BIOLOGICAL INDICATORS OF COPPER-INDUCED STRESS IN SOIL

By

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

SUMMARY

The concentrations of copper (Cu) in vineyard soils of the Western Cape range from 0.1 to 20 ppm. However, more than 160 tons of the fungicide copper oxychloride are annually being sprayed on these vineyards. This has raised concerns that Cu may accumulate in these soils, resulting in a negative impact on the soil biological processes, especially since the soils in the Western Cape are slightly acidic, making Cu more mobile and available for soil organisms than would have been the case in alkaline soils.

The goal of the initial part of this study was therefore to identify those soil microbial communities indigenous to the Western Cape, which are most susceptible to Cu-induced stress as a result of the addition of copper oxychloride. These potential bioindicators of Cu-induced stress were first searched for in uncultivated agricultural soil from Nietvoorbij experimental farm. Consequently, a series of soil microcosms was prepared by adding various concentrations of Cu as a component of copper oxychloride, to each of eight aliquots of soil: 0 (control), 10, 20, 30, 40, 50, 100, 500 and 1000 ppm. The resulting concentrations of exchangeable Cu in these microcosms were found to be 2 (control), 12, 23, 34, 42, 59, 126, 516 and 1112 ppm. Selected microbial communities in each microcosm were subsequently monitored over a period of 245 days. It was found that the culturable microbial numbers did not provide a reliable indication of the effect of Cu on community integrity. However, analyses of terminal-restriction fragment length polymorphism (T-RFLP) community fingerprints and especially analyses of the whole community metabolic profiles, revealed that shifts in the soil microbial communities took place as the Cu concentration increased. Direct counts of soil protozoa also revealed that the addition of Cu to the soil impacted negatively on the numbers of these eukaryotes.

To confirm these findings in other soil ecosystems, the impact of copper oxychloride on whole community metabolic profiles and protozoan numbers were investigated in soils from Koopmanskloof commercial farm and Nietvoorbij experimental farm. These potential bioindicators were subsequently monitored in a series of soil microcosms prepared for each soil type by adding the estimated amounts of 0 (control), 30, 100 and

1000 ppm Cu as a component of copper oxychloride to the soil. The results confirmed the findings that elevated levels of copper impact negatively on the metabolic potential and protozoan numbers of soil.

Consequently, it was decided to investigate a combination of protozoan counts and metabolic profiling as a potential bioindicator for Cu-induced stress in soil. Data collected from all the microcosms containing exchangeable Cu concentrations ranging from 1 ppm to 1112 ppm was used to construct a dendrogram using carbon source utilization profiles in combination with protozoan counts. It was found that the microcosms grouped into clusters, which correlated with the concentration of exchangeable Cu in the soil. Under the experimental conditions used in this study, the combination of protozoan counts and metabolic profiling seemed to be a reliable indicator of Cu-induced stress. However, this bioindicator must be further investigated in other soil types using other types of stress inducing pollutants.

In addition to the above findings it was also found that the numbers of soil protozoa was particularly susceptible to Cu-induced stress in soils with a low soil pH. This is in agreement with the findings of others on the bio-availability of heavy metals in low pH soils. In these soils, nutrient cycling as a result of protozoan activity, may therefore be particularly susceptible to the negative impact of copper to the soil.

OPSOMMING

Die konsentrasies van koper (Cu) in wingerdgronde van die Wes-Kaap wissel tussen 0.1 en 20 dpm. Meer as 160 ton van die fungisied koper-oksichloried word egter jaarliks op dié wingerde gespuit, wat kommer laat ontstaan het oor die moontlike akkumulasie van Cu in dié grond en die gevaar van 'n negatiewe impak op die biologiese prosesse in die grond. Die gevaar word vererger deur die feit dat die Wes-Kaapse grond effens suur is, wat Cu meer mobiel en beskikbaar maak vir grondorganismes as wat die geval sou wees in alkaliese grond.

