2.00	0.43	-3.47	-6.21	6.30	-1.34	11.69
Derek’s Exit	0.85	4.75	4.55	6.29	5.00	1.16	6.48	0.35	0.51	432.11	1.66	12868.88	-1.16	1.33	1.27	1.74	1.41	-0.70	1.78	-2.42	-1.88	7.84	-0.19	12.74
2nd Sampling Interval																								
Kent’s Entrance	0.54	5.51	24.21	9.47	16.57	1.50	4.44	1.46	0.66	545.59	15.55	22414.64	-2.10	1.24	3.38	2.02	2.83	-0.63	0.93	-0.68	-1.83	7.87	2.74	13.23
Keyhole	0.31	3.72	7.42	6.14	9.05	1.28	3.46	0.58	1.19	360.61	8.49	19592.79	-2.47	1.12	2.11	1.84	2.40	-0.42	1.01	-1.56	-0.53	7.72	2.31	13.48
Main Entrance	0.31	3.45	6.51	5.60	6.86	0.98	3.95	0.53	0.13	283.10	6.43	19587.85	-2.03	1.45	2.37	2.15	2.45	-0.36	1.65	-1.26	-3.28	7.81	2.35	13.93
Fault Passage	0.62	6.78	11.17	14.47	19.44	1.73	4.94	1.21	0.31	726.82	18.23	74865.76	-1.45	2.00	2.72	3.09	3.52	0.03	1.54	-0.49	-2.46	8.75	3.43	15.43
Pristine Chamber	1.35	20.21	31.55	15.49	40.71	3.46	11.65	4.17	0.63	754.26	38.18	789079.77	-1.35	2.56	3.20	2.17	3.57	0.01	1.76	0.28	-2.45	7.78	3.48	17.81
Elevation Pit	0.48	0.55	6.70	19.26	1.77	1.87	6.90	0.82	0.53	473.10	1.66	2950.38	-2.80	-2.59	1.01	2.54	-0.91	-0.83	1.06	-2.01	-2.63	7.16	-1.00	9.80
NE Section	2.05	4.42	6.84	7.87	7.62	1.76	14.75	0.71	0.22	402.48	7.15	31106.89	-1.11	0.00	0.63	0.83	0.78	-1.33	1.74	-2.64	-4.35	6.51	0.69	12.78
Cave Inflow Area	0.74	7.95	18.65	15.55	20.81	1.82	6.59	1.87	0.19	770.33	19.52	63215.08	-2.13	1.30	2.53	2.27	2.69	-0.83	1.03	-0.79	-4.08	7.90	2.60	14.26
Wonderf. Canal	0.96	3.64	6.38	6.47	7.37	0.95	6.51	0.90	0.04	385.94	6.91	28968.74	-2.34	-0.41	0.40	0.42	0.60	-2.36	0.43	-2.44	-7.09	6.32	0.51	12.55
Stoffels Dam	0.69	0.95	1.43	1.53	2.26	1.37	4.56	0.50	0.01	101.24	2.12	30227.46	-3.21	-2.75	-2.15	-2.05	-1.49	-2.21	-0.48	-3.68	-9.04	3.99	-1.58	12.22

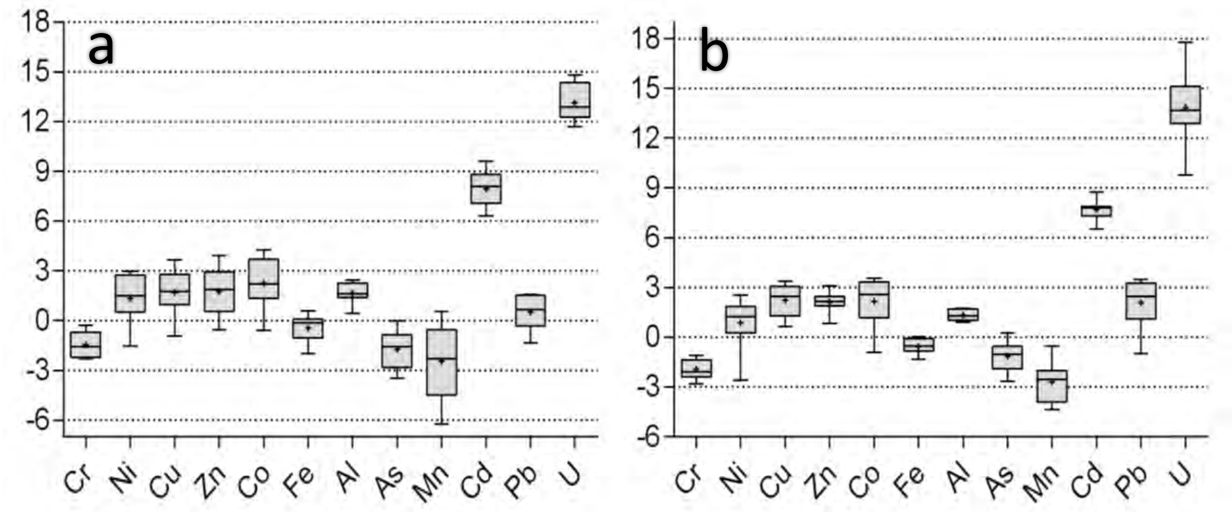

Figure 3.3: Box-and-whisker plots illustrate the variability and skewness of mean I_{geo} values calculated for sediment samples collected during the a) 1st (April 2013) and b) 2nd (September 2013) sampling intervals from the Wonderfontein Cave.

3.2.2.3 Relationships between metal enrichment and characteristics of substrate samples collected from the Wonderfontein Cave and Spruit

An ordination diagram (Figure 3.4), created as a PCA, illustrates the relationships that existed between metal enrichment, substrate characteristics (particle size distribution and TOC) and the respective substrate (soil and sediment) samples collected from the Wonderfontein Cave and Spruit during the 1st and 2nd sampling intervals. The eigenvalues for axis 1 and 2 are 0.89 and 0.04, respectively, while the cumulative percentage of the explained variance was calculated as 89.6 % and 93.5 % for the same axes. The PCA revealed that there existed a positive relationship between many of the sediment samples (collected during both sampling intervals) and Fe, Zn, Al, Ni, Pb, Cu, Co, Cd and U enrichment, as well as clay, silt and TOC percentages. The same sediment samples presented a negative relationship to especially Mn, but also Cr and As enrichment. However, a few sediment samples collected from Keyhole, Pristine Chamber and Cave Inflow Area during the 1st sampling interval and from Stoffels Dam during the 2nd sampling interval, presented a negative relation to metal enrichment.

On the contrary, most of the soil samples presented a positive relation to only Mn and Cr enrichment, while soil samples collected from North-Eastern Section during both sampling intervals and from Elevation Pit during the 2nd sampling interval, presented a strong positive relation to Cr and As enrichment. Most of the soil samples, however, presented a negative relation to the enrichment of the remaining metals.

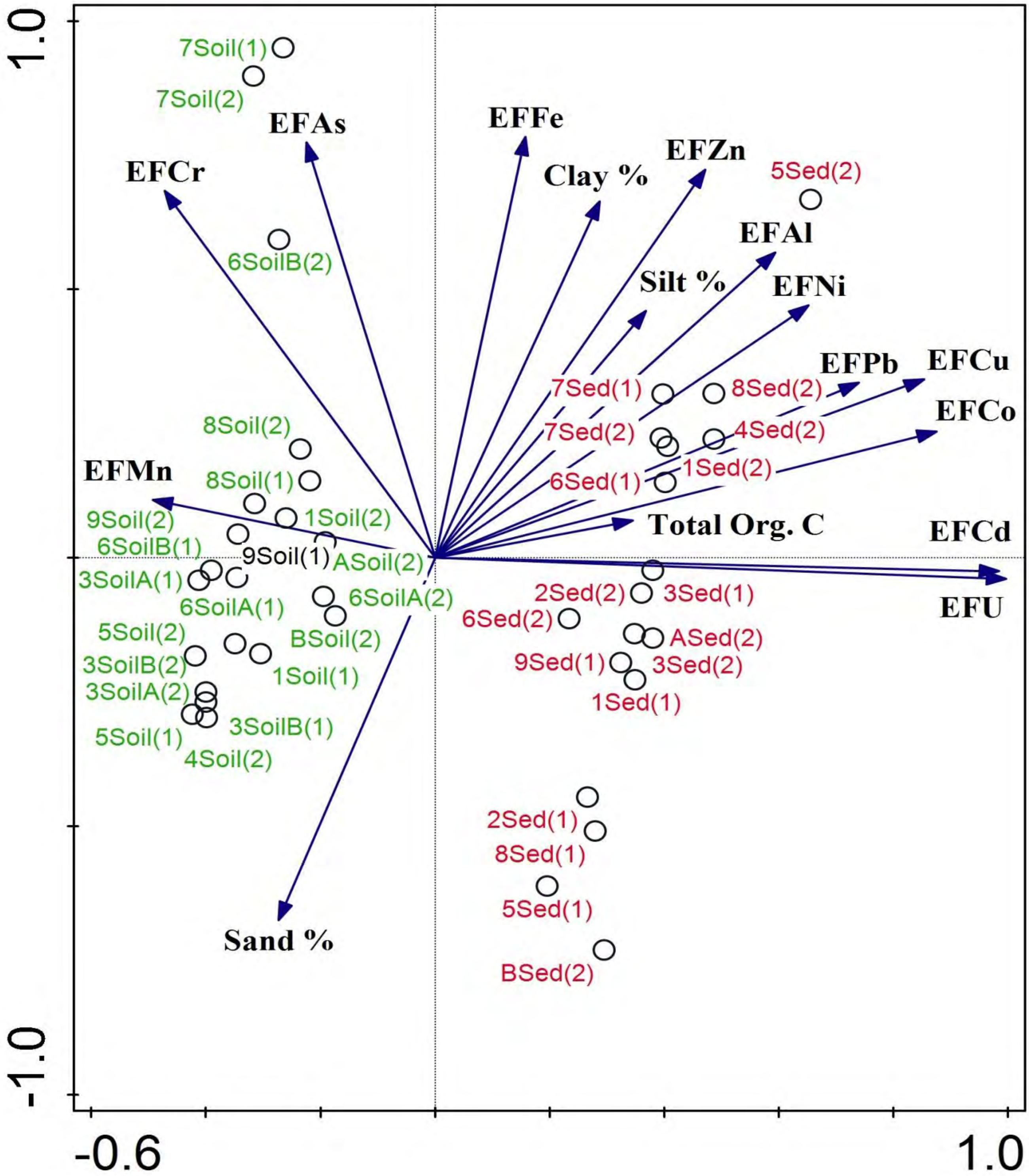

Figure 3.4: A principal component analysis (PCA) illustrates the relationships that existed between metal enrichment factors (EF), substrate characteristics (particle size distribution and total organic carbon) and the respective substrate (soil and sediment) samples collected from the Wonderfontein Cave and Spruit during the 1st (April 2013) and 2nd (September 2013) sampling intervals. The ordination explained 89.6 % variance on the first axis and 93.5 % variation on the second axis. The number (either 1 or 2) in parenthesis is representative of the 1st and 2nd sampling intervals, respectively. Also, for illustrative purposes, each site has been assigned to either a numeric number or letter as follows: 1 = Kent's Entrance; 2 = Keyhole; 3 = Main Entrance; 4 = Fault Passage; 5 = Pristine Chamber; 6 = Elevation Pit; 7 = North-Eastern Section; 8 = Cave inflow Area; 9 = Derek's Exit; A = Wonderfontein Canal; B = Stoffels Dam.

Furthermore, the PCA revealed that minimal temporal variation in metal enrichment existed in soil samples between the respective sampling intervals. In contrast, substantial temporal variation was presented in metal enrichment between the respective sampling intervals for most of the sediment samples. The only sediment samples that presented minimal temporal variation were collected from North-Eastern Section.

3.2.3 Biological significance of anthropogenic metal enrichment of water, soils and sediments

The concentrations of metals (see Appendix A) in water, soil and sediment samples collected from the Wonderfontein Cave and Spruit were compared against environmental quality guidelines in order to assess the toxicity hazard potential of the subterranean abiotic environment. For water comparisons, the Canadian Water Quality Guidelines (CWGQ) for the Protection of Aquatic Life (CCME, 2014), as well as the South African Water Quality Guidelines for Aquatic Ecosystems (DWAF, 1996) were used. For soil comparisons, the Canadian Soil Quality Guidelines (CSQG) for the Protection of Environmental Health (CCME, 2014), as well as the South African National Norms and Standards for the Remediation of Contaminated Land and Soil Quality (DEA, 2014), were used. The Canadian Environmental Quality Guidelines (CEQG) for water and soil, as listed above, were also used in this study as these provide a comprehensive list of pollutant threshold levels, are up-to-date, globally recognized, well substantiated and allow uniform comparisons.

Metal concentrations in sediments were compared to consensus-based sediment quality guidelines (SQG) proposed by MacDonald *et al.* (2000). Furthermore, mean probable effect concentration (PEC) quotients were calculated, which provides the means of taking into account the threat posed by a mixture of chemicals (metals) associated with a single sediment sample (MacDonald *et al.*, 2000; Long *et al.*, 2006). This index also provides a single numerical value that enables easy comparison between different sites and/or samples.

3.2.3.1 Water quality guidelines as a measure of toxicity hazard potential in Wonderfontein Cave water

All the necessary normalization steps were applied as required by the respective water quality guidelines, which includes considering the pH and hardness of each water sample. While the CWQG provide a single guideline value, the South African Water Quality Guidelines provide chronic effect values (CEV), as well as acute effect values (AEV), for the different metals. All three these water quality guideline criteria were considered, however, it should be noted that only the metal values that exceeded at least one of the water quality guideline criteria were reported in this chapter. Furthermore, for illustrative purposes, any water quality guideline criteria (value) that well exceeded the highest reported metal concentration per sample were omitted from the respective figure.

Metals that exceeded the CWQG and/or South African Water Quality Guidelines included Cr, Cu, Al, As, Pb and U (Figure 3.5). The CWQG for Cr (Figure 3.5 a) was only exceeded in a single water sample collected at Kent's Entrance during the 1st sampling interval. Although Cr was also measured at Keyhole and Main Entrance during the same sampling interval, negligible concentrations were measured at the remaining sites and during the 2nd sampling interval. While both Cu and Al concentrations (Figures 3.5 b, c) exceeded the CWQG and CEV in water samples collected from Fault Passage during the 2nd sampling interval, the same metals exceeded all the water quality guideline criteria in water samples collected from the Cave Inflow Area during the same sampling interval. Also, the concentration of Cu at Kent's Entrance exceeded the CWQG and CEV during the 1st sampling interval, while the concentration of Al at the Wonderfontein Canal and Stoffels Dam exceeded the CEV during the 2nd sampling interval. Aluminium concentrations associated with the latter sampling site also exceeded the CWQG during the 2nd sampling interval.

In contrast, As (Figure 3.5 d) and U (Figure 3.5 f) concentrations exceeded the CWQG in all of the water samples collected during both sampling intervals. Arsenic metal concentrations also exceeded the CEV in all of the water samples collected during the 2nd sampling interval, except for at Stoffels Dam. While Pb (Figure 3.5 e) concentrations exceed the CEV in all of the collected water samples during the 1st sampling interval, the same samples complied with the CWQG.

Although minimal variation was observed in metal concentrations between the sampling sites during the same sampling interval, in most cases higher metal concentrations were observed during the 2nd sampling interval. Lead presented the only scenario where substantially higher concentrations were observed in all of the water samples collected during the 1st sampling interval. When considering only the sites directly associated with the Wonderfontein Cave, As concentrations ranged between 7.7 and 8.9 µg/l during the 1st sampling interval and between 19.7 and 24 µg/l during the 2nd sampling interval. Uranium concentrations ranged between 27.1 and 32.2 µg/l during the 1st sampling interval and between 54 and 60.5 µg/l during the 2nd sampling interval. In contrast, the highest concentration of Pb during the 2nd sampling interval was measured at only 0.1 µg/l, while all of the measured concentrations exceeded 5 µg/l during the 1st sampling interval. These findings clearly indicate temporal variation in the concentrations of the studied metals.

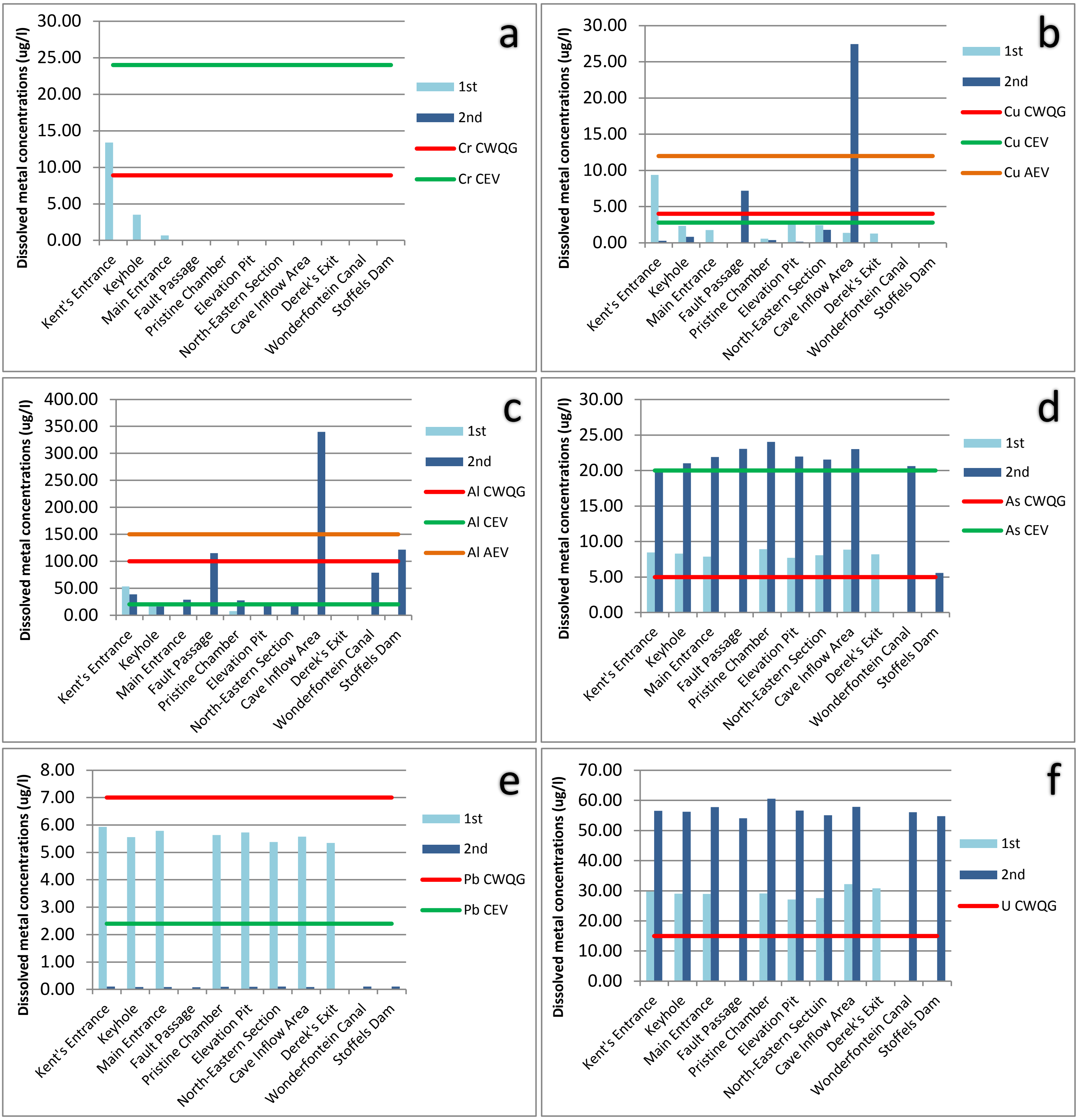

Figure 3.5: The concentrations of selected metals including a) Cr, b) Cu, c) Al, d) As, e) Pb and f) U in water samples collected from the Wonderfontein Cave and Spruit during the 1st (April 2013) and 2nd (September 2013) sampling intervals are provided. Also, the metal guideline values, as stated in the Canadian Water Quality Guidelines (CWGQ) for the Protection of Aquatic Life as well as the chronic effect (CEV) and acute effect values (AEV), as stated in the South African Water Quality Guidelines for Aquatic Ecosystems, are indicated where applicable.

3.2.3.2 Soil quality guidelines as a measure of toxicity hazard potential in the Wonderfontein Cave soils

As with the water quality guidelines, only the metals that exceeded the Canadian Soil Quality Guidelines (CSQG) for the Protection of Environmental Health (CCME, 2014) and/or the South African metal soil screen values (SSV) (DEA, 2014), as published by the South African Department of Environmental Affairs, are reported. Furthermore, for illustrative purposes, any soil quality guideline criteria (values) that well exceeded the highest reported metal concentration were omitted from the respective figure.

Metals that exceeded the CSQG and/or SSV included Cr, Ni, Cu, Co As, Mn and Pb as illustrated in Figures 3.6 and 3.7. These metals presented some variability between both the sampling sites and/or intervals. Chromium (Figure 3.6 a) concentrations well exceeded CSQG in soil samples collected during the 1st sampling interval at Main Entrance (soil sample A), during the 2nd sampling interval from Elevation Pit (soil sample B) and during both sampling intervals at Main Entrance (soil sample b), North-Eastern Section and Cave Inflow Area. Soils collected at North-Eastern Section during the 2nd sampling interval measured the highest Cr concentration of 180.59 mg/kg, while the CSQG for Cr is only 64 mg/kg. When considering the Ni (Figure 3.6 b) concentrations, most samples exceeded the CSQG, however, only soils collected from Kent’s Entrance during both sampling intervals and from Main Entrance (soil sample B) and Elevation Pit (soil sample A) during the 2nd sampling interval, exceeded the SSV. Soil samples collected from Elevation Pit (soil sample B) and Stoffels Dam complied with all of the Ni soil quality guideline criteria.

While Cu (Figure 3.6 c) concentrations in soil samples collected from most of the sampling sites, except for at Wonderfontein Canal, exceeded the SSV, only the soil sample collected at Kent’s Entrance during the 2nd sampling interval did not comply with the CSQG. Similarly, Co (Figure 3.6 d) concentrations were well below the CSQG during both sampling intervals, except for soil collected at Kent’s Entrance during the 2nd sampling interval. Although none of the remaining metals (As, Mn and Pb) exceeded the CSQG in soil samples collected from the Wonderfontein Cave and Spruit during both sampling intervals, the concentrations of these metals did exceed the SSV. While only Mn (Figure 3.7 b) concentrations in soil samples collected from the Cave Inflow Area (1st and 2nd sampling intervals) complied with the SSV, most of the As (Figure 3.7 a) concentrations in soil samples were below the SSV during the 1st sampling interval. Generally, the metal concentrations associated with the soil samples collected from the Wonderfontein Spruit were lower than those associated with the Wonderfontein Cave. Also, high variability and thus temporal variation was evident with higher concentrations mostly recorded during the 2nd sampling interval.

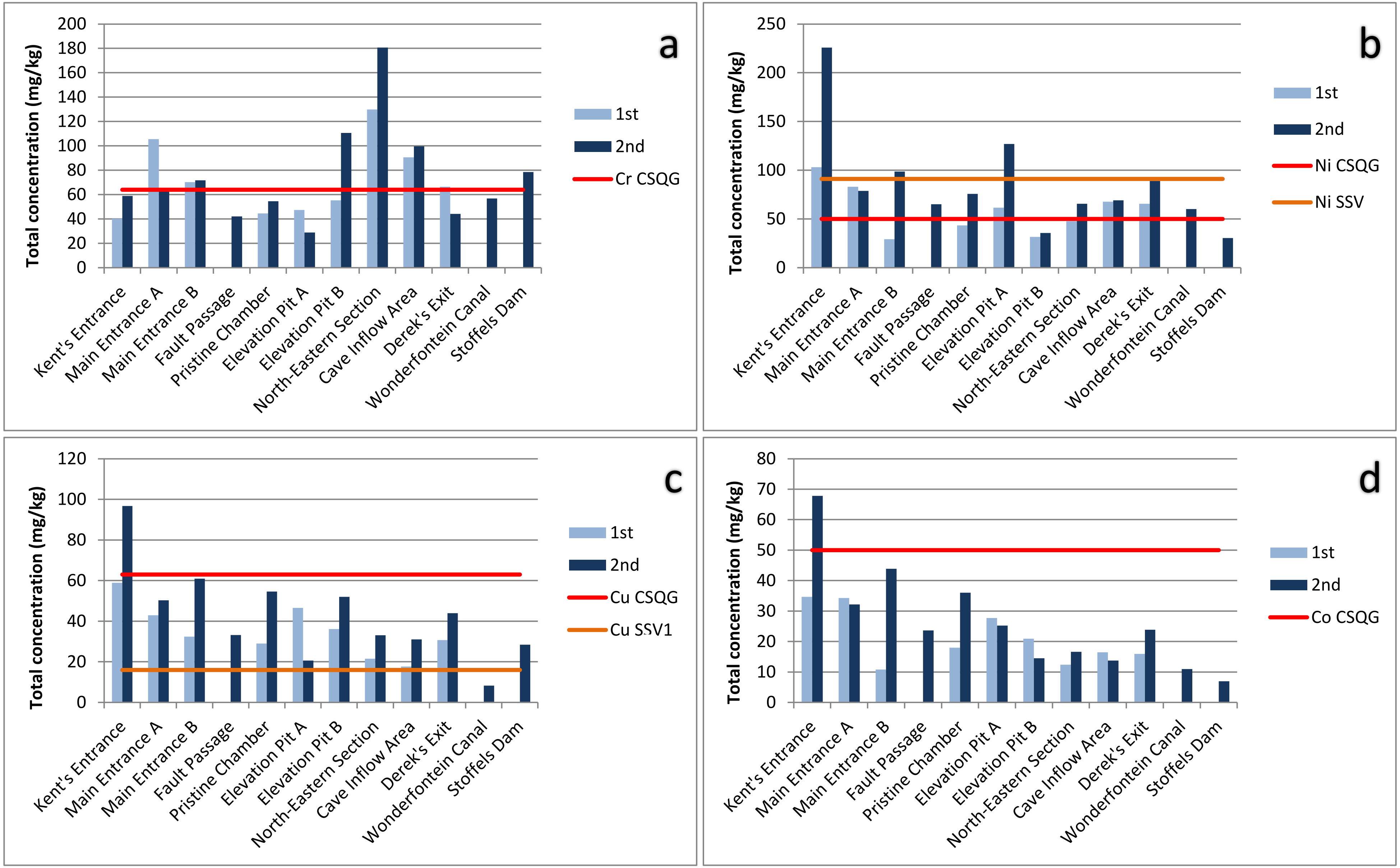

Figure 3.6: The concentration of selected metals including a) Cr, b) Ni, c) Cu and d) Co in soil samples collected from the Wonderfontein Cave and Spruit during the 1st (April 2013) and 2nd (September 2013) sampling intervals are provided. Also, the metal guideline values, as stated in the Canadian Soil Quality Guidelines (CSQG) for the Protection of Environmental Health as well as the soil screen values (SSV), as published by the South African Department of Environmental Affairs, are indicated where applicable.

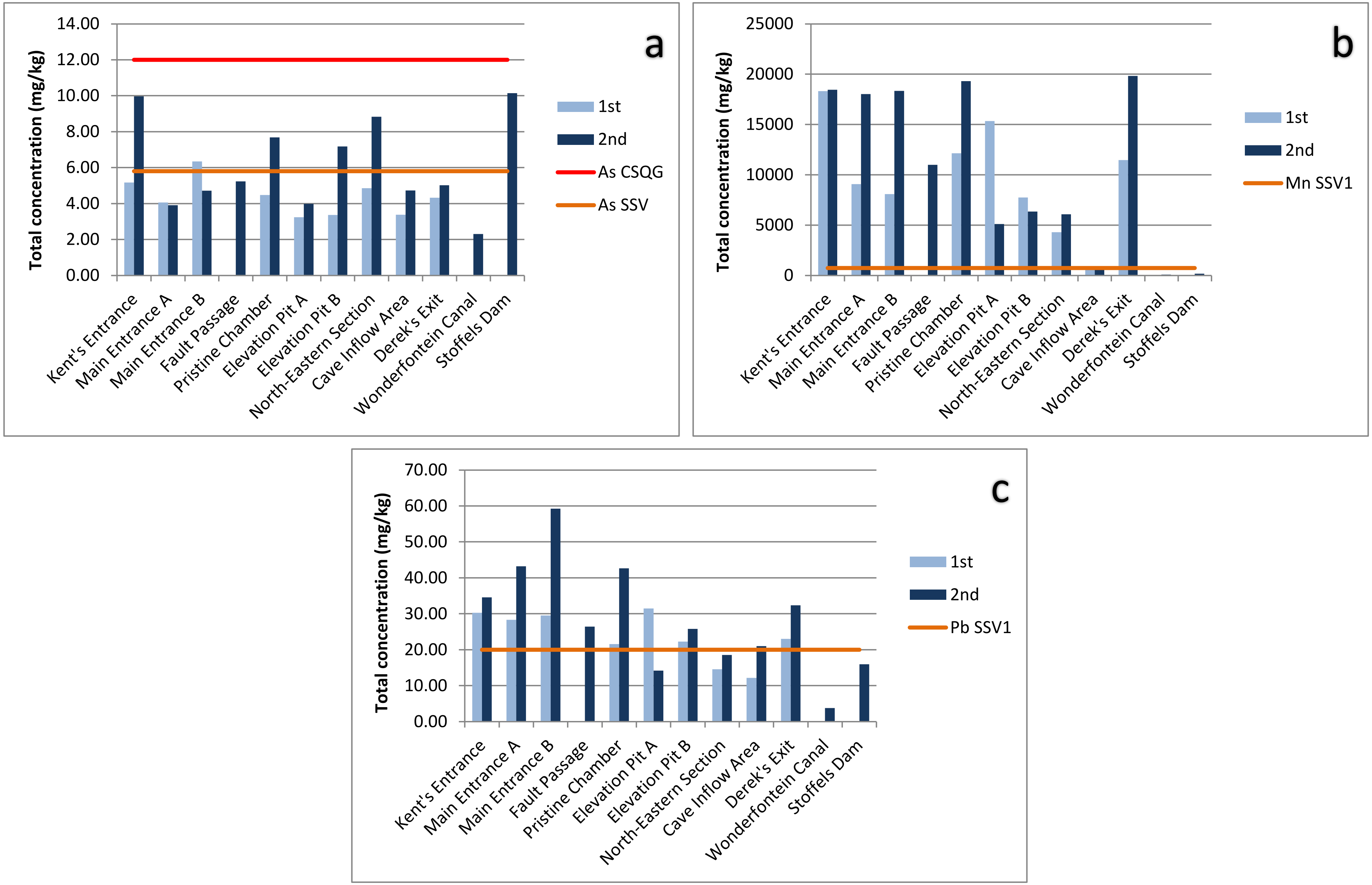

Figure 3.7: The concentration of selected metals including a) As, b) Mn and c) Pb in soil samples collected from the Wonderfontein Cave and Spruit during the 1st (April 2013) and 2nd (September 2013) sampling intervals are provided. Also, the metal guideline values, as stated in the Canadian Soil Quality Guidelines (CSQG) for the Protection of Environmental Health as well as the soil screen values (SSV), as published by the South African Department of Environmental Affairs, are indicated where applicable.

3.2.3.3 Consensus-based sediment quality guidelines as a measure of toxicity hazard potential in the Wonderfontein Cave sediments

Consensus-based Sediment Quality Guidelines (SQG) proposed by MacDonald *et al.* (2000) have been widely applied in environmental health assessments as it provide both threshold effect concentrations (TEC) and probable effect concentrations (PEC) for metal and organic pollutants (MacDonald *et al.*, 2000; McCready *et al.*, 2006; Farkas *et al.*, 2007). Figure 3.8 illustrates that Cr, Ni, Cu, Zn, As and Pb concentrations in sediment samples collected from the Wonderfontein Cave and Spruit exceeded the respective TEC and/or PEC values. Unfortunately, MacDonald *et al.* (2000) did not provide any guideline values for Al and U concentrations in sediment samples.

Figure 3.8 further illustrates the variation observed in metal concentrations between different sampling sites, as well as between the two sampling intervals. As previously mentioned, the metals associated with the soils (Figures 3.6, 3.7) generally presented higher concentrations during the 2nd sampling interval. However, no such order is associated with the sediments as samples collected during both intervals presented high metal concentrations. Chromium (Figure 3.8 a) concentrations in samples collected at Main Entrance, Elevation Pit, North-Eastern Section and Derek's Exit during the 1st sampling interval and Fault Passage, Pristine Chamber and North-Eastern Section during the 2nd sampling interval, well exceeded the PEC value of 111 mg/kg. Sediments sampled at Elevation Pit and North-Eastern Section during the 1st sampling interval presented the highest Cr concentrations of 265.7 and 331.7 mg/kg, respectively. However, Cr concentrations in sediments sampled from the Wonderfontein Canal and Stoffels Dam did not exceed the PEC value.

Nickel (Figure 3.8 b) presented exceptionally high concentrations that well exceeded the PEC value of 49 mg/kg. The only sediment sample that did not exceed the PEC value was collected at Stoffels Dam during the 2nd sampling interval. Sediments collected at Main Entrance and Elevation Pit during the 1st sampling interval presented Ni concentrations of 1850 and 1818.2 mg/kg, respectively, while Ni concentrations in sediments collected at Pristine Chamber during the 2nd sampling interval neared 1400 mg/kg. However, concentrations were highly variable as only 374.2 mg/kg Ni was present in sediments collected during the 1st sampling interval from the same site. Similarly, Cu (Figure 3.8 c) concentrations of 324.9 and 442.5 mg/kg were present in sediment collected at Pristine Chamber (2nd sampling interval) and Elevation Pit (1st sampling interval), respectively, which both exceeded the PEC value of 149 mg/kg. High Cu concentrations were also recorded at Main Entrance (271.5 mg/kg) during the 1st sampling interval and Kent's Entrance (367 mg/kg), Fault Passage (233.18 mg/kg) and Cave Inflow Area (204.2 mg/kg) during the 2nd sampling interval. Zinc (Figure 3.8 d), As (Figure 3.8 e) and Pb (Figure 3.8 f) were present in lower concentrations as only sediment collected at Elevation Pit during the 1st sampling interval exceeded the respective Zn (456 mg/kg) and As PEC (33 mg/kg) values. Sediment collected at Pristine Chamber during the 2nd sampling interval also exceeded the PEC value for As. Although Pb concentrations in some sediments, especially those collected during the 1st sampling interval, well exceeded the TEC value of 36 mg/kg, none exceeded the PEC value of 128 mg/kg. It should also be noted that sediments collected from the two surface sites, Wonderfontein Canal and Stoffels Dam, rarely exceeded PEC and even TEC values.

3.2.3.4 Mean probable effect concentration quotients as a measure of the biological significance of a mixture of metals

Mean PEC quotients (Table 3.6) were used in order to allow for easy comparison between the different sediment samples as the index provides a single numeric value that reflects the biological significance of metal mixtures (MacDonald *et al*., 2000). According to the latter authors this index is useful for predicting whether a sediment sample is toxic or non-toxic. Mean PEC quotients with a value less than 0.5 are considered to be non-toxic, while sediments with values larger than 0.5 are regarded as toxic. The threshold value for mean PEC quotients is thus 0.5.

Based on the mean PEC quotients values presented in Table 3.6, all of the sediment samples were toxic to biota with the exception of sediments collected at Elevation Pit (2nd sampling interval), Cave Inflow Area (1st sampling interval) and Stoffels Dam (2nd sampling interval). Generally, the sediments sampled during the 1st sampling interval presented higher toxicity than those sampled during the 2nd sampling interval, with the exception of sediments sampled at Pristine Chamber and Cave Inflow Area.

Table 3.6: Mean PEC quotients provide insight into the biological significance of a mixture of metals by predicting whether a sediment sample is toxic ($x > 0.5$) or non-toxic ($x < 0.5$).

	Kent's Entrance	**Keyhole**	**Main Entrance**	**Fault Passage**	**Pristine Chamber**	**Elevation Pit**	**North-Eastern Section**	**Cave Inflow Area**	**Derek's Exit**	**Wonderfontein Canal**	**Stoffels Dam**
1st	3.35	2.44	5.48	-	1.25	5.82	1.36	0.36	1.92	-	-
2nd	2.05	1.69	2.10	3.10	4.35	0.37	0.93	1.97	-	0.65	0.17

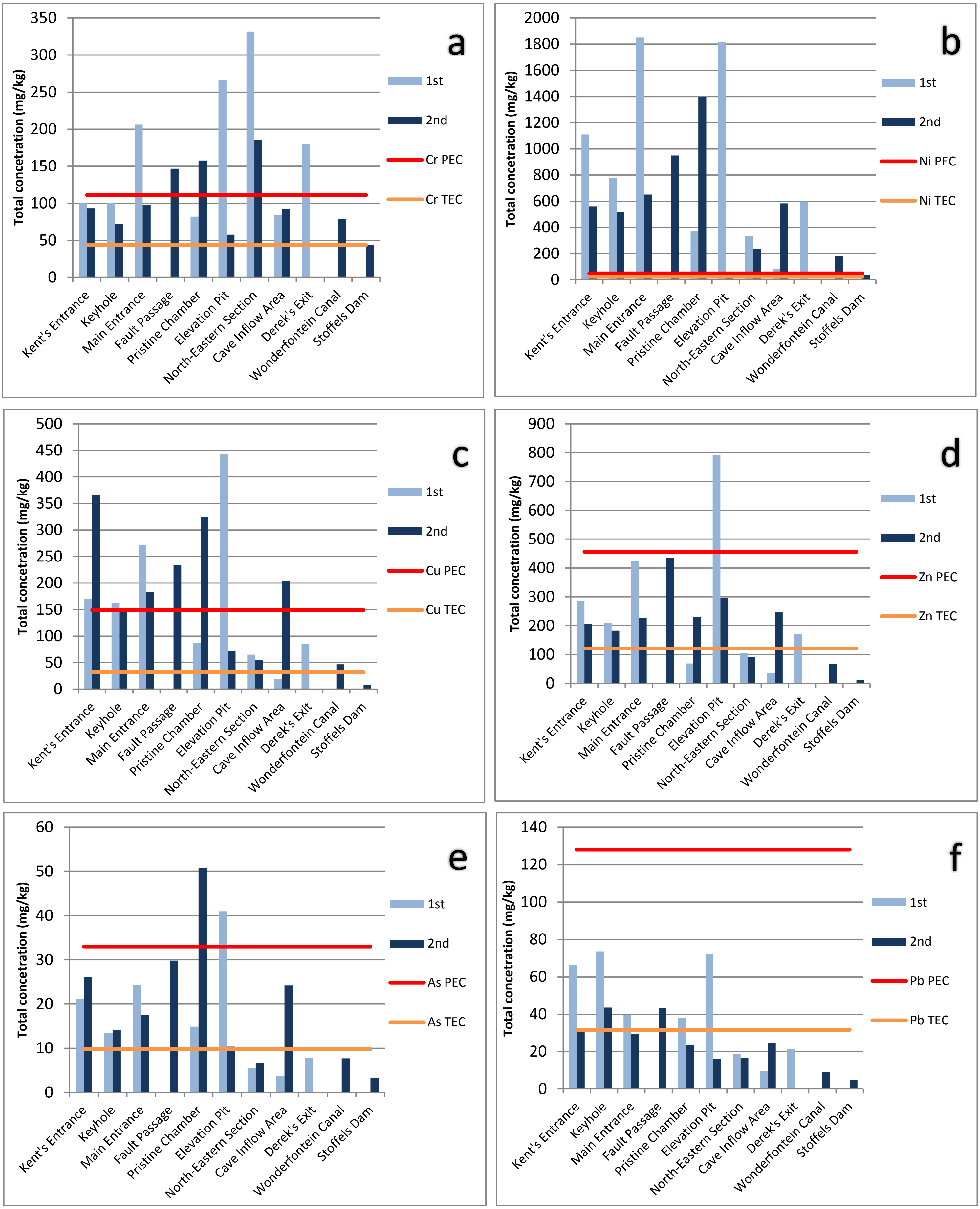

Figure 3.8: The concentration of selected metals including a) Cr, b) Ni, c) Cu, d) Zn, e) As and f) Pb in sediment samples collected from the Wonderfontein Cave and Spruit during the 1st (April 2013) and 2nd (September 2013) sampling intervals are provided. Also, the threshold effect concentrations (TEC) and probable effect concentrations (PEC), as stated in MacDonald *et al*. (2000), are indicated where applicable.

3.3 Discussion

3.3.1 The source of pollution and the bioavailability of metals

It is generally accepted that substrate (water, soil and sediment) characteristics play an important role in the concentration and bioavailability of metals (Reuther, 1986). The Piper diagram analysis, according to criteria provided by Hounslow (1995), revealed that all of the collected water samples presented hardness with enriched SO_4^{2-} and Cl^- concentrations. Akcil and Koldas (2006) stated that pollution sourced from mines may result in high concentrations of especially SO_4^{2-} anions being present in the associated environment. Furthermore, a study by Campaner *et al.* (2014) presented similar Piper diagram results, which were indicative of SO_4^{2-}enrichment in stream waters associated with mine effluent. Also, acid mine drainage (AMD) is commonly associated with mining activities and low pH values ranging between two and four (Akcil & Koldas, 2006). Although the Wonderfontein Spruit is likely subjected to AMD as a result of waste water effluent, tailings runoff and decanting shafts (Coetzee *et al.*, 2006; Hamman & Van Rensburg, 2012), the pH of the water samples collected from the Wonderfontein Spruit and Cave ranged between 8.39 and 9.15 and were classified as very hard. This is not unusual since mine effluent is commonly treated with lime and other chemicals in order to neutralize the pH (Akcil & Koldas, 2006), while the karst (carbonate) landscape also serves as a natural buffer (Webb & Sasowsky, 1994).