Die eerste doelstelling van hierdie studie was dus om die mikrobiese gemeenskappe in die grond, wat inheems is aan die Wes-Kaap, te identifiseer wat die meeste vatbaar is vir Cu-geïnduseerde stres as gevolg van die toevoeging van koper-oksichloried. Hierdie potensiële bioindikatore van Cu-geïnduseerde stres is eerstens gesoek in onbewerkte landbougrond van die Nietvoorbij-proefplaas. 'n Reeks grondmikrokosmosse is gevolglik berei deur verskillende konsentrasies Cu, as 'n komponent van koper-oksichloried, by elk van agt hoeveelhede grond te voeg naamlik 0 (kontrole), 10, 20, 30, 40, 50, 100, 500 en 1000 dpm. Die gevolglike konsentrasies van uitruilbare Cu in hierdie mikrokosmosse was 2 (kontrole), 12, 23, 34, 42, 59, 126, 516 en 1112 dpm. Geselekteerde mikrobiese gemeenskappe in elke mikrokosmos is vervolgens oor 'n tydperk van 245 dae bestudeer. Daar is gevind dat die kweekbare mikrobiese tellings nie 'n betroubare aanduiding kon gee van die uitwerking van Cu op gemeenskapsintegriteit nie. Die ontledings van terminale-restriksie fragment lengte polymorfisme (T-RFLP) gemeenskapsvingerafdrukke en veral van die metaboliese profiele van die totale gemeenskap, het getoon dat verskuiwings in die grondmikrobiese gemeenskappe plaasgevind het met 'n toename in Cu-konsentrasies. Direkte tellings van grondprotosoë het ook aangedui dat die toevoeging van Cu tot die grond 'n negatiewe uitwerking op die getalle van hierdie eukariote gehad het.

Om dié resultate te bevestig, is die impak van koper-oksichloried op die metaboliese profiele van totale gemeenskappe en protosoë-getalle in ander grond-ekosisteme

vervolgens bestudeer deur grond van die kommersiële plaas Koopmanskloof en die Nietvoorbij-proefplaas te gebruik. Dié potensiële bioindikatore is vervolgens bestudeer in 'n reeks grondmikrokosmosse, wat vir elke grondtipe voorberei is deur die toevoeging van beraamde hoeveelhede van 0 (kontrole), 30, 100 en 1000 dpm Cu as 'n komponent van koper-oksichloried. Die resultate het die bevindings bevestig dat verhoogde vlakke van Cu 'n negatiewe uitwerking het op die metaboliese potensiaal en op die protosoë-getalle in die grond.

Daar is gevolglik besluit om 'n kombinasie van protosoë-tellings en metaboliese profiele te ondersoek as 'n potensiële bioindikator van Cu-geïnduseerde stres in grond. Data van al die mikrokosmosse wat uitruilbare Cu bevat, wisselend van 1 dpm tot 1112 dpm, is gebruik om 'n dendrogram te konstrueer wat koolstofbronbenuttingsprofiele in kombinasie met protosoë tellings gebruik. Daar is gevind dat die mikrokosmosse groepe vorm wat korrelleer met die konsentrasie uitruilbare Cu in die grond. Onder die eksperimentele kondisies wat in dié studie gebruik is, wil dit voorkom of die kombinasie van protosoë-tellings en metaboliese profiele 'n betroubare indikator van Cu-geïnduseerde stres is. Hierdie bioindikator moet egter verder in ander grondtipes en met ander tipes stres-induserende besoedeling ondersoek word.

By bogenoemde bevindings is daar ook gevind dat die getalle grondprotosoë besonder gevoelig is vir Cu-geïnduseerde stres in grond met 'n lae pH. Dit is in ooreenstemming met die bevindings van andere met betrekking tot die bio-beskikbaarheid van swaar metale in grond met 'n lae pH. In dié tipe grond mag nutriëntsiklering as gevolg van protosoë aktiwiteit besonder gevoelig wees vir die negatiewe uitwerking van koper in die grond.