The water quality parameters from both sampling intervals, as presented in the Piper diagrams, were unexpectedly uniform. Arguably, the different hydrological settings associated with the Wonderfontein Cave and Spruit would alter the chemistry of the water. Nevertheless, the similar character of water collected from both the Wonderfontein Cave and Spruit are likely the result of high interconnectivity, not only between the surface and subterranean environments, but also within the cave system itself. It should also be considered that the passages accessible by human are not necessarily the only pathways by which water is transported within the cave system. As karst landscapes are generally highly interconnected at multiple depths (Ford & Williams, 2007), water may be transported below the main cave development level. However, in order to truly assess the effect of the hydrological setting on the water quality parameters of the Wonderfontein Cave, further investigations are required that were not included in the aims of this study.

The total metal concentrations associated with the collected soils and sediments were determined in order to compare the results to the respective environmental quality guidelines. However, total concentrations provide little information on the bioavailability of metals (Traina & Laperche, 1999). Subsequently, factors influencing the bioavailability of metals should be considered, which include the pH, chemical complexation with organic and inorganic ligands and particle size distribution (Violante *et al.*, 2010). According to Abdu (2010) lower pH values increase the mobility of positively charged metals. Although most of the pH values remained above seven, the few samples that measured low pH values likely presented higher bioavailable concentrations of most of the studied metals. The low pH values associated with soils collected from Elevation Pit are, however, not unusual as bat guano is known to present low pH values (Bowell *et al.*, 1996) and was found in the vicinity of the soils. A study by Akan *et al.* (2013) concluded that an increase in metal mobility can also be related to an increase in TOC. One reason for this is the ability of organic matter to chelate with metals (Chesworth, 2008). Subsequently, the relatively high TOC observed in some sediments, especially those associated with the Main Entrance, Elevation Pit and Cave Inflow Area sampling sites, may have increased the mobility of the metals as well as their bioavailability to benthic organisms (USEPA, 2002). Furthermore, the high TOC observed in soils associated with the Cave Inflow Area, Derek's Exit and Stoffels Dam may also have resulted in the increased bioavailability of metals to soil biota.

It is generally accepted that higher metal concentrations are associated with finer particles, rather than coarser fractions, as the former present a greater surface area and contain more clay and organic matter (Li *et al.*, 2014). It should also be considered that finer particles are easily re-suspended in and transported by the water column. Re-suspended particles, associated with high organic content and metal concentrations, thus pose an ecological risk as it can be transported with a change in environmental conditions as well as ingested by biota (USEPA, 2002). Although most of the sediment and soil samples collected from the Wonderfontein Cave and Spruit presented low clay content, most were associated with high silt percentages, while the fraction percentages less than 2 mm generally remained below 50 %. Nevertheless, if the Wonderfontein Cave was suddenly exposed to intense flooding and/or turbulent water flow, it is possible that metals chelated to particles may become re-suspended in the water column and available to some biota.

3.3.2 Enrichment of soils and sediments associated with the Wonderfontein Cave

Unfortunately, the contamination of cave soils and sediments by anthropogenically introduced metals has not been well studied. Furthermore, most studies that report on the contamination of cave environments focused on organic pollutants (Simon & Buikema Jr, 1997; Graening & Brown, 2003; Wood *et al.*, 2008). There are, however, a few studies that provide background information on metal pollution in especially cave sediments (Gutierrez *et al.*, 2004; Doughty & Johnson, 2012). A study by Doughty and Johnson (2012) conducted in the United States of America (USA) revealed that a cave system associated with an urban environment, compared to that of an undisturbed watershed, presented elevated levels of Zn, Mn and Pb. Furthermore, they provided evidence that lowered metal concentrations in more pristine sections of the cave allowed the survival of aquatic troglobites, otherwise absent in the polluted section. Another cave system, known as Sloan's Valley Cave (Kentucky, USA), was subjected to pollutants from a leaching landfill. The local community became actively involved in preventing further pollution, highlighting the importance of public opinion and participation in conservation activities (Hopper, 1996).

When considering the current study, some soil samples and especially those collected at Kent's Entrance, Elevation Pit, North-Eastern Section and the Cave Inflow Area, exhibited significant anthropogenic enrichment of mainly Cr, Zn, Al and U. High contamination levels were observed at the same sites for Zn, Al and U. However, understanding the mechanism behind the enrichment of only certain soils is rather challenging. Yet, it would seem that soils associated with sites such as Kent's Entrance and North-Eastern Section, which are closely associated with the water column, presented higher enrichment and contamination levels.

Anthropogenically enriched metals associated with the sediments included Ni, Cu, Zn, Co and Al, while Cd and U were extremely enriched. High contamination levels of the same elements were observed at all of the sites, except for at Stoffels Dam where only Cd and U contamination was reported. Yet, the exceptionally high Cd and U contamination levels is not entirely surprising as Coetzee *et al.* (2006) reported Cd and U concentrations associated with the Wonderfontein Spruit at 700 and 720 times the natural background values, respectively. The latter authors also identified Cr, Co, Ni, Cu and As as metals associated with the Wonderfontein Spruit that were occurring in concerningly high concentrations. It should be noted that the latter authors used the same backgournd values (Klerkskraal Dam) that were used in the current study.

The sediments collected from the Wonderfontein Cave and Spruit presented substantially higher metal enrichment levels than that of the soils, as was revealed by the PCA. This is most likely the result of the sediments being directly associated with the water column and thus the influx of polluted water. It should be taken into consideration that mining companies built a weir in 1969 that allowed the disposal of water pumped directly from the Carletonville mines into the Wonderfontein Cave (Kent *et al.*, 1978). Unfortunately, no record was kept of the water levels within the cave. Yet, the concentration of metals associated with the sediments collected from the cave may represent the aggregated concentrations and thus the result of anthropogenic enrichment over an unknown time period (Gutierrez *et al.*, 2004). The PCA further revealed that there existed substantial temporal variation of metal enrichment in the sediment samples and minimal in the soil samples. This further supports the theory that sediments presented higher enrichment and variability as a result of being directly associated with the water column (metal transport medium). The reason for the high metal enrichment variation observed in the sediment samples, however, remains unclear, but might hold relation to the change in flow regime and/or the hydrological setting of the Wonderfontein Cave.

The high variability of metal contamination levels within the Wonderfontein Cave soils and sediments is further illustrated by the mean I_{geo} box-and-whisker plots. According to Hill (2010) a change in the flow of water may facilitate the precipitation of suspended particles. It is thus suggested that once water enters the Wonderfontein Cave, the flow velocity and turbidity of the water decreases as it is now subjected to an expanded network of passages. This could then allow particles to precipitate in different sections of the cave system and possibly explain the higher contamination levels observed within the Wonderfontein Cave. Furthermore, the observed variation in TOC and particle size distribution may also contribute to the observed variation (Birch *et al.*, 2001; Newman & Watling, 2007). According to Birch *et al.* (2001) temporal variation in aquatic systems naturally occur over a range of scales, including hours (water flow), months (seasonal) and years (climate). Although water levels within the Wonderfontein Cave weren't actively monitored, lower levels were observed during the 2nd sampling interval, which is subject to the associated low flow period. And although variation occurred between the subterranean sampling sites, an increase in the enrichment of some metals, including Ni, Cu, Co, Al and Pb, was recorded in sediments from the 1st to the 2nd sampling interval. This arguably substantiates the theory that metal concentrations might have accumulated over many years of exposure to the influx of polluted mine water.

3.3.3 Toxicity hazard potential of water, soils and sediments associated with the Wonderfontein Cave

Also of importance is the toxicity hazard potential of water, soils and sediments associated with the Wonderfontein Cave. The high concentration of toxicants (metals), as observed in especially the sediments associated with the Wonderfontein Cave, may pose a severe threat to the ecosystem health of the subterranean environment. The metal concentrations in the collected water samples revealed, firstly, that especially As and U concentrations substantially exceeded the water quality guidelines and secondly, that the metal concentrations were, with the exception of Cr and Pb, higher during the 2nd sampling interval. It should, however, be noted that as a result of the dynamic nature of water chemistry, the metal concentrations only reflect the water chemistry at that point in time

(Newman & Watling, 2007). Nevertheless, the higher concentrations of most metals observed during the 2nd sampling interval may be the result of an increase in mine effluent and/or the reduced dilution effect as sampling occurred during the dry season (end of low flow period). According to DWAF (1996) the CEV portrays the metal concentration at which 5 % of the animal species associated with the aquatic community will experience chronic effects. Furthermore, continued exposure to this level of pollution could lead to death. If the CEV are exceeded, as was observed especially in Pb concentrations during the 1st sampling interval, the risk for acute toxic effects increases (DWAF, 1996). However, the high temporal variation observed between the two sampling intervals may indicate that the aquatic community are exposed to CEV levels for shorter time periods, the frequency of which remains unknown.

According to CCME (2014) As is equally toxic to fish species, such as the Rainbow trout, as to aquatic invertebrates including copepods and daphnids. Also, elevated U concentrations can affect aquatic invertebrates in a number of ways, however, the most significant include valve closure in bivalves. The latter adversely affects reproduction and can lead to higher mortality rates. Furthermore, some species of crustaceans are reportedly highly sensitive to U pollution (CCME, 2014), while Pb, even in low concentrations, in known to affect some fish species by inducing the formation of a mucous film over the gills and body, which could lead to suffocation and eventually death (DWAF, 1996).

When considering the metal concentrations in soil samples collected from the Wonderfontein Cave, Cr, Ni, Cu, As, Mn and Pb concentrations well exceeded soil quality guidelines. However, although it has been reported that Cr can accumulate in invertebrates, significant mortality rates of Oligochaeta (earthworm) species only occurred at much higher concentrations than what were recorded in the Wonderfontein Cave (CCME, 2014). Similarly, Oligochaeta LC50 values for Ni was only reported at substantially higher concentrations, however, lower Ni concentrations may still have an effect on the microbial community by reducing nitrification, mineralization and respiration rates. While Cu pollution can result in reduced cocoon production in some earthworm species, lethal As concentrations is soil environments were only reported from 100 mg/kg. Similarly, LC50 values for the earthworm species, *Eisenia fetida*, were much higher than what were observed in this study (CCME, 2014). Furthermore, a study conducted by Ekschmitt and Korthals (2006) on the effect of different metal concentrations on a broad spectrum of nematode genera (PPN and NPN) revealed that most nematodes presented a negative correlation to the enrichment of multiple metals including Ni and Cu.

The observed elevated levels of especially Cr, Ni, Cu above the consensus-based PEC in the sediments of the Wonderfontein Cave is particularly relevant. According to CCME

(2014), elevated concentrations of Cr, Cu, Zn and As can have deleterious effects on benthic organisms, which include decreased diversity and abundance, increased mortality rates as well as behavioural changes. Chromium toxicity in amphipods has been reported in concentrations as low as 95 mg/kg, while gastropods were less abundant in sediment with a Cu concentration of 52.2 mg/kg when compared to more pristine environments. Adverse effects, induced by toxic Zn concentrations, were reported in a variety of benthic taxa including Gastropoda, Amphipoda, Echinodermata and Annelida. Elevated Pb concentrations in sediments may result in increased mortality rates, a decrease in diversity and abundance of benthic organisms as well as abnormal development (CCME, 2014). Although amphipods are sensitive to high concentrations of Pb, the Wonderfontein Cave sediments presented concentrations well below the PEC of Pb. According to Madoni (2000) the toxicity of Ni in aquatic ecosystems in not well known, however, Wright and Welbourn (2002) states that elevated Ni concentrations may reduce growth and reproduction of invertebrates. The severity of the pollution threat posed by metal concentrations associated with the sediments of the Wonderfontein Cave is further substantiated as most of the sediments were classified as being toxic.

3.4 Conclusion

The hypotheses are accepted as evidence is provided that the Wonderfontein Cave is subjected to metal enrichment as a result of the influx of polluted water from the Wonderfontein Spruit. This poses a severe threat to biota residing within the Wonderfontein Cave as water, soil and sediment quality guidelines were exceeded by some metals, while most sediments were classified as being toxic. Also, the temporal variation in metal enrichment and contamination observed in especially the sediments is likely attributed to the complex hydrological system associated with the Wonderfontein Cave, as well as the substrate characteristics.

3.5 References

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CHAPTER 4

BENEFICIAL/NON-PARASITIC NEMATODES AS INDICATORS OF ECOSYSTEM HEALTH AND THEIR ASSOCIATION WITH METALS IN SOIL AND SEDIMENT FROM THE WONDERFONTEIN CAVE

4.1 Introduction

To avoid any confusion, all beneficial and/or non-parasitic nematodes will be referred to as NPN and not free-living nematodes. Nematodes (Phylum: Nematoda), also known as round- or eelworms, inhabit a diverse range of environments and represent 80 % of the world's metazoans (Platt, 1994; Ferris & Bongers, 2009). Although nematode pests may cause significant damage to crop yields, their beneficial counterparts fulfill a vital role in maintaining soil health. Neher (2001) stated that NPN do so by altering the metabolic activity of microbes, as well as the size of the prevailing microbial community. Thus, the rate of decomposition is partially controlled by nematodal activities. Furthermore, NPN indirectly allow nutrient mineralization (nitrogen cycling) by feeding on soil microbes, immobilizing nitrogen in biomass and defecating ammonium. As a result of their abundance and the ecosystem services they provide, NPN are considered to be the most important bioindicator among soil fauna (Neher, 2001; Ekschmitt & Korthals, 2006; Ferris, 2010).

The use of NPN as bioindicators of pollution and especially indicators of toxic-metal contamination has been well documented (Ekschmitt & Korthals, 2006; Pen-Mouratov *et al.*, 2008; Šalamún *et al.*, 2012). The latter authors agree that metal contamination has a severe effect on the community structure of NPN. Thus, general community and nematode specific indices can be applied as an alternative to common chemical approaches in determining the pollution effect. However, these indices have most likely never before been tested in cave habitats, nor is there information available on the ecosystem function of NPN that prevail in cave environments. Durand *et al.* (2012) was the first to include nematode assemblages (PPN and NPN) in the ecological study of a cave environment in South Africa.

By applying selected community (Shannon diversity, Margalef species richness and Pielou's evenness) and nematode specific (MI, EI, SI and metabolic footprint) indices for both PPN and NPN, it was the main aim of this study to determine the state of the ecosystem associated with the Wonderfontein Cave. Also, the possible effect of mining-associated pollutants on such ecosystems was investigated. Eight subterranean sites and three surface sites associated with Wonderfontein Cave and Spruit were selected for investigations. Soil and sediment samples were collected during two sampling intervals (April and September 2013) and the nematodes extracted, identified to genus level and counted. Nematode specific indices were calculated and the results confirmed by making use of NINJA: An automated calculation system for nematode-based biological monitoring (Sieriebriennikov *et al.*, 2014).

It is hypothesized that 1) the state of the ecosystems associated with the soils and sediments of the Wonderfontein Cave are severely degraded, mainly as a result of the influx of polluted water from the Wonderfontein Spruit and 2) that NPN assemblages can be used as bioindicators of ecosystem disturbance in this subterranean environment.

4.2 Results

Although various substrate types (soil, sediment, guano and water) were collected from the Wonderfontein Cave and Spruit, only the NPN data from the soils and sediments are included in this chapter. Unfortunately, the NPN community structure of the guano samples cannot provide any insight into the pollution effect as it has no direct association with the Wonderfontein Spruit. Also, guano provides a unique niche for specific NPN assemblages (Durand *et al.*, 2012). The latter author further stated that it is probable for the entire NPN nematode assemblage of a guano sample to consist of a single or two genera such as *Panagrolaimus and Diplogasteroides*. Guano substrates naturally contain high quantities of organic material which favours bacterivores. Using the NPN assemblages associated with guano samples as a measure of disturbance would thus be unfounded and bias. Nematode data gathered from analysing water samples are also not included as some samples contained very few specimens, while most had none. Relating the low number of NPN that were present in such samples to the pollution effect would thus be inaccurate and inconsistent. Although constrained by the near unmanageable environment of the cave, it is proposed that when future sampling is undertaken, an adequate amount of water is filtered *in situ*. Although not reported in this study, the nematode data of the guano samples are listed in Appendix B as the average number of individuals per genus per sample.

4.2.1 Identified nematode genera from soil and sediment samples

In total 60 nematode genera, including PPN and NPN, were identified from both the surface and cave environments from which soil and sediment samples were obtained (Tables 4.1, 4.2). These nematode assemblages were represented by nine PPN genera (herbivores) and 51 NPN genera. The latter consisted of 31 bacterivores, two eukaryotic feeders, four fungivores, 10 omnivores and four carnivores. Bacterivores identified from soil and sediment samples during this study dominated in terms of their occurrence and numbers. Coloniser-persister values for the NPN genera identified in soil and sediment samples ranged from 1 to 5.

It should be noted that two sites, the Wonderfontein Canal and Stoffels Dam, were only sampled during the 2nd sampling interval. Both of these sites represented the surface environment associated with the Wonderfontein Spruit and served as a control for the Wonderfontein Cave. Also, Fault Passage was only sampled during the 2nd sampling interval since difficulty entering the site was experienced during the 1st interval. Although sediment was collected from Derek's Exit during the 1st interval, none was collected during the second as the water had dried up most likely as a result of seasonal trends. Ultimately, four tables are provided in Appendix B that list the average number of individuals per genus identified and counted from soil and sediment samples collected during the 1st (April 2013) and 2nd (September 2013) sampling intervals. Furthermore, the average number of nematodes, TOC and pH of each substrate is also listed per site. If no nematodes were found in a sample, it was excluded from the list.

4.2.2 The application of general community indices

Data generated from the identification and counting of both PPN and NPN that inhabited soil and sediment samples obtained from the Wonderfontein Cave and Spruit, during both sampling intervals, were compared by using different community indices, namely Shannon's diversity, Margalef's species richness and Pielou's evenness (Neher & Darby, 2009). Shannon's diversity index revealed that the soils (Figure 4.1 a) presented inconsistent diversity in terms of PPN and NPN over both a temporal and spatial scale. Soil sample A from Elevation Pit had the highest calculated PPN and NPN diversity for both the 1st (2.45) and 2nd (2.53) sampling intervals, while no nematodes were presented in the soils of Pristine Chamber. The diversity index of the sediment samples (Figure 4.1 b) exhibited a more even distribution as most of the samples had a value of above 1. However, the Cave Inflow Area presented a very low diversity (0.4) for both sampling intervals. Two sites (Cave Inflow Area and Wonderfontein Canal) associated with the surface environment had a low diversity (below 0.4), while Stoffels Dam further downstream presented a higher diversity (1.4).

Table 4.1: A list of plant-parasitic (PPN) and non-parasitic (NPN) nematode genera found in soil and sediment samples collected from the Wonderfontein Cave and Spruit during the 1st (April 2013) sampling interval. Trophic group and cp-value classification of nematode genera was based on entries listed in the Nemaplex online database (Available at http://plpnemweb.ucdavis.edu/nemaplex).

		1st SAMPLING INTERVAL (APRIL)														
		SOILS							SEDIMENTS							
		Kent's Entrance	Main Entrance A	Main Entrance B	Elevation Pit A	Elevation Pit B	Cave Inflow Area	Derek's Exit	Kent's Entrance	Keyhole	Main Entrance	Pristine Chamber	Elevation Pit	North-Eastern Section	Cave Inflow Area	Derek's Exit
HERBIVORES																
Aphelenchoides		x									x		x	x		
Ditylenchus					x										x	
Hemicycliophora										x						
Longidorella					x			x								
Meloidogyne					x	x		x								
Paratylenchus					x						x					
Rotylenchus																
Psilenchus																
Tylenchus					x		x		x							
BACTERIVORES	**CP-Value**															
Acrobeloides	2			x	x			x								
Acrobeles	2			x	x	x										
Anaplectus	2															
Aulolaimus	3															
Bunonema	1				x											
Butlerius	1															
Cephalobus	2															
Cervidellus	2				x			x								
Chiloplacus	2			x		x		x								
Chronogaster	3									x						
Chromadorina	3									x						
Cylindrolaimus	3															x
Desmolaimus	3															
Diploscapter	1	x			x		x									
Ethmolaimus	3													x		
Eucephalobus	2	x		x										x		
Mesorhabditis	1				x		x	x			x					
Microlaimus	3									x		x				
Monhystera	1				x	x	x				x				x	x
Monhystrella	1															
Odontolaimus	3															
Panagrolaimus	1	x			x		x									

Table 4.1: Continued

		1st SAMPLING INTERVAL (APRIL) - CONTINUED														
		SOILS							SEDIMENTS							
		Kent's Entrance	Main Entrance A	Main Entrance B	Elevation Pit A	Elevation Pit B	Cave Inflow Area	Derek's Exit	Kent's Entrance	Keyhole	Main Entrance	Pristine Chamber	Elevation Pit	North-Eastern Section	Cave Inflow Area	Derek's Exit
BACTERIVORES	**CP-Value**															
Plectus	2		x	x							x	x			x	x
Prismatolaimus	3									x	x	x		x		x
Rhabditis	1	x			x		x									
Rhabdolaimus	3															
Seleborca	2				x											
Stegelleta	2				x											
Teratocephalus	3															
Wilsonema	2															
Zeldia	2															
EUKARYOTIC FEEDERS																
Achromadora	3											x				
Chromadorita	3										x					
FUNGIVORES																
Aphelenchus	2		x													
Dorylaimoides	4				x		x	x								
Nothotylenchus	2															
Paraphelenchus	2															
OMNIVORES																
Aporcelaimus	5	x														
Actinolaimus	4															
Allodorylaimus	4															
Campydora	4															
Dorylaimus	4															
Eudorylaimus	4															
Kochinema	4				x											
Mesodorylaimus	5						x									
Paraxonchium	5								x							
Pungentus	4				x			x								
CARNIVORES																
Mononchus	4															
Mylonchulus	4	x														
Tobrilus	3															
Tripyla	3				x											

Table 4.2: A list of plant-parasitic (PPN) and non-parasitic (NPN) nematode genera found in soil and sediment samples collected from the Wonderfontein Cave and Spruit during the 2nd (September 2013) sampling interval. Trophic group and cp-value classification of nematode genera was based on entries listed in the Nemaplex online database (Available at http://plpnemweb.ucdavis.edu/nemaplex).

		2nd SAMPLING INTERVAL (APRIL)																				
		SOILS											SEDIMENTS									
		Kent's Entrance	Main Entrance A	Fault Passage	Pristine Chamber	Elevation Pit A	Elevation Pit B	North-Eastern Section	Cave Inflow Area	Derek's Exit	Wonderfontein Canal	Stoffels Dam	Kent's Entrance	Keyhole	Main Entrance	Fault Passage	Pristine Chamber	Elevation Pit	North-Eastern Section	Cave Inflow Area	Wonderfontein Canal	Stoffels Dam
HERBIVORES																						
Aphelenchoides		x										x							x			
Ditylenchus									x													
Hemicycliophora									x				x									
Longidorella						x																
Meloidogyne		x				x																
Paratylenchus																						
Rotylenchus						x																
Psilenchus									x			x										
Tylenchus		x				x	x		x			x						x		x		
BACTERIVORES	**CP-Value**																					
Acrobeloides	2	x	x			x		x	x	x		x						x				x
Acrobeles	2	x				x																
Anaplectus	2									x		x				x	x				x	
Aulolaimus	3	x														x						
Bunonema	1																					
Butlerius	1								x													
Cephalobus	2											x					x					
Cervidellus	2																					
Chiloplacus	2								x	x												
Chronogaster	3											x										
Chromadorina	3																					
Cylindrolaimus	3																				x	
Desmolaimus	3											x										
Diploscapter	1	x							x			x					x					
Ethmolaimus	3																					
Eucephalobus	2	x				x			x			x	x					x				x
Mesorhabditis	1	x				x		x	x		x	x			x							
Microlaimus	3																					
Monhystera	1	x	x	x	x	x				x	x				x	x	x	x	x	x	x	x
Monhystrella	1					x										x						x
Odontolaimus	3	x																				
Panagrolaimus	1	x				x	x			x		x					x					

Table 4.2: Continued

		2nd SAMPLING INTERVAL (APRIL)																				
		SOILS											SEDIMENTS									
		Kent's Entrance	Main Entrance A	Fault Passage	Pristine Chamber	Elevation Pit A	Elevation Pit B	North-Eastern Section	Cave Inflow Area	Derek's Exit	Wonderfontein Canal	Stoffels Dam	Kent's Entrance	Keyhole	Main Entrance	Fault Passage	Pristine Chamber	Elevation Pit	North-Eastern Section	Cave Inflow Area	Wonderfontein Canal	Stoffels Dam
BACTERIVORES	**CP-Value**																					
Plectus	2	x	x	x		x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x
Prismatolaimus	3	x				x		x				x		x	x		x	x	x			
Rhabditis	1	x		x		x	x									x						
Rhabdolaimus	3					x																x
Seleborca	2					x																
Stegelleta	2								x													
Teratocephalus	3		x			x			x	x												
Wilsonema	2								x													
Zeldia	2		x									x										
EUKARYOTIC FEEDERS																						
Achromadora	3																					
Chromadorita	3																					
FUNGIVORES																						
Aphelenchus	2																					
Dorylaimoides	4																					
Nothotylenchus	2													x								
Paraphelenchus	2	x				x			x			x	x					x				
OMNIVORES																						
Aporcelaimus	5																					
Actinolaimus	4											x										
Allodorylaimus	4											x										
Campydora	4					x	x												x			
Dorylaimus	4										x	x										
Eudorylaimus	4					x																
Kochinema	4					x																
Mesodorylaimus	5					x			x		x	x										
Paraxonchium	5																					
Pungentus	4																					
CARNIVORES																						
Mononchus	4	x															x					
Mylonchulus	4																					
Tobrilus	3											x			x				x	x		x
Tripyla	3							x										x				

Margalef's species richness index (Figure 4.2) revealed that only a few sites presented a high richness compared to a generally low richness, which was the norm in this case. Soil samples (Figure 4.2 a) collected from the Main Entrance, Cave Inflow Area and Derek's Exit presented low richness with minimal temporal variation in terms of PPN and NPN genera identified. Kent's Entrance, with values of 4.3 (1st sampling interval) and 5.3 (2nd sampling interval) and Elevation Pit soil sample A with values of 5.7 (1st sampling interval) and 6.9 (2nd sampling interval), presented higher PNP and NPN richness, but also with minimal variation. However, although Elevation Pit soil sample B presented PPN and NPN richness higher than 7 for the 2nd sampling interval, soil collected during the 1st sampling interval was calculated to be just above 2. And although no richness was calculated for North-Eastern Section during the 1st sampling interval, soil collected during the 2nd sampling interval presented a richness nearing 8. Pristine Chamber followed the latter trend with virtually no richness in term of NPN and PPN recorded during the 1st sampling interval. Sediments (Figure 4.2 b) sampled from Keyhole and Main Entrance, during the 1st sampling interval, presented richness of higher than 5.5 and 7, respectively. On the contrary, sediments sampled during the 2nd sampling interval from Main Entrance had a considerably lower calculated PPN and NPN richness of 2. Other sediment samples presented low richness in terms of PPN and NPN assemblages, however, with minimal variation. Temporal variation with regard to PPN and NPN communities was thus evident from both substrate types, as well as from a number of sites.

Pielou's evenness index presented some considerable variation between the soil (Figure 4.3 a) samples. Soil collected from Kent's Entrance and Main Entrance presented an evenness higher than 0.85 during the 1st sampling interval, but below 0.8 and even as low as 0.48 for Main Entrance soil sample A during the 2nd sampling interval. On the contrary, soils collected during the 2nd sampling interval from Elevation Pit (soil sample A and B) and North-Eastern Section presented a higher evenness compared to that calculated for the 1st sampling interval. When considering the sediments (Figure 4.3 b), an average PPN and NPN evenness of 0.9 with minimal variation was calculated for only the Wonderfontein Cave. However, the surface sites were different as the Cave Inflow Area had very low PPN and NPN evenness values (1st sampling interval: 0.19; 2nd sampling interval: 0.21) calculated for both intervals. The Wonderfontein Canal had a low evenness of 0.29, while Stoffels Dam presented a higher evenness of 0.75.

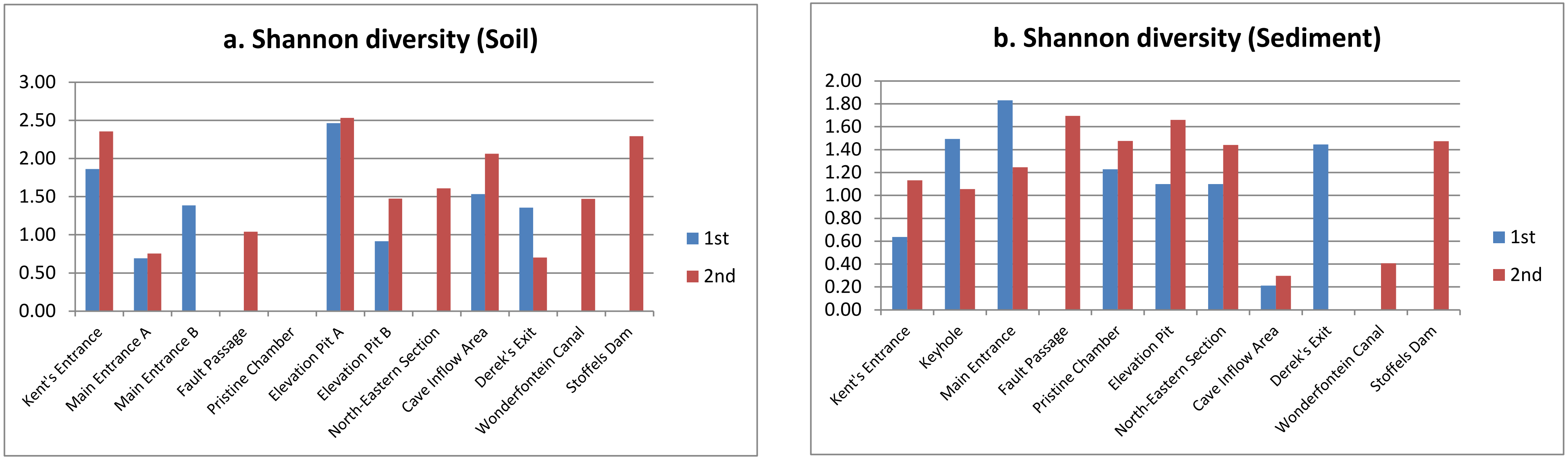

Figure 4.1: Shannon diversity index of nematode assemblages associated with a) soil and b) sediment samples collected from the Wonderfontein Cave and Spruit during the 1st (April 2013) and 2nd (September 2013) sampling intervals.

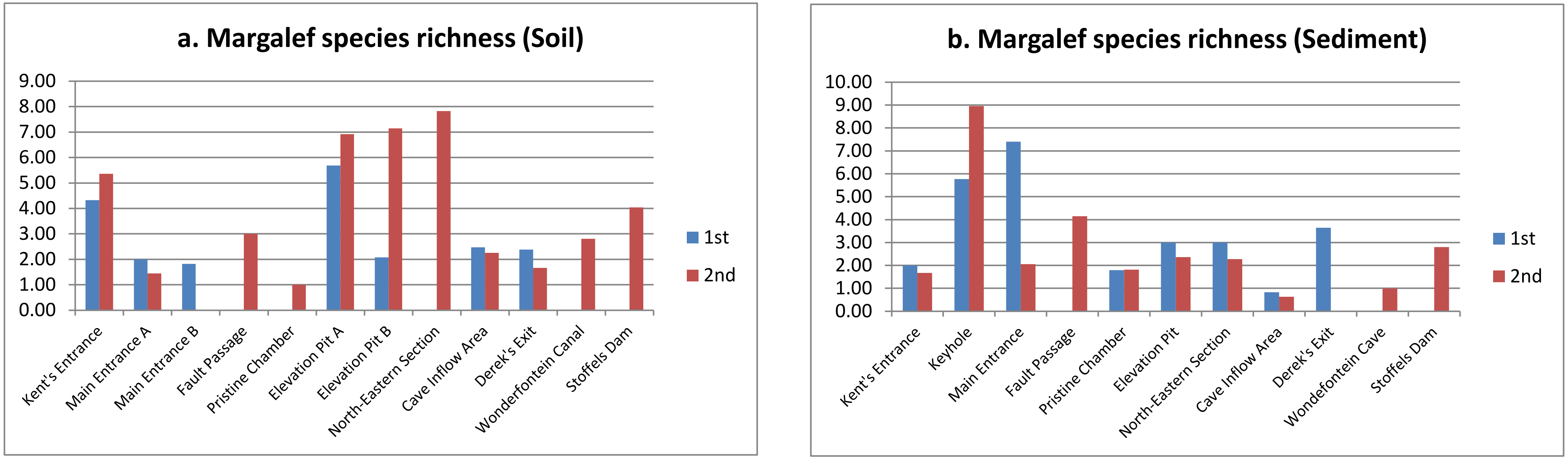

Figure 4.2: Margalef species richness index of nematode assemblages associated with a) soil and b) sediment samples collected from the Wonderfontein Cave and Spruit during the 1st (April 2013) and 2nd (September 2013) sampling intervals.

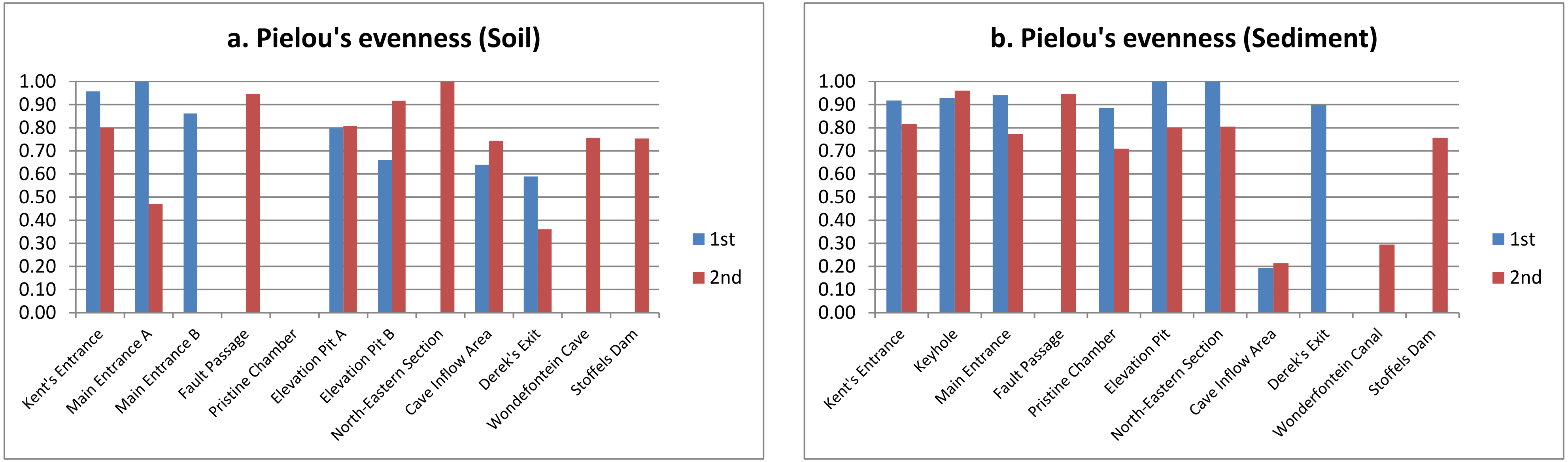

Figure 4.3: Pielou’s evenness index of nematode assemblages associated with a) soil and b) sediment samples collected from the Wonderfontein Cave and Spruit during the 1st (April 2013) and 2nd (September 2013) sampling intervals.

4.2.3 Trophic group structure and cp-value classification of nematode assemblages

The trophic group structures are provided in Figures 4.4 a, b and 4.5 a, b, while the identified nematodes were classified as either herbivores (Her), bacterivores (Ba), eukaryotic feeders (Eu), fungivores (Fu), omnivores (Om) or carnivores (Ca).

When considering the soils (Figure 4.4) sampled from both the 1st (Figure 4.4 a) and 2nd (Figure 4.4 b) sampling intervals, NPN communities dominated with bacterivores being the predominant trophic group. Furthermore, herbivores (PPN) were recorded during the 1st sampling interval only in soil samples from Kent's Entrance, Elevation Pit (soil sample A and B), Cave Inflow Area and Derek's Exit. During the 2nd sampling interval, herbivores were present in soil samples from Kent's Entrance, Elevation Pit (soil sample A and B), Cave Inflow Area and Stoffels Dam. In terms of fungivores, they were present in soil samples obtained from four of the sites during the 1st sampling interval [Main Entrance (Soil sample A), Elevation Pit (Soil sample A), Cave Inflow Area and Derek's Exit] with this trophic group consisting of 50 % of the total nematode community for the site Main Entrance (soil sample A). During the 2nd sampling interval, fungivores also occurred in four of the sites [Kent's Entrance, Elevation Pit (Soil sample A), Cave Inflow Area and Stoffels Dam] with population levels substantially lower than those recorded during the 1st sampling interval.

Although sporadically found, carnivores were also recorded from the soil samples obtained from three sites during both sampling intervals. During the 1st sampling interval carnivores were recorded in soil samples from Kent's Entrance, Elevation Pit (soil sample A) and Cave Inflow Area, opposed to Kent's Entrance, Northern-Eastern Section and Stoffels Dam during the 2nd sampling interval. Soil samples obtained from the latter site best represented the carnivores that constituted 32 % of the total nematode community. Omnivores were present in soil samples from four sites [Kent's Entrance, Elevation Pit (soil sample A), Cave Inflow Area and Derek's Exit] during the 1st sampling interval and in soil samples from four sites [Elevation Pit (soil samples A and B), Cave Inflow Area, Wonderfontein Canal and Stoffels Dam] during the 2nd sampling interval.

Colonizer-persister values ranged from 1 to 5 as each value is assigned to nematode groups with similar life strategies. For a review of each life strategy associated with the cp-values, please refer to Chapter 1 (section 1.4.5.2). The nematode assemblages recorded from the 1st sampling interval were mainly classified, with the exception of a few sites (Kent's Entrance and Cave Inflow Area), as belonging to cp-2 (Figure 4.4). Nematodes that are classified as cp-2 present longer generation times and lower fecundity levels as cp-1 nematodes, are tolerant to unfavourable conditions and are dominated by bacterivores and fungivores (Ferris *et al.*, 2001). The 2nd sampling interval (Figure 4.4 d) was dominated by nematodes that were both classified as cp-1 and cp-2, while cp-3 nematodes were found to be associated with soils especially from North-Eastern Section and Stoffels Dam. Higher cp (4 and 5) nematodes were sporadically found during both sampling intervals with the highest, being cp-4, constituting 25 % of the total nematode community identified from Cave Inflow Area sampling site during the 1st sampling interval.

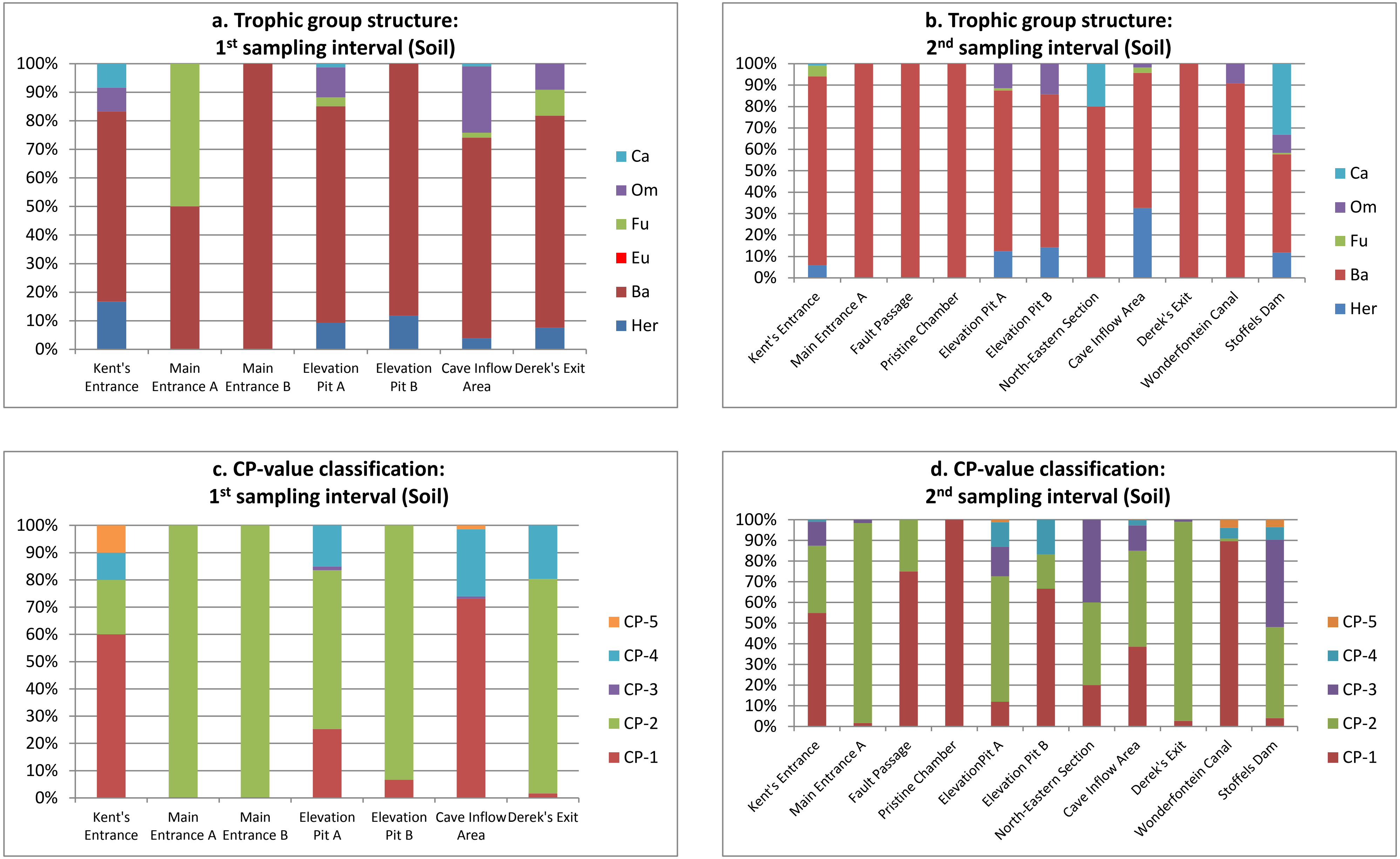

Figure 4.4: Trophic group structure (a and b) and colonizer-persister (cp) classification (c and d) of nematode assemblages associated with soil samples collected from the Wonderfontein Cave and Spruit during the 1st (April 2013) and 2nd (September 2013) sampling intervals.