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CHAPTER 1

INTRODUCTION

CHAPTER 2

THE IMPACT OF COPPER OXYCHLORIDE ON SELECTED EUKARYOTIC AND PROKARYOTIC MICROBIAL POPULATIONS IN SOIL

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CHAPTER 1

INTRODUCTION

1.1. Background

Copper (Cu)-containing compounds have widely been used in agricultural systems as ingredients of fertilizers (Baker & Senft, 1995) and fungicides (Flores-Vélez *et al.*, 1996) and resulted in Cu accumulation in soils. The accumulation of heavy metals such as Cu generates many types of stresses on the environment, resulting in many organisms and communities of organisms displaying specific injury symptoms or shifts in community composition (Chaphekar, 1978). These responses to specific stresses display the concept of biological indication (Mhatre & Pankhurst, 1997) or biological indication, *i.e.* the use of a living organism, part of an organism or a community of organisms to obtain information about the quality of the environment (Wittig, 1993). Throughout this study the term 'bioindicator' was used as a synonym for the term 'biological indicator'.

There is a need to be able to asses how farming and industrial practices are affecting the capacity of the land to remain productive and how such practices are reducing or improving the health of soil (Pankhurst *et al.*, 1997). The soils on which the vineyards of the Western Cape were planted, originally contained a relatively low Cu concentration ranging from 0.1 to 20 ppm (Conradie, 2001). However, about 160 tons of copper oxychloride are annually being applied to these vineyards as a fungicide (Labuschagne, 2001). In general, the soils in the Western Cape are slightly acidic, making Cu more mobile and available for soil microorganisms (McBride, 1994). It is therefore important to estimate how elevated Cu concentrations, as a result of spraying with Cu-containing compounds such as copper oxychloride, will impact on the diversity of indigenous soil microbes.

Soil microflora including bacteria and fungi have the potential to be important indicators of soil quality or soil health (Parr *et al.*, 1992). Despite their wide occurrence and extensive role in soils, microorganisms have not been used widely as indicators of soil quality or soil health (Turco *et al.*, 1994). The most significant

reason for this is that, because of technical limitations, only a relatively small number of all the microorganisms in the soil have been cultured or identified (Hawksworth & Mound, 1991). Studies comparing ribosomal RNA (rRNA) methodology with culture techniques have estimated that at least 90% of microbial species have not been identified in soil (Embley & Stackebrandt, 1996) or aquatic environments (Ward *et al.*, 1990). Although molecular fingerprinting techniques have gained popularity, there is evidence that culture techniques such as plate count methods, are useful in comparative studies of specific microbial populations (Harris & Birch, 1992). Utilizing culture techniques, it has been found that fungi are less sensitive to metals than bacteria and actinomycetes (Doelman, 1985; Hiroki, 1992).

Protozoa have been found to be sensitive bioindicators because of their rapid growth and delicate external membranes (Foissner, 1994). Although Mejstrík & Lepsová (1993) concluded that many species of fungi can function as indicator species, despite their different sensitivities to heavy metals and their different abilities in accumulating them, Wondratschek & Röder (1993) proposed extreme caution in the use of higher fungi for biomonitoring soil pollution. They claimed that different species show highly variable responses to heavy metals and that even if the level of substrate contamination by heavy metals is related to their uptake by some fungal species, the use of fungi as bioindicators is not always advisable.

It is known that diversity measurements are useful for monitoring changes due to pollution, since microbial diversity changes in response to environmental stress (Atlas, 1984). Some recently developed approaches for measuring the biodiversity of soil microbial communities include analysis of community functioning based on utilization of substrates (Biolog™ system) and analysis of community structure based on extraction and amplification of soil DNA (Pankhurst, 1997). DNA-based detection of bacterial populations helps to overcome a major limitation of microbial ecology and soil biology, through the fact that one can now specifically monitor an individual population of microbes in an environment, and in the presence of almost the entire microbial community (Holben, 1994).

With the above as background it was the aim of this study to identify potential bioindicators of Cu-induced stress, by studying the responses of different soil microbial communities indigenous to the Western Cape, to elevated levels of this heavy metal added to soil in the form of copper oxychloride. Consequently, the impact of copper oxychloride levels on a wide diversity of soil microbial populations was investigated in pristine soil originating from Nietvoorbij experimental farm, Stellenbosch. Potential bioindicators were again tested on experimentally contaminated soil from Koopmanskloof commercial farm, Stellenbosch and on potting soil from Nietvoorbij experimental farm.

1.2. Copper

In the metal state, copper (Cu) is reddish coloured and takes on a bright metallic luster (Baker & Senft, 1995). Copper alloys can be reddish-yellow through gold, to pale yellow, white and even purple. The theoretical density of pure copper is 8.96g/cm^3 at 20^{0}C, making it one of the heavy metals. The melting point of the metal is 1083^{0}C and the boiling point 2567^{0}C. Copper has an atomic number of 29, an atomic weight of 63.54 and its atomic radius is 1.275Å with 1 and 2 valencies. The ionic radii for Cu^{+2} and Cu^{+1} are 0.72Å and 0.96Å, respectively. Copper is divided into subgroup 1B in the periodic table; the same group as gold and silver. Copper combines readily with many other elements and with acid radicals, but its chemical properties make it suitable for a wide variety of purposes (West, 1982). The principal uses of Cu are in the production of wire and of brass and bronze alloys (Baker & Senft, 1995).

Copper (Cu) is also one of the most important elements for plants and animals (Baker & Senft, 1995). This heavy metal is an essential transition element that plays a fundamental role in the biochemistry of all aerobic organisms (Hung *et al.*, 1997). Proteins exploit the unique redox nature of this metal to undertake a series of facile electron transfer reactions utilizing Cu as a cofactor in a series of critical enzymatic pathways. The function of these enzymes is essential for cellular respiration, iron homeostasis, pigment formation, neurotransmitter production, peptide biogenesis, connective tissue biosysnthesis and antioxidant defense.

However, copper can also be found associated with inorganic material. Copper ores are found in most parts of the world and they may either be at great depths which necessitates underground mining or near the surface, making open cast mining possible (West, 1982). Copper is not an abundant metal in the earth's crust; it only occurs to the extent of about 7 parts per million. In addition to the common minerals, Cu in nature occurs in dispersed forms in ordinary rocks, sediments and soils (Baker & Senft, 1995).

1.3. Soil

Soil is a complex and dynamic system, consisting of inorganic mineral particles, living organisms and dead or senescing organic matter interacting to form a porous matrix (O'Donnell & Görres, 1999). Soil plays a vital role in food and timber production, as a reservoir for water and as a buffer and filter for pollutants. In terms of global change, soils store almost twice as much carbon as does the atmosphere and are important links in the natural cycle that determines atmospheric CO_2 levels. Soil consists of mineral particles of various sizes, shapes and chemical characteristics (Paul & Clark, 1996). It also consists of plant roots, living animal and microbial populations, and an organic matter component at various stages of decomposition. Soil gases, soil water and dissolved minerals complete the soil habitat.

Soil aggregate formation is initiated when microflora and roots produce fibrils, filaments and polysaccharides that combine with clays to form organomineral complexes (Paul & Clark, 1996). Soil structure is created when physical forces such as drying, shrink-swell, freeze-thaw, root growth, animal movement and compaction mold the soil into aggregates. The particles involved in aggregate formation include fine clays and organic molecules measurable in nanometers; microorganisms, coarse clays and silt measurable in micrometers; and sand, small metazoans and small rootlets measurable in millimeters (Figure 1). Clay particles (< 20 μm) are bound into domains by a mixture of clay microstructures, biopolymers and microorganisms (Oades & Waters, 1991). Domains are bound into microaggregates (20-250 μm) by

plant debris, and microaggregates into macroaggregates (> 250 μm) by roots of fungal hyphae.

Heavy metals such as Cu have large effects on processes important for soil fertility by affecting structure and function of microbial communities (Giller *et al.*, 1998). Copper, like all trace elements in soil, can be associated with various soil components (Baker & Senft, 1995).