The trophic group structure of the sediments (Figures 4.5 a and b), collected during both intervals, was similar to that of the soils as bacterivores dominated most. While herbivores were dominant in sediment samples collected from Kent's Entrance and Elevation Pit during the 1st sampling interval, they were also found in low numbers at Keyhole, Main Entrance, North-Eastern Section and Cave Inflow Area during the same sampling interval. On the contrary, herbivores were substantially less represented and only found at Kent's Entrance, Elevation Pit, North-Eastern Section and Cave Inflow Area during the 2nd sampling interval, with the highest occurrence recorded from North-Eastern Section (12 %). Neither fungivores nor carnivores were recorded from sediments during the 1st sampling interval. However, while fungivores were represented at Kent's Entrance, Keyhole and Elevation Pit during the 2nd sampling interval, carnivores dominated Cave Inflow Area (94 %) during the same sampling interval. Carnivores were also represented, though in minimal percentages (< 10 %), in sediment samples from Main Entrance, Pristine Chamber and Elevation Pit during the 2nd sampling interval. Eukaryotic feeders were only recorded in sediment samples from Main Entrance (11 %) and Pristine Chamber (25 %) during the 1st sampling interval.

It should be noted that temporal variation was evident as no bacterivores were recorded from Kent's Entrance during the 1st sampling interval, yet, made up more than 90 % of the nematode assemblage during the 2nd sampling interval. Also, as was noted above, herbivores were only sporadically found during the 2nd sampling interval, while dominating sediment samples collected from Main Entrance and Elevation Pit during the 1st sampling interval.

The NPN cp classification of the sediments (Figures 4.5 c and d) differ somewhat from that of the soils (Figures 4.4 c and d). While the soils were mostly dominated by cp-1 and cp-2 nematodes, the sediments, especially those from the 1st sampling interval, were dominated by cp-3 nematodes, except for at Kent's Entrance, Elevation Pit and Cave Inflow Area. The sediments from the 2nd sampling interval were largely represented by cp-2 and cp-3 nematodes. Nematodes that are classified as cp-3 also present, similarly to cp-2, longer generation times, however, are more sensitive to unfavourable conditions and are typically represented by fungivores, bacterivores and carnivores (Ferris *et al.*, 2001). Only Fault Passage hosted nematodes of which more than 40 % were classified as cp-1. From both sampling intervals, only two sites, Pristine Chamber (4 %) and Elevation Pit (31 %), hosted nematodes with a classification of cp-4 and only Kent's Entrance with nematodes classified as cp-5.

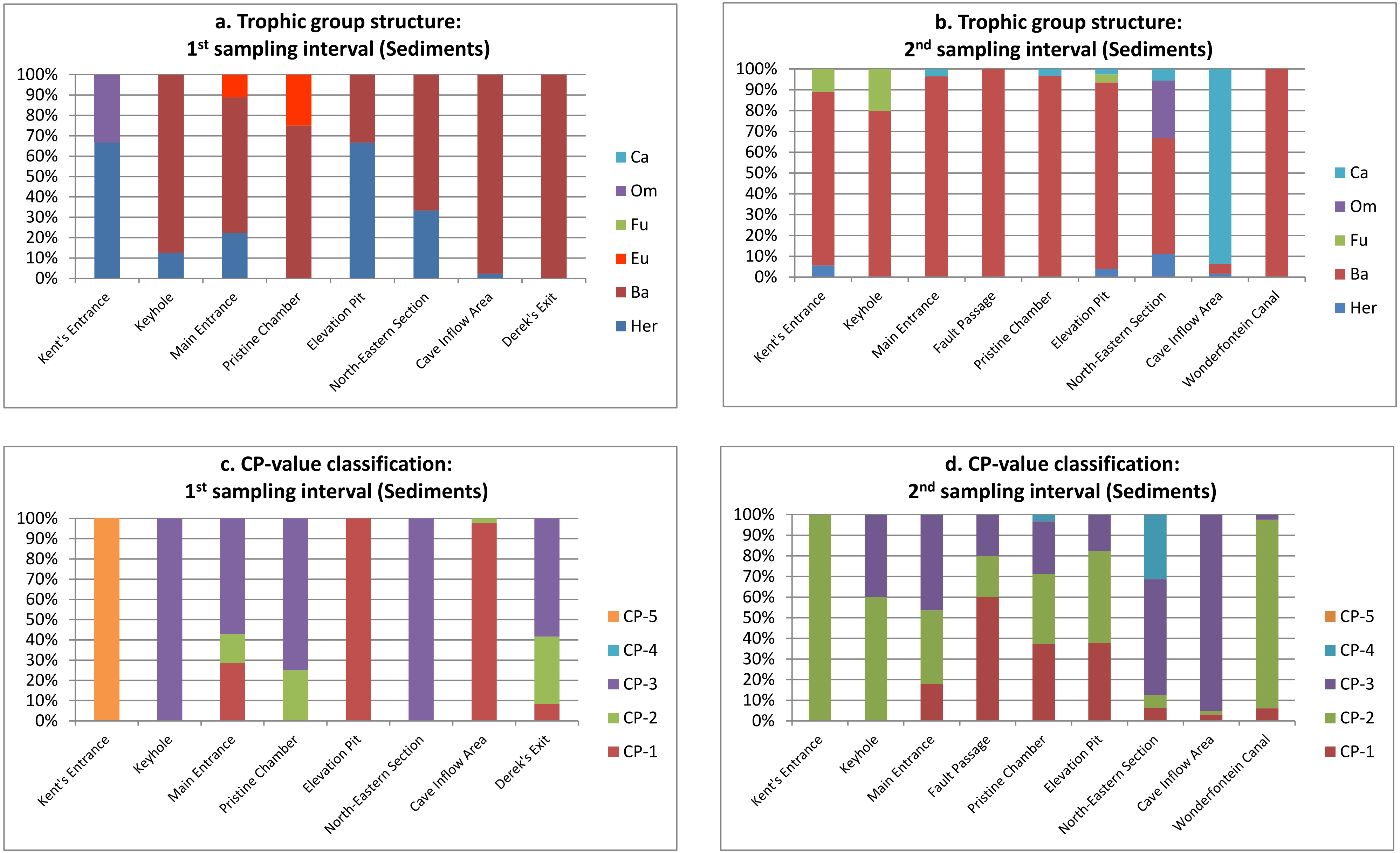

Figure 4.5: Trophic group structure (a and b) and colonizer-persister (cp) classification (c and d) of nematode assemblages associated with sediments sampled from the Wonderfontein Cave and Spruit during the 1st (April 2013) and 2nd (September 2013) sampling intervals.

4.2.4 The application of nematode specific indices

4.2.4.1 Maturity index

The MI serves as a set of tools that measures the disturbance of an ecosystem on a scale of 1 to 5 (Bongers, 1999). A nutrient-enriched, but disturbed environment, would rate lower (± 2) and a pristine/stable environment higher (± 4). The MI and MI_{2-5} were applied to evaluate the level of disturbance of both soil and sediment samples collected from the Wonderfontein Cave and Spruit during this study. Table 4.3 lists the scoring of the MI and MI_{2-5} for all the soil samples collected from both sampling intervals. Samples in which no nematodes were found is marked with a dash. Most of the soil samples from the 1st sampling interval had relatively low MI and MI_{2-5} values. For the 1st sampling interval, MI values ranged from 1.93 (Kent's Entrance) to 2.37 (Derek's Exit), while MI_{2-5} values ranged from 2 (Main Entrance) to 3.33 (Kent's Entrance). Soil samples from the 2nd sampling interval presented a similar trait with MI values ranging from 1.62 (Elevation Pit: Soil sample B) to 2.57 (Stoffels Dam), and MI_{2-5} values from 2 (Fault Passage and Pristine Chamber) to 3.33 (Wonderfontein Canal). The surface sites presented some variance as Wonderfontein Canal had both the lowest MI (1.32) and highest MI_{2-5} (3.33) value. The Cave Inflow Area scored lower for both indices than Stoffels Dam.

The MI and MI_{2-5} values of the sediments, collected during both sampling intervals, are listed in Table 4.4. Generally, the sediment values of the 1st sampling interval were higher than that of the soils, but with some considerable and irregular variation. Although most of the MI and MI_{2-5} values were very similar during the 1st sampling interval and ranged between 2 and 3, Kent's Entrance presented an exceptionally high value of 5 for both indices. This value is highly improbable and most likely the result of a poorly represented sample. When considering the sediments collected during the 2nd sampling interval, variance between different sites was evident. The MI values of the cave sediments ranged from 1.83 (Fault Passage) to 3.04 (North-Eastern Section) and the MI_{2-5} values from 2.13 (Fault Passage) to 3.04 (North-Eastern Section). Contrary to the observations made from the surface soils, the sediment values of the Cave Inflow Area are the highest at 2.95 for both indices, while Wonderfontein Canal scored the lowest at 2.03. Stoffels Dam presented slightly lower MI (2.29) and MI_{2-5} (2.29) values than the soils collected from the same site.

Table 4.3: The calculated MI and MI_{2-5} values of the soil samples collected from the Wonderfontein Cave and Spruit during the 1st (April 2013) and 2nd (September 2013) sampling intervals. Each value is followed by the calculated standard deviation with not applicable values (N/A) also indicated.

		Kent's Entrance	Main Entrance A	Main Entrance B	Fault Passage	Pristine Chamber	Elevation Pit A	Elevation Pit B	North-Eastern Section	Cave Inflow Area	Derek's Exit	Wonderfontein Canal	Stoffels Dam
April	**MI**	1.93 ± 0.12	2.00 ± N/A	2.00 ± 0	-	-	2.10 ± 0.25	2.00 ± 0	-	2.28 ± 0.3	2.37 ± 0.05	-	-
	MI2	3.33 ± 1.53	2.00 ± N/A	2.00 ± 0	-	-	2.43 ± 0.17	2.00 ± 0	-	2.53 ± 0.21	2.39 ± 0.07	-	-
Sept	**MI**	1.74 ± 0.34	2.01 ± 0.03	-	1.83 ± NA	2.00 ± N/A	2.33 ± 0.18	1.62 ± 0.75	2.33 ± 0.58	1.78 ± 0.12	2.00 ± 0	1.32 ± 0.19	2.57 ± 0.30
	MI2	2.31 ± 0.29	2.01 ± 0.03	-	2.00 ± NA	2.00 ± N/A	2.42 ± 0.15	3.00 ± N/A	2.50 ± 0.50	2.25 ± 0.15	2.01 ± 0.02	3.33 ± 0.94	2.64 ± 0.33

Table 4.4: The calculated MI and MI_{2-5} values of the sediment samples collected from the Wonderfontein Cave and Spruit during the 1st (April 2013) and 2nd (September 2013) sampling intervals. Each value is followed by the calculated standard deviation with not applicable values (N/A) also indicated.

		Kent's Entrance	Keyhole	Main Entrance	Fault Passage	Pristine Chamber	Elevation Pit	North-Eastern Section	Cave Inflow Area	Derek's Exit	Wonderfontein Canal	Stoffels Dam
April	**MI**	5.00 ± N/A	3.00 ± N/A	2.42 ± 0.65	-	2.72 ± 0.25	2.00 ± N/A	2.67 ± N/A	2.00 ± 0	2.64 ± 0.26	-	-
	MI2	5.00 ± N/A	3.00 ± N/A	2.54 ± 0.42	-	2.72 ± 0.25	2.00 ± N/A	2.67 ± N/A	2.00 ± 0	2.64 ± 0.26	-	-
Sept	**MI**	2.00 ± 0	2.33 ± N/A	2.48 ± 0.38	1.83 ± 0.29	2.27 ± 0.05	2.18 ± 0.21	3.04 ± 0.05	2.95 ± 0.06	-	2.03 ± 0.06	2.29 ± 0.28
	MI2	2.00 ± 0	2.33 ± N/A	2.65 ± 0.31	2.13 ± 0.23	2.34 ± 0.04	2.18 ± 0.21	3.04 ± 0.05	2.95 ± 0.06	-	2.03 ± 0.06	2.29 ± 0.28

4.2.4.2 Faunal analysis

The EI (y-axis) and SI (x-axis) for nematodes sampled during this study were also calculated for both substrates (soil and sediment) and plotted on two separate scatter diagrams (Figures 4.6 and 4.7). Four quadrants, each representing a different state of the associated ecosystem, are defined by dashed lines drawn on the 50 % mark of both axes. It should be noted that soil samples sampled from some sites were not plotted as they contained no nematodes, which included Pristine Chamber and North-Eastern Section during the 1st sampling interval and Main Entrance (Soil sample B) during the 2nd sampling interval.

Figure 4.6 illustrates that the collective scenario for soil samples from the different sites were representative of all four quadrants of the nematode food web (faunal analysis) as published by (Ferris *et al.*, 2001). Only soil samples obtained during the 2nd sampling interval from Kent's Entrance and Cave Inflow Area were representative of quadrat A; which depicted disturbed and enriched nematode ecosystems that were mostly inhabited by bacterivores. Samples obtained during the first 1st sampling interval from Kent's Entrance and Elevation Pit, as well as those obtained from the latter site and Wonderfontein Canal during the 2nd sampling interval, plotted in quadrat B. In terms of the status of their nematode food webs, such ecosystems are classified as matured, enriched and are dominated by bacterivores. The only cave soils that represented a matured and fertile ecosystem (quadrat C), in terms of nematode food webs, were collected during the 1st sampling interval from the Cave Inflow Area site. The same scenario was evident for soils collected during the 2nd sampling interval from Elevation Pit, North-Eastern Section and Stoffels Dam. Soils collected during the 1st sampling interval from Main Entrance (soil samples A and B), Elevation Pit (soil sample B) and Derek's Exit, were plotted in quadrat D, which represents minimal enrichment and structure in terms of nematode food webs that existed during that specific time. Also, soil samples obtained during the 2nd sampling interval from Fault Passage, Derek's Exit, Pristine Chamber and Main Entrance (soil sample A), plotted in the same quadrat. The ecosystems associated with the soils of these sites were thus regarded as degraded and depleted in terms of their composition and population levels, while being dominated by fungivores.

Although Elevation Pit (soil sample A) was representative, during the 1st sampling interval, of a maturing and enriched ecosystem, it represented a matured and fertile nematode food web during the 2nd sampling interval. Also, the nematode food web associated with Elevation Pit (soil sample B) presented higher enrichment and structure during the 2nd sampling interval.

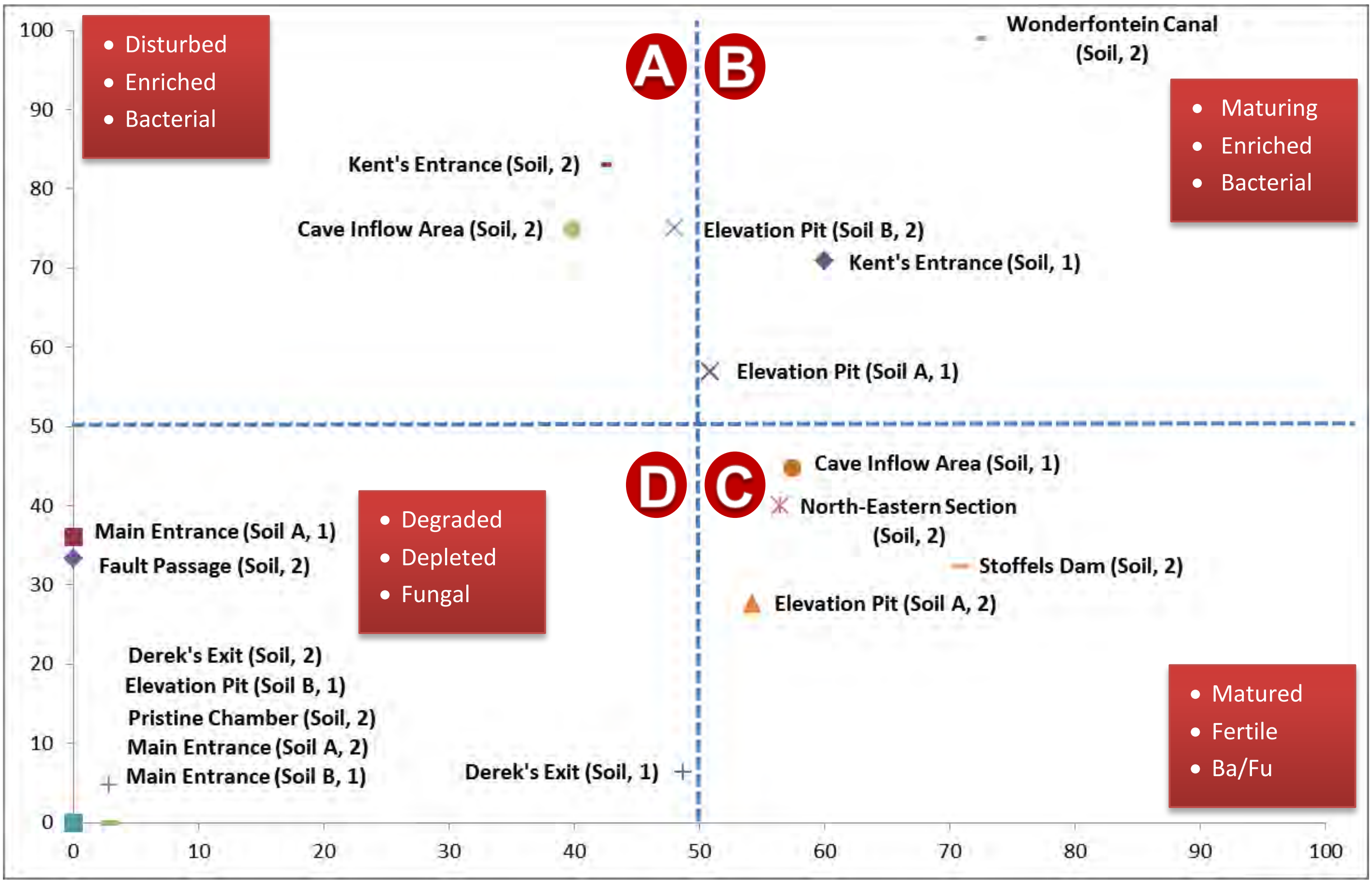

Figure 4.6: Faunal analysis of the non-parasitic nematode (NPN) assemblages associated with soils collected from the Wonderfontein Cave and Spruit during the 1st (April 2013) and 2nd (September 2013) sampling intervals. Site names are indicated with the substrate type and sampling interval in brackets.

On the contrary, soil collected from Kent's Entrance, during the 1st sampling interval, was representative of a maturing ecosystem, while being disturbed during the 2nd sampling interval. Also, the nematode food web associated with soil collected at Derek's Exit showed less structure during the 2nd sampling interval, than during the 1st, while soil collected from Cave Inflow Area shifted from being representative of a matured and fertile ecosystem (1st sampling interval), to being representative of a disturbed and enriched ecosystem (2nd sampling interval). This data further provides evidence of temporal variation with regard to the nematode assemblages associated with the respective sites. Although it became evident that over a temporal scale, some sites, such as Elevation Pit, presented a shift towards a more mature and fertile ecosystem, others, such as Cave Inflow Area and Kent's Entrance, showed degradation.

Generally, the faunal analyses of the sediments (Figure 4.7) presented less enrichment in terms of nematode food webs than their soil counterparts. However, structural variation was evident among the food-web status of nematodes identified from some sediment samples. Also, the temporal variation observed for nematodes identified from sediment samples from

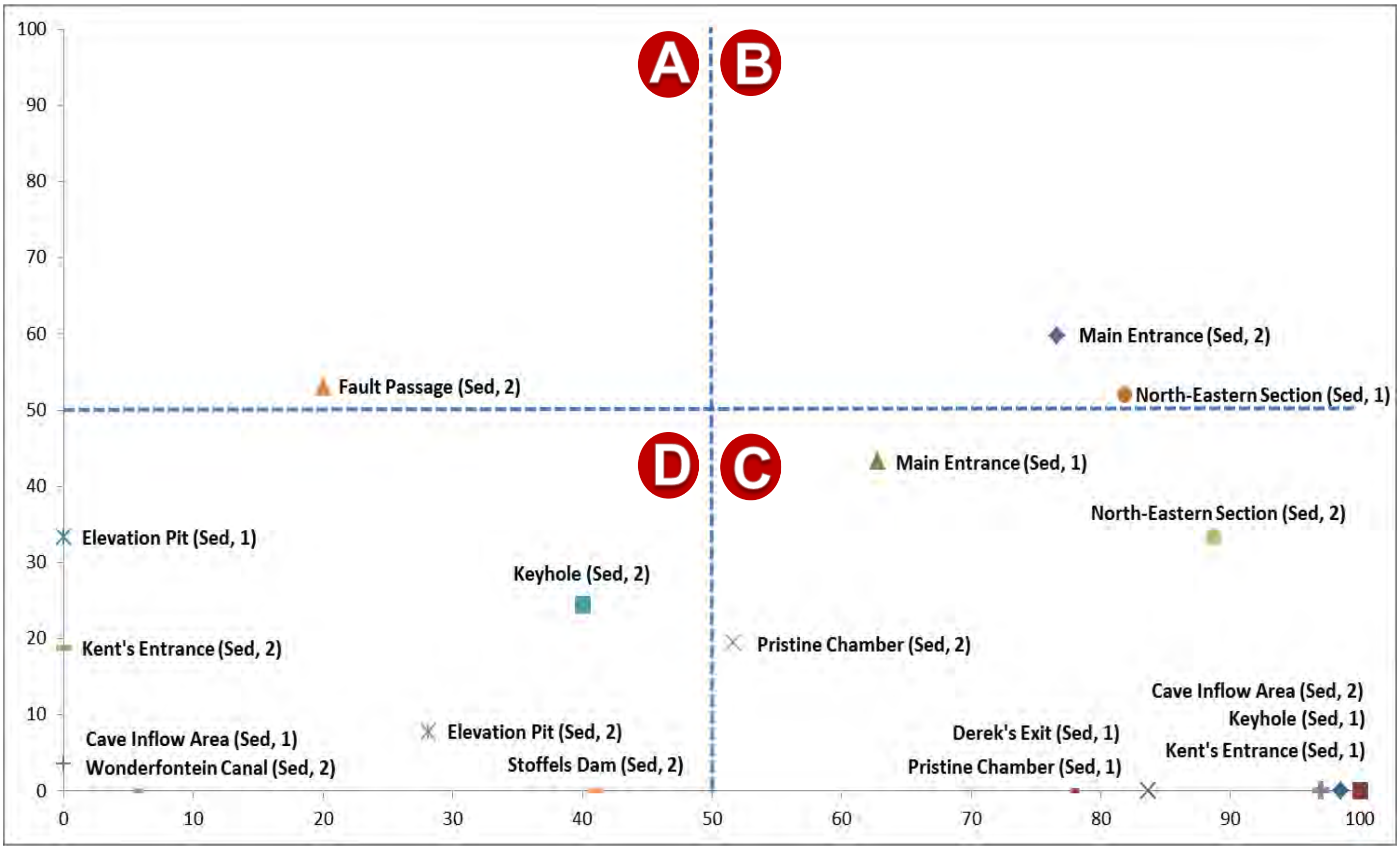

Figure 4.7: Faunal analysis of the non-parasitic nematode (NPN) assemblages associated with sediments collected from the Wonderfontein Cave and Spruit during the 1st (April 2013) and 2nd (September 2013) sampling intervals. Site names are indicated with the substrate type and sampling interval in brackets. Each quadrat represents a food web structure as follows: A = disturbed, enriched and dominated by bacterivores; B = maturing, enriched and dominated by bacterivores; C = mature, fertile and dominated by bacterivores and fungivores; D = degraded, depleted and dominated by fungivores.

different sites might have been the result of the low nematode abundance recorded in some samples. The latter was evident for a number of sediment samples collected, during the 1st sampling interval, from sites including Kent's Entrance and North-Eastern Section (average of 1 specimen per sample), Keyhole (average of 2 specimens per sample) and Elevation Pit (average of 0.75 specimens per sample). Also, during the 2nd sampling interval, a low number of nematodes were recorded for Kent's Entrance (average of 6 specimens per sample), Keyhole (average of 1.25 specimens per sample), Fault Passage (average of 3.33 specimens per sample) and North-Eastern Section (average of 9 specimens per sample). The average number of nematodes recorded per sample for each site and substrate type is provided in Appendix B.

Sediment samples collected from Fault Passage during the 2nd sampling interval, as illustrated by Figure 4.7, were representative of a disturbed and enriched ecosystem (quadrat A), while during the same sampling interval, sediment obtained from Main Entrance presented a maturing (quadrat B) nematode food web dominated by bacterivores. Also, the nematode assemblage associated with North-Eastern Section presented the same food web structure as the latter during the 1st sampling interval. A number of sites were associated with a matured and fertile ecosystem (quadrat C) and included Main Entrance, Derek's Exit, Pristine Chamber, Keyhole and Kent's Entrance during the 1st sampling interval and North-Eastern Section, Pristine Chamber and Cave Inflow Area during the 2nd sampling interval. However, there were also a number of degraded and depleted (quadrat D) ecosystems represented at Elevation Pit and Cave Inflow Area during the 1st sampling interval, and Kent's Entrance, Keyhole, Elevation Pit, Wonderfontein Canal and Stoffels Dam during the 2nd sampling interval.

Temporal variation in the food web structure of sediment was more evident for some sites, such as Kent's Entrance, Keyhole and Cave Inflow Area, than others. The latter site shifted from being representative of a degraded and depleted ecosystem (1st sampling interval), to that of a mature and fertile ecosystem (2nd sampling interval). Also, Kent's Entrance and Keyhole shifted from being representative of mature and fertile ecosystems, to that of degraded and depleted ecosystems during the same sampling intervals. Other sites that presented substantial structural variation include Pristine Chamber and Elevation Pit, while Main Entrance and North-Eastern Section presented some structural and enrichment variation.

4.2.4.3 Metabolic footprint

Quantifying the metabolic footprints of the NPN assemblages associated with the soils and sediments provides additional insight into the state of the associated ecosystems (Ferris, 2010). Since the data plots (EI and SI) for the Wonderfontein Cave and Spruit substrates have already been provided (Figures 4.6 and 4.7), this section will only list the metabolic (enrichment and structure) footprints of selected substrate samples. Generally, the NPN assemblages associated with the soils collected during both sampling intervals produced a greater metabolic footprint (Figures 4.8 and 4.9) than that of the sediments. Yet, the only Wonderfontein Cave NPN assemblage that produced a substantial metabolic footprint was collected during the 1st sampling interval from Elevation Pit (soil sample A). Other measureable footprints were recorded from two surface sites, the Cave Inflow Area and Stoffels Dam. The former produced a greater structure than enrichment footprint during the 1st sampling interval, but a greater enrichment footprint during the 2nd sampling interval.

When considering the sediments from both sampling intervals, minimal metabolic activity (Figures 4.10 and 4.11) was produced by the associated nematode assemblages. Although the assemblages associated with the sediments collected from the Main Entrance and North-Eastern Section did present some structure activity, miniscule enrichment activity was recorded. Pristine Chamber produced only a structure footprint, while other sediments from the 1st sampling interval presented no measurable metabolic footprint. Furthermore, no metabolic footprint was measured for any of the sediments collected during the 2nd sampling interval, with the exception of the Cave Inflow Area that presented only structure activity.

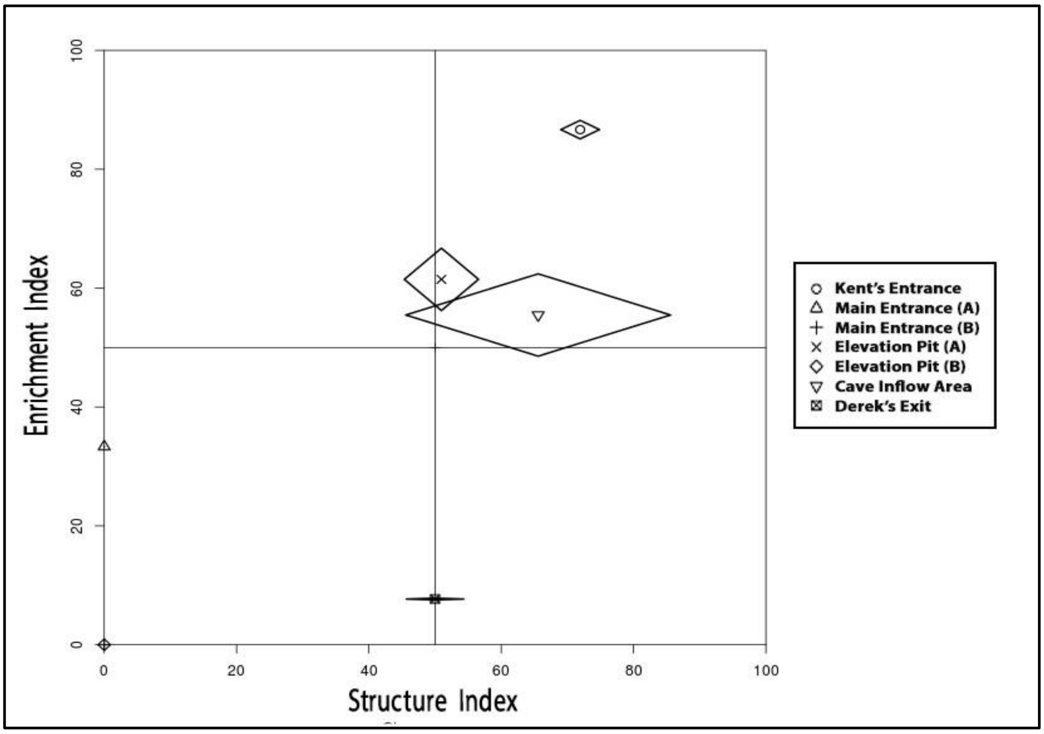

Figure 4.8: Metabolic footprints of non-parasitic nematode (NPN) assemblages associated with soils collected during the 1st (April 2013) sampling interval from the Wonderfontein Cave and Spruit.

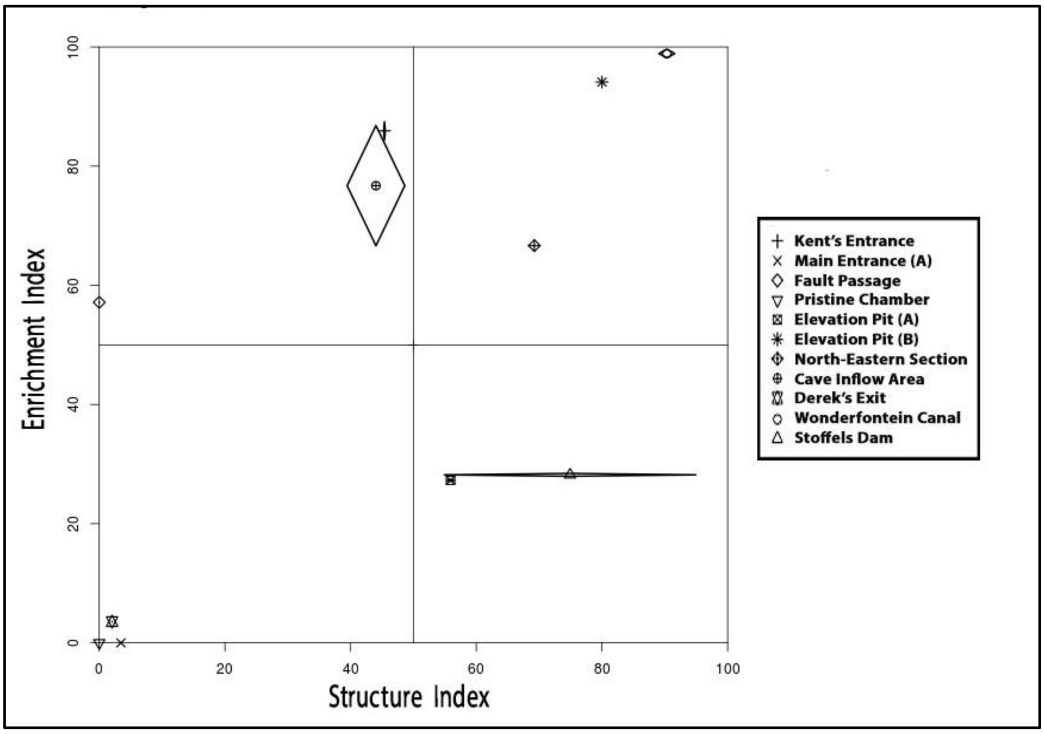

Figure 4.9: Metabolic footprints of non-parasitic nematode (NPN) assemblages associated with soils collected during the 2nd (September 2013) sampling interval from the Wonderfontein Cave and Spruit.

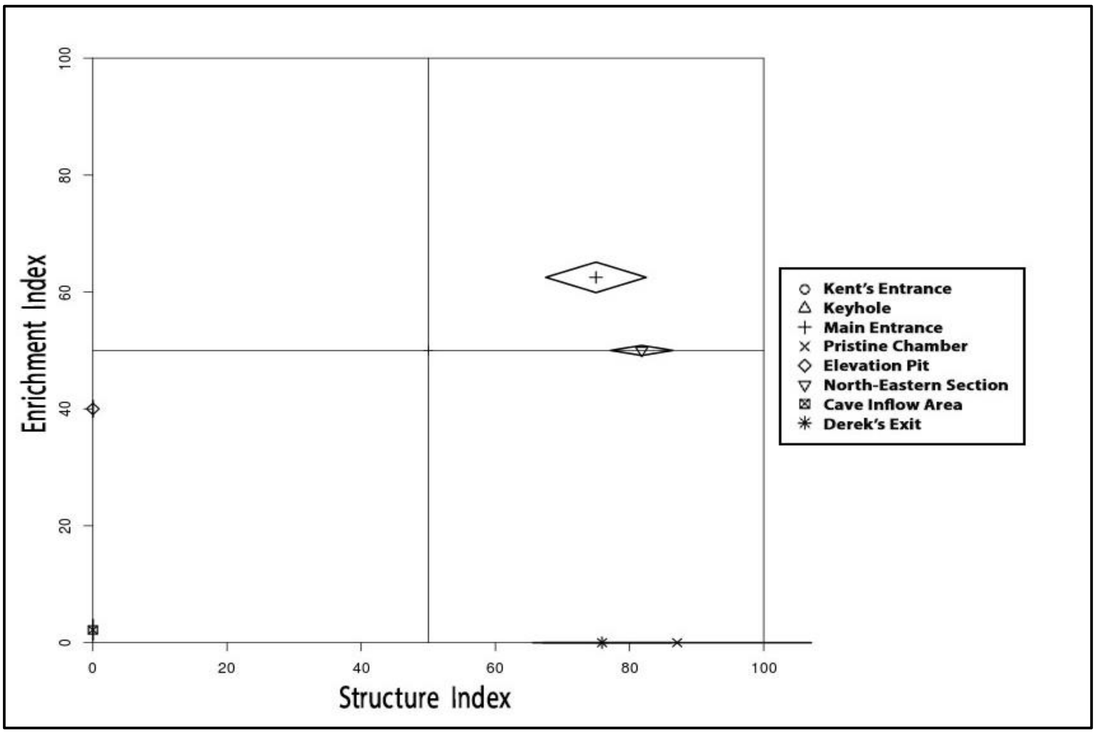

Figure 4.10: Metabolic footprints of non-parasitic nematode (NPN) assemblages associated with sediments collected during the 1st (April 2013) sampling interval from the Wonderfontein Cave and Spruit.

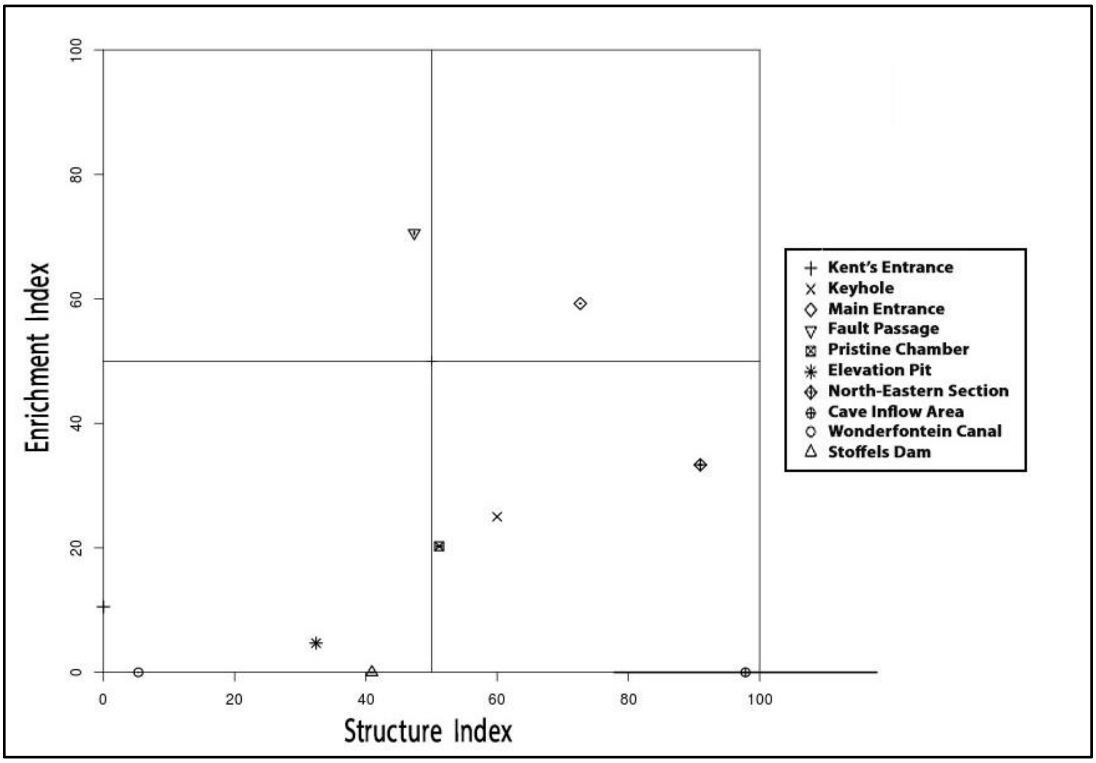

Figure 4.11: Metabolic footprints of non-parasitic nematode (NPN) assemblages associated with sediments collected during the 2nd (September 2013) sampling interval from the Wonderfontein Cave and Spruit.

4.2.5 Applied multivariate statistical analysis of nematode assemblages and abiotic conditions

Ordination diagrams created by a canonical correspondence analysis (CCA), or any similar model, enable researchers to study the relationship between species data and environmental variables. As CCA is a constrained analysis, the variance in species composition is explained by the environmental variables (Ter Braak & Smilauer, 2012). Since the NPN assemblage data presented a high diversity of genera and low abundance of individuals, it would be more appropriate to use nematode groupings. The cp classification holds relation to the sensitivity/tolerance of the associated nematode taxa to environmental disturbances and therefore, this grouping system was related (Figures 4.12 and 4.13) to the metal enrichment factors (EF) provided in Chapter 3 (section 3.2.2). Since the data was not suited for a CCA, an unconstrained principal component analysis (PCA) was performed where species composition is interpreted with the help of environmental variables (Ter Braak & Smilauer, 2012). Although a PCA does not constrain the species data with the environmental variables, relationships between biotic (NPN assemblage data) and abiotic components (metal EF) can still be studied. It should be noted that the combined data from both sampling intervals from only the Wonderfontein Cave samples was used to generate the respective soil and sediment PCA.

The PCA analysis of the Wonderfontein Cave soils (Figure 4.12) presented the relationships between the cp-groups of the NPN assemblages and the metals (Cr, Zn, Fe, Al, Cd and U) identified as being enriched. The eigenvalues for axis 1 and 2 are 0.61 and 0.23, respectively, while the cumulative percentage of the explained variance was calculated as 61 % and 83.9 % for the same axes. The ordination diagram presented a positive relation between cp-5 NPN and all of the metal EF, with the exception of U. Furthermore, cp-5 NPN presented a positive relation to clay and silt percentages and a negative relation to TOC and sand percentages. The cp-1, cp-3 and cp-4 NPN presented a positive relation to only U enrichment and TOC, as well as a negative relation to fraction percentage larger than 2 mm. A positive relation between CP-2 NPN and sand percentage was also observed. While cp-1 and cp-3 NPN presented minimal positive relation to Cd enrichment, cp-2 and cp-4 NPN presented a negative relation to most of the metal EF.

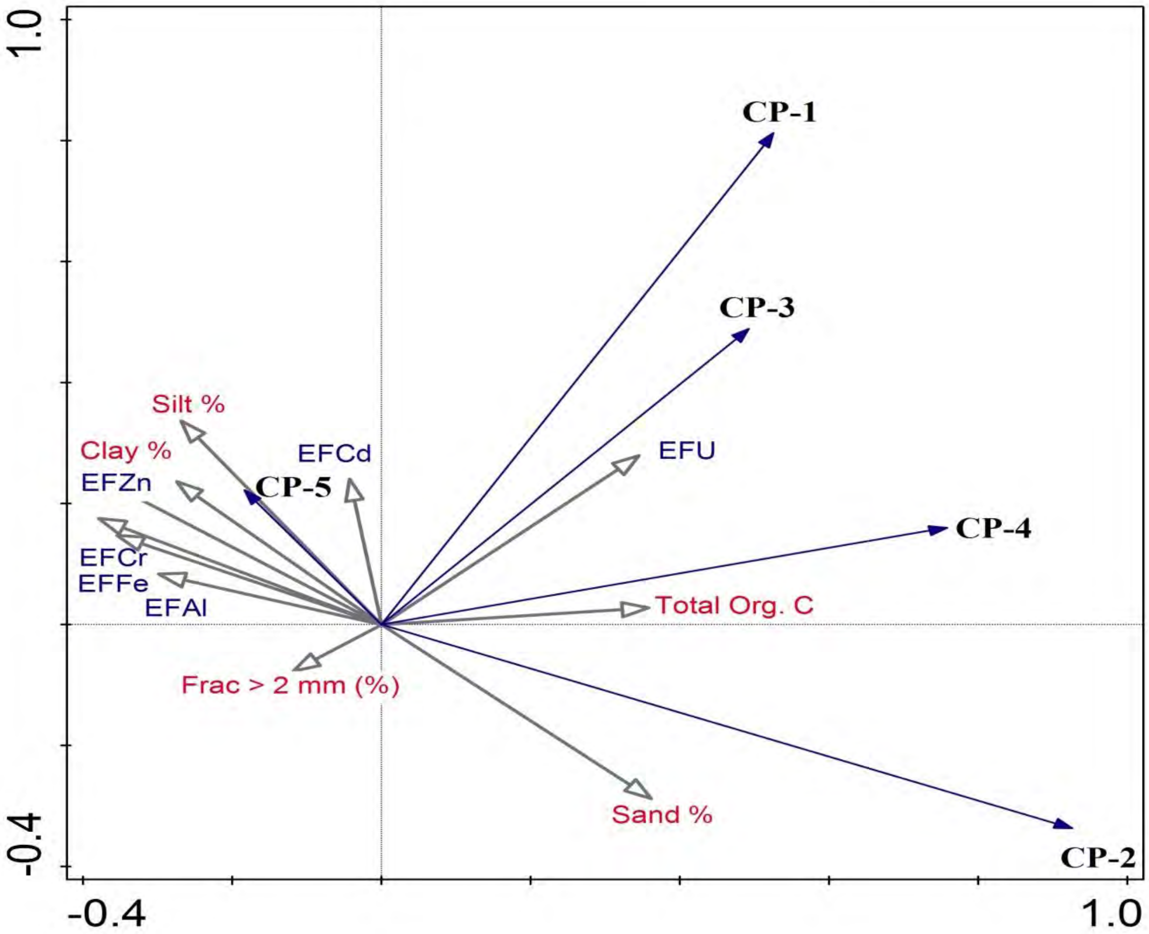

Figure 4.12: A principal component analysis (PCA) illustrates the relation between cp-groups of non-parasitic nematodes (NPN) and metal [Aluminium (Al), Cadmium (Cd), Chromium (Cr), Iron (Fe), Zinc (Zn) and Uranium (U)] enrichment factors (EF) associated with the soils of the Wonderfontein Cave. The relation of these parameters to the particle size distribution and total organic carbon (TOC) is also presented. This analysis was performed on the combined generated data from both sampling intervals (April and September 2013). The ordination explained 61 % variance on the first axis and 83.9 % variation on the second axis.

The PCA analysis of the Wonderfontein Cave sediments (Figure 4.13) presented the relationships between the cp-groups of the NPN assemblages and the metals (Cr, Ni, Cu, Zn, Co, Al, Cd, Pb and U) identified as being enriched. The eigenvalues for axis 1 and 2 are 0.73 and 0.14, respectively, while the cumulative percentage of the explained variance was calculated as 73.2 % and 87.2 % for the same axes. The ordination diagram presented a strong positive relation between cp-1-4 NPN and most of the metal EF, with some cp-groups more strongly related (positive) to certain metal EF. While, cp-1 and cp-4 NPN were positively related to Pb, Ni, U and Al enrichment, cp-3 NPN were positively related to only Al enrichment. Al three these cp-groups were also positively related to percentage clay and negatively related to TOC, sand percentage and fraction percentage larger than 2 mm.

Furthermore, cp-2 NPN presented a strong positive relation to Cu, Co, Pb and Ni enrichment and a negative relation to silt percentage. Finally, cp-5 NPN presented a negative relation to most of the metal EF and a positive relation to TOC, sand percentage and fraction percentage larger than 2 mm.

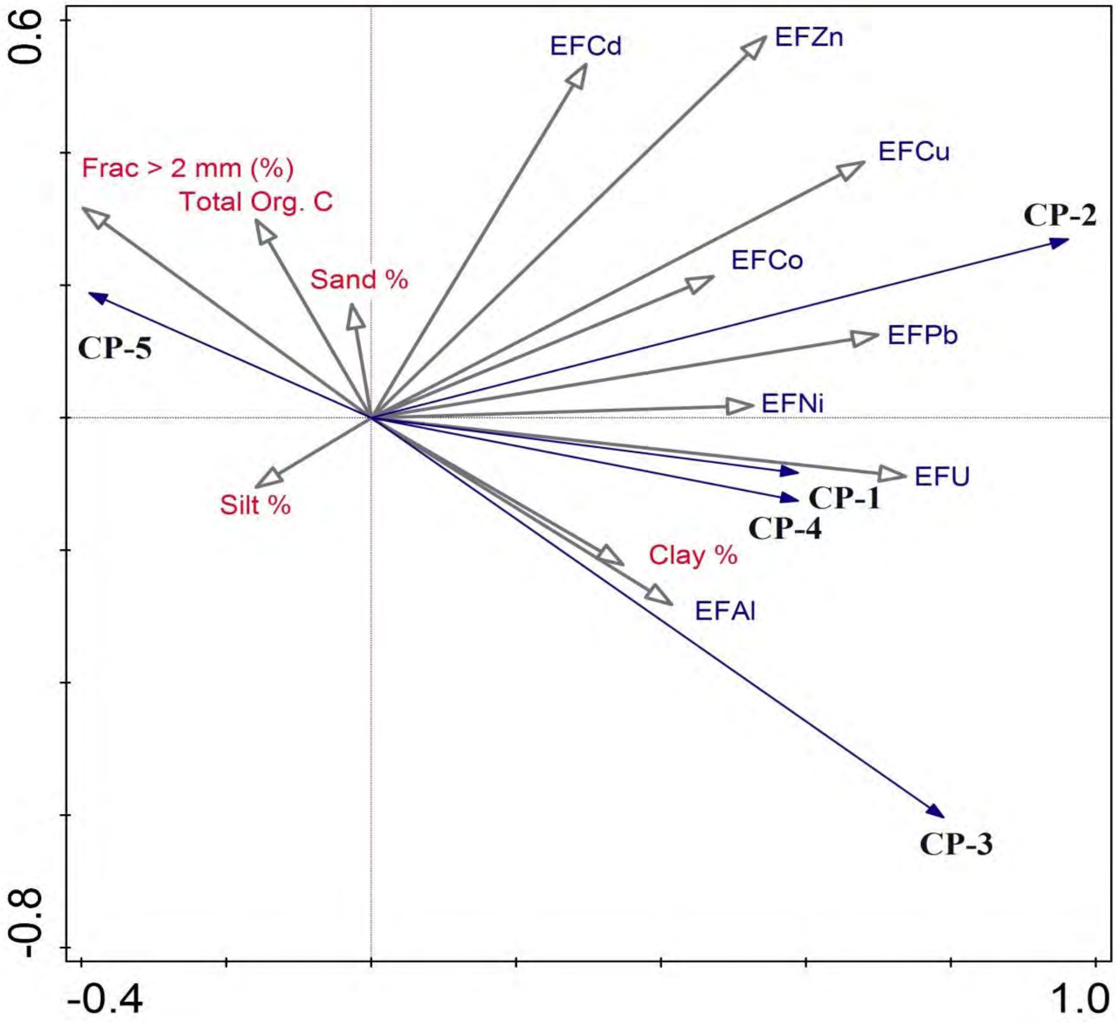

Figure 4.13: A principal component analysis (PCA) illustrates the relation between cp-groups of non-parasitic nematodes (NPN) and metal [Aluminium (Al), Chromium (Cr), Cobalt (Co), Copper (Cu), Iron (Fe), Nickel (Ni), Zinc (Zn) and Uranium (U)] enrichment factors (EF) associated with the sediments of the Wonderfontein Cave. The relation of these parameters to the particle size distribution and total organic carbon (TOC) is also presented. This analysis was performed on the combined generated data from both sampling intervals (April and September 2013). The ordination explained 73.2 % variance on the first axis and 87.2 % variation on the second axis.

4.3 Discussion

4.3.1 Occurrence of nematode assemblages in the Wonderfontein Cave

That the knowledge of nematology regarding NPN is vastly lacking, compared to that of PPN, is still true (Neher, 2010). However, the importance and ecological value of NPN has long since been recognized (Banage, 1963). Nevertheless, information on the existence of cavernicolous (cave-inhabiting) nematodes, their diversity and distribution in subterranean environments and the ecosystem services they provide, remains extremely limited (Durand *et al.*, 2012). The current study was thus undertaken not only to determine the effect of mining-associated pollutants on nematode assemblages, but also to establish baseline data for future studies.

During the course of this study 60 nematode genera, including PPN and NPN, were identified from soils and sediments collected from the Wonderfontein Cave. Hodda *et al.* (2006) provided a list of cavernicolous nematodes sourced from literature mostly published during the 1940' to 1960's. In total, 16 out of the 28 cavernicolous nematode genera listed by the latter authors, were also recorded from the Wonderfontein Cave. Non-parasitic nematodes dominated in soil and sediment samples collected from the Wonderfontein Cave and Spruit, with bacterivores that belong to genera such as *Acrobeloides*, *Chronogaster* and *Plectus* being the most dominant. Yet, some higher trophic nematode taxa, namely *Mononchus* and *Mylonchulus*, were also recorded during this study. During a local study, Durand *et al.* (2012) listed 27 nematode genera from samples obtained from the Bakwena Cave (South Africa), representative of the major trophic groups and the complete cp classification series. Most intriguingly, however, was the discovery that four nematode genera including *Aulolaimus, Campydora, Chromadorita and Ethmolaimus* recorded from the Wonderfontein Cave, have never before been recorded in South Africa. Furthermore, these genera are not listed to be found to date in Africa (Swart, 2014). *Desmolaimus*, another unrecorded genus, was identified from the soils of Stoffels Dam, which is located downstream of the Cave Inflow Area. Listing of these unrecorded nematode genera is, however, not surprising since South African caves and especially the fauna they host, are very poorly studied (Durand *et al.*, 2012).

However, many of the nematodes recorded during this study and especially the ones recorded for the first time, might have been accidental inhabitants of the Wonderfontein Cave. Entry was likely gained through the influx of water and/or by one of the many surface entrances. Furthermore, herbivores are unlikely to be permanent residents of subterranean environments since caves are not able to facilitate autotrophic production by plants (Hodda *et al.*, 2006). Herbivore nematodes may, however, reside in cave environments for a considerable time as roots and decomposing organic material is sometimes available (Romero, 2009), as was observed in the Wonderfontein Cave at several of the sites. Yet, data from this study revealed that herbivores were mostly recorded from the sediments, which are directly associated with the influx of water from the Wonderfontein Spruit. It is thus likely that the water column served as the transport medium (Romero, 2009; Culver & Pipan, 2010) for individuals from this nematode trophic group. Also, herbivore (PPN) taxa (*Aphelenchoides*, *Meloidogyne* and *Tylenchus*) were recorded from the soils of Kent's Entrance, which also had a high exposure to the influx of water from the Wonderfontein Spruit, as well as from an overlying vertical shaft entrance. Furthermore, *Ditylenchus*, *Longidorella*, *Meloidogyne*, *Paratylenchus*, *Rotylenchus* and *Tylenchus* specimens, all classified as herbivores, were recorded from Elevation Pit (soil sample A), which was occasionally exposed to infiltrating rainfall water entering via Absolute Elevation Pit. It is thus evident that many of the nematode genera, especially herbivores, were unlikely to be permanent residents of the Wonderfontein Cave. Unfortunately, it is currently not possible to determine the amount of time that these nematodes have resided within the Wonderfontein Cave. Other aspects to consider, which could account for the observed nematode food web structures, include the possibility that caves may host multiple microhabitats that are relatively isolated from other parts of the cave (Wynne, 2013). Also, that food resources might be unevenly distributed with a patchy occurrence (Culver *et al.*, 2004).

4.3.2 Interpretation of nematode food web structures

Cave environments and the communities they host are generally considered to be relatively stable (Di Russo *et al.*, 1997; Romero, 2009). However, the community indices applied on nematode data obtained from substrate samples collected from the Wonderfontein Cave, do not complement the latter statement. It was revealed that substantial variation occurred both over a temporal and spatial scale and was possibly influenced by a number of factors. Karst landscapes, such as the one associated with the Wonderfontein Cave, are interconnected systems that sometimes facilitate the exchange of fauna between the surface and subterranean landscape (Stokes *et al.*, 2010). This is most likely the reason, as previously mentioned, why soil sample A collected from Elevation Pit had a higher average species diversity and richness than soil sample B, as the former was exposed to infiltrating surface waters. Another example is that of Pristine Chamber, which was initially perceived as a potential biodiversity hotspot since the site was sufficiently elevated above the water level. This site would thus not have been previously exposed to mining-associated pollutants. Evidence of this is provided in Chapter 3 (section 3.2.2) as no significant enrichment of metals was associated with this site. Furthermore, bat guano and spider webs were found on the cave floor, which indicated the likely presence of also other fauna (Ferreira *et al.*, 2007). From what seemed to be a pristine environment, only a single *Monhystera* specimen was found in soils collected from both sampling intervals (see Appendix B). It can thus be reasoned that this phenomenon was the result of zero interaction with the water column and thus no introduction of nematodes.

Other factors that might have resulted in the observed nematode food web structures include the lack of food and sufficient habitat, as well as the composition of the host substrate (Neher, 2010). And although the evidence does not directly support this theory, the pollution effect is expected to have had some effect as it did in other studies (Ekschmitt & Korthals, 2006; Šalamún *et al.*, 2012). However, it should be noted that the observed lower nematode species diversity and richness in the sediments, if compared to that of the soils, is a well-documented natural phenomenon (Heyns, 1976; Bongers, 1999) and not necessarily the result of anthropogenic influence. As from the above, it is not possible to associate the variance observed in species richness and diversity as a direct cause of a single or combination of these factors since no other known cave exists that could have served as a control. Similarly, the interpretation of the trophic structures of the nematode assemblages is subject to the same reasoning. Delineating the observed structures as a result of one or two specific factors, would be unfounded. It is, however, notable that most of the soils and sediments were dominated by bacterivores, as was also reported by Durand *et al.* (2012) from the Bakwena Cave. Furthermore, only a few specimens that were characterised as cp-4 and cp-5 nematodes, were recorded. Shao *et al.* (2008) found that heavy metal pollution might be the cause of the absence of higher nematode trophic groups. Nevertheless, for the reasons stated above and taking into consideration the aims of this study, the possible effects of mining-associated pollutants on the nematode assemblages were further evaluated with the faunal and metabolic footprint analyses, as well as the PCA, which will subsequently be discussed.

The MI, applied to evaluate the nematode assemblages associated with the soils and sediments of the Wonderfontein Cave and Spruit, has been successfully implemented before as a routine measure to quantify the level of disturbance induced by metal pollution (Sánchez-Moreno & Navas, 2007; Šalamún *et al.*, 2012). Although the MI was the original index developed by Bongers (1990), the adapted $MI_{2\text{-}5}$ is more suitable when measuring the disturbance in NPN community structures caused by metal pollution (Bongers & Ferris, 1999; Ferris & Bongers, 2009). According to the latter authors pollutants may serve as a food source for the microbial community, which subsequently acts as a resource for enrichment opportunistic, cp-1 NPN. For reasons stated above and considering the aims of this study, only the values of the $MI_{2\text{-}5}$ index will be discussed. The MI values were provided to serve as reference for future studies. The soil MI_{2-5} values, mostly ranging between 2-3, were relatively low and indicative of disturbed systems in terms of NPN food webs that are known for terrestrial habitats. Nagy *et al.* (2004) reported similar MI_{2-5} value ranges when the effect of microelement concentrations on soil nematode assemblages, associated with artificially contaminated agricultural fields, were studied. The high temporal variation in NPN assemblages observed during this study between the soils collected during both sampling intervals from Kent's Entrance, might thus be the result of close interaction with the influx of surface water. As with Durand *et al.* (2012), it is difficult to truly assess the degree of disturbance of the soils as there exists no reference values for cave environments. However, valuable data have been recorded by Durand *et al.* (2012) and also as a result of this study. Such data can already serve as baseline data for similar, future studies.

When considering the sediments, it is important to note that caution should be exercised when interpreting MI values. Bongers and Ferris (1999) warned that the structure of NPN assemblages associated with sediments can be inconsistent and variable. It is possible that the observed structure is the result of frequent introduction of NPN via the water column, rather than trophic interactions. Also, Bongers (1999) stated that trophic interaction in sediments might not even exist as nematode diversity are typically very low. Nevertheless, the sediments collected from the Wonderfontein Cave presented disturbed systems with some considerable, yet, inconsistent variation. Data obtained from the surface sediments also generally indicated nematode-food web systems as being disturbed. Furthermore, taking into consideration that the Wonderfontein Spruit sediments are known to be polluted as a result of mining activities (Coetzee *et al.*, 2006; Durand *et al.*, 2010; Hamman & Van Rensburg, 2012), it is likely that the cave sediments suffer the same fate.

According to Shao *et al.* (2008) the faunal analysis provide a valued assessment of soil system health and is more suitable than other approaches such as the MI. This is especially true in instances where fewer NPN individuals were recorded. Comparison of the faunal analyses of the soils and sediments showed that they represented different faunal NPN structures. Generally, the soils were more enriched, yet, presented more temporal stability, while the sediments were more structured with more temporal variation. The temporal variation in the sediments was especially noticeable in the systems closer to the inflow area (Cave Inflow Area, Kent's Entrance and Keyhole Entrance), owning to the Bongers and Ferris (1999) statement that sediments can be inconsistent and variable. Evidently, NPN data showed that the two substrates had different faunal structure functions. Sánchez-Moreno and Navas (2007) and Šalamún *et al.* (2012) stated that under heavy metal pollution, an ecosystem shift is visible in the faunal analysis since typically polluted sites are less structured and more enriched in terms of NPN assemblages. The faunal analysis diagrams of the Wonderfontein Cave and Spruit substrates present similar traits to that of Sánchez-Moreno and Navas (2007) under polluted conditions as the plots were less clustered with only soils from a few sampling sites, in terms of nematode food webs, being classified as mature and fertile.

The metabolic footprint analysis is an additional component of the faunal analysis that provide further insight into the functioning of and services provided by the nematode assemblages (Ferris, 2010). The use of metabolic footprints as an indicator of the response of nematodes to metal pollution was successfully implemented for the first time in a study by Rodríguez Martín *et al.* (2014), which focused on the effect of mining residue on nematode assemblages in mining, natural and agricultural areas in the Cartagena-La Union district (Spain). However, as expected from the nematode assemblages that were identified from the Wonderfontein Cave sediments, the metabolic footprint analysis revealed that the associated assemblages presented minimal ecosystem functioning. Although structure footprints were recorded from some systems, no enrichment footprints were presented. As with the sediments, the soils presented some metabolic activity, however, only from a few systems that were mostly associated with the surface environment. The only cave soils (Elevation Pit) that presented some metabolic activity had been exposed to infiltrating water, which supports the postulation that the nematodes associated with that system was mostly introduced. Evidence provided by the faunal and metabolic footprint analyses suggested that the nematodes in the sediment samples from the various sites were not only subjected to inconsistent temporal stability, but also non-functioning. And although the cave soils were more stable over a temporal scale, it is evident that most of the associated systems were either enriched or degraded with minimal maturity. It is likely that the harsh conditions of the cave environment limited the functioning of intricate faunal food webs.

The data presented up to now confirmed that the Wonderfontein Cave hosted nematode assemblages under stress, however, the cause of which remains unclear. The use of ordination diagrams, with the purpose of identifying associations between specific nematode groups and environmental conditions, have been successfully implemented in multiple studies (Gyedu-Ababio *et al.*, 1999; Georgieva *et al.*, 2002; Shao *et al.*, 2008). For this reason the NPN cp-groups were related to metal EF in order to assess whether the variance in species composition and the observed state of ecosystem health was related to anthropogenic metal pollution.

4.3.3 Relation of non-parasitic nematode (NPN) trophic groups to metal enrichment

The PCA of the Wonderfontein Cave soils revealed that most of the cp-groups, except for cp-5 NPN, related negatively to metal enrichment and positively to TOC. This is contrary to what would be expected as cp-1-2 NPN are known to be more tolerant to environmental disturbance (adverse conditions) than cp-3-5 NPN (Bongers & Ferris, 1999; Ferris *et al.*, 2001). However, the PCA of the Wonderfontein Cave sediments presented a positive relation between cp-1-4 NPN and metal enrichment and a negative relation between cp-5 NPN and metal enrichment. Although it is expected that cp-1-2 NPN would be associated with metal enrichment (environmental disturbance), cp-3-4 NPN are also known to be sensitive to environmental disturbances (Ferris *et al.*, 2001). The relationships between NPN cp-groups and metal EF, as illustrated by the PCA, are thus an unlikely scenario, which further suggests that anthropogenic metal pollution is not the direct cause of variance observed in species composition and ecosystem disturbance.

4.4 Conclusion

Although caves are naturally considered as stable environments, they can be unforgiving even without the influence of anthropogenic activities. It is concluded that although it is highly likely that metal pollution contributed towards the deteriorated state of the soil and sediment ecosystems associated with the Wonderfontein Cave, it was probably not the main factor at play. Thus, although the ecosystems associated with the soils and sediments of the Wonderfontein Cave are degraded, the hypotheses are rejected as no concrete evidence was obtained to illustrate the effect of metal pollution on the nematode assemblages, nor could the NPN assemblage data be related to and used as an indication of ecosystem health.

There is a pressing need to study the fauna associated with South African caves as it would provide intricate knowledge on the ecosystem functioning of the local subterranean environments. Furthermore, this would provide baseline data for future studies. Ultimately, the proposed efforts will contribute towards the conservation of subterranean environments that are currently neglected.

4.5 References

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CHAPTER 5

METAL BIOACCUMULATION AND BIOMARKER RESPONSES IN *CLARIAS GARIEPINUS* OF THE WONDERFONTEIN CAVE AND AN EPIGEAN POPULATION

5.1 Introduction

Fish occur in nearly all aquatic systems and play a major role in the ecology of food webs. They are sensitive to environmental change and are thus considered to be among the most important of aquatic bioindicators (Van der Oost *et al.*, 2003; Naigaga *et al.*, 2011; Jia & Chen, 2013). Various techniques can be applied in order to assess the influence of environmental pollution on fish populations and thus the associated aquatic ecosystem. Widely used techniques include determining the bioaccumulation of toxic substances in fish tissue (Kotze *et al.*, 1999; Crafford & Avenant-Oldewage, 2010), quantifying oxidative stress and exposure biomarkers (Van der Oost *et al.*, 2003), as well as evaluating physical health by means of the HAI (Adams *et al.*, 1993) and gross body indices such as the CF and HSI (Van Dyk et al., 2012; McHugh et al., 2013).

The Wonderfontein Cave consists of a network of subterranean passages, which was flooded at the commencement of this study in October 2012 and has remained so until the present. This system is associated with the banks of the Wonderfontein Spruit from which water drains into the subterranean network (Kent *et al.*, 1978). Residing within the Wonderfontein Cave is a *Clarias gariepinus* (African sharptooth catfish) population that utilize the subterranean environment most likely for shelter, foraging and possibly even spawning (Romero, 2009). It is unclear whether this population of cave fish has been isolated from the surface aquatic environment and what its state of health might be. In this part of the study the degree of isolation is considered by making use of the cytochrome b (mitochondrial DNA) molecular marker and the health of the Wonderfontein Cave inhabiting population compared to that of a epigean (surface) population (Stoffels Dam) associated with the Wonderfontein Spruit by applying the various assessments named above. The latter also provided insight into the ecosystem health of the Wonderfontein Cave and the subsequent threat posed by the influx of anthropogenic derived metal toxicants of which evidence was provided in Chapter 3.

Firstly, it is hypothesized that the *C. gariepinus* population within the Wonderfontein Cave is genetically diverged from the epigean population and secondly, that there are no differences in fish population health as both populations are subjected to the same source of pollution.

5.2 Results

5.2.1 Genetic structure of *Clarias gariepinus* populations

The genetic analysis of the cyt b gene [mitochondrial (mt) DNA] was performed in order to determine whether the Wonderfontein Cave population have been isolated, thus, preventing gene flow. Although there are a number of factors that could be considered regarding the structure of the respective (Wonderfontein Cave and Stoffels Dam) populations, the aim of this analysis was to assess if the Wonderfontein Cave population has diverged from the epigean populations and subsequently, whether it can be considered as permanent residents of the cave system.

The *C. gariepinus* sequences of the specimens sampled from the Wonderfontein Cave (n = 9) and Stoffels Dam (n = 10) were approximately 600 bp long. Also, cyt b sequences available on GenBank for North [Accession No. HQ70170(0-4).1] and East [Accession No. HQ7016(88-91).1, HQ7016(94-99).1] African *C. gariepinus* clades were used for comparative purposes. The Wonderfontein Cave and North African populations, as interpreted from the nucleotide diversity (Table 5.1), illustrated a higher degree of polymorphism than the Stoffels Dam and East African populations. Also, higher haplotype diversity was observed in the former populations.

Table 5.1: Measures of genetic variance in the *Clarias gariepinus* populations associated with the Wonderfontein Cave and Stoffels Dam compared to the North and East African populations.

	n	**Number of haplotypes**	**Haplotype diversity (h)**	**Nucleotide diversity (π)**
Wonderfontein Cave	9	5	0.81	0.0035
Stoffels Dam	10	4	0.75	0.0088
North Africa	5	3	0.80	0.0077
East Africa	10	4	0.78	0.0035

The pairwise F_{ST} fixation index (Table 5.2) was used to determine the genetic distances between the respective *C. gariepinus* populations. The significance of each comparison was calculated by following 16 000 permutations with a 95 % confidence interval. The F_{ST} analysis produced significant ($p < 0.05$) high values between the North and East African populations, as well as between the latter populations and the Wonderfontein Cave and Stoffels Dam populations, respectively. This indicated that most of the observed molecular variance was attributed to differentiation between and not within the populations. However, zero ($p > 0.05$) genetic distance was observed between the Wonderfontein Cave and Stoffels Dam populations. Therefore, 100 % of the observed molecular variance was within the Wonderfontein Cave and Stoffels Dam populations, respectively.

A haplotype (median-joining) network (Figure 5.1) was created to illustrate the genetic divergence (structure) between the different *C. gariepinus* populations. The network clearly illustrates that the North and East African populations (clades) were genetically diverged from each other, as well as from the Wonderfontein Cave and Stoffels Dam populations. In contrast, it was evident that most of the haplotypes from the Wonderfontein Spruit clade occurred in both the Wonderfontein Cave and Stoffels Dam populations; only one haplotype was unique to the Wonderfontein Cave population.

Table 5.2: Pairwise F_{ST} values indicating the genetic distances between the Wonderfontein Cave, Stoffels Dam, North and East African *Clarias gariepinus* populations. Significant ($p < 0.05$) F_{ST} values are indicated with an asterisk (*).

F_{ST}		**(1)**	**(2)**	**(3)**	**(4)**
Wonderfontein Cave	(1)	-			
Stoffels Dam	(2)	0.0	-		
East Africa	(3)	0.96*	0.97*	-	
North Africa	(4)	0.95*	0.95*	0.83*	-

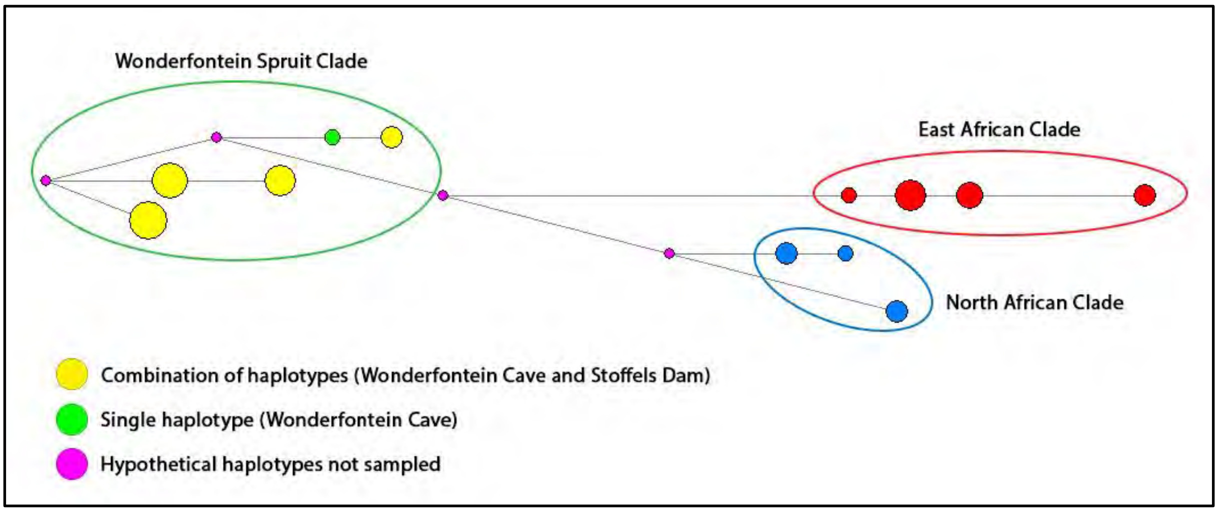

Figure 5.1: A haplotype (median-joining) network illustrating the genetic divergence (structure) between the North African, East African and Wonderfontein Spruit *Clarias gariepinus* clades. The size of the circles is proportional to the haplotype frequency.

An analysis of molecular variance (AMOVA; Table 5.3) was performed based on the genetic structure illustrated by Figure 5.1. For this analysis the Wonderfontein Cave and Stoffels Dam populations were grouped together, while each of the North and East African populations were representative of its own group. For each comparison, a fixation index (F_{CT}, F_{SC} and F_{ST}) was calculated to test the significance of the analysis. The AMOVA analysis revealed that no significant variance was observed among populations within groups (F_{SC}).

Table 5.3: Analysis of molecular variance (AMOVA) between the Wonderfontein Spruit (Wonderfontein Cave and Stoffels Dam populations), North African and East African *Clarias gariepinus* clades. Significant ($p < 0.05$) fixation index values are indicated with an asterisk (*).

Source of variation	**Df**	**Sum of squares**	**Variance components**	**Percentage of variation**	**Fixation Index**
Among groups	2	976.04	50.16	**96**	$F_{CT} = 0.96$
Among populations within groups	1	1.44	-0.07	**0**	$F_{SC} = 0.0$
Within populations	29	61.75	2.12	**4**	$F_{ST} = 0.96$*
Total	32	1039.24	52.22		

5.2.2 Metal bioaccumulation in muscle tissue of *Clarias gariepinus* populations

The metals Cr, Ni, Cu, Zn, Co, Fe, Al, As, Mn, Pb, U, Ti, Selenium (Se), Gold (Au) and Thorium (Th) were selected as literature provide evidence of their relevance in fish health assessments (Deacon & Stephens, 1998; Kotze *et al.*, 1999; Liang *et al.*, 1999; Avenant-Oldewage & Marx, 2000; Polak-Juszczak, 2009; Murtala *et al.*, 2012). Cadmium was not included as concentrations in all of the analysed fish muscle samples were below the detection limit (0.04 mg/kg).

There were no significant ($p < 0.05$) differences recorded for Cr (Figure 5.2 a), Cu (Figure 5.2 c), Al (Figure 5.3 a), As (Figure 5.3 b), Pb (Figure 5.3 d), U (Figure 5.3 e), Au (Figure 5.4 b) and Th (Figure 5.4 c) between the Wonderfontein Cave (2nd sampling interval) and Stoffels Dam populations, as well as between the 1st and 2nd sampling intervals in the Wonderfontein Cave population. Copper (3.09 mg/kg), Al (10.71 mg/kg), As (0.69 mg/kg), U (0.03 mg/kg) and Au (0.19 mg/kg) concentrations were the highest in muscle tissue of the *C. gariepinus* population associated with the Wonderfontein Cave (2nd sampling interval), while higher concentrations of Cr (0.55 mg/kg), Pb (0.15 mg/kg) and Th (0.18 mg/kg) were recorded in muscle tissue of the Stoffels Dam population.

Significant ($p < 0.05$) differences were, however, recorded between the two sampling intervals in the Wonderfontein Cave population for Zn (Figure 5.2 d) and Co (Figure 5.2 e) bioaccumulation levels in the muscle tissue of *C. gariepinus*, as well as between the Wonderfontein Cave (2nd sampling interval) and Stoffels Dam populations for Ni (Figure 5.2 b), Zn, Co, Mn (Figure 5.3 c), Ti (Figure 5.3 f) and Se (Figure, 5.4 a).

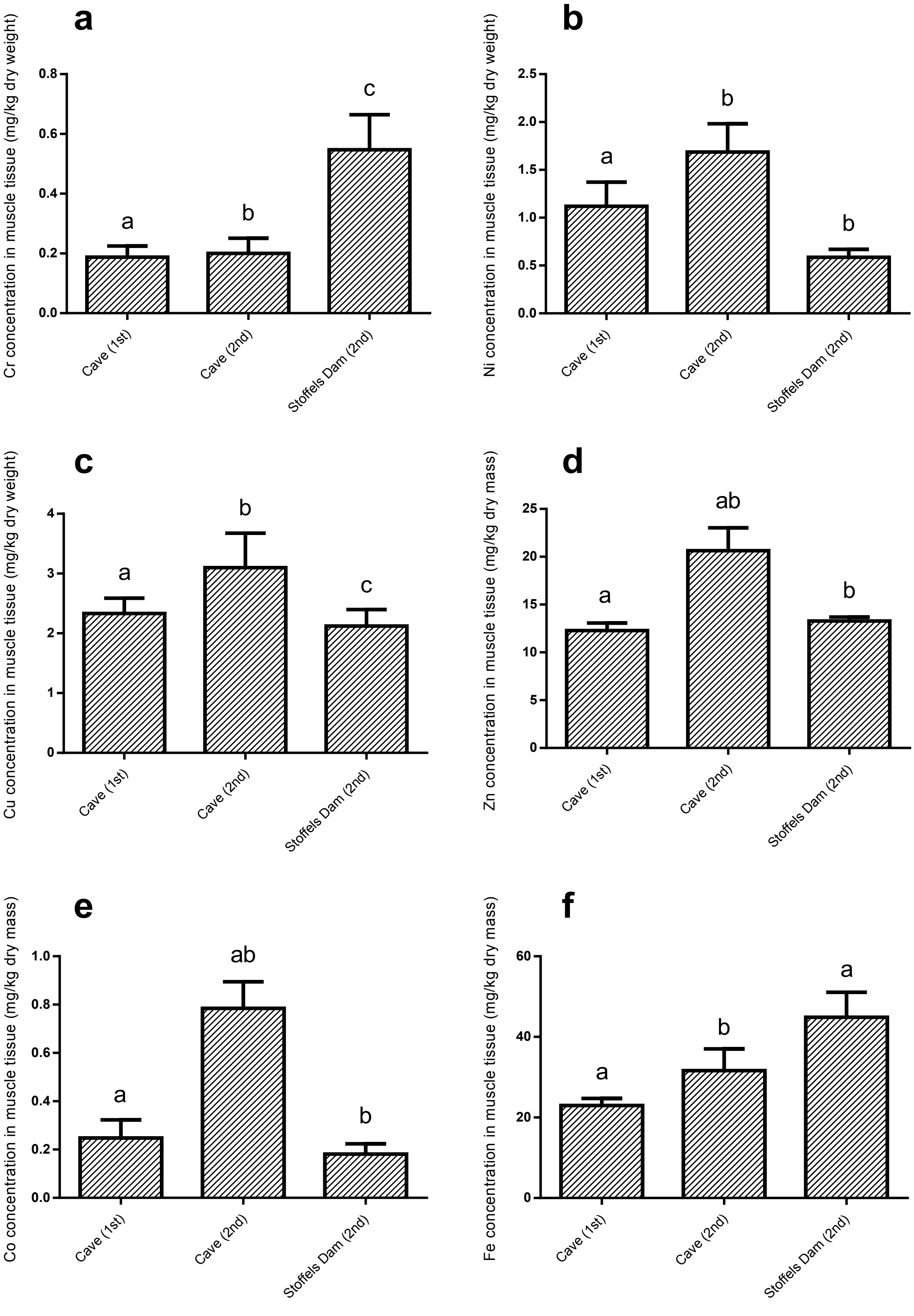

Figure 5.2: Bioaccumulation of a) Cr, b) Ni, c) Cu, d) Zn, e) Co and f) Fe in muscle tissue (mg/kg dry weight) of *Clarias gariepinus* populations sampled from the Wonderfontein Cave and Stoffels Dam during the 1st (April 2013) and 2nd (September 2013) sampling intervals. These sampling intervals represent the end of the high and low flow periods, respectively. Bars with common superscript present significant ($p < 0.05$) differences.

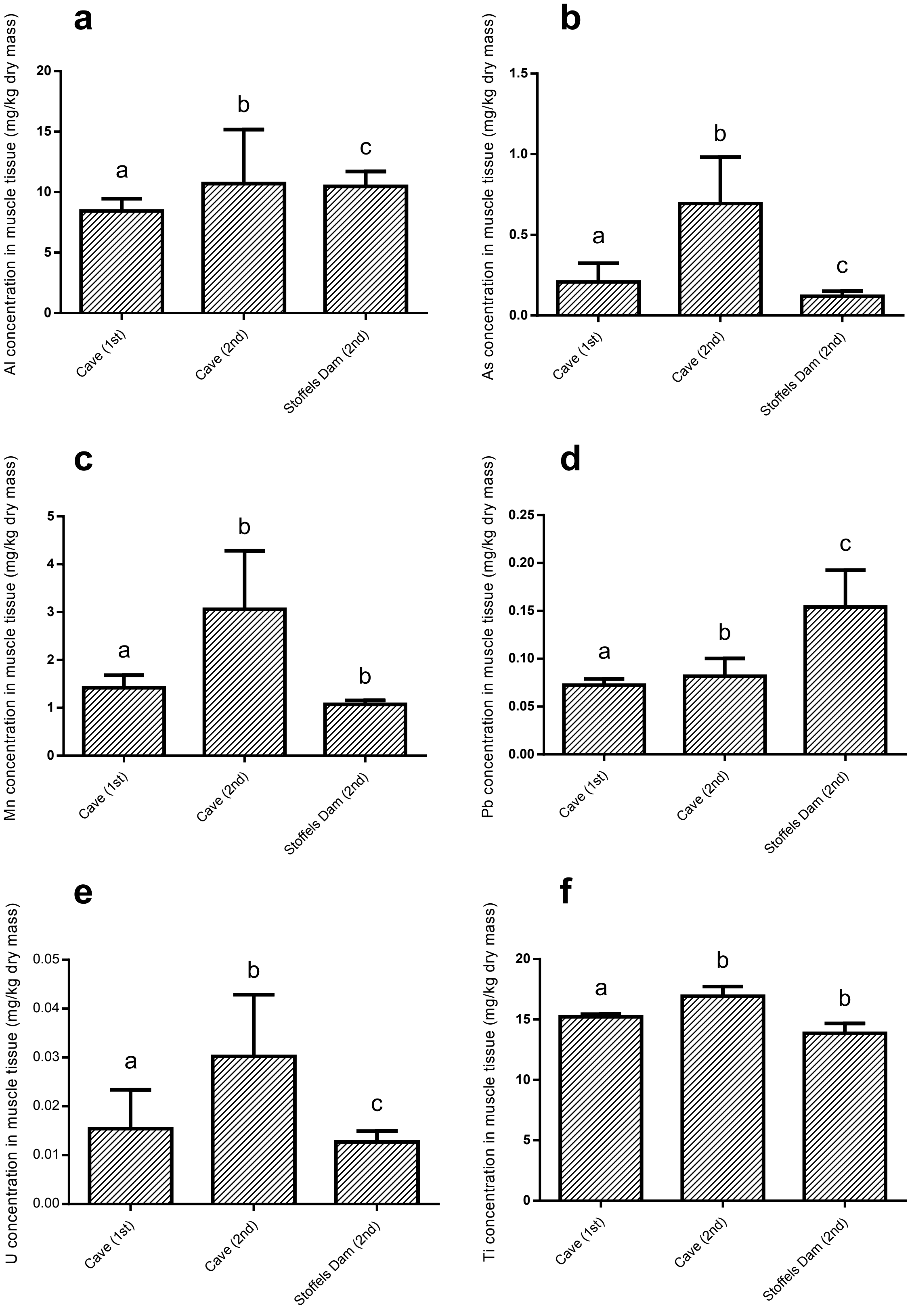

Figure 5.3: Bioaccumulation of a) Al, b) As, c) Mn, d) Pb, e) U and f) Ti in muscle tissue (mg/kg dry weight) of *Clarias gariepinus* populations sampled from the Wonderfontein Cave and Stoffels Dam during the 1st (April 2013) and 2nd (September 2013) sampling intervals. These sampling intervals represent the end of the high and low flow periods, respectively. Bars with common superscript present significant ($p < 0.05$) differences.