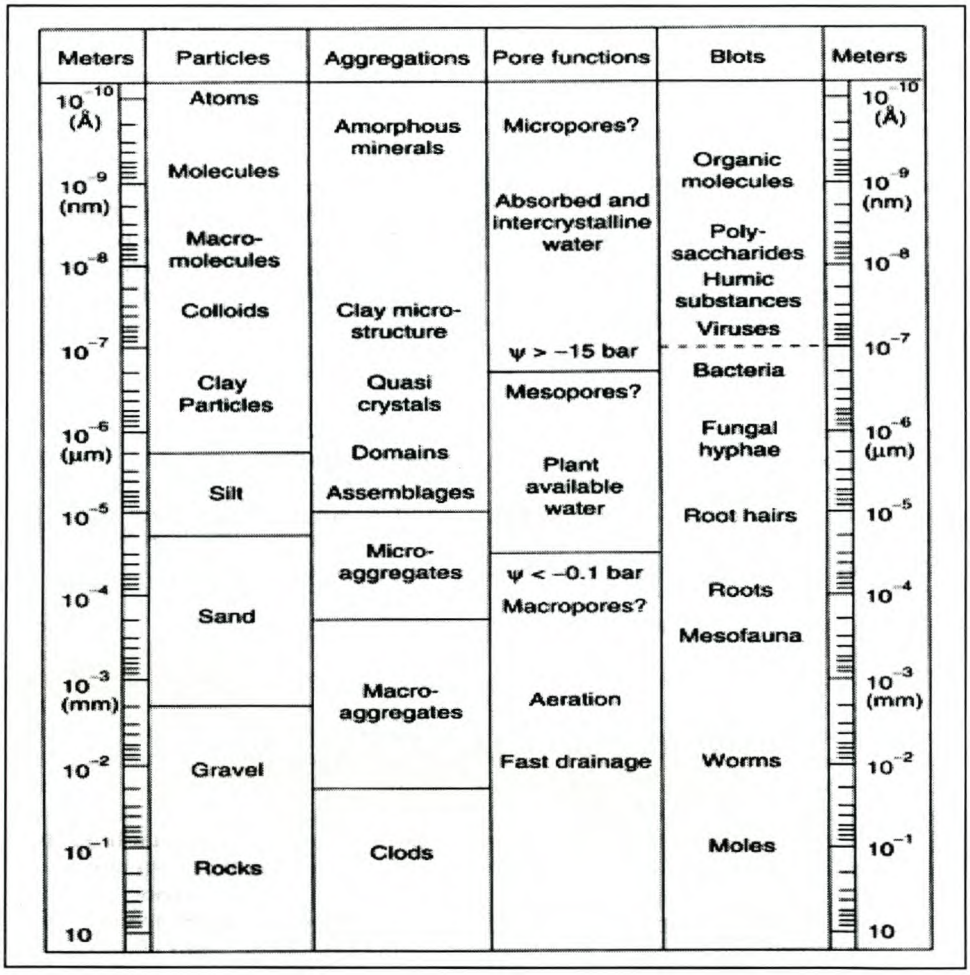


Figure 1. Size scale for soil particles. (Waters and Oades, 1991)

1.4. Copper in soil

Copper can be adsorbed on surfaces of clay, iron and manganese oxyhydroxides or complexed with soil organic matter (Baker & Senft, 1995). It may also be present in the lattice of secondary minerals like carbonates, phosphates, sulphates or oxides, be

occluded in amorphous materials such as iron and manganese oxyhydroxides, and in dead organic material. Copper may also be present in the lattice of primary minerals such as chalcite (Cu), covellite (CuS), bornite ($Cu5FeS_4$) or chalcopyrite ($CuFeS_2$). (Tessier & Campbell, 1988; McBride, 1981). The worldwide average concentration for Cu in soils of the world has recently been reported as 30 mg/kg (Baker & Senft, 1995).

The most important chemical processes affecting the behaviour and bio-availability of metals in soils are those concerned with the adsorption of metals from the liquid phase on to the solid phase (Alloway, 1995). Several mechanisms can be involved in the adsorption of metal ions, including cation exchange (non-specific adsorption), specific adsorption, co-precipitation and organic complexation. These processes control the concentrations of metal ions and complexes in the soil solution and thus exert a major influence on their uptake by microorganisms.

Extractable (available) Cu refers to an amount of this element in the soil that correlates statistically with concentrations absorbed and assimilated by plants (Baker & Senft, 1995). This 'availability' of Cu refers to the readiness with which the available ion $[Cu(H_2O)_6]^{2+}$ is absorbed by plants in acid soils and $Cu(OH)_2^0$ in neutral and alkaline soils. The level and distribution of total and extractable Cu in the soil profile vary with soil type and parent material. Copper is specifically absorbed or 'fixed' in soils, making it one of the trace elements which leaches the least through the soil. The levels of Cu in soil are affected by soil and crop treatments including fungicides, indiscriminant use of Cu containing fertilizers, livestock manure, sewage sludge and atmospheric deposition.

1.5. Agricultural importance of copper

Copper-containing compounds have been widely used in agricultural systems as fertilizers (Baker & Senft, 1995). The relative high toxicity of Cu-containing compounds to plant pathogens, low cost, and low toxicity to mammals, have made them economically important (Cha & Cooksey, 1991). Copper-containing

compounds are the most common bactericides for control of plant bacterial diseases, especially since antibiotics are not registered for use on most edible crops. The most common Cu fertilizer source is hydrated copper sulphate ($CuSO_4 \bullet 5H_2O$), although other compounds, mixtures, and chelates are also used. Hydrated $CuSO_4$ is compatible with most fertilizer materials (Baker & Senft, 1995). Other sources of Cu used for crop production include Cuprite (Cu_2O), Tenorite (CuO), Covellite (CuS) and Chalcocite (Cu_2S).

Copper-containing compounds have also been used as fungicides (Flores-Vélez *et al.*, 1996). Copper sulphate (Bordeaux mixture) has been used as a fungicide against mildew in vineyards for more than a hundred years. This treatment has resulted in Cu accumulation in soils. Copper fungicides are also used on citrus fruit, hops and vegetables to counter downy mildew (Adriano, 1986), and have been used extensively in Kenya since the 1930's to control leaf rust and since the 1960's to control berry disease in coffee. Kenyan soils have been found to contain more than 500 kg Cu per ha as a result of fungicide spraying (Flores-Vélez *et al.*, 1996).

Although the soil on which vineyards of the Western Cape were planted, originally contained a relative low Cu concentration ranging from 0.1 to 20 ppm (Conradie, 2001), more than 160 tons copper oxychloride are annually being sprayed on these soils (Labuschagne, 2001). These soils are slightly acidic, making Cu more mobile and available for soil microorganisms than would have been the case in alkaline soils. At low pH, H^+ associates with phenolic groups which suppresses Cu^{2+} adsorption to organic matter and soil particles (McBride, 1994).

From the above it can be expected that Cu will accumulate in the surface of soils as a result of fungicide application and soil amendments. In addition, Cu can also accumulate in soil as a result of the accumulation of crop residues containing this heavy metal.

1.6. Toxicity of copper

Virtually all metals, essential or inessential, can exhibit toxicity above certain threshold concentrations. This concentration may be extremely low for highly toxic metal species, *e.g.* Ag^{+} ions (Gadd, 1992). It may vary depending on the type of heavy metal and the specific microbial strain involved, *e.g. Pseudomonas pickettii* showed resistance to up to 2 mM of Cu, while it showed resistance to up to 25 mM of zinc on gluconate minimal medium (Gilotra & Srivastava, 1996). In general, toxic effects include blocking of functional groups of important molecules, *e.g.* enzymes, polynucleotides, transport systems for essential nutrients and ions. It also causes the displacement and/or substitution of essential ions from cellular sites, denaturation and inactivation of enzymes, and disruption of cell- and organellar membrane integrity (Ochiai, 1987).

Heavy metals such as Cu also have effects on processes important for soil fertility, by affecting structure and function of microbial communities (Giller *et al.*, 1998). Heavy metal contamination inhibits key functions in soil processes such as mineralization of organic material and nitrogen fixation and may lead to a reduction of total microbial biomass, a decrease in numbers of specific populations or to shifts in microbial community structure (Sandaa *et al.*, 1999).

1.7. Biological indicators

As mentioned above, many organisms and communities of organisms display specific injury symptoms or shifts in community composition when they are exposed to stresses, *e.g.* excess Cu, in the environment (Chaphekar, 1978). These responses of organisms or communities of organisms to specific stresses, display the concept of bioindication (Mhatre & Pankhurst, 1997). Bioindication can be defined as the use of a living organism, part of an organism or a community of organisms to obtain information about the quality of the environment (Wittig, 1993). Accumulation of hazardous substances by microbes has become an important component of

bioindication, as it allows the presence of low levels of such chemicals in the environment to be identified and quantified (Mhatre & Pankhurst, 1997).