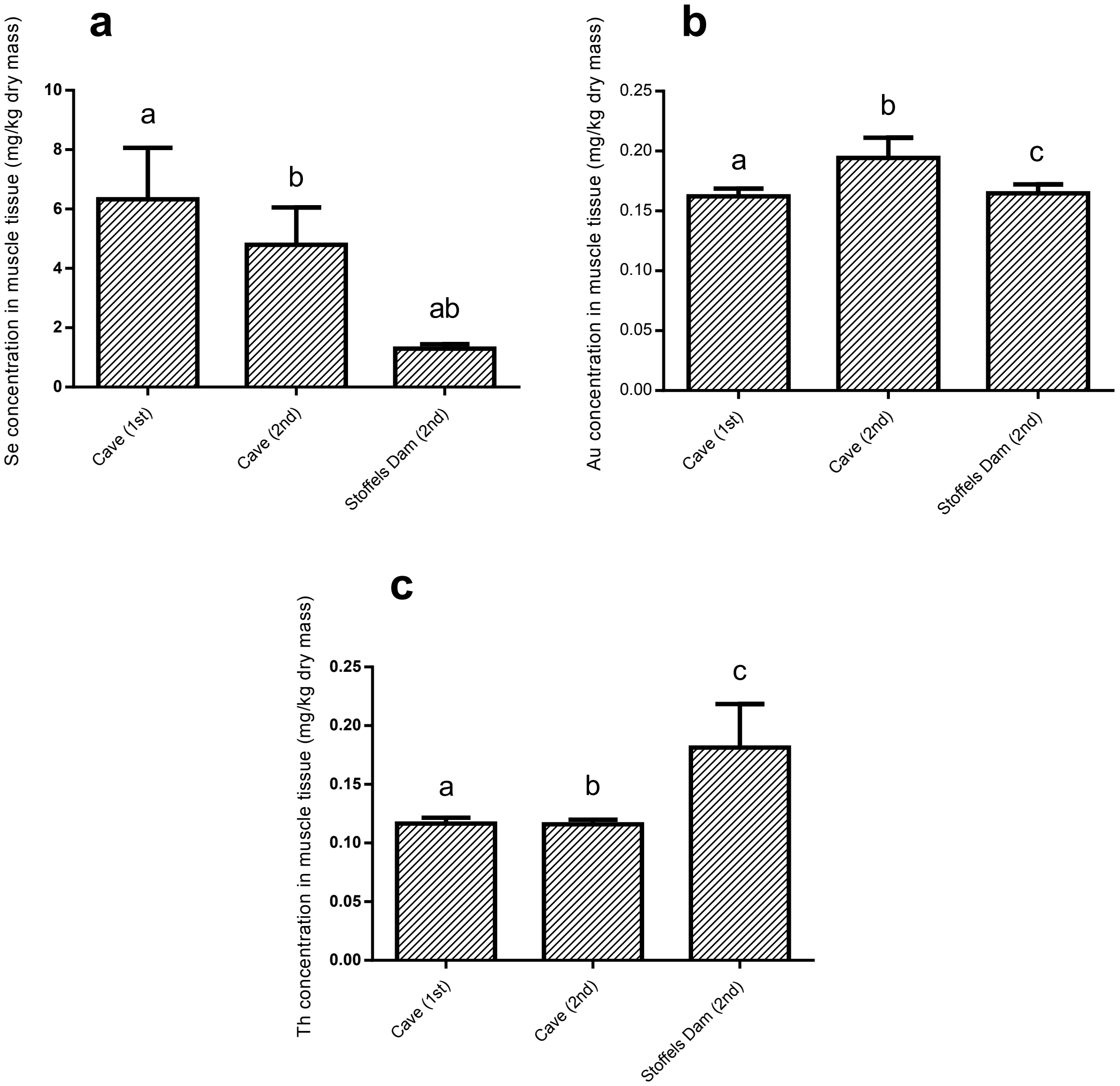

Figure 5.4: Bioaccumulation of a) Se, b) Au and c) Th in muscle tissue (mg/kg dry weight) of *Clarias gariepinus* populations sampled from the Wonderfontein Cave and Stoffels Dam during the 1st (April 2013) and 2nd (September 2013) sampling intervals. These sampling intervals represent the end of the high and low flow periods, respectively. Bars with common superscript present significant ($p < 0.05$) differences.

When considering only the metals that presented significant ($p < 0.05$) concentration differences between sampling intervals and/or populations, the highest metal concentrations of Se (6.33 mg/kg) (1st sampling interval) and Ni (1.69 mg/kg), Zn (20.65 mg/kg), Co (0.78 mg/kg), Mn (3.06 mg/kg) and Ti (16.92 mg/kg) (2nd sampling interval) were recorded in the muscle tissue of the Wonderfontein Cave *C. gariepinus* population, while Fe was the only metal with the highest concentration recorded in the muscle tissue of the Stoffels Dam population.

5.2.3 Health assessment index (HAI) and gross body indices

The HAI values (Table 5.4) of the Wonderfontein Cave population collected during the 1st and 2nd sampling intervals were calculated as zero and 15, respectively, while the HAI value of the Stoffels Dam population was 16. Although no significant ($p < 0.05$) differences were recorded in the CF values (Figure 5.5 a) between both the two sampling intervals and the respective populations, a significant ($p < 0.05$) difference was recorded in the HSI values (Figure 5.5 b) between the Wonderfontein Cave (2nd sampling interval) and Stoffels Dam populations. The Wonderfontein Cave population (2nd sampling interval) presented the highest HSI value of 1 ± 0.14 (Table 5.4). Furthermore, significant ($p < 0.05$) differences were recorded in the mean body weight (Figure 5.5 c) and length (Figure 5.5 d) between the Wonderfontein Cave (2nd sampling interval) and Stoffels Dam populations.

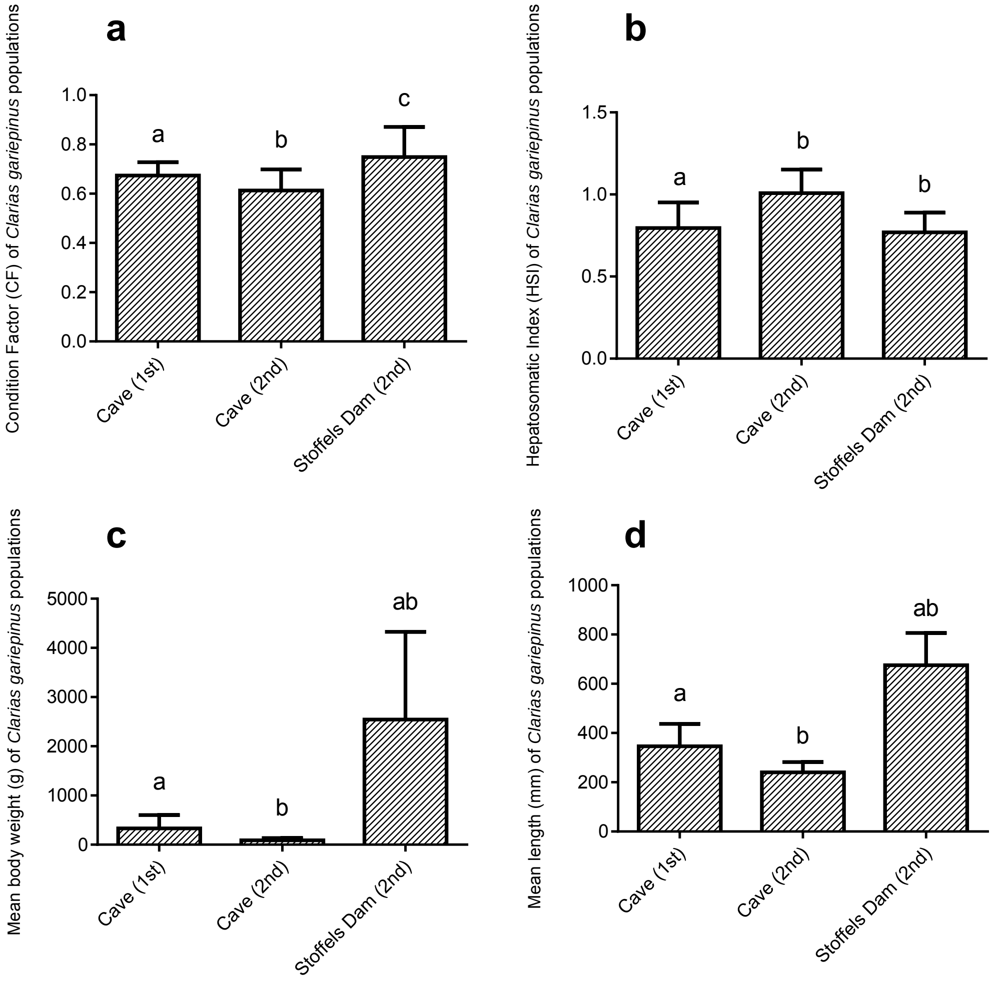

Figure 5.5: Gross body indices [condition factor (CF) and hepatosomatic index (HIS)] and mean body weight and length of *Clarias gariepinus* populations sampled from the Wonderfontein Cave and Stoffels Dam during the 1st and 2nd sampling intervals. These sampling intervals represent the end of the high and low flow periods, respectively. Bars with common superscript present significant ($p < 0.05$) differences.

Table 5.4: The mean (± SD) total weight and length, condition factor (CF), hepatosomatic index (HSI) and health assessment index (HAI) values of *Clarias gariepinus* populations sampled from the Wonderfontein Cave and Stoffels Dam during the 1st (April 2013) and 2nd (September 2013) sampling intervals. These sampling intervals represent the end of the high and low flow periods, respectively.

Sampling Interval	**n**	**Total Body Weight (g)**	**Total Length (mm)**	**CF**	**HSI**	**HAI**
Cave (1st)	5	332.8 ± 268.37	346.2 ± 90.86	0.67 ± 0.05	0.79 ± 0.15	0
Cave (2nd)	4	89.64 ± 46.62	240 ± 42.43	0.61 ± 0.09	1 ± 0.14	15 ± 30
Stoffels Dam (2nd)	10	2543 ± 1782	675 ± 131.17	0.75 ± 0.12	0.76 ± 0.12	16 ± 22.2

5.2.4 Biomarkers of oxidative stress and exposure

The biomarkers of oxidative stress, which includes CAT (Figure 5.6 a), SOD (Figure 5.6 b) and PC (Figure 5.6 c), as well as MT (Figure, 5.6 d), the biomarker of exposure, present some significant ($p < 0.05$) differences between sampling intervals and/or the respective *C. gariepinus* populations. Significant ($p < 0.05$) differences in this regard between the two sampling intervals in the Wonderfontein Cave population were recorded for all the biomarkers. The highest concentrations of CAT (12.77 µmol H_2O_2/min/mg protein) and PC (586.68 nmol carbonyls/mg protein) were recorded in liver tissue in the Wonderfontein Cave *C. gariepinus* population during the 2nd sampling interval, while the highest concentrations of SOD (0.12 ng SOD ng/mg protein) and MT (3.81 nM/mg protein) were recorded in liver tissue in the same population, however, only during the 1st sampling interval.

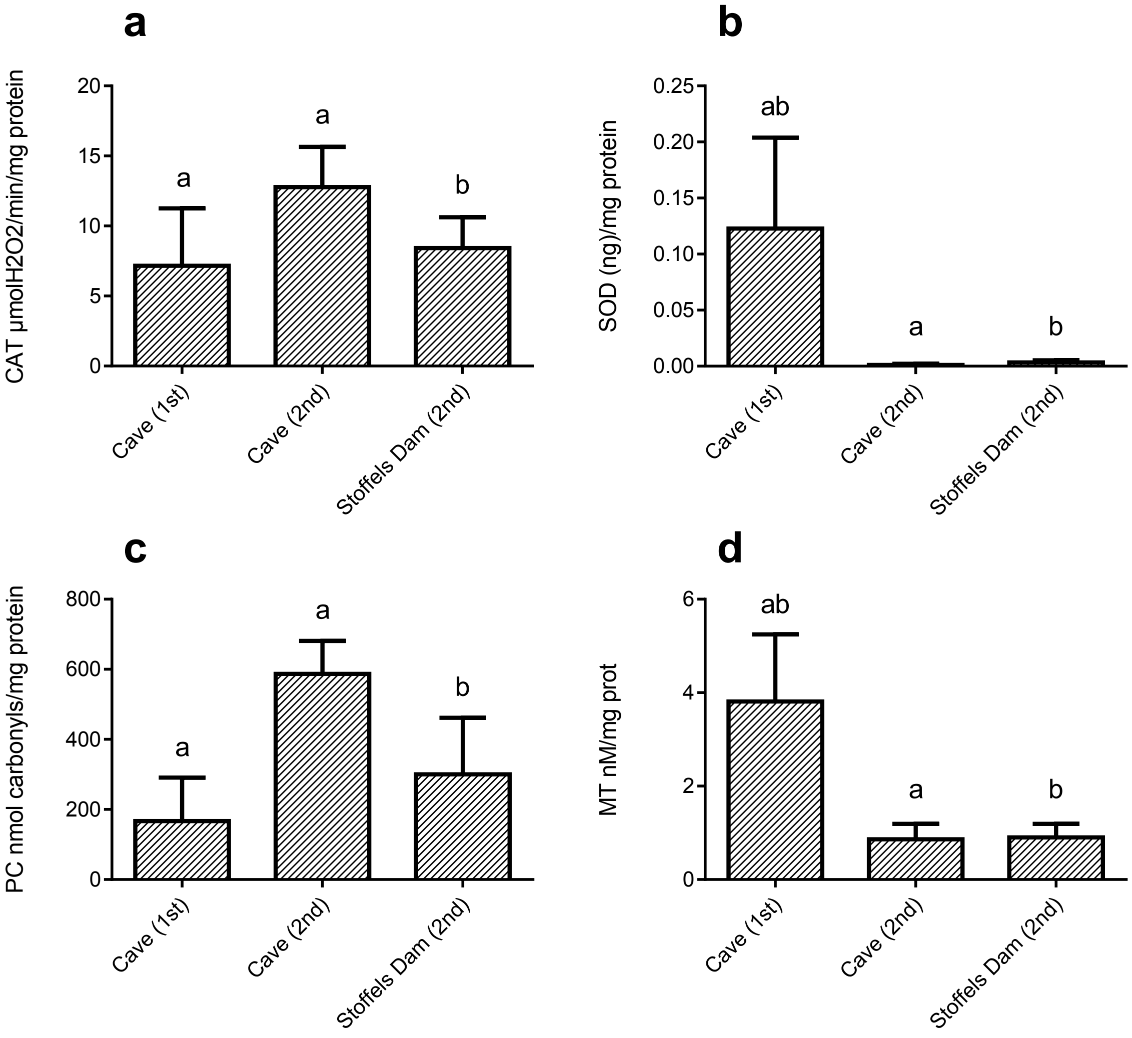

Figure 5.6:Biomarkers of oxidative stress [catalase (CAT), superoxide dismutase (SOD) and protein carbonyl (PC)] and exposure [methallothioneins (MT)] concentrations in the liver tissue of *Clarias gariepinus* populations sampled from the Wonderfontein Cave and Stoffels Dam during the 1st (April 2013) and 2nd (September 2013) sampling intervals. These sampling intervals represent the end of the high and low flow periods, respectively. Bars with common superscript present significant ($p < 0.05$) differences.

5.2.5 Relationships between metal bioaccumulation, biomarkers of oxidative stress and exposure and gross body indices associated with *Clarias gariepinus* populations

An ordination diagram (Figure 5.7), created as a PCA, illustrates the relationships that existed between metal bioaccumulation, biomarkers of oxidative stress and exposure, as well as the CF and HSI of the respective *C. gariepinus* populations sampled during the 1st and 2nd sampling intervals. The eigenvalues for axis 1 and 2 are 0.79 and 0.2, respectively, while the cumulative percentage of the explained variance was calculated as 79.8 % and 100 % for the same axes. The PCA revealed that there existed clear temporal variation in the Wonderfontein Cave population between the two sampling intervals, as well as spatial variation between the Wonderfontein Cave and Stoffels Dam populations. While these fish populations were strongly associated with certain metals, the Wonderfontein Cave population (1st sampling interval) presented a strong relation to MT and SOD. Furthermore, the CF presented a strong relationship to the Stoffels Dam population, while the Wonderfontein Cave population (2nd sampling interval) were related to PC, CAT and the HSI. Metals that were related to the former population included Fe, Al, Pb, Cr and Th, while Zn, Au, U, Co, As, Mn and Cu showed relation to the latter population. Titanium and Ni were also related, although not as prominently, to the Wonderfontein Cave population (2nd sampling interval). Selenium presented a negative relationship to the Stoffels Dam population.

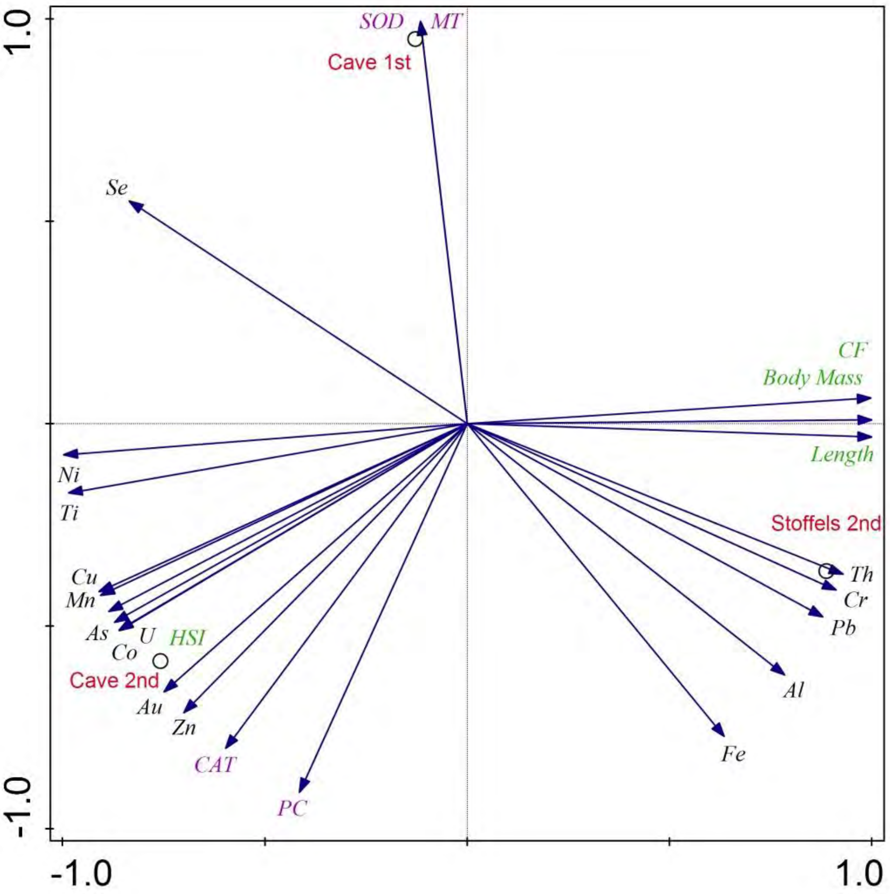

Figure 5.7: A principal component analysis (PCA) illustrating the relation between metal bioaccumulation, biomarkers of oxidative stress and exposure, gross body indices and mean body weight and length of *Clarias gariepinus* populations sampled from the Wonderfontein Cave and Stoffels Dam during the 1st (April 2013) and 2nd (September 2013) sampling intervals. These sampling intervals represent the end of the high and low flow periods, respectively. The ordination explained 79.8 % variance on the first axis and 100 % variation on the second axis.

5.3 Discussion

5.3.1 Genetic structure of the *Clarias gariepinus* population associated with the Wonderfontein Cave

Subterranean fish species have been reported from many cave systems and are sometimes endemic to a single locality (Proudlove, 2010). One remarkable subterranean catfish species, *Clarias cavernicola*, is known from only a few underground lakes in Namibia (Berti & Messana, 2010). Unfortunately, this unique species is threatened by anthropogenic activities such as groundwater abstraction that lowers the water table, subsequently threatening its habitat (Bruton, 1995; Proudlove, 2001).

According to Romero (2009) many cave-dwelling species diverged from epigean populations. Determining whether the *C. gariepinus* population associated within the Wonderfontein Cave has diverged from the epigean population serves more than one purpose. Firstly, if the Wonderfontein Cave population has diverged from the epigean population with gene flow not permitted, then the cave population should be regarded as singular. Theoretically, if left undisturbed for a number of years, this population could adapt to become a true subterranean fish species. It should be, however, noted that preadaptation is regarded by many scientists as the first step in the evolution of cave fish (Proudlove, 2010). Secondly, the genetic structure analysis provided insight into whether the Wonderfontein Cave population could serve as a bioindicator of ecosystem health of only the Wonderfontein Cave, or the surface environment as well. Lastly, it provided insight into whether any ‘sizeable’ connections existed between the subterranean and surface aquatic systems.

The study of population genetics has been widely applied to various fish species, including *C. gariepinus* (So *et al.*, 2006; Nwafili & Gao, 2007; Roodt-Wilding *et al.*, 2010; Sousa-Santos *et al.*, 2014). The genetic distance (F_{ST} and AMOVA) analyses revealed that there existed zero to minimal genetic divergence between the *C. gariepinus* populations associated with Wonderfontein Cave and Stoffels Dam. Also, the haplotype network analysis revealed that most of the identified *C. gariepinus* haplotypes from the Wonderfontein Spruit clade were present within both the Wonderfontein Cave and Stoffels Dam populations.

It is thus evident that the Wonderfontein Cave population has not diverged from the Stoffels Dam (Wonderfontein Spruit) population and that gene flow between the subterranean and surface environments, with respect to *C. gariepinus*, is likely still possible. According to a study by Porter *et al.* (2007) the evolution of *Astyanax mexicanus*, a blind Mexican cave fish, might have extended over a maximum of two million years. It is thus also conceivable that if the Wonderfontein Cave *C. gariepinus* population has only recently been isolated, divergence might still occur over many years to come. Yet, it remains unclear for how long *C. gariepinus* individuals have been utilizing the Wonderfontein Cave environment. Nevertheless, the *C. gariepinus* population associated with the Wonderfontein Cave was still used as a bioindicator of ecosystem health as the population has resided within the Wonderfontein Cave at least since the commencement of initial investigations. Furthermore, individuals of this fish species have been observed in most parts of the extensive, partially flooded cave system and have subsequently been exposed to the abiotic conditions associated with the Wonderfontein Cave

5.3.2 Variation in metal bioaccumulation levels between *Clarias gariepinus* populations and the risk posed to human health

Bioavailable persistent chemicals, such as metals, may accumulate in fish tissue as uptake may occur via ingestion of contaminated particles/food or via the gills and skin from water (Van der Oost *et al.*, 2003). According to the latter authors metal bioaccumulation should be regarded as a hazard criterion as adverse effects resulting from it may only become noticeable during a later stage in a fish's life cycle. This not only poses a threat to the health of the fish and the associated aquatic ecosystem, but also to any consumer, including humans (Yi *et al.*, 2011). The bioaccumulation of metals in *C. gariepinus* populations has been reported in a number of studies (Avenant-Oldewage & Marx, 2000; Crafford & Avenant-Oldewage, 2010; Murtala *et al.*, 2012). In this study the muscle tissue of the respective *C. gariepinus* populations were analysed for metal bioaccumulation as this species serves as a food source for some local communities (Du Preez *et al.*, 1997; Avenant-Oldewage & Marx, 2000).

The significant temporal and spatial variation in metal bioaccumulation in muscle tissue observed between the *C. gariepinus* populations associated with the Wonderfontein Cave and Stoffels Dam, as well as between the respective sampling intervals, may have resulted from a number of factors (Kotze *et al.*, 1999; Avenant-Oldewage & Marx, 2000; Polak-Juszczak, 2009). Variation among the *C. gariepinus* individuals, as illustrated by the standard error bars, should also be considered. According to Avenant-Oldewage and Marx (2000) variation in metal bioaccumulation between individuals may be the result of age, size, health and genetic variability. Furthermore, the time of residence in the particular sampling area, as well as the degree of stress experienced during capture and retrieval, may also contribute towards variation within a single population (Avenant-Oldewage & Marx, 2000). Some, or even most of the above factors might have contributed towards the higher variation observed in bioaccumulation of Cu, Al, As, Mn and U among individuals of the Wonderfontein Cave population during the 2nd sampling interval.

There were significant temporal differences in metal bioaccumulation in the Wonderfontein Cave population, with a strong relationship existing between specific metals and the Wonderfontein Cave population (2nd sampling interval). According to Crafford and Avenant-Oldewage (2011) factors that should be considered as a cause for temporal variation in the concentration of metals in fish tissue include both biotic (water quality parameters) and abiotic (fish health and physiological state) factors. Also, a study conducted by Kotze *et al.* (1999) on the bioaccumulation of Cu and Zn in *C. gariepinus* revealed that temporal variation in metal bioaccumulation occurred as a result of changes in the flow regime. It was reasoned that since higher bioaccumulation levels were observed during the high flow periods, metals bound to sediments were re-suspended with an increase in water flow and thus available for uptake. Temporal variation may also relate to differences in climatic conditions (Kotze *et al.*, 1999). However, when considering that the present study provides evidence of higher Zn and Co metal bioaccumulation levels during the 2nd sampling interval (representing the end of the low flow period) the re-suspension of metals are unlikely to be the cause of the observed temporal variation. Also, with no significant differences observed in the mean body weight and length, CF and HSI in the *C. gariepinus* population associated with the Wonderfontein Cave between the two sampling intervals, it is unlikely that these factors contributed towards the observed temporal variation. Furthermore, data obtained suggested that the Wonderfontein Cave *C. gariepinus* population was not associated with the CF, nor the mean body weight and length, during any of the sampling intervals.

Although it can be reasoned that the observed temporal variation was minimal as only Zn and Co (out of 15 studied metals) presented significant temporal variation in the Wonderfontein Cave *C. gariepinus* population, the PCA statistics suggested otherwise. The ordination diagram clearly illustrated that a much stronger relationship existed between bioaccumulated metals and the Wonderfontein Cave *C. gariepinus* population during the 2nd sampling interval, than during the 1st sampling interval. The reason for this remains unclear, however, may hold relation to less dilution occurring during the low flow period (Guasch *et al.*, 2010). According to the latter authors higher metal exposure, uptake and accumulation may be the result of, respectively, less dilution, higher retention and greater exposure time associated with low flow (water scarcity) periods.

Spatial variation was observed with significant differences in Ni, Zn, Co, Mn, Ti and Se bioaccumulation in muscle tissue of *C. gariepinus* between the Wonderfontein Cave (2nd sampling interval) and Stoffels Dam populations. Kotze *et al.* (1999) observed that spatial variation in Cu and Zn bioaccumulation in muscle tissue of *C. gariepinus* was caused by a variation in the degree of Cu and Zn pollution between sites. Other factors that might have contributed to the observed spatial variation in metal bioaccumulation in the present study include both biotic and abiotic factors previously mentioned (Crafford & Avenant-Oldewage, 2011). This is supported by significant differences in the HSI, mean body weight and length values observed between the *C. gariepinus* populations associated with Wonderfontein Cave (2nd sampling interval) and Stoffels Dam. In every instance where significant differences in metal bioaccumulation values between the two fish populations were observed, the Wonderfontein Cave population presented the highest mean metal concentration value, as well as the strongest relationship to the identified metals. Furthermore, the PCA statistics revealed a strong relation between some of the gross body indices (mean body weight, mean length and CF) and the *C. gariepinus* population associated with Stoffels Dam. Subsequently, it can be questioned whether the significant increase in mean body weight and length observed in the Stoffels Dam population could account for the observed lower metal bioaccumulation levels. A study conducted by Liang *et al.* (1999) on the bioaccumulation of metals in the organs of freshwater fish revealed that Zn, Cu, Cd, Cr, Ni and Pb concentrations were negatively correlated to the body length. Wood *et al.* (2011) stated that a negative correlation between Zn and body size, as observed in a number studies, most likely relate to the lower metabolism associated with older (larger) fish. Furthermore, in a study by Zhang and Wang (2007) aimed at studying the bioaccumulation of metals in marine fish tissue concluded that body size is an important factor influencing metal uptake from water and dietary phases and related negatively to the ingestion rate. From this it seems possible that the observed weaker relation to significantly elevated metal bioaccumulation levels in the Stoffels Dam *C. gariepinus* population may be explained by the bigger mean body size.

The Wonderfontein Spruit has been extensively subjected to anthropogenic pollution, which subsequently poses a severe threat to the health of the associated ecosystem (Coetzee *et al.*, 2006; Van Veelen, 2009; Hamman & Van Rensburg, 2012). Furthermore, there exists a considerable threat to human health as polluted water is used to irrigate farmlands and as drinking water for livestock (Van Eeden *et al.*, 2008). Also, *C. gariepinus* is a major food source for many local communities, thus, there is an increasing concern that the consumption of contaminated fish could serve as an exposure pathway for toxicants (Barnhoorn *et al.*, 2015). The European Food Safety Authority (EFSA) produced a report that provided provisionally tolerable weekly intake (PTWI) limits for various contaminants, including selected metals, in fish tissue (EFSA, 2005). According to EFSA (2005) the PTWI concentrations for As and Pb are 0.015 and 0.025 mg/kg, respectively. Concentrations of these metals in the muscle tissue of *C. gariepinus* populations associated with both the Wonderfontein Cave (1st and 2nd sampling intervals) and Stoffels Dam exceeded the PTWI values. Furthermore, Food Standards Australia New Zealand (FSANZ) provides generally expected levels (GEL) of metal contaminants in different food types (FSANZ, 2001). The proposed GEL for Cu (0.5 mg/kg), Se (0.5 mg/kg) and Zn (5 mg/kg) in fish were also reported (FSANZ, 2001). Metal concentrations in the muscle tissue of both the Wonderfontein Cave (1st and 2nd sampling intervals) and Stoffels Dam populations exceeded the GEL for all three metals. It is thus evident that the *C. gariepinus* populations associated with the Wonderfontein Cave and Stoffels Dam could pose a risk to human health if consumed.

5.3.3 Variation in measured biomarkers in *Clarias gariepinus* populations

When considering the whole organism condition (HSI, CF and HAI) parameters of the respective *C. gariepinus* populations, only spatial variation was observed as a significant difference in HSI values between the Wonderfontein Cave (2nd sampling interval) and Stoffels Dam populations. The HSI is generally associated with the energetic state and metabolic activity of a fish (Pyle *et al.*, 2005) and has been shown to increase as a result of detoxification activities in response to toxicant exposure (Pereira *et al.*, 1993; Fernandes *et al.*, 2008; Kopecka & Pempkowiak, 2008). This is supported by the findings of this study as the HSI was strongly related to the bioaccumulation of specific metals (Cu, Mn, As, Co, U, Au and Zn) in the Wonderfontein Cave population (2nd sampling interval). This suggests that the observed elevated levels of metal bioaccumulation in the liver tissue of the Wonderfontein Cave *C. gariepinus* population (2nd sampling interval) might have induced antioxidant enzymatic activities, which protects the fish from damage caused by ROS. Subsequently, metabolites are produced causing an increase in liver size and thus the HSI (Fernandes *et al.*, 2008). However, a study by Van Dyk *et al.* (2012) reported the mean HSI value of various *C. gariepinus* populations sampled from unpolluted sites (South Africa) as 0.6, while polluted aquatic systems scored 1.2 and above. Also, Marchand *et al.* (2009) reported a lower HSI (1.1) value for a *C. gariepinus* population bred in an controlled laboratory environment and higher values (1.2 and above) for populations associated with two polluted dams (South Africa). Both the Wonderfontein Cave (1st and 2nd sampling intervals) and Stoffels Dam populations presented similar HSI values to those associated with the uncontaminated *C. gariepinus* populations reported above. This suggests that although elevated metal bioaccumulation levels were observed in the Wonderfontein Cave population (2nd sampling interval), the induced physical effect remained minimal.

The studied biomarkers of oxidative stress and exposure presented significant differences over a temporal scale in the Wonderfontein Cave population between the two sampling intervals. However, no significant differences were observed over a spatial scale between the Wonderfontein Cave (2nd sampling interval) and Stoffels Dam populations. The reason for this is likely the result of the observed relationships that existed between the studied oxidative stress and exposure biomarkers (CAT, SOD, PC and MT), whole organism condition parameters and metal bioaccumulation in muscle tissue, which will be further discussed below.

Studies conducted on bivalves have shown that a positive correlation between MT and specific metals, such as Cd, existed (Ivanković *et al.*, 2005). It could be reasoned that MT should present a strong relation to some of the studied metals as MT are known to be induced by specific metals including Ni, Cu, Zn and Co (Van der Oost *et al.*, 2003; Wood *et al.*, 2011). However, PCA statistics revealed that there existed a negative relation between MT and most of the studied metals. When considering that a significantly higher mean MT concentration was observed in the liver tissue of the Wonderfontein Cave *C. gariepinus* population (1st sampling interval), it is suggested that the metals were effectively excreted by MT and other defence mechanisms before bioaccumulation in the muscle tissue could occur (Wang & Rainbow, 2010). The same is suggested for SOD, which is regarded as the first line of defence against especially Cu and Zn contamination (Okamoto & Colepicolo, 1998; Van der Oost *et al.*, 2003). No relation between SOD and bioaccumulated metals was observed with significantly higher SOD concentrations reported in the liver tissue of the Wonderfontein Cave *C. gariepinus* population (1st sampling interval). Based on the PCA relationships, the elevated concentration of PC in the Stoffels Dam *C. gariepinus* population most likely related to the higher HSI values as was previously reported. Metabolic activities induced by metal bioaccumulation may result in an increase in liver size and since PC (metabolites) accumulate in the liver as a result of the oxidation of amino acid residues (Dalle-Donne *et al.*, 2003), it may account for PC being related (PCA) to the HSI.

5.4 Conclusion

Although the *C. gariepinus* population associated with the Wonderfontein Cave has not diverged from the epigean populations, it can still be considered as representative of metal bioaccumulation and biomarker responses induced by the abiotic environment associated with the Wonderfontein Cave. From the evidence provided it is suggested that varying metal bioaccumulation, biomarkers of oxidative stress and exposure, as well as gross body indices variation occurred over both a temporal and spatial scale. Furthermore, it is likely that water scarcity and fish size might hold relation to temporal and spatial variation in metal bioaccumulation, respectively. Although the bioaccumulation of metals in *C. gariepinus* populations was strongly associated with specific sites and sampling intervals, the evidence suggested that minimal variation in fish condition (health) occurred between populations. Furthermore, the gross body indices presented similar values to reference/uncontaminated populations reported in other studies. It could thus, however, not be established whether the Wonderfontein Cave population are at threat as a result of anthropogenic metal pollution. Nevertheless, all of the *C. gariepinus* populations investigated during this study pose a threat to human health if consumed on a regular basis.

Since no genetic distance was reported between the *C. gariepinus* populations associated with the Wonderfontein Cave and Stoffels Dam, the first hypothesis is rejected. Although significant differences in the HSI were recorded between the Wonderfontein Cave and Stoffels Dam populations, the CF presented minimal spatial variation, thus, the second hypothesis is accepted. It is recommended that further investigations are required to fully assess the potential of the Wonderfontein Cave population as an indicator of ecosystem health. Also, alternative methods to assess the latter should be evaluated, as no known control to the Wonderfontein Cave exists.

5.5 References

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CHAPTER 6

GENERAL CONCLUSION AND RECOMMENDATIONS

Africa’s karst landscapes are at threat of degradation as a result of anthropogenic activities. Subterranean environments, such as those hosted by caves, are especially sensitive to environmental change and exposure to toxicants. Furthermore, caves may host uniquely adapted species that are endemic to a single cave system. Subsequently, it is important to consider not only the ecosystem health of the surface environment, but also that located subterraneanly. However, studying cave ecosystems may proof to be especially challenging due to confined spaces, cave debris and vertical shafts typically associated with such systems. Although this presents a challenge for active sampling and monitoring programs, it remains crucial that our focus as conservationists includes nature as a whole.

The nature of this study remains unprecedented in the karst landscapes of the Witwatersrand Basin. With the ultimate goal of promoting the conservation of Africa’s subterranean environments, public awareness can now be created and efforts made to mitigate anthropogenic influences on such environments. For this reason, the findings reported in this study will be published in a series of internationally accredited scientific journals, enabling the data presented to serve as a baseline for future studies.

From the evidence provided in this study the first general hypothesis is accepted as the Wonderfontein Cave has been subjected to the influx of metal pollution from the Wonderfontein Spruit. Substantial enrichment of especially Ni, Cu, Zn, Co, Al, Cd, Pb and U was observed in sediments associated with the Wonderfontein Cave, while many of the studied metals also exceeded the respective water, soil and sediment environmental quality guidelines. Thus, also taking into consideration that most of the sediments were classified as being toxic, a severe threat is posed to the health of the associated biota.

However, the second and third hypotheses are rejected as no evidence was obtained that the nematode taxa and *C. gariepinus* population associated with the Wonderfontein Cave are isolated within the cave system. Although 60 nematode genera were identified from the soils and sediments associated with the Wonderfontein Cave and Spruit, it was concluded that these genera were likely only temporary residents of the subterranean environment that were introduced from the surface. It is thus also unlikely that the observed nematode food web structures were the direct result of the abiotic conditions presented by the Wonderfontein Cave soils and sediments. Also, no genetic divergence was reported between the *C. gariepinus* populations associated with the Wonderfontein Cave and Stoffels

Dam. Compared to the North and East African *C. gariepinus* clades, with the haplotypes presented by each diverged from any other clade, the Wonderfontein Cave and Stoffels Dam shared the majority of the haplotypes associated with the Wonderfontein Spruit clade. Only the Wonderfontein Cave *C. gariepinus* population presented a single haplotype that was not also present within the Stoffels Dam population.

The fourth hypothesis is partially accepted as minimal variation in biomarker responses, however, significant variation in metal bioaccumulation was observed between the Wonderfontein Cave (2nd sampling interval) and Stoffels Dam populations. Findings indicate that especially the Wonderfontein Cave population, sampled during the 2nd sampling interval, presented oxidative biomarker responses to metal bioaccumulation. The latter population also presented relation to the HSI, which was likely the result of an increase in antioxidant enzymatic activities induced by the bioaccumulation of metals. However, no significant differences in the fish condition was observed between the Wonderfontein Cave (2nd sampling interval) and Stoffels Dam populations.

The final hypothesis is also accepted as 1) total metal concentrations associated with most substrate samples varied between sampling intervals, 2) nematode assemblage and food web structure variations was evident and 3) clear differentiation in most biomarker responses and some bioaccumulated metals were presented in the Wonderfontein Cave population sampled during the 1st and 2nd sampling intervals.

Although the evidence obtained in Chapter 4 did not support the hypothesis that metal enrichment of the Wonderfontein Cave substrates posed a threat to ecosystem health, undoubtedly, the presence of these toxicants is disadvantageous to the subterranean environment and the biota associated with it. For any future studies carried out on the subterranean environments and ecosystems hosted by South Africa's karst landscapes, it is recommended that the following should be considered:

- Recruit the help of cavers/volunteers to manage and transport samples throughout the cave system.
- Undertaking frequent sampling to ensure data is representative of the subterranean environment over a well-defined temporal scale.
- Sample and analyse water samples frequently and periodically in order to clearly identify fluctuations in water quality parameters (pH, temperature, dissolved oxygen and ionic concentrations).
- Determine whether subterraneanly sampled biota are permanent or temporary resident of a cave system by also sampling from the surface environment.

- If a subterranean water system is studied of which the flow path is unknown, use an environmentally friendly tracer dye or other appropriate method to gain a better understanding of the hydrological setting.
- Follow a holistic scientific approach that includes studying the various trophic interactions typically associated with subterranean ecosystems as this will provide a better understanding of ecosystem function.

Theodore Roosevelt once said that “We should not forget that it will be just as important to our descendants to be prosperous in their time as it is to us to be prosperous in our time.” Humans, as the prime of nature, have a responsibility to ensure the sustainability of Earth’s treasures and in order to do so, we must consider conserving every aspect of nature.

APPENDIX A

Table 1: Ionic concentrations (µm/l) in water samples collected during the 1st (April 2013) sampling interval from the Wonderfontein Cave and Spruit.

	SO_4^{2}	NO_3^-	Cl^-	HCO_3^-
Kent's Entrance	350140.00	14150.00	60520.00	216606.45
Keyhole	350440.00	15760.00	61180.00	216606.45
Main Entrance	340410.00	17140.00	60990.00	216606.45
Pristine Chamber	344760.00	18940.00	61520.00	219657.24
Elevation Pit	338560.00	19110.00	61250.00	222708.04
North-Eastern Section	336710.00	19100.00	61020.00	225758.83
Cave Inflow Area	349210.00	16920.00	62670.00	222708.04
Derek's Exit	344260.00	16510.00	60460.00	222708.04

Table 2: Ionic concentrations (µm/l) in water samples collected during the 2nd (September 2013) sampling interval from the Wonderfontein Cave and Spruit

	SO_4^{2-}	NO_3^-	Cl^-	HCO_3^-
Kent's Entrance	227540 ± 2370	25260 ± 0220	59920 ± 0580	240200 ± 1960
Keyhole	228790 ± 0630	27970 ± 0090	61560 ± 0180	237150 ± 1410
Main Entrance	216730 ± 2520	26790 ± 0390	61520 ± 0530	244470 ± 0700
Fault Passage	217550 ± 2450	27830 ± 0360	62040 ± 0720	237960 ± 0000
Pristine Chamber	210860 ± 0910	28520 ± 0230	63050 ± 0160	240200 ± 1410
Elevation Pit	216840 ± 0650	28440 ± 0170	62440 ± 0120	237960 ± 0000
North-Eastern Section	217380 ± 0780	27910 ± 0240	62310 ± 0230	236130 ± 1610
Cave Inflow Area	230340 ± 3920	27130 ± 1310	61350 ± 1200	235930 ± 1760
Wonderfontein Canal	232720 ± 0780	26200 ± 0470	60750 ± 0710	215590 ± 1760
Stoffels Dam	250760 ± 2000	2720 ± 0050	66040 ± 0440	219660 ± 0000

Table 3: Dissolved metal content (µm/l) and pH of water samples collected during the 1st (April 2013) sampling interval from the Wonderfontein Cave and Spruit

	Cr	**Ni**	**Cu**	**Zn**	**Co**	**Fe**	**Al**	**As**	**Mn**	**Cd**	**Pb**	**U**	**pH**
Kent's Entrance	13.38	26.52	9.37	0.01	11.25	626.5	53.58	8.46	83.37	0.00	5.93	29.72	9.15
Keyhole	3.53	29.13	2.35	0.08	10.56	529	20.75	8.31	43.24	0.00	5.56	29.03	8.51
Main Entrance	0.69	31.10	1.78	0.08	10.53	488.2	0.69	7.88	40.2	0.00	5.79	28.99	8.63
Pristine Chamber	0.02	28.38	0.57	0.11	9.957	480.3	7.59	8.91	32.6	0.00	5.64	29.10	8.46
Elevation Pit	0.03	31.86	2.98	0.08	9.656	466	0.06	7.70	21.71	0.00	5.73	27.13	8.41
North-Eastern Section	0.03	33.28	2.44	0.08	9.695	453.8	0.07	8.07	21.29	0.01	5.38	27.58	8.39
Cave Inflow Area	0.03	29.90	1.37	0.10	11.15	455.8	0.04	8.87	17.27	0.00	5.58	32.25	8.7
Derek's Exit	0.04	18.99	1.29	0.10	10.6	465.9	0.10	8.21	20.71	0.00	5.35	30.79	8.92

Table 4: Dissolved metal content (µm/l) and pH of water samples collected during the 2nd (September 2013) sampling interval from the Wonderfontein Cave and Spruit.

	Cr	Ni	Cu	Zn	Co	Fe	Al	As	Mn	Cd	Pb	U	pH
Kent's Entrance	0.03 ± 0.00	35.46 ± 0.44	0.28 ± 0.35	0.16 ± 0.01	10.21 ± 0.26	341.33 ± 44.99	38.79 ± 35.25	19.76 ± 0.31	30.09 ± 3.59	0.01 ± 0.00	0.10 ± 0.01	56.59 ± 2.01	8.7
Keyhole	0.03 ± 0.00	40.70 ± 2.89	0.82 ± 0.64	0.19 ± 0.01	10.09 ± 0.40	274.97 ± 14.22	16.28 ± 10.50	21.02 ± 0.12	22.09 ± 12.77	0.01 ± 0.00	0.09 ± 0.00	56.27 ± 0.61	8.64
Main Entrance	0.03 ± 0.00	33.65 ± 1.31	0.03 ± 0.00	0.21 ± 0.02	8.97 ± 0.06	276.37 ± 22.77	29.02 ± 40.97	21.90 ± 0.26	8.89 ± 1.26	0.01 ± 0.00	0.09 ± 0.00	57.82 ± 0.50	8.54
Fault Passage	0.02 ± 0.01	47.37 ± 15.79	7.21 ± 7.33	0.11 ± 0.07	12.13 ± 2.94	369.63 ± 108.28	114.93 ± 71.17	23.06 ± 0.72	78.98 ± 50.95	0.01 ± 0.00	0.08 ± 0.01	54.09 ± 6.72	8.59
Pristine Chamber	0.03 ± 0.00	39.79 ± 3.16	0.37 ± 0.30	0.17 ± 0.01	9.56 ± 0.56	272.03 ± 17.75	27.58 ± 10.77	24.05 ± 0.72	44.34 ± 17.03	0.01 ± 0.00	0.09 ± 0.00	60.59 ± 0.62	8.45
Elevation Pit	0.03 ± 0.00	38.01 ± 1.88	0.15 ± 0.21	0.19 ± 0.02	9.53 ± 0.32	262.63 ± 10.17	17.74 ± 4.21	21.95 ± 0.24	31.05 ± 7.55	0.01 ± 0.00	0.10 ± 0.00	56.65 ± 0.27	8.45
North-Eastern Section	0.03 ± 0.00	41.15 ± 5.88	1.78 ± 1.25	0.20 ± 0.04	9.48 ± 1.04	252.57 ± 33.59	23.41 ± 24.05	21.53 ± 0.63	22.51 ± 16.73	0.01 ± 0.00	0.10 ± 0.00	55.12 ± 2.03	8.4
Cave Inflow Area	0.02 ± 0.01	90.35 ± 55.12	27.45 ± 34.29	19.08 ± 26.78	21.67 ± 12.96	604.47 ± 339.82	339.69 ± 345.60	23.02 ± 2.53	192.31 ± 162.53	0.01 ± 0.00	0.08 ± 0.02	57.86 ± 1.79	8.55
Wonderfontein Canal	0.03 ± 0.00	49.84 ± 1.16	0.04 ± 0.05	0.17 ± 0.02	12.63 ± 0.18	361.57 ± 18.40	79.03 ± 5.62	20.64 ± 0.17	66.20 ± 2.37	0.01 ± 0.00	0.10 ± 0.00	56.13 ± 0.28	8.39
Stoffels Dam	0.03 ± 0.00	9.68 ± 0.38	0.05 ± 0.01	0.24 ± 0.01	5.57 ± 0.07	413.20 ± 54.60	121.45 ± 52.40	5.56 ± 0.13	65.97 ± 16.45	0.01 ± 0.00	0.11 ± 0.00	54.74 ± 0.58	8.59

Table 5: Total concentration of metals (mg/kg), as well as total organic carbon (TOC) and fraction percentages larger than 2 mm in soils and sediments collected during the 1st (April 2013) sampling interval from the Wonderfontein Cave and Spruit.

	Cr	Ni	Cu	Zn	Co	Fe	Al	As	Mn	Cd	Pb	U	Org. C %	Frac > 2 mm %
							SOILS							
Kent's Entrance	40.18	103.23	58.88	16.40	34.65	16002.5	9640.00	5.17	18320	0.11	30.25	2.84	0.91	57.64
Main Entrance A	105.53	83.05	42.90	56.95	34.3	21352.5	15605.00	4.07	9075	0.10	28.33	0.45	0.41	21.19
Main Entrance B	70.25	29.15	32.35	28.43	10.7925	28525	13657.50	6.34	8092.5	0.07	29.55	1.12	0.31	25.96
Pristine Chamber	44.58	43.48	28.98	15.71	17.9675	20537.5	4362.50	4.48	12147.5	0.10	21.55	0.34	0.40	7.69
Elevation Pit A	47.40	61.63	46.58	28.25	27.7	16827.5	5595.00	3.25	15340	0.15	31.45	0.99	2.55	7.11
Elevation Pit B	55.33	31.55	36.13	43.93	20.915	19417.5	8142.50	3.36	7747.5	0.09	22.26	0.47	0.11	28.02
North-Eastern Section	129.85	47.68	21.48	33.83	12.365	26000	13315.00	4.86	4307.5	0.06	14.59	0.86	0.37	3.57
Cave Inflow Area	90.60	67.65	17.66	39.35	16.45	10027.5	14325.00	3.38	815.5	0.12	12.17	7.22	4.08	1.38
Derek's Exit	66.40	65.50	30.73	57.20	15.9425	16422.5	10912.50	4.33	11460	0.14	23.04	0.95	4.22	25.48
							SEDIMENTS							
Kent's Entrance	100.98	1110.25	170.18	286.25	262.75	27725	19150.00	21.22	22657.5	0.57	66.15	17.41	2.21	59.51
Keyhole	100.60	776.75	163.20	209.40	187.925	31500	20395.00	13.42	45250	0.51	73.55	12.75	2.94	39.58
Main Entrance	206.25	1850.00	271.50	425.00	311.5	32725	37525.00	24.23	3050	0.72	39.60	37.75	5.88	4.15
Pristine Chamber	82.08	374.25	87.13	68.68	59.925	28725	19552.50	14.88	17065	0.20	38.15	6.68	0.73	34.65
Elevation Pit	265.75	1818.25	442.50	791.75	442.75	46000	40425.00	41.00	4702.5	1.18	72.30	43.05	4.86	52.05
North-Eastern Section	331.75	334.25	64.85	104.28	58.05	13530	28500.00	5.51	1055	0.21	18.84	9.65	0.65	13.82
Cave Inflow Area	83.83	83.23	18.70	34.63	15.2325	7625	9800.00	3.77	411	0.12	9.70	4.96	4.27	0.79
Derek's Exit	179.85	598.50	85.40	170.53	60.9	18777.5	25050.00	7.83	8282.5	0.34	21.50	10.24	3.30	12.18

Table 6: Total concentration of metals (mg/kg), as well as total organic carbon (TOC) and fraction percentages larger than 2 mm in soils and sediments collected during the 2nd (September 2013) sampling interval from the Wonderfontein Cave and Spruit.

	Cr	**Ni**	**Cu**	**Zn**	**Co**	**Fe**	**Al**	**As**	**Mn**	**Cd**	**Pb**	**U**	**Org. C%**	**Frac > 2mm**
Kent’s Entrance	58.97 ± 10.55	225.78 ± 56.13	96.78 ± 23.21	60.49 ± 18.70	67.80 ± 15.12	18297.50 ± 2621.21	10261.67 ± 1345.79	9.97 ± 1.86	18444.17 ± 4728.00	0.12 ± 0.04	34.54 ± 9.44	6.53 ± 1.61	1.47 ± 0.09	32.42 ± 6.07
Main Entrance A	63.18 ± 16.24	78.81 ± 19.58	50.27 ± 14.89	18.60 ± 3.18	32.20 ± 9.81	21087.50 ± 3141.38	7070.83 ± 616.21	3.92 ± 1.16	18021.67 ± 6476.28	0.07 ± 0.03	43.24 ± 11.11	0.82 ± 0.26	0.72 ± 0.03	33.22 ± 7.95
Main Entrance B	71.67 ± 13.24	98.68 ± 33.77	60.90 ± 19.99	49.01 ± 9.38	43.84 ± 17.75	20931.67 ± 2204.88	13815.00 ± 2486.57	4.72 ± 1.14	18336.67 ± 9827.36	0.09 ± 0.03	59.22 ± 25.43	2.28 ± 1.45	1.92 ± 0.24	29.30 ± 13.47
Fault Passage	42.08 ± 2.64	65.08 ± 5.11	33.17 ± 2.61	17.28 ± 2.52	23.66 ± 3.16	18800.00 ± 297.13	8393.33 ± 89.36	5.23 ± 1.14	10996.67 ± 671.45	0.05 ± 0.01	26.44 ± 3.91	1.07 ± 0.31	0.57 ± 0.19	16.78 ± 7.32
Pristine Chamber	54.62 ± 3.27	75.66 ± 5.93	54.63 ± 6.99	17.81 ± 0.55	36.05 ± 5.26	24589.17 ± 2217.23	7874.17 ± 878.65	7.68 ± 1.05	19301.67 ± 1820.96	0.07 ± 0.01	42.65 ± 7.60	0.63 ± 0.04	2.67 ± 2.95	18.15 ± 1.95
Elevation Pit A	28.93 ± 5.91	126.97 ± 124.91	20.63 ± 7.80	43.07 ± 23.92	25.20 ± 21.75	12293.33 ± 3669.57	6092.50 ± 1821.12	3.99 ± 1.57	5105.83 ± 1309.70	0.07 ± 0.05	14.19 ± 2.45	12.97 ± 16.43	1.40 ± 0.38	7.50 ± 1.85
Elevation Pit B	110.58 ± 54.52	35.73 ± 9.81	51.95 ± 8.49	163.79 ± 118.74	14.49 ± 4.05	22018.33 ± 7284.79	10720.83 ± 3417.85	7.18 ± 3.58	6354.42 ± 4302.98	0.21 ± 0.17	25.80 ± 5.93	1.45 ± 0.70	3.33 ± 1.53	22.86 ± 16.17
North-Eastern Section	180.59 ± 26.91	65.68 ± 28.00	33.08 ± 14.57	102.78 ± 66.54	16.62 ± 5.91	36796.67 ± 12961.01	24920.83 ± 2047.67	8.83 ± 5.97	6079.58 ± 5330.74	0.06 ± 0.04	18.54 ± 1.76	1.03 ± 0.43	2.26 ± 0.13	8.41 ± 11.15
Cave Inflow Area	99.77 ± 13.99	69.17 ± 26.68	30.98 ± 8.60	94.78 ± 43.35	13.75 ± 5.94	14740.83 ± 3699.88	20455.00 ± 1434.57	4.73 ± 1.85	845.67 ± 407.83	0.11 ± 0.04	21.02 ± 7.33	9.10 ± 4.76	6.14 ± 2.74	2.57 ± 1.48
Derek’s Exit	44.13 ± 2.60	88.95 ± 11.07	43.92 ± 6.70	63.46 ± 2.43	23.86 ± 2.64	16402.50 ± 718.94	9384.17 ± 427.89	5.02 ± 0.42	19835.00 ± 1830.03	0.14 ± 0.01	32.39 ± 1.68	1.47 ± 0.17	3.57 ± 0.45	29.39 ± 6.56
Wonderfontein Canal	56.90 ± 4.20	60.08 ± 10.21	8.33 ± 0.88	17.08 ± 0.88	10.97 ± 1.71	2020.42 ± 195.49	5225.00 ± 542.26	2.31 ± 0.20	93.37 ± 8.67	0.04 ± 0.00	3.80 ± 0.42	5.18 ± 0.28	0.97 ± 0.18	5.02 ± 2.40
Stoffels Dam	78.49 ± 1.29	30.52 ± 3.00	28.39 ± 0.89	35.51 ± 4.95	6.98 ± 1.81	16581.67 ± 1165.70	11415.83 ± 75.42	10.14 ± 3.64	186.78 ± 72.32	0.11 ± 0.05	15.96 ± 2.19	22.70 ± 1.81	7.08 ± 2.11	7.40 ± 2.38

Table 7: Total concentration of metals (mg/kg), as well as total organic carbon percentage and fraction percentage larger than 2 mm in sediments collected during the 2nd (September 2013) sampling interval from the Wonderfontein Cave and Spruit.

	Cr	**Ni**	**Cu**	**Zn**	**Co**	**Fe**	**Al**	**As**	**Mn**	**Cd**	**Pb**	**U**	**Org. C%**	**Frac > 2mm**
Kent's Entrance	93.48 ± 1.86	560.33 ± 38.54	367.00 ± 5.52	207.43 ± 9.17	163.17 ± 11.38	19669.17 ± 1149.08	13875.00 ± 865.45	26.08 ± 1.40	8596.67 ± 2407.52	0.35 ± 0.02	32.66 ± 6.34	14.41 ± 0.55	2.36 ± 0.14	32.62 ± 10.39
Keyhole	72.43 ± 19.20	514.83 ± 235.92	152.89 ± 58.87	182.85 ± 82.44	121.15 ± 26.48	22726.67 ± 2468.61	14702.50 ± 3766.14	14.12 ± 4.50	21150.00 ± 13071.45	0.32 ± 0.11	43.52 ± 8.75	17.13 ± 10.55	2.75 ± 1.37	21.55 ± 17.28
Main Entrance	98.00 ± 7.15	650.42 ± 93.16	183.04 ± 29.31	227.47 ± 34.82	125.16 ± 16.99	23795.83 ± 1765.40	22880.83 ± 1940.34	17.47 ± 2.16	3134.17 ± 522.18	0.34 ± 0.05	29.51 ± 3.01	23.35 ± 5.38	3.69 ± 0.50	4.20 ± 1.37
Fault Passage	146.64 ± 50.19	949.83 ± 443.31	233.18 ± 135.65	436.39 ± 324.05	263.62 ± 164.50	31165.83 ± 6756.40	21267.50 ± 5895.58	29.80 ± 9.91	5531.92 ± 2321.62	0.64 ± 0.45	43.27 ± 19.13	66.29 ± 43.37	2.67 ± 1.67	9.57 ± 8.14
Pristine Chamber	157.70 ± 40.65	1397.33 ± 869.47	324.98 ± 206.47	230.62 ± 97.32	272.38 ± 161.47	30750.00 ± 4333.16	24727.50 ± 10702.46	50.76 ± 23.89	5564.17 ± 1216.97	0.33 ± 0.16	23.58 ± 1.21	344.78 ± 281.71	1.36 ± 0.78	15.32 ± 2.89
Elevation Pit	57.65 ± 5.90	39.47 ± 13.63	71.43 ± 5.87	296.77 ± 60.14	12.25 ± 6.87	17214.17 ± 2734.10	15160.00 ± 347.01	10.37 ± 2.53	4913.67 ± 4190.24	0.21 ± 0.01	16.27 ± 5.28	1.33 ± 0.44	4.66 ± 1.17	24.21 ± 7.40
North-Eastern Section	185.47 ± 80.19	236.92 ± 61.87	54.66 ± 2.52	90.85 ± 16.19	39.54 ± 2.85	12095.00 ± 4100.17	24291.67 ± 10636.89	6.73 ± 0.43	1492.23 ± 920.01	0.14 ± 0.02	16.51 ± 5.54	10.54 ± 0.68	2.55 ± 1.18	20.91 ± 17.06
Cave Inflow Area	91.85 ± 10.40	584.06 ± 338.74	204.20 ± 106.82	246.05 ± 131.06	148.04 ± 77.30	17204.17 ± 7667.68	14884.17 ± 5316.19	24.18 ± 12.45	1809.08 ± 1056.87	0.36 ± 0.17	24.61 ± 12.10	29.37 ± 19.30	3.61 ± 2.17	6.57 ± 4.51
Wonderfontein Canal	79.17 ± 32.77	178.31 ± 98.66	46.58 ± 21.36	68.18 ± 46.49	34.94 ± 17.49	5952.50 ± 4082.23	9796.67 ± 5907.35	7.72 ± 3.76	224.48 ± 107.55	0.12 ± 0.07	8.90 ± 5.83	8.97 ± 5.25	2.45 ± 2.02	2.33 ± 2.12
Stoffels Dam	43.33 ± 2.35	35.33 ± 1.92	7.96 ± 0.97	12.32 ± 2.43	8.18 ± 0.90	6595.00 ± 2848.86	5229.17 ± 788.91	3.27 ± 0.62	57.78 ± 20.15	0.02 ± 0.00	4.62 ± 0.55	7.14 ± 1.89	1.17 ± 0.15	2.26 ± 1.12

Table 8: Total concentration of selected metals and Titanium (Ti) reference element in the Klerkskraal Dam (reference site) sediments as reported by Coetzee *et al.* (2006).

(mg/kg)	**Cr**	**Ni**	**Cu**	**Zn**	**Co**	**Fe**	**Al**	**As**	**Mn**	**Cd**	**Pb**	**U**	**Ti**
Klerkskraal Dam	267.205	158.232	23.575	34.065	15.314	20335.63	4859.277	27.855	20335.63	0.001	16.327	0.001	163.98

References

Coetzee, H., Winde, F. & Wade, P.W. 2006. An assessment of sources, pathways, mechanisms and risks of current and potential future pollution of water and sediments in gold-mining areas of the Wonderfonteinspruit Catchment. Pretoria: Water Research Commission. 202 p.

APPENDIX B

Table 1: Diversity and abundance (average of replicate samples) of nematode genera (PPN and NPN) found in soil samples collected from the Wonderfontein Cave and Spruit during the 1st (April) sampling interval. Trophic group and cp-value classification of nematode genera was based on entries listed in the Nemaplex online database (Available at http://plpnemweb.ucdavis.edu/nemaplex).

		Kent's Entrance	**Main Entrance A**	**Main Entrance B**	**Elevation Pit A**	**Elevation Pit B**	**Cave Inflow Area**	**Derek's Exit**
Herbivores								
Aphelenchoides		0.67 ± 0.58	0	0	0	0	0	0
Ditylenchus		0	0	0	0.5 ± 1	0	0	0
Longidorella		0	0	0	1.25 ± 1.89	0	0	1.33 ± 1.15
Meloidogyne		0	0	0	1 ± 1.15	0.5 ± 1	0	2 ± 3.46
Paratylenchus		0	0	0	0.5 ± 1	0	0	0
Tylenchus		0	0	0	0.5 ± 1	0	2.25 ± 2.87	0
Bacterivores	**CP-Value**							
Acrobeles	**2**	0	0	2.5 ± 3.32	10.25 ± 4.5	0.5 ± 0.58	0	0
Acrobeloides	**2**	0	0	3.75 ± 3.20	2.5 ± 3.70	0	0	2.67 ± 3.06
Bunonema	**1**	0	0	0	0.25 ± 0.5	0	0	0
Cervidellus	**2**	0	0	0	0.5 ± 1	0	0	0.67 ± 1.15
Chiloplacus	**2**	0	0	0.75 ± 1.5	0	3 ± 1.63	0	28.67 ± 7.57
Diploscapter	**1**	0.33 ± 0.58	0	0	0.25 ± 0.5	0	0.5	0
Eucephalobus	**2**	0.67 ± 1.15	0	1.5 ± 3	0	0	0	0
Mesorhabditis	**1**	0	0	0	3.5 ± 2.89	0	4.5 ± 3.87	0.67 ± 1.15
Monhystera	**1**	0	0	0	0.5 ± 1	0.25 ± 0.5	29.5 ± 22.66	0
Panagrolaimidae	**1**	0	0	0	0	0	1 ± 2	0
Panagrolaimus	**1**	1 ± 1.73	0	0	2.75 ± 1.5	0	1.5 ± 1.73	0
Plectus	**2**	0	0.33 ± 0.58	0.5 ± 0.58	0	0	0	0
Rhabditis	**1**	0.67 ± 1.15	0	0	2 ± 1.63	0	3 ± 3.46	0
Seleborca	**2**	0	0	0	7.5 ± 5.20	0	0	0
Stegelleta	**2**	0	0	0	0.5 ± 1	0	0	0
Fungivores								
Aphelenchus	**2**	0	0.33 ± 0.58	0	0	0	0	0
Dorylaimoides	**4**	0	0	0	1 ± 2	0	1 ± 2	3.33 ± 5.77
Leptonchidae	**4**	0	0	0	0.25 ± 0.5	0	0	0
Tylencholaimidae	**4**	0	0	0	0	0	0	0.67 ± 1.15
Omnivores								
Aporcelaimus	**5**	0.33 ± 0.58	0	0	0	0	0	0
Dorylaimidae	**4**	0	0	0	3 ± 3.56	0	12.5 ± 19.69	1.33 ± 2.31
Kochinema	**4**	0	0	0	1 ± 2	0	0	0
Mesodorylaimus	**5**	0	0	0	0	0	0.75 ± 1.5	0
Pungentus	**4**	0	0	0	0.25 ± 0.5	0	0	2.67 ± 4.62
Carnivores								
Mylonchulus	**4**	0.33 ± 0.58	0	0	0	0	0	0
Tobrilidae	**3**	0	0	0	0	0	0.5 ± 1	0
Tripyla	**3**	0	0	0	0.5 ± 1	0	0	0
TOTAL NEMATODES		**4.00 ± 1.00**	**0.67 ± 1.15**	**9.00 ± 2.16**	**40.25 ± 6.85**	**4.25 ± 2.36**	**57.00 ± 49.24**	**44.00 ± 10.58**

Table 2: Diversity and abundance (average of replicate samples) of nematode genera (PPN and NPN) found in sediment samples collected from the Wonderfontein Cave and Spruit during the 1st (April) sampling interval. Trophic group and cp-value classification of nematode genera was based on entries listed in the Nemaplex online database (Available at http://plpnemweb.ucdavis.edu/nemaplex).

		Kent's Entrance	**Keyhole**	**Main Entrance**	**Pristine Chamber**	**Elevation Pit**	**North-Eastern Section**	**Cave Inflow Area**	**Derek's Exit**
Herbivores									
Aphelenchoides		0	0	0.25 ± 0.5	0	0.25 ± 0.5	0.33 ± 0.58	0	0
Aphelenchoididae		0	0	0	0	0.25 ± 0.5	0	0	0
Ditylenchus		0	0	0	0	0	0	0.25 ± 0.5	0
Hemicycliophora		0	0.25 ± 0.5	0	0	0	0	0	0
Pratylenchus		0	0	0.25 ± 0.5	0	0	0	0	0
Tylenchus		0.67 ± 1.15	0	0	0	0	0	0	0
Bacterivores	**CP-Value**								
Chromadoridae	**3**	0	0	0	0	0	0	0	0.5 ± 1
Chromadorina	**3**	0	0.25 ± 0.5	0	0	0	0	0	0
Chronogaster	**3**	0	0.25 ± 0.5	0	0	0	0	0	0
Cylindrolaimus	**3**	0	0	0	0	0	0	0	0.25 ± 0.5
Ethmolaimus	**3**	0	0	0	0	0	0.33 ± 0.58	0	0
Mesorhabditis	**1**	0	0	0.25 ± 0.5	0	0	0	0	0
Microlaimus	**3**	0	0.5 ± 0.58	0	0.33 ± 0.58	0	0	0	0
Monhystera	**1**	0	0	0.25 ± 0.5	0	0	0	10.75 ± 12.20	0.25 ± 0.5
Monhysteridae	**1**	0	0	0	0	0.25 ± 0.5	0	0	0
Plectus	**2**	0	0	0.25 ± 0.5	1.33 ± 1.53	0	0	0.25 ± 0.5	1 ± 1.41
Prismatolaimus	**3**	0	0.75 ± 0.96	0.75 ± 0.5	2.33 ± 2.31	0	0.33 ± 0.58	0	1 ± 0.82
Eukaryotic Feeders									
Achromadora	**3**	0	0	0	1.33 ± 2.31	0	0	0	0
Chromadorita	**3**	0	0	0.25 ± 0.5	0	0	0	0	0
Omnivores									
Paraxonchium	**5**	0.33 ± 0.58	0	0	0	0	0	0	0
TOTAL NEMATODES		**1 ± 1.73**	**2 ± 1.41**	**2.25 ± 0.96**	**5.333 ± 3.51**	**0.75 ± 0.5**	**1 ± 1.73**	**11.25 ± 11.87**	**3 ± 1.41**

Table 3: Diversity and abundance (average of replicate samples) of nematode genera (PPN and NPN) found in soil samples collected from the Wonderfontein Cave and Spruit during the 2nd (September) sampling interval. Trophic group and cp-value classification of nematode genera was based on entries listed in the Nemaplex online database (Available at http://plpnemweb.ucdavis.edu/nemaplex).

		Kent's Entrance	**Main Entrance A**	**Fault Passage**	**Pristine Chamber**	**Elevation Pit A**	**Elevation Pit B**	**North-Eastern Sec**	**Cave Inflow Area**	**Derek's Exit**	**Wonderfontein Canal**	**Stoffels Dam**
Herbivores												
Aphelenchoides		0.5 ± 0.577	0	0	0	0	0	0	0	0	0	1 ± 2
Ditylenchus		0	0	0	0	0	0	0	11.75 ± 13.87	0	0	0
Hemicycliophora		0	0	0	0	0	0	0	30 ± 43.89	0	0	0
Longidorella		0	0	0	0	2 ± 2.83	0	0	0	0	0	0
Meloidogyne		0.25 ± 0.5	0	0	0	0.25 ± 0.5	0	0	0	0	0	0
Rotylenchus		0	0	0	0	0.25 ± 0.5	0	0	0	0	0	0
Psilenchus		0	0	0	0	0	0	0	13.5 ± 27	0	0	1.5 ± 3
Tylenchus		1 ± 2	0	0	0	0.5 ± 0.58	0.25 ± 0.5	0	195.25 ± 72.87	0	0	14.25 ± 14.93
Bacterivores	**CP-Value**											
Acrobeloides	**2**	2.25 ± 1.5	12.5 ± 4.65	0	0	0.25 ± 0.5	0	0.33 ± 0.58	178.75 ± 19.24	5.33 ± 9.24	0	5 ± 4.16
Acrobeles	**2**	0.5 ± 1	0	0	0	7.75 ± 2.87	0	0	0	0	0	0
Anaplectus	**2**	0	0	0	0	0	0	0	0	0.33 ± 0.58	0	1 ± 2
Aulolaimus	**3**	0.5 ± 1	0	0	0	0	0	0	0	0	0	0
Butlerius	**1**	0	0	0	0	0	0	0	13.5 ± 27	0	0	0
Cephalobus	**2**	0	0	0	0	0	0	0	0	0	0	8 ± 9.80
Chiloplacus	**2**	0	0	0	0	0	0	0	7.75 ± 15.5	29.33 ± 20.74	0	0
Chronogaster	**3**	0	0	0	0	0	0	0	0	0	0	2 ± 4
Desmolaimus	**3**	0	0	0	0	0	0	0	0	0	0	2 ± 4
Diplogasteridae	**1**	1.25 ± 1.89	0	0	0	0	0	0	0	0	0	0
Diploscapter	**1**	0.5 ± 1	0	0	0	0	0	0	26 ± 22.45	0	0	1.5 ± 3
Eucephalobus	**2**	2 ± 2.16	0	0	0	2 ± 2.31	0	0	20.5 ± 29.23	0	0	3 ± 3.83
Leptolaimidae	**3**	0.25 ± 0.5	0	0	0	0	0	0	0	0	0	0
Mesorhabditis	**1**	10 ± 10.17	0	0	0	0.75 ± 0.96	0	0.33 ± 0.58	160 ± 46.3	0	16.75 ± 6.08	2.25 ± 2.63
Monhystera	**1**	0.5 ± 0.58	0.25 ± 0.5	0.5 ± 0.58	0.33 ± 0.58	0.5 ± 0.58	0	0	0	0.67 ± 1.15	0.5 ± 1	0
Monhystrella	**1**	0	0	0	0	0.75 ± 1.5	0	0	0	0	0	0
Odontolaimus	**3**	1.5 ± 3	0	0	0	0	0	0	0	0	0	0
Panagrolaimus	**1**	0.5 ± 0.58	0	0	0	0.25 ± 0.5	0.75 ± 0.96	0	0	0.33 ± 0.58	0	1.25 ± 2.5
Plectus	**2**	2.75 ± 3.10	1 ± 0.82	0.25 ± 0.5	0	1 ± 0.82	0.25 ± 0.5	0.33 ± 0.58	0	0.33 ± 0.58	0.25 ± 0.5	19 ± 26.46
Prismatolaimus	**3**	1 ± 1.41	0	0	0	0.5 ± 0.58	0	0.33 ± 0.58	0	0	0	2 ± 4
Rhabditis	**1**	2.5 ± 2.08	0	0.25 ± 0.5	0	0.25 ± 0.5	0.25 ± 0.5	0	0	0	0	0
Rhabdolaimus	**3**	0	0	0	0	1.25 ± 1.89	0	0	0	0	0	0
Seleborca	**2**	0	0	0	0	1.5 ± 1.29	0	0	0	0	0	0
Stegelleta	**2**	0	0	0	0	0	0	0	6.75 ± 13.5	0	0	0
Teratocephalus	**3**	0	0.25 ± 0.5	0	0	1.25 ± 0.96	0	0	63.5 ± 32.05	0.33 ± 0.58	0	0
Wilsonema	**2**	0	0	0	0	0	0	0	6.75 ± 13.5	0	0	0
Zeldia	**2**	0	2 ± 1.83	0	0	0	0	0	0 ± 0	0	0	18 ± 36

Table 3: Continued.

		Kent's Entrance	Main Entrance A	Fault Passage	Pristine Chamber	Elevation Pit A	Elevation Pit B	North-Eastern Sec	Cave Inflow Area	Derek's Exit	Wonderfontein Canal	Stoffels Dam
Fungivores												
Paraphelenchus	**2**	1.5 ± 1.5	0	0	0	0.25 ± 0.5	0	0	19.25 ± 13.5	0	0	1 ± 2
Omnivores												
Actinolaimus	4	0	0	0	0	0	0	0	0	0	0	1.5 ± 3
Allodorylaimus	**4**	0	0	0	0	0	0	0	0	0	0	1.5 ± 3
Campydora	**4**	0	0	0	0	0.5 ± 1	0.25 ± 0.5	0	0	0	0	0
Dorylaimidae	**4**	0	0	0	0	1.5 ± 1	0	0	13.5 ± 27	0	0	0
Dorylaimus	**4**	0	0	0	0	0	0	0	0	0	1 ± 1.41	4.5 ± 4.12
Eudorylaimus	**4**	0	0	0	0	0.25 ± 0.5	0	0	0	0	0	0
Kochinema	**4**	0	0	0	0	0.25 ± 0.5	0	0	0	0	0	0
Mesodorylaimus	**5**	0	0	0	0	0.25 ± 0.5	0	0	0.25 ± 0.5	0	0.75 ± 0.96	4.5 ± 5.74
Carnivores												
Mononchus	**4**	0.25 ± 0.5	0	0	0	0	0	0	0	0	0	0
Tobrilus	**3**	0	0	0	0	0	0	0	0	0	0	47 ± 36.9
Tripyla	**3**	0	0	0	0	0	0	0.33 ± 0.58	0	0	0	0
TOTAL NEMATODES		**28.75 ± 14.77**	**16 ± 4.32**	**1 ± 1.41**	**0.33 ± 0.58**	**23.75 ± 2.63**	**1.75 ± 0.5**	**1.67 ± 1.15**	**767 ± 164.1**	**36.67 ± 11.59**	**19.25 ± 6.65**	**141.75 ± 36.92**

Table 4: Diversity and abundance (average of replicate samples) of nematode genera (PPN and NPN) found in sediment samples collected from the Wonderfontein Cave and Spruit during the 2nd (September) sampling interval. Trophic group and cp-value classification of nematode genera was based on entries listed in the Nemaplex online database (Available at http://plpnemweb.ucdavis.edu/nemaplex).

		Kent's Entrance	**Keyhole**	**Main Entrance**	**Fault Passage**	**Pristine Chamber**	**Elevation Pit**	**North-Eastern Section**	**Cave Inflow Area**	**Wonderfontein Canal**	**Stoffels Dam**
Herbivores											
Aphelenchoides		0	0	0	0	0	0	1 ± 0	0	0	0
Hemicycliophora		0.33 ± 0.58	0	0	0	0	0	0	0	0	0
Tylenchus		0	0	0	0	0	0.75 ± 1.5	0	1.75 ± 3.5	0	0
Bacterivores	**CP-Value**										
Acrobeloides	**2**	0	0	0	0	0	0.5 ± 0.58	0	0	0	0.25 ± 0.5
Anaplectus	**2**	0	0	0	0.33 ± 0.58	0.5 ± 0.71	0	0	0	0.25 ± 0.5	0
Aulolaimus	**3**	0	0	0	0.67 ± 1.15	0	0	0	0	0	0
Cephalobus	**2**	0	0	0	0	1 ± 1.41	0	0	0	0	0
Cylindrolaimus	**3**	0	0	0	0	0	0	0	0	0.5 ± 1	0
Diploscapter	**1**	0	0	0	0	0.5 ± 0.71	0	0	0	0	0
Eucephalobus	**2**	2.33 ± 4.04	0	0	0	0	1.75 ± 2.87	0	0	0	0.25 ± 0.5
Mesorhabditis	**1**	0	0	1 ± 0.82	0	0	0	0	0	0	0
Monhystera	**1**	0	0	0.25 ± 0.5	0.33 ± 0.58	15.5 ± 7.78	7 ± 5.29	0.5 ± 0.71	3.5 ± 4.04	1.25 ± 2.5	3 ± 2.45
Monhystrella	**1**	0	0	0	0.67 ± 1.15	0	0	0	0	0	0.25 ± 0.5
Panagrolaimus	**1**	0	0	0	0	1.5 ± 2.12	0	0	0	0	0
Plectus	**2**	2.67 ± 3.06	0.5 ± 1	2.5 ± 2.08	0.33 ± 0.58	14.5 ± 16.26	5.25 ± 3.77	0.5 ± 0.71	1.75 ± 3.5	18.5 ± 27.39	2.75 ± 5.5
Prismatolaimus	**3**	0	0.5 ± 1	3 ± 2.45	0	12 ± 0	2.75 ± 3.40	4 ± 5.66	0	0	0
Rhabditis	**1**	0	0	0	1 ± 1	0	0	0	0	0	0
Rhabdolaimus	**3**	0	0	0	0	0	0	0	0	0	0.25 ± 0.5
Fungivores											
Nothotylenchus	**2**	0	0.25 ± 0.5	0	0	0	0	0	0	0	0
Paraphelenchus	**2**	0.67 ± 0.58	0	0	0	0	0.75 ± 0.96	0	0	0	0
Omnivores											
Campydora	**4**	0	0	0	0	0	0	2.5 ± 0.71	0	0	0
Carnivores											
Mononchus	**4**	0	0	0	0	1.5 ± 0.71	0	0	0	0	0
Tobrilus	**3**	0	0	0.25 ± 0.5	0	0	0	0.5 ± 0.71	106 ± 17.57	0	1.75 ± 2.06
Tripyla	**3**	0	0	0	0	0	0.5 ± 0.58	0	0	0	0
TOTAL NEMATODES		**6 ± 5**	**1.25 ± 1.5**	**7 ± 4.32**	**3.33 ± 3.21**	**47 ± 7.07**	**18.5 ± 4.93**	**9 ± 5.66**	**113 ± 15.3**	**20.5 ± 26.03**	**8.5 ± 2.38**

Table 5: Diversity and abundance (average of replicate samples) of nematode genera (NPN) extracted from guano samples collected from the Wonderfontein Cave during the 1st (April) sampling interval. Trophic group and cp-value classification of nematode genera was based on entries listed in the Nemaplex online database (Available at http://plpnemweb.ucdavis.edu/nemaplex).

		Main Entrance	**Elevation Pit**
Bacterivores	**CP-Value**		
Acrobeloides	2	141.75 ± 111.56	0
Chiloplacus	2	3.5 ± 7	0
Monhystera	1	0	0.3333 ± 0.5774
Eukaryotic Feeders			
Chromadorita	3	0	0.3333 ± 0.5774
TOTAL NEMATODES		145.25 ± 115.61	0.6667 ± 1.1547

Table 6: Diversity and abundance (average of replicate samples) of nematode genera (NPN) extracted from guano samples collected from the Wonderfontein Cave during the 2nd (September) sampling interval.. Trophic group and cp-value classification of nematode genera was based on entries listed in the Nemaplex online database (Available at http://plpnemweb.ucdavis.edu/nemaplex).

		Pristine Chamber
Bacterivores	**CP-Value**	
Acrobeloides	2	376.5 ± 132.3
Monhystera	1	1 ± 2
Panagrolaimus	1	0.25 ± 0.5
Plectus	2	0
Carnivores		
Tobrilus	3	0.25 ± 0.5
TOTAL NEMATODES		378 ± 131.2

APPENDIX C

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Hairy Stalagmites, a new biogenic root speleothem from Botswana

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Abstract: Ngamiland in northwestern Botswana hosts the Gcwihaba Caves which present unique subterranean environments and host speleothems never before recorded. Cave atmospheric conditions can be extreme with temperatures as high as 28°C and relative humidity nearing 99.9%. Within Dimapo and Diviner's Caves peculiar root speleothems that we named 'Hairy Stalagmites' were found. These stalagmites are closely associated with the roots of Namaqua fig (Ficus cordata) trees that enter the cave environment in search of water. Pieces of broken stalagmites were sampled from Dimapo Cave for further investigations. Stereo and electron microscopy revealed that the Hairy Stalagmites consist of multiple intertwined tubes created when thin films of $CaCO_3$ are deposited around fine lateral roots. The importance of the roots is substantiated with evidence of calcified epidermal cells, apical meristems and epidermal imprints. The development of these stalagmites starts when roots accumulate on the cave floor in the vicinity of a water drip and a root nest is created to capture the water. From this point the roots grow upwards (positive hydrotropism) allowing the development of the calcite structure, and as CO_2 diffusion and evaporation occurs, $CaCO_3$ is deposited. The environmental conditions necessary for the growth of Hairy Stalagmites, as well their developmental mechanism, are discussed and illustrated.

Keywords: Botswana; root speleothem; Hairy Stalagmites; Namaqua fig tree; developmental mechanism; scanning electron microscopy

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INTRODUCTION

The hypothesis that biological interactions may somehow play a role in the deposition of secondary chemicals in caves was first suggested by Beaumont (1676) and was later described by Tournefort (1704). Speleothems were even considered, due to the presence of inner growing layers, as true 'rock plants'. At the end of the 18th and beginning of the 19th centuries, the most common speleothem types were often represented as parts of trees with stalactites as roots, stalagmites and columns as trunks, and helictites as leaves or flowers (Forti, 2001). However, at the beginning of the 20th century, any biogenic involvement in the formation of speleothems was ruled out as a better understanding of the main chemical mechanisms was achieved. Yet, in time, it became evident that micro-organisms may play some role in the formation of speleothems (Hill, 1976), while roots were reported to induce the development of peculiar speleothems such as showerheads and rootsticles (Hill & Forti, 1997). Today, it's generally accepted that plants (mainly roots) can passively advance the development of speleothems by enhancing capillary migration of water to structures where evaporation occurs (Forti, 2001). Also, it has recently been shown that microbial communities can actively influence the genesis and development of certain subaqueous and/or vadose speleothems (Melim et al., 2001; Barton & Northup, 2007; Dodge-Wan & Deng Hui Min, 2013).

Roots are the single portion of a tree which may somehow contribute towards speleothem development, however, it is presumably rare in deeper parts of caves since roots generally do not reach depths greater than several metres. Nevertheless, the morphological effects induced by roots over speleothems are evident. The surfaces of roots that enter cave voids may serve as pathways for the flow of seeping water and, if environmental conditions are favourable, the precipitation of $CaCO_3$ (calcium carbonate). This mechanism causes the development of peculiar stalactites and columns with a tilted and

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often anastomosed shape over which several pseudo-helictites grow. These speleothems have globally been observed with the same characteristics and are normally called ‘rootsicles’ (Hill & Forti, 1997). In wet tropical environments the root apparatus of large trees may become the main driving force for the development of peculiar cone-shaped stalactites known as ‘Showerheads’, which were first described from Brazilian caves (Lino, 1989) and later observed in many other tropical areas (Hill & Forti, 1997). Winkelhofer (1975) was the first to describe root stalagmites from sandstone caves in Germany which were later reported from the Czech Republic and other countries of Central Europe and Northern America (Bunnell, 2010; Pavuza & Cech, 2013). In all of these cases the root stalagmites either developed in the twilight zone or in total darkness. They were described as conical and/or cylindrical dense networks of several coniferous roots that may reach up to 60 cm in height. The voids that formed within the networks consisted mainly of living terminal roots often coated by symbiotic fungi, and were filled with sandy grains and organic matter; the latter being the product of mucilaginous excretion and/or decay of the roots. A smaller part of the network was formed by thicker and/or skeletal roots directly linked to the mother tree. Owing to the chemical composition of the host rock, no part of the root stalagmite was covered by a calcite crust.

The developmental mechanism of root stalagmites was initially described by Jenik & Kopecky (1992). Kopecky & Jenik (2001) recognized the dripping of water over a sand floor as the fundamental factor allowing for the development of these biogenic speleothems. In fact, only where dripping water impacts a root, do thin new roots grow upwards and towards the water drip (positive hydrotropism). This allows for the development of conical and/or cylindrical root stalagmites of which the height and diameter depends mainly on the energy of the water splashing on them, as well as on the availability of sand grains. Until recently, root stalagmites have globally been discovered in about 40 locations including from the Czech Republic, Poland, Austria, Slovakia, Hungary, Sweden, Spain, South Africa, Australia, and the United States (Mlejnek, 2010). Most of these caves were formed in sandstone or in other non-carbonate rocks (for example granite and gneisses). Root stalagmites were first discovered in a carbonate environment in 1987 during the exploration of Pofaddergat limestone cave (Namibia), of which a written report was only published nine years later by Marais et al. (1996). These root stalagmites developed below dripping points, were typically 20 cm tall, 3-4 cm wide, and consisted of densely packed thin rootlets. However, the presence of calcite incrustations was not reported. A few other limestone (or marble) caves (Bunnell, 2010) also host root stalagmites of which only one or two are partially covered by thin calcite crusts (Pavuza & Cech, 2013). Nevertheless, the organic component of the stalagmites is by far the most dominant.

Peculiar root stalagmites, called Hairy Stalagmites due to the fineness of their roots, have recently been discovered in dolomite caves in Botswana. These root stalagmites are peculiar since they are almost entirely composed of calcite with roots only visible on top of still-active growing speleothems. In the present paper the microclimate of the relevant cave, the morphology and chemical composition of the Hairy Stalagmites, as well as the proposed mechanism responsible for the development of this new type of root stalagmite, are discussed.

GEOLOGICAL SETTING AND CHARACTERISTICS OF STUDY AREA

Ngamiland: Botswana’s host of subterranean realms

The Gcwihaba Caves, first explored in 1932 by Marthinus Drotsky and initially known as Drotsky’s Caverns, are located in Ngamiland in the northwestern part of Botswana (Cooke, 1975). These caverns have facilitated various scientific expeditions, including paleoclimatic (Cooke, 1975; Railsback et al., 1994), paleontological (Robbins et al., 1996) and mineralogical (Martini, 1996) studies, but have, however, only been visited by a small number of tourists. Subsequently, the government of Botswana initiated the Gcwihaba Caves Project which aims at discovering new cave systems for the purpose of establishing an economically viable tourist destination. As part of the project, ground gravimetric surveys were conducted in order to identify cavities isolated below surface. Entry into these cavities was gained by drilling 600 mm diameter vertical shafts that penetrate on average 50 m below surface (Ellis, 2011). Until the beginning of 2014, five new caves have been penetrated of which three are extensive systems. Within two of these systems, Dimapo (20°1’12.34” S, 21°21’38.41” E) and Diviner’s (20°8’32.20” S, 21°12’36.60” E) caves, Hairy Stalagmites were discovered.

Diviner’s and Dimapo caves are located in the Koanaka and Gcwihaba Hills respectively; both hills form part of the Koanaka Group stratigraphy (Key & Ayres, 2000; Ellis, 2011). The latter is located in the Precambrian Damara Sequence of Ngamiland (Carney et al., 1994; Williams et al., 2012) and consists of greenschist-facies marble (Singletary et al., 2003). The surface landscape forms part of the Kalahari Desert which is classified as a semi-arid region with shrub savannah vegetation. Rainfall in the desert varies from 400 mm to 450 mm per annum and temperatures from -8.5°C to 42.2°C (Kennedy et al., 2012). Namaqua fig (Ficus cordata) trees naturally occur in the region, however, are mainly associated with the named hills. Preliminary field investigations have revealed that the trees found on Koanaka Hill directly overly Diviner’s and Mongongo (20°8’47.05” S, 2°12’27.08” E) caves. It is perceived that the specific location of the trees holds relation to their usage of the subterranean cavities as a water source, thus, ensuring their survival in the desert. Fig tree roots have been reported at a maximum depth of 120 meters below surface in Echo Caves (Ohrigstad, South Africa) (Kutschera, 1991).

Characteristics and features of Dimapo Cave

Dimapo Cave developed along a fault line as shallow as 40 meters below surface, and represents the main system where Hairy Stalagmites were sampled and studied. As illustrated by Fig. 1, the system is characterized by vast chambers, arrays of parallel passages, flowstone formations such as 'Calcite Mountain' (Fig. 2), slopes of infiltrating Kalahari sands, and extensive boulder fields. Dimapo Cave, as well as the other drilled caves, present microclimates atypical to southern Africa's caves. Durand et al. (2012) reported average temperatures of 17°C in the Bakwena

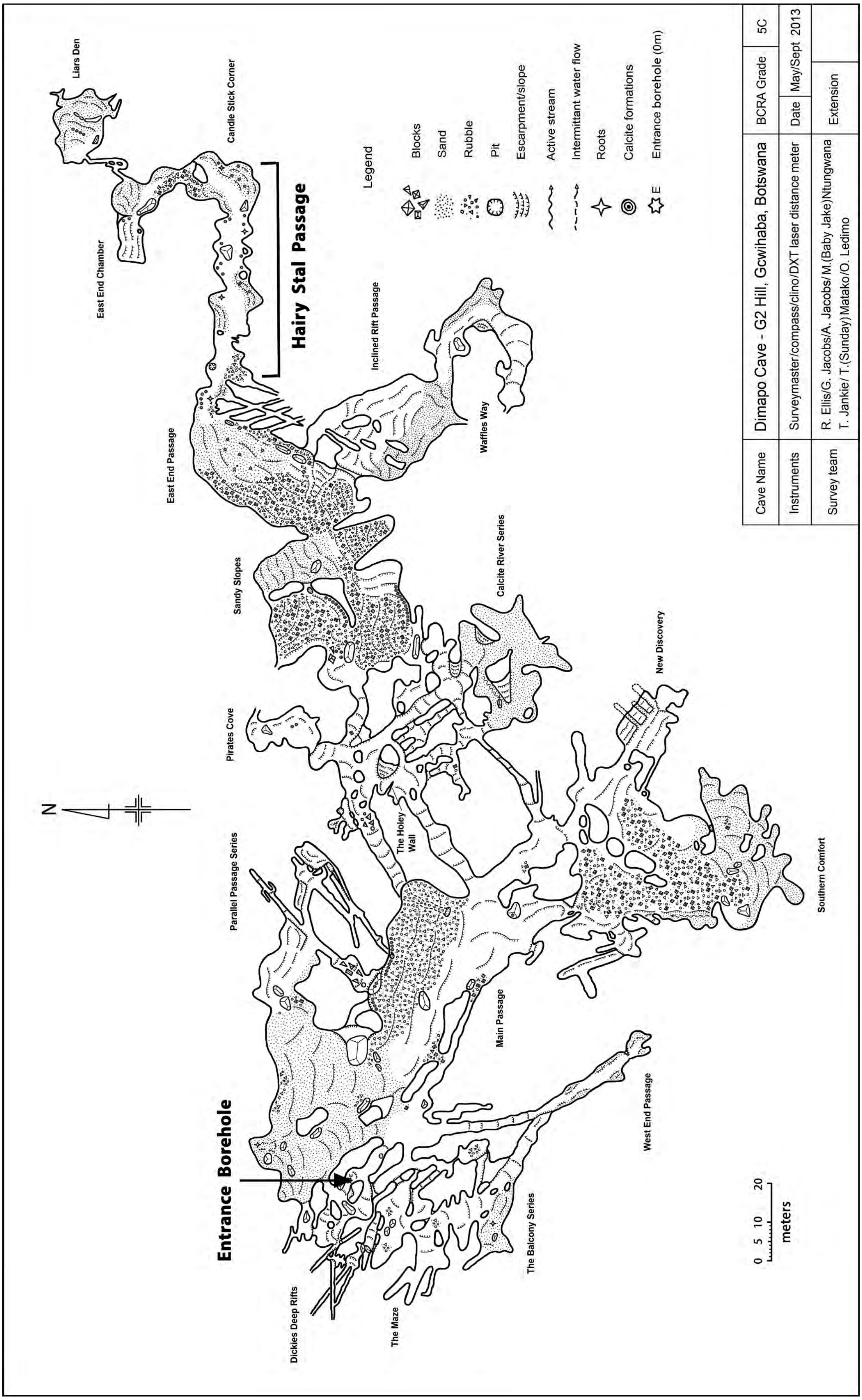

Fig. 1. Map of Dimapo Cave with the location of the Hairy Stalagmites ('Hairy Stal Passage') (Drawing provided by Roger Ellis).

Fig. 2. A flowstone formation referred to as ‘Calcite Mountain’ in Dimapo Cave (Photo by Anton Jacobs).

Cave (Irene, South Africa). However, temperatures within the drilled caves (Botswana) can reach as high as 28°C with 99% relative humidity, which nears 99.9% in boulder areas associated with Hairy Stalagmites. It is believed that the Namaqua fig trees utilize the subterranean environment, mainly due to the high humidity levels that result in water flowing along the roots. However, there are numerous water drips throughout Dimapo Cave, which are ‘hunted’ by roots extending over the cave floor. These roots, in association with the respective water drips, facilitate the development of the Hairy Stalagmites.

The Hairy Stalagmites are found within Dimapo Cave at the northeastern end of the cave system; a 10 to 15 m wide passage known as ‘Hairy Stal Passage’ (Fig. 1) which extends 60 m in an easterly direction, after which it continues 30 m north and ends in an alcove. On both sides of the passage are banks of infiltrating sand overlain by roots.

METHODS

Inactive and partially broken Hairy Stalagmite samples were carefully collected during September (spring) 2013 from Dimapo Cave (Botswana) by using a hand spade. Care was taken to avoid any direct skin contact. The cave atmospheric conditions were measured with a calibrated Vaisala thermo hygrometer (HMI41) which was left undisturbed for 15 minutes to ensure stable and accurate readings. The samples were stored in cushioned plastic containers, sealed, and transported to the North-West University (Potchefstroom, South Africa) and University of Bologna (Italy) for further analyses.

During all analyses, samples were handled using latex gloves and stored at room temperature in airtight containers. In order to study the surface features of the speleothem more closely, a Nikon AZ-100 (Amsterdam, Netherlands) stereo microscope and FEI Quanta 250 FEG (Bruno, Czech Republic) scanning electron microscope (SEM) with integrated software was used. Stereo light micrographs were taken with a digital camera by making use of the Nikon NIS-Elements software package that creates multilayered-image micrographs. For SEM, smaller pieces of speleothem were sputter-coated with gold/palladium. A small piece of uncoated Hairy Stalagmite was used to analyse the chemical composition of the speleothem substrate with energy-dispersive spectroscopy (EDS) by using an Oxford X-max 20 SDD detector and INCA software.

RESULTS AND DISCUSSION

The Hairy Stalagmites

Most of the Hairy Stalagmites found in Dimapo Cave are ‘dead’ (Fig. 3); calcite deposition has ceased most likely due to climatic variability and is indicated by the absence of Namaqua fig tree roots associated with the structure of the speleothems. It was noted that more than 90% of the speleothems were stagnant, however, some were ‘alive’, as roots were directly associated and intertwined with their structure (Fig. 4). Typically, roots travel along the cave strata and where intercepted by a water drip, give rise to many smaller rootlets and root hairs; an interconnected web of roots is created which plays a vital role in the development of the speleothem. The dimensions of the different Hairy Stalagmites vary greatly; some are only a few centimetres tall, while others tower over one meter. Generally, the Hairy Stalagmites are three to five centimetres in diameter. The latter, as well as the speed of growth of the speleothems, is most likely affected by: (1) the sustainability and volume of the water source, (2) the concentration of dissolved $CaCO_3$, and (3) the kinetic energy locked within each water drop. The amount of kinetic energy, determined by the distance of travel and diameter of a water drop, affects the dispersion of it upon impact (Salles et al., 2002) and thus possibly the initial base thickness of the speleothem.

Fig. 3. A ‘dead’ Hairy Stalagmite that has collapsed and regrown over time with roots associated with the base of the speleothem.

Fig. 4. Namaqua fig tree roots associated with an active Hairy Stalagmite. The roots create a nest at the top of the speleothem which captures the water from the drip (Photo by Anton Jacobs).

Microscopy investigations

Multilayered-imaging micrographs revealed that the entire structure consists of multiple intertwined tubes (Fig. 5). The tubes were initially formed around a network of lateral roots that originated from several horizontally-growing roots on the cave floor. The initial Hairy Stalagmites, also referred to as nests, consist of a network of modified roots that grow upwards. Lamont & Lange (1976) referred to them as stalagmiform roots.

Fig. 5. Micrograph of the external surface of a Hairy Stalagmite illustrating the intertwined calcite tubes that constitute its structure.

The individual tubes were studied more closely with the SEM. The inner surfaces of the calcite tubes showed the presence of both calcified epidermal cells, as well as epidermal root imprints (Fig. 6) that were sporadically found in close proximity to one another. Rarely, entire calcified root fragments (Fig. 7) were observed inside the Hairy Stalagmites. Intact calcified cells in the tubes were mostly root apical meristems (Fig. 8). Root caps appeared to be well preserved as their anatomical attributes were clearly visible as a population of thin-walled, isodiametrically-shaped cells (Fig. 8). The coarse granular texture of the root tips points to the calcification of a mucilaginous substance called mucigel that is normally secreted by root cap cells. Further back from the root tip, root imprints were predominantly observed and only occasionally intact calcified epidermal cells. In root fragments there was no evidence of calcified cortex cells, but the epidermis appeared to be multiseriate and the cell layer underneath the outermost epidermal layer was also calcified (Fig. 7). A cross section (Figs. 9; 10) revealed that no organic matter remains within the tubes because of the complete decomposition of dead roots. This process is important for nutrient recycling as it constantly makes nutrients available for new root growth and has important implications for the maintenance of the cave and above-ground ecosystem.

A possible explanation for the presence of both replicas and calcified cells is that some roots were alive when calcification occurred, while others were already dead. Calcified cells point towards rapid $CaCO_3$

biomineralization while the roots were still alive. Replicas are the consequence of physical precipitation of calcite around already dead roots. The dead roots subsequently disintegrate and leave epidermal imprints on the calcite. The presence of both types of rhizoliths may highlight the role of the root itself in the calcite deposition process. Living, respiring roots add CO_2 to their surroundings, which may produce H_2CO_3 in the presence of H_2O. Subsequently, higher acidity levels will produce a carbonate rich solution. This solution may penetrate intercellularly and also impregnate cell walls. As the calcite reprecipitates, it effectively preserves cell structure relatively quickly.

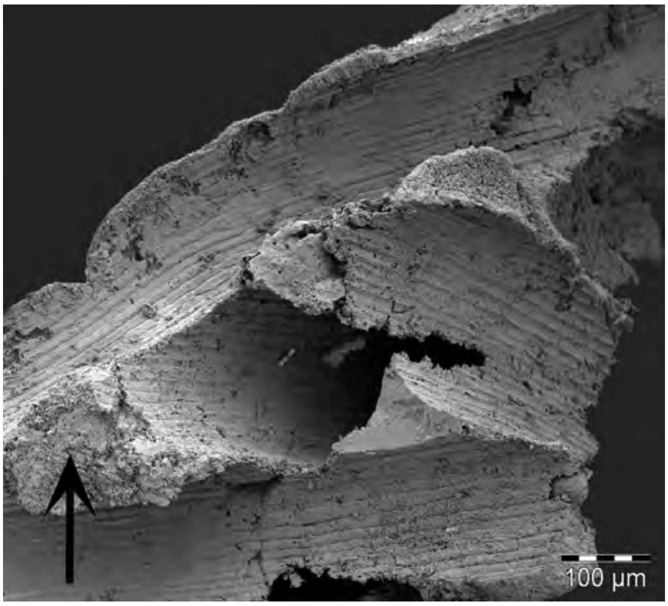

Fig. 6. Scanning electron micrograph showing root epidermal cell imprints created as $CaCO_3$ was deposited over the roots. Inter-tube cavities are sometimes partially filled with calcite (arrow).

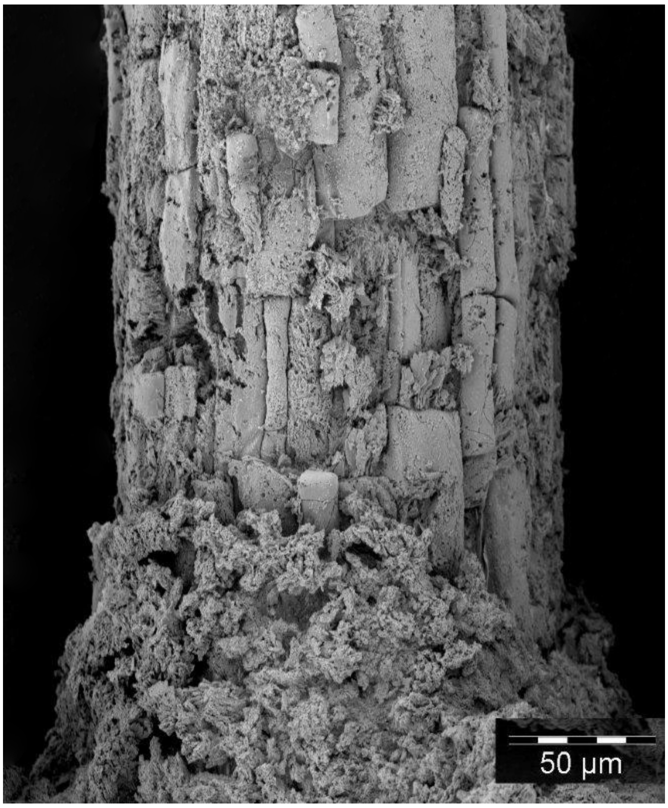

Fig. 7. Scanning electron micrograph showing intact calcified root fragment with calcite partially surrounding the bottom part of it.

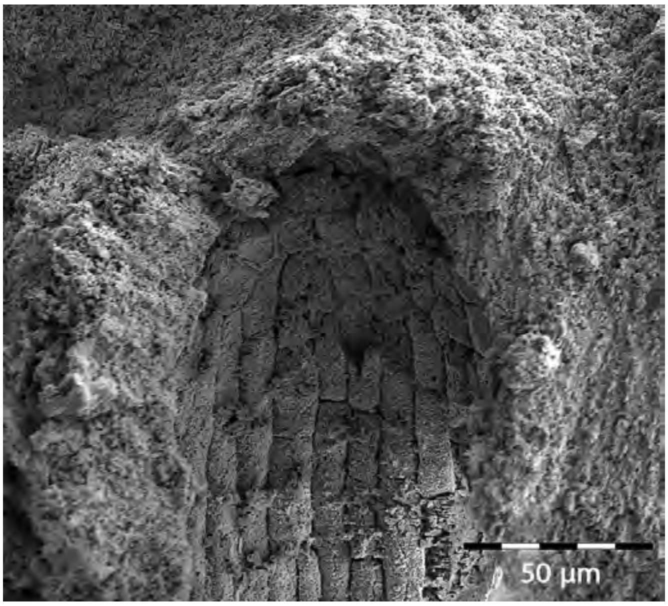

Fig. 8. Scanning electron micrograph showing root tip that contains calcified and isodiametrically-shaped thin-walled root cap cells and young epidermal cells in the zone of cell division.

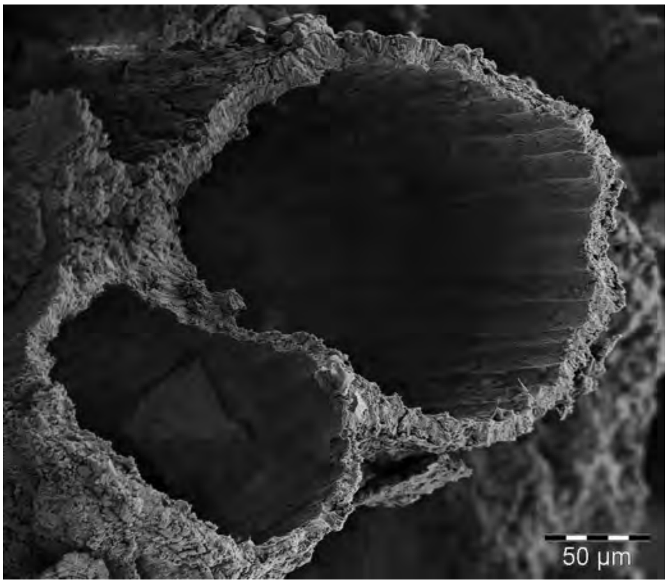

Fig. 10. Scanning electron micrograph of a cross section through calcite tubes shows that no organic matter remains within the tubes.

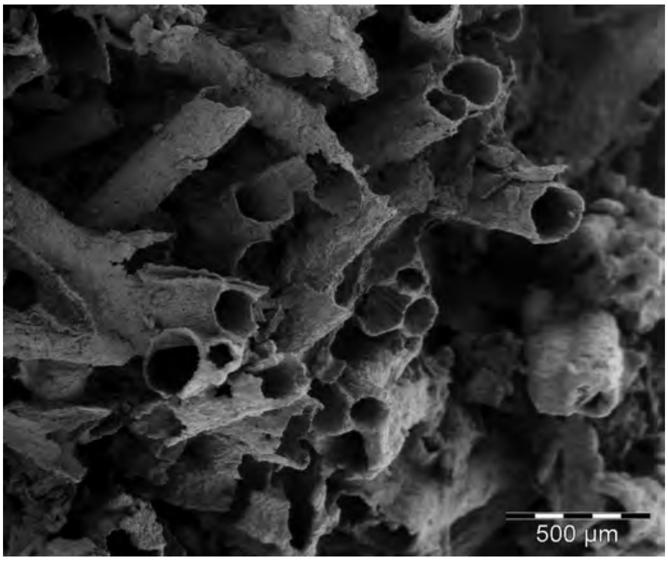

Fig. 9. Scanning electron micrograph of a cross section of multiple calcite tubes showing that all organic matter has either been decomposed or calcified.

However, water removal through absorption by mature living roots may concentrate the carbonate solution and also contribute to $CaCO_3$ precipitation, but mostly to the formation of calcite tubes surrounding the roots. Since there is no or limited impregnation and reprecipitation of cell walls and intercellular spaces by the carbonate solution, only epidermal imprints are left after the decomposition of organic material. The zone of cell division in the root tip consists of a population of actively dividing cells and hence high respiration rates. The more common preservation of the root cap cells in root tips, rather than older cells in mature root regions, points to the role of CO_2 from respiring roots during calcifications of cell structures. However, although biogenic processes may be involved in the calcification of some cells, it is probably a less dominant process than the physical processes involved during the precipitation of $CaCO_3$ around individual roots. The calcified tube walls are thin (10-20 μm) which indicates an overall slow rate of precipitation around each root. The EDS analysis (47.8% CaO, 3.8% MgO, 0.7% SiO_2, and 47.8% CO_2) revealed that the tubes consist of more than 95% of $CaCO_3$.

The developmental mechanism of a Hairy Stalagmite

When considering the development of a Hairy Stalagmite, several conditions are necessary for both the deposition of calcite, as well as the calcification of roots. The following conditions, however, are generally required for the development of any root stalagmite:

1. Trees and/or bushes of which the roots are capable of penetrating the underlying cave must cover the aboveground landscape. Thus, the depth of cave development may not exceed the limit of root penetration.
2. The cave environment must serve as a sufficient and sustainable water source.
3. Dripping water should contain dissolved $CaCO_3$.
4. Dripping inside the cave must be constant and provide sufficient water to avoid the desiccation of the roots, however, the cave floor must remain dry enough to induce positive root hydrotropism.

The following additional and specific conditions are required for the development of a Hairy Stalagmite:

5. Dripping water must either be slightly undersaturated or slightly oversaturated with $CaCO_3$. This is required to facilitate the development of Hairy Stalagmites and not typical speleothems (stalagmites and/or flowstones). If high supersaturation conditions were induced, it would result in rapid calcite deposition, thus burying the roots inside the formed structure.
6. Water flow, as a result of water dripping, should be minimal to allow for the deposition of calcite and to avoid the transport of deposited particles.
7. The relative humidity of the cave atmosphere must remain high in order to prevent the desiccation of the root tips, however, below 100% to avoid condensation of CO_2 rich water over the roots, which will hinder calcite deposition.
8. Dying-off and degradation of the roots associated with the Hairy Stalagmites should be slow enough in order to facilitate adequate calcite deposition and structure forming.

The development of a Hairy Stalagmite can only occur when all of these conditions are met, explaining why these speleothems are a rare phenomenon. The developmental mechanism can be sub-divided into five stages of which a description follows:

Stage one is initiated when roots from the surface penetrate the cave and travel along the cave walls and floor until they reach a constant water dripping point (Figs. 11 A; 12). The water stimulates the growth of several small hydrophilic upwards (positive hydrotropism) growing roots, creating a rounded root nest (Figs. 11 B; 13). As root growth continues, the nest enlarges, while the impact of the droplets creates a deepening depression in the center. The concave shape of the nest and its increasing central depth progressively reduce the impact of the water drops, which subsequently prevents droplets from escaping it. The lateral expansion of the nest stops when all of the dripping water is contained within it. Furthermore, the lateral roots facilitate capillary migration of water towards the external part of the nest where calcite deposition processes are active. Nearing the end of this stage, most, if not all of the droplets, are kept within the root nest, which is substantiated by the absence of calcite deposition in the form of layered structures over the external surface or around the base of the stalagmite.

During the second stage (Fig. 11 C), the rounded root nest with a central depression captures and disperses water by capillary action throughout the nest. Supersaturation of $CaCO_3$ is possibly induced by several different reactions. Within this peculiar micro-environment, CO_2 diffusion and H_2O evaporation may not be the only mechanisms involved in calcite deposition, especially since the high relative humidity and CO_2 partial pressure of the cave atmosphere will inhibit these processes. Other biologically driven mechanisms, including selective water uptake by roots through a biological film (Klappa, 1980) and mineralizing microbial colonies within the biological film around the roots (Cacchio et al., 2012), may also facilitate the deposition of a thin layer of calcite around the roots, thus, creating calcite tubes (Fig. 11 D). The force of the water drops impacting the structure causes the partial detachment of some of the newly formed calcite crust. The detached calcite grains accumulate on the cave floor along the perimeter of the nest. A conical-shaped sandy deposit is formed, becomes enlarged and hardened, and ultimately serves as a protection barrier that prevents the detachment of the thin calcite layers from the individual lateral roots. Calcite deposition continues towards the core of the nest, covering new roots, thickening calcite films surrounding others, and fuses the root structures together, while also partially filling inter-tube cavities (Fig. 6). Since water is constantly introduced at the center of the nest, supersaturation of $CaCO_3$ and thus calcite deposition is significantly inhibited. This, together with the impact force of the water drops, causes the core structure to be weaker than the rest of the stalagmite.

In the third stage a true Hairy Stalagmite is formed (Figs. 11 E; 4), characterized by a constant external diameter and vertically growing roots near the core. The calcite films inhibit the growth of the roots by clogging the vascular system, which prevents the roots from conducting water and food. This ultimately leads to the death of most of the roots. However, since the external diameter of the stalagmite remains relatively constant, it is evident that the roots die-off at a specific rate as root lifespan and radial growth is inhibited by the calcification process. After the organic matter has decomposed, the remaining calcite films (10-20 µm thickness) become an intricate network of interconnected hollow tubes (Fig. 10). Some root epidermal cells and root tips are calcified (Figs. 7; 8), while other roots have died before any calcification could take place, as only root surface imprints are visible inside the calcite tubes (Fig. 6).

The described process of Hairy Stalagmite formation may last for several years, creating speleothems over 1 m tall. Discontinuities visible as indentations in the external diameter (Fig. 11 F) are most likely induced by the reduced frequency of dripping during the dry season, which will also lower the relative humidity of the cave atmosphere. Thus, H_2O uptake by roots, evaporation, and CO_2 diffusion are enhanced close to the external surface (Fig. 11 F1). These combined processes are also responsible for the deposition of

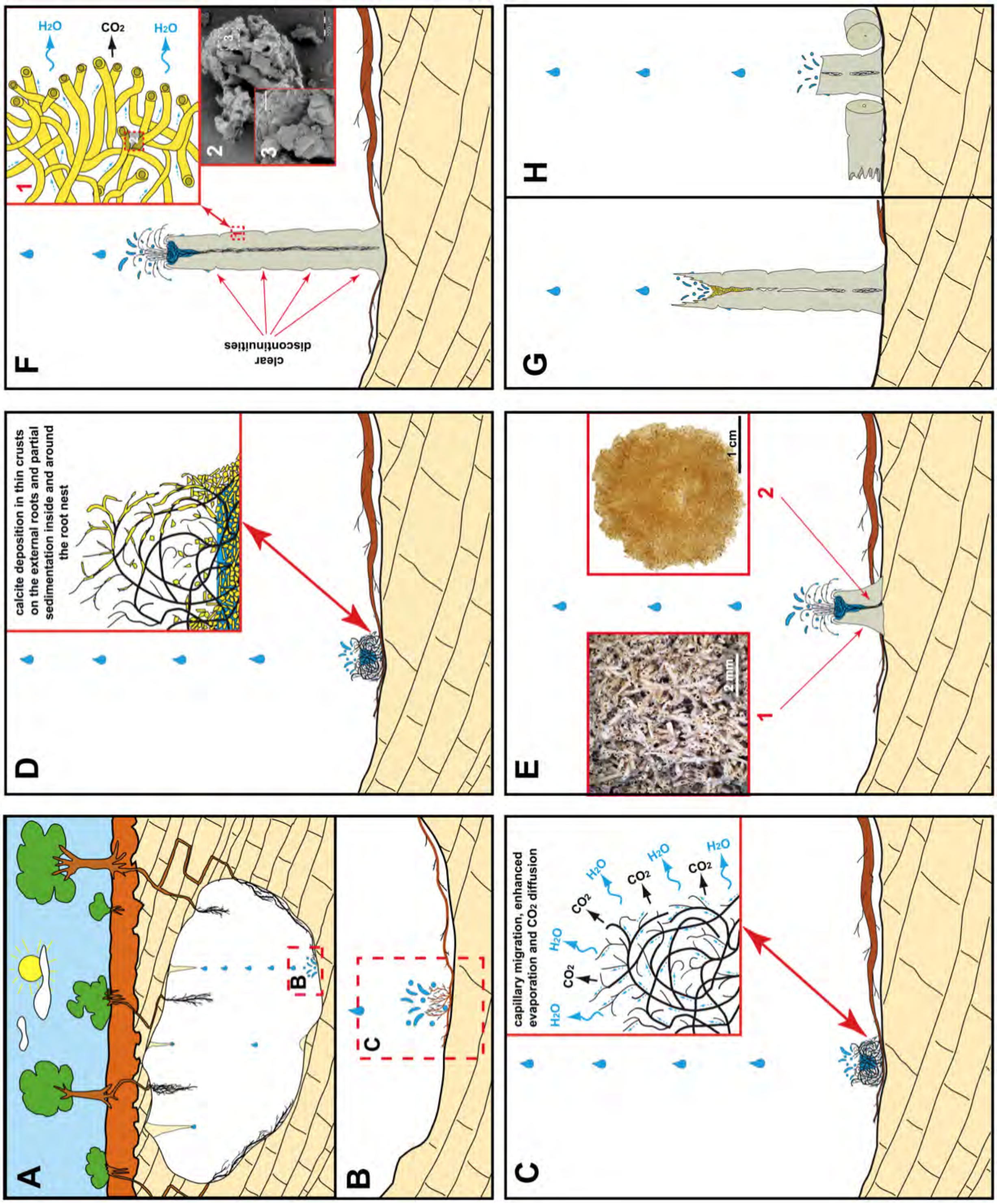

Fig. 11. The developmental stages of the Hairy Stalagmites: A) Roots of Namaqua fig trees migrate along the cave floor in search of water; B) Associated with a constant water drip, fine lateral roots grow upward creating a nest which traps the water; C, D) Evaporation and CO_2 diffusion allows for calcite precipitation within the nest; E) The process leads to the development of a Hairy Stalagmite of which the entire structure consists of calcite tubes (1), with no remaining organic material (2); F) Alternating dry and wet periods were recorded within the structure of the Hairy Stalagmite as variations in its diameter with the deposition of calcite euhedral crystals in the inter-tube cavities (1-3); G, H) After the internal roots have died, the upper nest disappears and the dripping starts eroding the inner part of the Hairy Stalagmite leading to its collapse.

Fig. 12. Dripping point on the cave floor 'hunted' by roots, which marks the start of the development of a Hairy Stalagmite.

Fig. 13. Root nest created during the first stage of development of a Hairy Stalagmite.

calcite crystals (Fig. 11 F2-3) in the voids that exist between the formed calcite tubes. While the calcite linings over the roots consist of small elongated crystals with a highly porous structure, the calcite deposited in the inter-tube cavities and on the surface of the Hairy Stalagmites has an entirely different morphology. The former is the result of alternating deposition and re-dissolution of calcite most likely controlled by biogenic processes that subsequently give rise to a high amount of CO_2. On the contrary, the calcite in the cavities and on the surface of the Hairy Stalagmite consist of euhedral non-porous crystals (Figs. 14; 15) which suggests a slow but continuous deposition process controlled by evaporation and/or enhanced H_2O uptake by roots during the dry season. As a result, the external surface of the stalagmite is more hardened. Due to the above described process, only the roots growing vertically along the dripping line survive. However, as they reach the open space above the partially hardened nest, they create an elaborate umbrella structure (Fig. 4) which allows the further development of the Hairy Stalagmite.

The fourth stage (Fig. 11 G) is related to the decay of the Hairy Stalagmite, which is influenced by the availability of water, the rate of calcite hardening, and the height of the structure. As the Hairy Stalagmite becomes taller, the roots within the structure may break as they become more fragile and exposed especially during the dry season. Also, if water dripping ceased for a period of time, the roots may become petrified. Consequently, the root nest at the top of the stalagmite will die and disintegrate, leaving the stalagmite exposed to the impact of dripping water. Direct contact between the water drops and the calcite structure will progressively degrade it and block the tubes with calcite fragments.

Finally, during the fifth stage (Fig. 11 H), the Hairy Stalagmites become unstable without the support of living roots. Furthermore, the impact of falling water drops is no longer cushioned by the root nest. If enough force is generated, the Hairy Stalagmite may break and collapse (Fig. 3), most likely at an area of weakness closer to the base of the speleothem. An area of weakness is probably the result of incomplete calcification and may also explain the discontinuities (Fig. 11F) that were observed in the external diameter of the stalagmite. If conditions again become favorable, the portion of the Hairy Stalagmite left standing may be recolonized by roots. This marks the start of the development of a new Hairy Stalagmite continuing from the third stage.

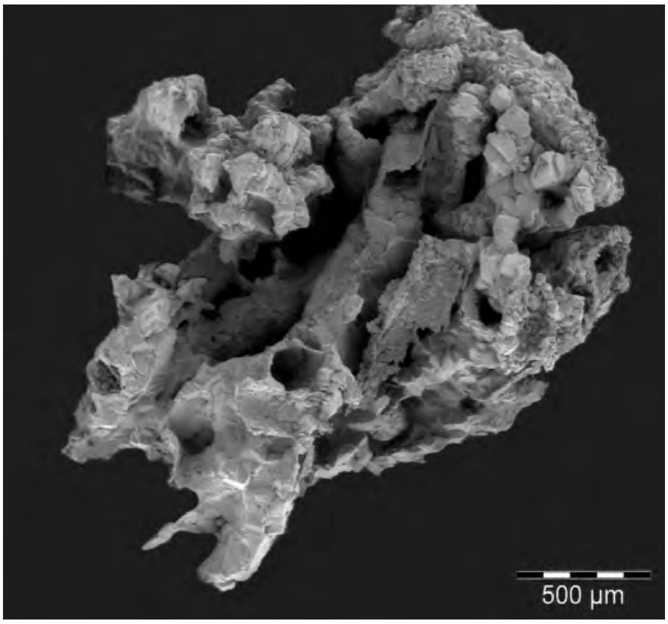

Fig. 14. Scanning electron micrograph showing euhedral calcite crystals that formed near the surface of a Hairy Stalagmite.

FINAL REMARKS

Hairy Stalagmites, a new type of biogenic speleothem, consist of both calcite tubes and calcified root cells, and are peculiar specimens representing biogenically-formed speleothems. Their development is strongly controlled by an intricate set of both atmospheric and abiotic conditions, most likely sensitive to

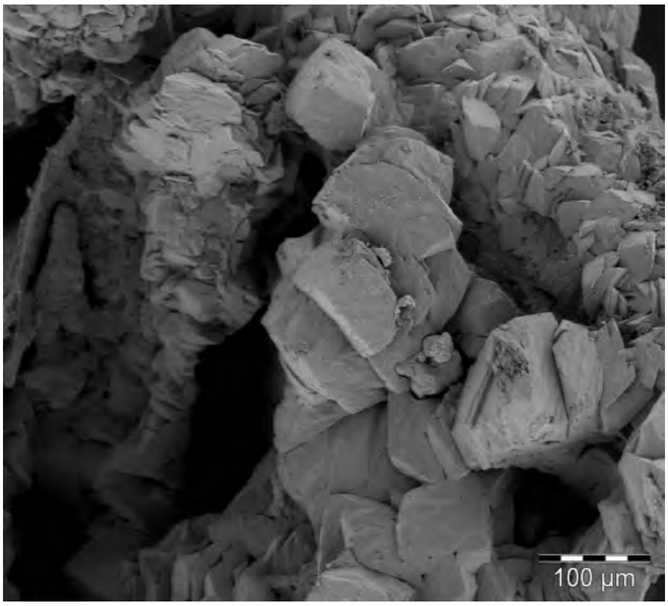

Fig. 15. Close-up view of calcite crystals formed on a Hairy Stalagmite.

climatic variations. The required conditions explain why the Hairy Stalagmites have only been observed in caves previously sealed. In order to preserve this unique environment together with this new type of speleothem, it is necessary to conserve not only the cave atmospheric conditions, but also the surface landscape that hosts the Namaqua fig trees. Therefore, with the Botswana government's efforts to create a tourist destination, adequate measures should be considered to insure the sustainability and function of the caves and surface landscape.

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A new troglobitic ideoroncid pseudoscorpion (Pseudoscorpiones: Ideoroncidae) from southern Africa

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Abstract. The first blind African species of Ideoroncidae is described from a cave in northwestern Botswana, *Botswanoncus ellisi*, representing a new genus and a new species. Apart from the complete lack of eyes, it is also unusual in having the lowest recorded number of trichobothria of any adult ideoroncid with 17 on the fixed finger and nine on the movable finger.

Keywords: Taxonomy, morphology, Botswana, new genus

The pseudoscorpion family Ideoroncidae comprises 13 genera and 70 species found in Africa, Asia and the Americas (Harvey 2013; Harvey & Muchmore 2013). The majority occur in tropical ecosystems, but some occur in drier regions such as the southwestern USA and Mexico, Chile and Argentina, and the deserts of the Middle East. Ideoroncids have the combination of two unique morphological features, supernumerary trichobothria and the sub-basal position of the median maxillary lyrifissure (Harvey 1992; Harvey & Muchmore 2013). Another unusual feature, the division of the median genital sac of the male genitalia into two distinct parts, is also found in some species currently attributed to the family Syarinidae (e.g., Vachon 1938, 1954, 1969; Chamberlin 1952; Mahnert 1980; Harvey 1992).

The African ideoroncid fauna (Fig. 3) consists of four genera, *Afroroncus* Mahnert 1981, *Dhanus* Chamberlin 1930, *Nannoroncus* Beier 1955 and *Negroroncus* Beier 1931 (Harvey 2013). The genera *Nannoroncus* and *Afroroncus* are restricted to forested habitats in Kenya (Mahnert 1981). Three species of the genus *Dhanus* are known from the island of Socotra located off the Somali coast (Mahnert 2007). This genus is otherwise known from the Middle East (Afghanistan and Iran), India and southeast Asia (Harvey 2013). *Negroroncus* is the most widespread African genus, being found throughout Kenya, eastern Democratic Republic of Congo and northern Tanzania, as well as individual outlying species from Zimbabwe and the Republic of Congo (Mahnert 1981; Harvey 2013).

Among a small collection of pseudoscorpions taken from a cave in the Gcwihaba region of northwestern Botswana, the junior author found a small ideoroncid that completely lacks eyes (Fig. 4). Eyeless ideoroncids are elsewhere only known from the New World: *Albiorix anophthalmus* Muchmore 1999 from a cave in Arizona, USA (Muchmore & Pape 1999), *Ideoroncus anophthalmus* Mahnert 1984 and *I. cavicola* Mahnert 2001 from Brazil (Mahnert 1984, 2001) and five species of *Typhloroncus* 1979 from caves in Mexico and epigean ecotypes in the US Virgin Islands (Muchmore 1979, 1982, 1986; Harvey & Muchmore 2013). The only cave-dwelling ideoroncids from Africa are *Negroroncus aelleni* Vachon 1958 from the Republic of Congo and *N. jeanneli* Vachon 1958 from Tanzania (Vachon 1958), but neither species is completely eyeless, even though the eyes are small (Vachon 1958).

An initiative by the Botswana government to discover and explore unknown caves with the aim to promote caving in the tourism industry led to the discovery of several caves with no natural openings in the northwestern Gcwihaba region of Botswana. These caves were discovered through gravimetrical surveys of dolomitic outcrops. This technique identifies isolated subterranean cavities that are subsequently penetrated by means of drilling 700 mm vertical shafts. In 2010 efforts led to the discovery of a cave system named Diviners Cave. The drilled shaft, with a surface entrance altitude of 1056 m a.s.l., penetrates through 50 meters of rock into the cave. Exploration revealed an extensive cave system (Fig. 1) at several levels, with chambers of up to up 180 meters in diameter. Within the sealed cave we found areas where the roots of wild fig trees (*Ficus cordata*) penetrate the caves associated with sand and water drips. In these areas we found a diversity of invertebrates including diplurans, centipedes and termites. Although the cave does not reach the water table, the relative humidity exceeds 95% with a fairly constant temperature of 27°C (±2°C). Prior to drilling to open the cave, the system was under a high CO_2 pressurized atmosphere, making the circumstances in which the cavernicoles survived quite different from other natural caves in southern Africa.

Our study of the specimens revealed a number of unusual features, including the lowest number of trichobothria thus far recorded for an adult ideoroncid (Table 1), and a short arolium that lacks a ventral hook-shaped process. Apart from being a distinctive new species, we also suggest that it represents a previously undescribed genus. Therefore, we here provide a description and name the species to highlight its distinctive morphology and habitat.

Figures 1, 2.—1. The type locality of *Botswanoncus ellisi*, Diviners Cave, Botswana (Photo: Gerhard Jacobs); 2. Roots of the fig tree (*Ficus cordata*): the specimens were collected from the soil below the roots.

METHODS

The specimens examined in this study were mainly sampled with pitfall traps. Each trap was neatly buried in the soft sand associated with wild fig tree roots (Fig. 2) and half-filled with 75% ethanol. Also, some specimens were caught by hand or extracted from soil samples using Berlese funnels. These specimens are lodged in the Western Australian Museum, Perth (WAM) and the KwaZulu-Natal Museum, Pietermaritzburg (NMSA), and were studied using temporary slide mounts prepared by immersion of the specimen in lactic acid at room temperature for several days. They were then mounted on microscope slides with a 10 mm coverslip supported by small sections of 0.25 or 0.35 mm diameter nylon fishing line. After study the specimens were returned to 75% ethanol with the dissected portions placed in 12 × 3 mm glass genitalia microvials (BioQuip Products, Inc.). Specimens were examined with a Leica MZ–16A dissecting microscope and a Leica DM2500 compound microscope, the latter fitted with interference contrast, and illustrated with the aid of a drawing tube attached to the compound microscope.

Measurements were taken at the highest possible magnification using an ocular graticule. Terminology and mensuration mostly follow Chamberlin (1931), with the exception of the nomenclature of the pedipalps and legs, and with some minor modifications to the terminology of the trichobothria (Harvey 1992), chelicera (Judson 2007) and faces of the appendages (Harvey et al. 2012).

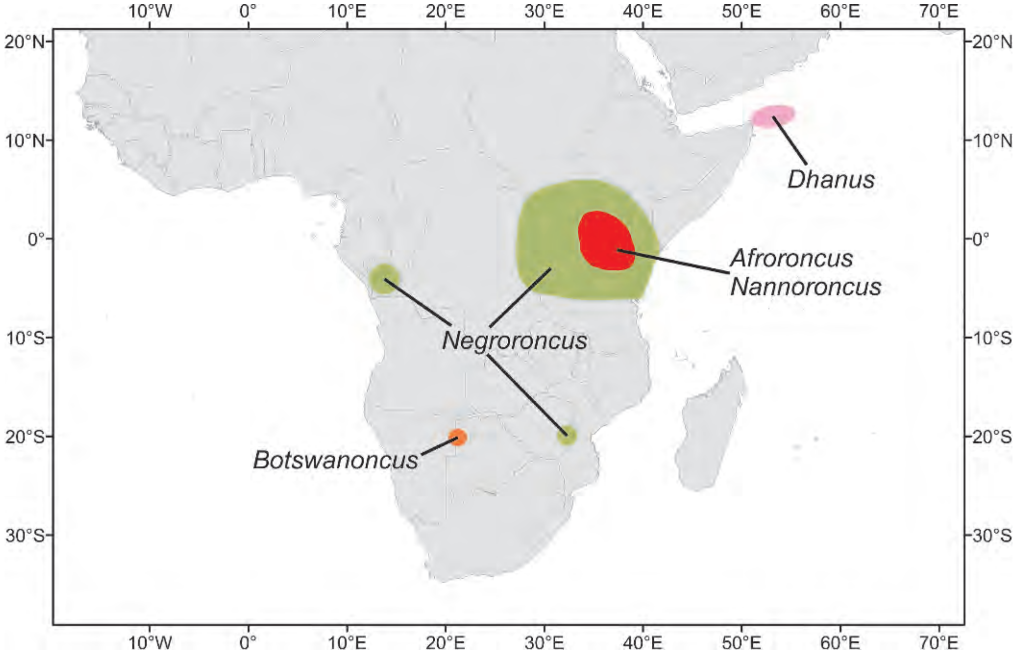


Figure 3.—Distribution of ideoroncid genera in Africa.

Figure 4.—*Botswanoncus ellisi*, new species, female holotype, prior to dissection, dorsal aspect.

The description was compiled using the DELTA (DEscription Language for Taxonomy) Editor computer program, version 1.0-Beta (available at http://code.google.com/p/open-delta/) (Dallwitz et al. 1999).

SYSTEMATICS

Family Ideoroncidae Chamberlin 1930
Genus **Botswanoncus** gen. nov.

Type species.—*Botswanoncus ellisi* new species

Diagnosis.—*Botswanoncus* is the only ideoroncid genus with a short arolium that lacks a ventral hook on the arolium. It also differs from other ideoroncids by the presence of only 17 trichobothria on the fixed chelal finger and nine trichobothria on the movable chelal finger (Table 1) (Figs. 11, 12), and from all other African ideoroncids by the complete lack of eyes (Fig. 5).

Description.—*Adult female (male unknown)*: Chelicera (Fig. 9): hand with 5 setae; movable finger with 1 sub-distal seta; all setae acuminate; galea present, simple, long and slender; lamina exterior absent; serrula exterior connected to chelicera finger for only part of length; not modified to form velum; rallum (Fig. 10) with 4 blades, all with anterior spinules, basal and sub-basal blades shorter than others.

Pedipalp (Fig. 8): long and slender. Fixed finger with 17 trichobothria (Figs 11, 12): *eb*, *esb*, *et*, *isb* and *it* regions each with 1 trichobothrium; *est* region with 6 trichobothria; *ib* region with 3 trichobothria; *ist* region with 3 trichobothria; *et* slightly distal to *it*. Movable finger with 9 trichobothria (Figs. 11, 12): *b*, *sb* and *st* regions each with 1 trichobothrium; *t* region with 6 trichobothria. Chelal teeth (Fig. 11) juxtadentate, with fixed finger chelal teeth low and flattened, and movable finger chelal teeth low; venom apparatus present in both chelal fingers; venom ducts of medium length, terminating midway between *it* and *est* in fixed finger and basal to *t* in movable finger; nodus ramosus not inflated.

Cephalothorax: carapace (Fig. 5) sub-rectangular; anterior margin slightly convex; with 4 setae on anterior margin and 2 on posterior margin; furrows absent; eyes completely absent. Manducatory process somewhat pointed, with 2 apical setae; median maxillary lyrifissure situated sub-basally.

Legs: femora I and II much longer than patellae I and II, respectively; femora III and IV about same size as patellae III and IV, respectively; metatarsi shorter than tarsi; subterminal tarsal seta acuminate; arolium about same length as claws, with slight medial division (Figs. 6, 7); ventral hook-shaped process absent.

Abdomen: tergite I with 2 setae; spiracular plates each with 1 seta; medial sternites without suture line; pleural membrane uniformly longitudinally striate; stigmatic helix present; anus situated between tergite XI and sternite XI.

Genitalia: female: with gonosac covered in small acetabula.

Remarks.—The arolium of *B. ellisi* is about the same length as the claws (Figs. 6, 7), and therefore resembles the New World genera *Typhloroncus* and *Xorilbia* Harvey and Mahnert 2006, the African *Negroroncus aelleni* Vachon 1958, and the Asian *Dhanus siamensis* (With 1906), which have arolia that are slightly shorter than the claws. The remaining ideoroncid genera have arolia that are clearly longer than the claws. It differs from these genera with short arolia by lacking the ventral hook of the arolium. It further differs from all other ideoroncids by the reduced number of trichobothria, with only 17 on the fixed finger and nine on the movable finger (Table 1, Figs. 11, 12).

Etymology.—The genus is named for its occurrence in Botswana, combined with the last five letters of *Roncus*, a common pseudoscorpion stem which has been thought to be derived from the Latin *runco*, "living in weeds" (Parker 1982). It is to be treated as masculine.

Botswanoncus ellisi sp. nov.
Figs. 4–12

Material examined.—**Botswana**: *North-western District*: holotype female, Diviners Cave, Gcwihaba region, 20°08′32.2″S, 21°12′36.6″E, 19 October 2011, G. Du Preez (WAM T125604). Paratypes: 1 female, same data as holotype except 13 March 2012 (WAM T130804); 1 female, same data as paratype (NMSA-Pse 026870).

Diagnosis.—As for genus.

Table 1.—The recorded number of trichobothria found in adults of genera of Ideoroncidae. Variant numbers are shown in brackets.

	eb	*esb*	*est*	*et*	*ib*	*isb*	*ist*	*it*	*b*	*sb*	*st*	*t*	Fixed finger, total	Movable finger, total	Reference
Afroroncus	1	1	6	1	4	1	5	1	2	1	1	6	20	10	Mahnert (1981)
Albiorix	1	1	6	1	4–5	1	5–6 (4)	1	2	1	1	6	20–22	10	Harvey & Muchmore (2013)
Botswanoncus	1	1	6	1	3	1	3	1	1	1	1	6	17	9	This study
Dhanus	1–3	1	5–9	1	3–6	1	5–12	1	2–4	1	1	6–8	23–31	11–14	Mahnert (1984, 2007)
Ideoroncus	1	1	6	1	4 (5)	1	4–6	1	2	1	1	6	20–21 (22)	10	Mahnert (1984, 2001); Harvey & Muchmore (2013)
Mahnertius	1	1	6	1	5	1	6	1	2	1	1	6	22	10	Harvey & Muchmore (2013)
Muchmoreus	1	1	6	1	4	1	5	1	2	1	1	6	20	10	Harvey & Muchmore (2013)
Nannoroncus	1	1	6	1	4	1	5	1	2	1	1	6	20	10	Mahnert (1981)
Negroroncus	1–2	1	6–10	1	4	1	5–6	1	2–3	1	1	6–7	20–26	10–12	Mahnert (1981); Vachon (1958)
Nhatrangia	2	1	9	1	6	1	9–10	1	4	1	1	8	30–31	14	Mahnert (1984)
Pseudalbiorix	1	1	6	1	4	1	5	1	2	1	1	6	20	10	Harvey et al. (2007)
Shravana	1	1	6	1	5	1	7	1	3	1	1	7	23	12	Mahnert (1984)
Typhloroncus	1	1	6	1	4–5	1	6–7	1	2	1	1	6	22	10 (11)	Muchmore (1986); Harvey & Muchmore (2013)
Xorilbia	1	1	6	1	5	1	6	1	2	1	1	6	22	10	Mahnert (1984, 1985)

Description.—*Adult*: Color: pedipalps, carapace, chelicerae and coxae light red-brown, tergite I and legs pale brown and remainder light yellow-brown (Fig. 4).

Setae and cuticle: setae long, mostly straight and acicular; most cuticular surfaces smooth and glossy, with exception of pedipalps and chelicera, which are finely granulate.

Chelicera (Fig. 9): ca. 50% length of carapace; surface reticulate; cheliceral hand with 5 setae; movable finger with 1 sub-distal seta; all setae acuminate; galea present, simple, very long, slender and slightly curved; fixed finger ca. 7 teeth, each approximately same size, small; movable finger with ca. 8 teeth, each approximately same size, small; exterior and interior condylar lyrifissures present; serrula interior with 18 blades; lamina exterior absent; rallum (Fig. 10) with 4 blades, all with anterior spinules; basal and sub-basal blades shorter than others.

Pedipalp (Fig. 8): long and slender; trochanter and femur granulate, prolateral margin of patella and chelal hand granulate, retrolateral surfaces of fingers granulate, all other surfaces smooth; setae acicular, straight or nearly so; trochanter with anterior margin rounded, 2.85 x; femur cylindrical, without trichobothria, 4.57–5.01 x; patella cylindrical, with strongly pronounced pedicel, with 3 lyrifissures, 2 at distal end of pedicel and 1 in middle of pedicel, 2.99–3.25 x; chelal hand ovoid, external chelal condyle small and rounded, internal chelal condyle small and rounded, chela (with pedicel) 3.91–4.14 x, chela (without pedicel) 3.71–3.92 x, hand (without pedicel) 1.26–1.32 x, movable finger 1.93–2.54 x longer than hand (without pedicel). Fixed finger with 17 trichobothria; movable finger with 9 trichobothria (Figs. 11, 12); *eb*, *esb* and *isb* in straight row at base of finger; *ib* region situated dorsally in the middle of chelal hand; *eb*, *esb*, *et*, *isb* and *it* regions each with 1 trichobothrium; *est* region with 6 trichobothria; *ib* region with 3 trichobothria; *ist* region with 3 trichobothria; *et* slightly distal to *it*; *b, sb* and *st* regions each with 1 trichobothrium; *t* region with 6 trichobothria; not ventrally displaced, or *st* situated much closer to *b* than to *t*; trichobothrium *t* acuminate. Both fingers straight in lateral view; chelal teeth juxtadentate (Figs. 11, 12); fixed finger chelal teeth low and flattened; venom apparatus present in both chelal fingers, venom duct of medium length, terminating midway between *it* and *est* in fixed finger and basal to *t* in movable finger; nodus ramosus not inflated.

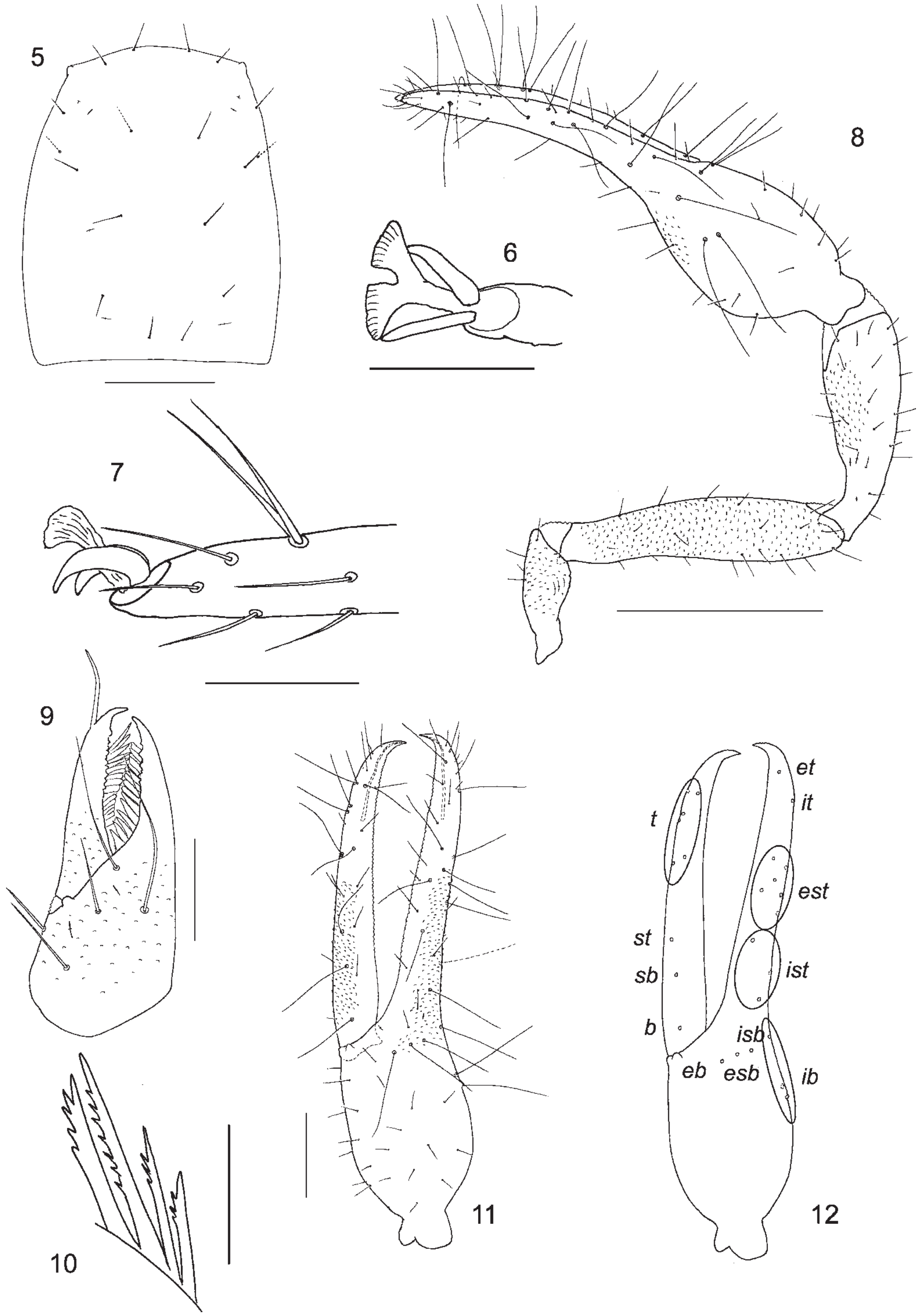
Cephalothorax: carapace (Fig. 5) sub-rectangular, 1.20–1.39 x longer than broad; anterior margin slightly convex; epistome absent; lateral margins slightly convex; posterior margin straight; with 18 setae, arranged 4: 4: 4: 2: 2: 2; setae subequal in length; furrows absent; eyes completely absent. Manducatory process somewhat pointed, with 2 apical setae, both setae approximately equal in length; maxilla with 5 additional setae; maxillary shoulder absent; median maxillary lyrifissure present, situated sub-basally, strongly curved, U-shaped; posterior maxillary lyrifissure present, strongly curved. Coxa I about same width as coxa IV; anterior margin of coxa I with process near foramen; coxae I–IV of ♀ with setae arranged 4: 5: 4: 4.

Legs: femora I and II much longer than patellae I and II, respectively; femur I and II without basal swelling; femora I and II with primary slit sensillum directed transversely; femora III and IV about same size as patellae III and IV, respectively; femur + patella IV 3.89 x longer than broad; tibiae III and IV without obvious tactile seta; metatarsi III and IV with long tactile seta, situated medially; tarsi III and IV without tactile seta; metatarsi and tarsi of all legs not fused; metatarsi shorter than tarsi; subterminal tarsal seta acuminate; claws smooth; arolium about same length as claws, with slight medial division (Figs. 6, 7); ventral hook-shaped process absent (Fig. 7).

Abdomen: tergites straight, without suture line, setal formula ♀, 2: 4: 4: 4: 5: 6: 6: 6: 6: 6: 6 (arranged T1TT1T): 2; arranged in single rows; sternites, without suture line, setal formula ♀, 6: (1) 2 (1): (1) 4 (1): 8: 8: 8: 8: 8: 8: 6 (arranged 1T2T1): 2; setae of anterior genital operculum (sternite II) of ♀ very small; posterior tergites and sternites with several tactile setae; glandular setae absent; pleural membrane uniformly longitudinally striate.

Genitalia: female: with gonosac covered in small acetabula.

Dimensions (mm): Female holotype (with paratypes in parentheses, where applicable). Body length 2.28 (2.16–2.25). Chelicera 0.313/0.132, movable finger length 0.200. Pedipalp:



Figures 5–12.—*Botswanoncus ellisi*, new species, female holotype: 5. Carapace, dorsal; 6. Tip of left tarsus IV showing claws and arolium, dorsal; 7. Distal end of left tarsus IV, lateral; 8. Right pedipalp, dorsal; 9. Left chelicera, dorsal; 10. Rallum; 11. Left chela, lateral; 12. Left chela, lateral, showing trichobothrial pattern. Scale lines = 0.05 mm (Figs. 6, 7), 0.1 mm (Figs. 9, 10), 0.2 mm (Figs. 5, 11, 12), 0.5 mm (Fig. 8).

trochanter 0.314/0.110, femur 0.685/0.150 (0.659–0.722/0.141–0.144), patella 0.545/0.182 (0.502–0.550/0.163–0.169), chela (with pedicel) 1.182/0.302 (1.136–1.250/0.295–0.302), chela (without pedicel) 1.12 (1.072–1.184), hand (without pedicel) 0.380 (0.390–0.397), movable finger length 0.732 (0.689–0.768). Carapace 0.564/0.471 (0.584–0.596/0.419–0.435). Leg I: femur 0.330/0.080, patella 0.160/0.070, tibia 0.250/0.049, metatarsus 0.152/0.039, tarsus 0.337/0.032. Leg IV: femur + patella 0.521/0.140, tibia 0.324/0.071, metatarsus 0.228/0.046, tarsus 0.328/0.040.

Remarks.—*Botswanoncus ellisi* exhibits some moderate modifications for cave existence, including the complete lack of eyes (Figs. 4, 5) and pallid coloration (Fig. 4). The appendages, however, are not especially elongated as in the other troglobitic ideoroncids *Albiorix anophthalmus* Muchmore 1999 from Arizona, USA and several *Typhloroncus* species from Mexican caves, which have long, slender appendages consistent with a permanent cave-dwelling lifestyle (Muchmore 1982, 1986; Muchmore & Pape 1999; Harvey & Muchmore 2013).

Only two other pseudoscorpion species have been recorded from Botswana. *Nanolpium subgrande* (Tullgren 1908) and *Beierolpium deserticola* (Beier 1964) (Tullgren 1908; Beier 1964), both in the family Olpiidae, making it one of the least known countries for pseudoscorpion diversity (Harvey 2013).

Etymology.—This species is named for the renowned caver Roger Ellis, who facilitated GDP's trip to Botswana. Roger has devoted over 40 years to the discovering, surveying and conserving of caves in southern Africa.

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Cover photos (some photos were adjusted/cropped)
Top left – José Bidegain, on his way for the recovery Marcel Loubens' body. Author unknown. For details see the paper by A.A. Cigna.
Top right – "Walking Mammoth" – a prehistoric drawing from the Kapova Cave, Russia. Photo by O. Minnikov. For details see the paper by Y. Lyakhnitsky et al.
Bottom left – "Astronaut" David Saint-Jacques (CSA) collecting microbiological samples for the scientific programme of the ESA CAVES course. Photo by V. Crobu. For details see the paper by Bessone et al.
Bottom right – The long-legged cave centipede Thereuopoda longicornis – a typical species of Lao caves. Photo by H. Steiner. For details see the paper by H. Steiner.

TERRESTRIAL MESOFAUNA BIODIVERSITY IN UNIQUE KARST ENVIRONMENTS IN SOUTHERN AFRICA

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Subterranean environments play host to unique and vastly undiscovered habitats, creating niches for distinctively adapted biota. These environments, in particular cave systems, allow the minds of evolutionists, ecologists and taxonomists to dwell nearly endlessly; allowing interesting finds and knowledge gained about the structured life of bizarre and singular species. In this study, a zoological approach was followed in determining the biodiversity of terrestrial mesofauna from six caves of which four are in South Africa and two in Botswana. Each of these six caves is distinctive from one another, but one cave (Diviner's cave in northern Botswana) is exceptional. Isolated and locked away 50 meters below surface, with no natural opening and a hot, humid and carbon dioxide pressurized atmosphere most likely never before studied habitat, was found. By applying several sampling and extraction methods, various trophic groups of nematoda, arachnida, myriapoda and hexapoda were collected from the six caves. Peculiar specimens of organisms found were sent to various specialists all over the world for identification. To date at least five expected and two confirmed new species of organisms were recorded as a result of this study.

1. Introduction

A great diversity of fauna such as birds, insects, mites, spiders, crayfish, harvestmen, centipedes, millipedes, reptiles, nematodes and even mammals naturally occur in caves (Romero 2009). Nematodes, generally known as round worms, play an important role as part of the food chain in caves. Some species feed on bacteria found in bat guano and in turn serve as a food source for mites and other arthropods. Although free-living nematodes are commonly found in karst environments (Romero 2009) such as the Bakwena Cave (Irene, South Africa), Durand et al. (2012) also listed a range of plant-parasitic nematodes that were present in the latter cave. Parasitic nematodes are, however, believed to enter cave systems by accident as a result of host plants and/or water that get flushed into cave openings (Romero 2009; Durand et al. 2012). Mites and spiders (Class Arachnida) constitute another diverse class of organisms that occurs in cave systems worldwide. Various species have been recorded from Southern African caves, e.g. mites such as *Chiropturopoda coprohila*, *Laelaps* sp., *Sancassania* sp. as well as 41 spider species of which only 10 were classified as true cave dwellers (Dippenaar-Schoeman and Myburgh 2009). Arachnid cave representatives commonly present troglobitic characteristics and their feeding habits include predation, parasitism and consumption of detritus (Romero 2009). Other organisms identified from the local Bakwena Caves in South Africa are insects such as *Monopis cf. transeans* (guano moth: Lepidoptera) and Crustacea (Amphipoda, Copepoda and Ostracoda) as well as bat flies (family Nycteriibidae). In terms of microbial organisms, eight fungal genera and some bacteria have also been identified from this cave (Durand et al. 2012). Another group of organisms found in cave environments, are the worm-like arthropods. They are classified as Myriapoda and are generally associated with decaying material (Romero 2009). Hexapoda represents the final group of cave organisms and is the greatest representative complement. Hexapod cave representatives include diplurans, insects and springtails (collembola) of which some species are abundantly found in cave environments (Romero 2009).

Since limited information on cave-dwelling organisms is available in Southern Africa, this study was conducted. The study follows a zoological approach by focusing on the biodiversity of five groups of microorganisms that are associated with caves, namely chelicerata, crustacea, hexapoda and myriapoda as well as nematodes, in unique karst environments. Six caves, including three from the old Transvaal region in South Africa and three from the Gcwihaba district in Botswana were selected for investigations. One of the caves in Botswana, named Diviner's cave (Figure 1), was only recently drilled open.

Figure 1. Surface opening of borehole penetrating Diviner's Cave. (Photo: Roger Ellis 2012)

This enabled significant atmospheric interchange possibly for the first time in thousands of years. The result of being insulated for thousands of years created a carbon dioxide enriched, hot and humid environment. Also, gene flow between epigean and hypogean species was restricted, if not completely disrupted.

It was hypothesised that a great diversity of species, some being true cave dwellers, would be identified. In addition, it was predicted that new and peculiar species would be found especially in the unique cave environment of Diviner's Cave. The value of caves, especially in Southern Africa, has to the opinion of the author, not been fully encompassed as part of conservation efforts and public awareness campaigns. By gaining information about these unique environments and the bios hosted within, it is perceived that contributions towards the better understanding and greater appreciation for subterranean environments will be made.

2. Geography, geology and site description

All of the caves relevant to this study occur in dolomitic rock and extends both horizontally and vertically. It should be noted that the depth of development, dimensions and extent of each cave are unique to that system.

All of the South African caves relevant to this study are natural caves and permit interaction with the surface environment and epigean biota. Most of these caves have similar atmospheric conditions. The average temperature of these cave systems were measured at 18ºC with a possible 2-3ºC fluctuation by using a simple thermometer during sampling periods. Although normal oxygen concentrations are found in most of the cave systems, it should be noted that elevated levels of carbon dioxide occur in the deeper sections of Gatkop 2 Cave. This was confirmed by monitoring carbon dioxide concentrations with a gas meter as well as observed heavier breathing by the cavers. The terrestrial environments of the South African caves are mostly semi-arid like with the possibility of some water drips present. Guano heaps are abundant and usually found in association with established bat colonies. An estimated 2000 m^2 lake can be found in Knockingshop Cave and some rimstone pools in Gatkop 2 Cave.

Bone Cave and Blue Cave, located in the Gcwihaba district of north-western Botswana, are both natural caves that also permit interaction with surface environments and biota. Although guano is frequently found in both systems, the deeper section of Bone Cave contains none and mostly consists of sandy slopes; a drastic difference compared to the rest of the cave's typical environment.

Diviner's Cave is unique and completely separable from all the other caves. This is the only cave that does not have a natural opening, but rather a 600 mm man-made borehole that allows limited interaction with the surface atmosphere. A relative humidity and temperature meter was used to determine the atmospheric conditions. With an average temperature of 28ºC and relative humidity of 96%, the conditions in Diviner's Cave are much more extreme than that of the other cave systems. Before penetration, the cavity was pressurized with a carbon dioxide enriched atmosphere and remnants of these elevated levels of carbon dioxide can be found in the lower lying areas of the cave system.

3. Methods

A general biodiversity sampling approach was implemented; aimed at sampling most cave biota (bats, fungi and bacteria were not included) present in each cave system. Sampling commenced at the entrance/twilight zone of each system and continued until the respective turnaround point for each cave was reached.

3.1 Sampling of Nematoda

Nematodes were sampled by acquiring soil and guano samples by using a small hand shovel and placing 500 g of substrate in a sealable plastic bag. A sample was made up of a representative portion of the first 30 cm of topsoil/guano. In order to avoid contamination between samples from different sites, a clean plastic cover/bag was placed over the shovel with each sampling instance. After sampling was completed, each sample was labelled, placed in an insulation box and transported to the Nematology Laboratory (NWU, Potchefstroom campus) for extraction and analysis. Nematodes were extracted within three days of sampling by using the decanting and sieving method, followed by the sugar-flotation method (Hooper et al. 2005), as well as by means of the modified Baerman-tray method (Hooper and Evans 1993).

Nematode data for the genera, species and or families identified from each of the caves were used for the calculation of prominence values (PV). The following equation was used to calculate the PV (Keikantsemang et al. 2012):

$$PV = population\ density\ x\ \sqrt{Frequency\ of\ occurence}\ /10$$

3.2 Sampling of Arthropoda

All arthropods were sampled using a simple 'catch by hand' technique, as well as by stationed baited/non-baited pitfall traps and baiting areas as described by Hunt and Millar (2001). When using the 'catch by hand technique' invertebrates with a size of 1 mm and larger were mostly caught. A sufficient light source, synthetic fine-haired brushes, research tweezers and vials/jars filled with a 70-80% ethanol solution were used to sample and preserve organisms directly from all surfaces (Hunt and Millar 2001). By using dissection microscopes, the contents of each jar and vial were separately analysed at the NWU (Potchefstroom campus).

Peculiar invertebrate specimens were sent to various taxonomic specialists all over the world to assist with identifications.

4. Results and discussion

4.1. Nematoda

Nematodes were found in four of the six caves, representing various groups from both plant-parasitic and free-living (bacteriovores, predators and fungivores) trophic levels (Table 1).

Table 1. Trophic classification of nematode groups identified from samples obtained from all six caves.

Nematode representatives	Trophic group	Nematode representatives	Trophic group
Aphelenchoides	Fungivore	*Neoactinolaimus*	Predator
Cephalobus	Bacteriovore	*Panagrolaimus*	Bacteriovore
Criconema	Plant-parasitic	*Paracrobeles*	Bacteriovore
Dorylaimida	Predator	*Prismatolaimus*	Bacteriovore
Helicotylenchus	Plant-parasitic	*Rhabditis*	Bacteriovore
Meloidogyne	Plant-parasitic	*Xiphinema*	Plant-parasitic
Monhysteridae	Predator	*Zeldia*	Bacteriovore

The diversity analysis of the gathered data (Figure 2) indicated a great difference in diversity and prominence of nematode groups when the different cave systems are plotted together. When considering Diviner's Cave, a relative high diversity of nematode trophic groups was present, but only a few groups were prominent. Similar results were found by Jansen van Rensburg (2010) since a total of 27 nematode genera were recorded from the six sampling sites in Bakwena Cave. Some of the latter species found are new to science and two species from the genera *Panagrolaimus* and *Plectus*, respectively, were described.

The data from Diviner's Cave further indicated that *Prismatolaimus* spp. (bacteriovores) and *Panagrolaimus* spp. (bacteriovores) proved to be equally prominent. Also, compared to the natural caves, Diviner's Cave had an exceptionally high diversity of nematode groups with more than one prominent group. Although the *Panagrolaimus* (bacteriovores) population found in Gatkop 2 Cave is the most prominent of all samplings, the cave had a very low nematode diversity and was dominated by this single genus. Ultimately bacteriovores dominated the community structures of all the cave systems included in this study.

Taking into consideration the results discussed above, it is clear that the nematode community structure of Diviner's Cave is much different from that of a typical natural cave and irrelevant to its geographic location. It seems that in natural caves various nematode genera may be found, but most likely only one will dominate the system. This contradicts the findings from Diviner's Cave since it was not dominated by a single nematode group, but rather by a few. The data suggests that a complex and dynamic community structure exists in Diviner's Cave. This creates the opportunity for investigations through which a better understanding of the community interactions and interactions with other biota can be gained as well as determining by what means these specific nematodes utilize energy sources.

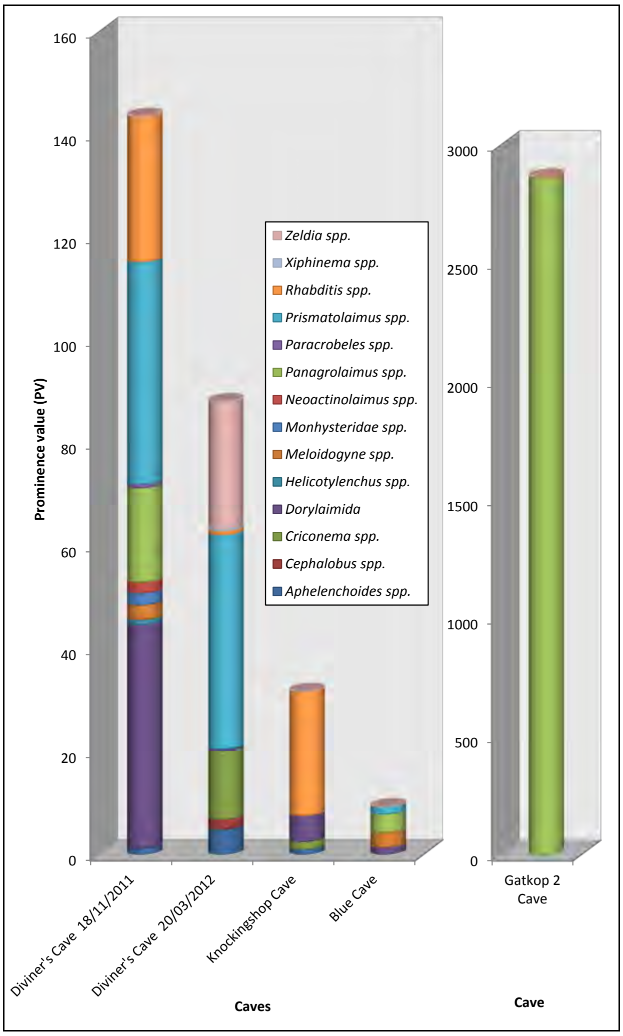

Figure 2. Graph illustrating prominence values of nematodes in the caves sampled during this study.

Figure 12: Graph illustrating prominence values of nematodes in all of the caves sampled during this study.

It is clear that further research aimed at studying nematode communities in cave systems is required. This especially since the literature on cave nematodes are scarce (Durand et al. 2012) and an international publication by Romero (2009) states that only 20 nematode species are worldwide known to occur in caves systems. Jansen van Rensburg (2010) suggested that indeed a vast number of cave nematode species have yet to be discovered. In recent times Borgonie et al. (2011) found a new bacteriovore nematode species, *Halicephalobus mephisto*, 3.6 km underground in mine water in South Africa. This discovery was groundbreaking since it was the first multicellular organisms to be obtained from such depths and serves as proof that not only caves, but also other subterranean environments provide scientists with the opportunity to make great and new discoveries.

4.2. Arthropoda

When the collective findings are considered, representatives of 18 families were sampled and represented by four Arthropod subphylums namely Chelicerata, Crustacea, Hexapoda and Myriapoda. Hexapods, belonging to 11 different families, occurred in three caves. In Bone Cave representatives of the families Blaberidae, Carabidae, Formicidae, Gryllidae and Reduviidae were found; in Diviner's Cave from Japygidae and Termitidae and in Gatkop 2 Cave from Blattidae, Nicolettidae and Staphylinidae. According to Romero (2009) Hexapoda is one of the most well represented taxonomical groups in caves and consists of numerous orders represented in the hypogean environment. Chelicerata were represented by six families in four caves. From Bone Cave an *Argas* sp. (Argasidae) was retrieved; in Diviner's Cave a representative from Ideoronicidae; in Gatkop 2 Cave from Uropodoidea and in Blue Cave three different Araneae species namely *Loxosceles* sp. (Sicariidae); *Selenops kruegeri* (Selenopidae) and *Smeringopus sambesicus* (Pholcidae) were found. Both the Crustacea and Myriapoda subphylums were represented by a single family and only found in Diviner's Cave. These two families are Cryptopidae and Platyarthridae respectively. According to Gunn (2004) representatives from several of the groups relevant to this study have been found in Southern African caves, but mostly only once or on a few occasions. This is most likely due to the lack of research in African subterranean environments. There is thus a great need to further explore Southern Africa's hypogean environments in search for new and peculiar species.

Various peculiar specimens were sent to numerous taxonomists all over the world. Currently three probable and two confirmed new species are considered. All of these specimens were collected from a single cave namely Diviner's Cave. The *Cryptops* sp., Japygidae representatives and *Microtermes* sp. (Chilopoda) are most likely new to science and with further efforts to identify the species, this might be confirmed. Gunn (2004) states that only one troglobitic Japygidae representative has been recorded in Zaire and no Centipedes (Myriapoda) from Africa. It is thus most likely that these specimens sampled are new to science and the first to be found in southern Africa. The *Trichorhina* sp. (Figure 3), as well as the Ideoronicidae representative (Figure 4) are confirmed new species and the manuscript for the publication describing the latter is currently being written. During sampling and identification it became clear that various caves host a great diversity of invertebrates. However, if the collection of organisms found in one cave is compared to that of another, it seems that the community is endemic to a specific system. Also, when the biota from Bone Cave and Blue Cave are considered, initial investigations indicate that similar level of adaptation and taxonomical groups are present if these systems are compared. However, the biota found in Diviner's Cave illustrate troglobitic characteristics such as depigmentation and the loss of eyesight; and are seemingly completely unique to the general karst landscape. Since all three of these caves are located in the same area, these results suggest that the geographical location does not play a significant role in the occurrence of single characteristics in the cave biota of Diviner's Cave, but rather the unique environment present in the system. At present it is suggested that the main drive resulting in the higher level of adaptation of species found in Diviner's Cave, is the very limited opportunity for gene flow to occur.

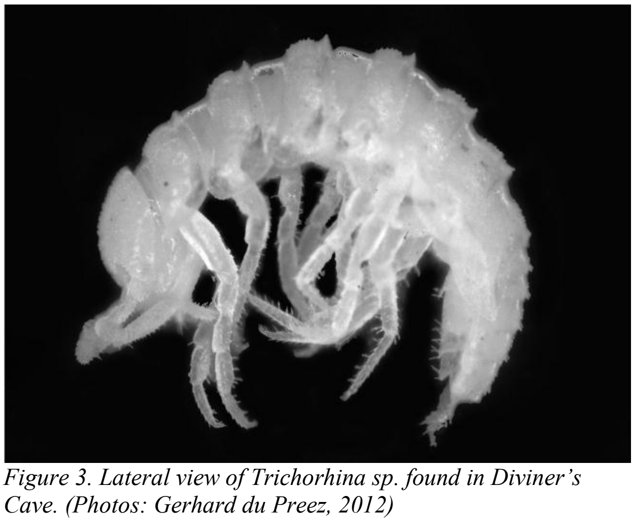

Figure 3. Lateral view of Trichorhina sp. found in Diviner's Cave. (Photos: Gerhard du Preez, 2012)

Figure 4. Ideoronicidae representative found in Diviner's Cave. (Photo: Gerhard du Preez, 2012)

5. Conclusion

The findings from this study clearly indicated that a wide diversity of unique and peculiar species occur in subterranean environments in southern Africa. Biospeleology is one of the most understudied fields of science and presents many opportunities for new and singular discoveries. It is concluded by stating that a great diversity of species were sampled of which some present peculiar characteristic traits and may be/are new species to science.

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