Bioindicators include changes in microbial biomass, microbial activity (*e.g.* soil respiration and respiration quotient), populations of microflora (*e.g.* bacteria, actinomycetes and fungi), microbial communities (*e.g.* composition and functional diversities), soil enzymes (*e.g.* dehydrogenases, phosphatases, sulphatases and peptidases), microfauna (*e.g.* abundance of protozoa and nematodes), mesofauna (*e.g.* abundance, diversity and physiotype of collembola and mites), macrofauna (*e.g.* abundance and diversity of earthworms), lower plants (*e.g.* lichens) and diversity and growth of higher plants (Pankhurst *et al.*, 1997). Soil physical and chemical properties can also be used as indicators of soil health and soil quality. Soil chemical indicators include pH, electric conductivity and cation exchange capacity of soil, as well as organic matter and heavy metals in soil.

It is unlikely that ecosystem health can be defined by a single measure because of the multitude of components in a system which could be diseased (Elliot, 1997). The suspected key processes/components must be targeted, and measurements must be made of them, which are indicative of the disease. Numerous recent studies (Chander & Brooks, 1993; Yeates *et al.*, 1994; Frostegård *et al.*, 1996) have shown the impact of heavy metal contamination on various measures of microbial biomass and activity. Pesticide pollution (Harden *et al.*, 1993) and industrial contaminants in soils (Rowell & Florence, 1993) have also been detected with bioindicators.

The ideal bioindicator of soil health would be simply measured, work equally well in all environments and reliably reveal what problems existed where (Elliot, 1997). A diverse spectrum of bioindicators are discussed in this chapter, and include protozoa and nematodes (eucarya), as well as purple bacteria (*Pseudomonas*) and gram positive bacteria (actinomycetes) (Figure 2). In addition, general microbial diversity as bioindicator will be discussed.

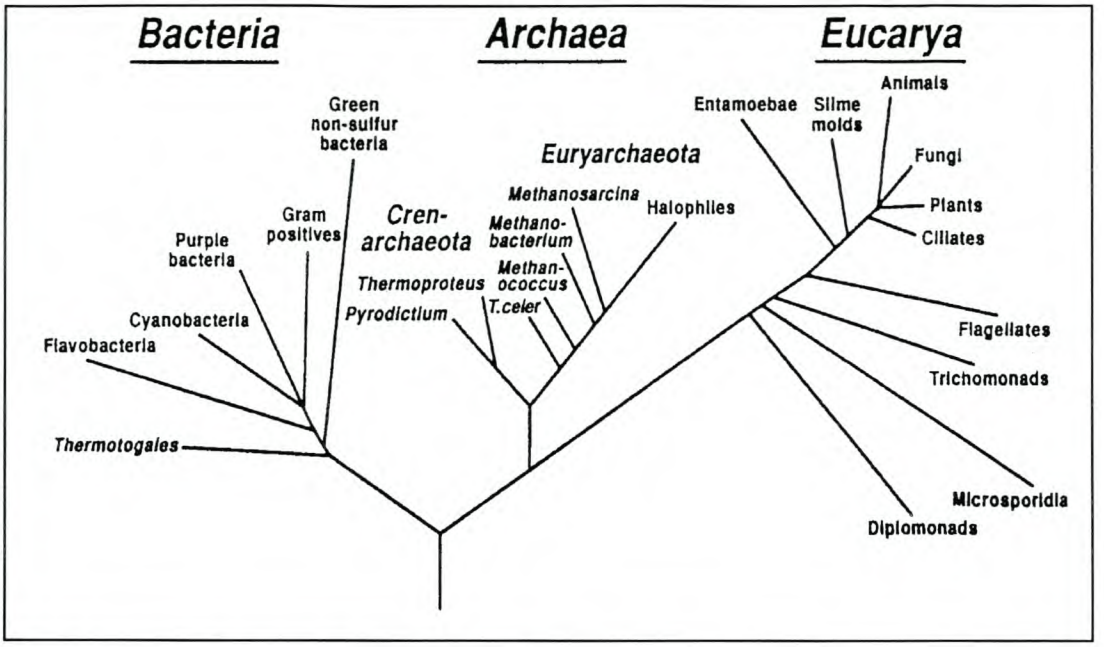
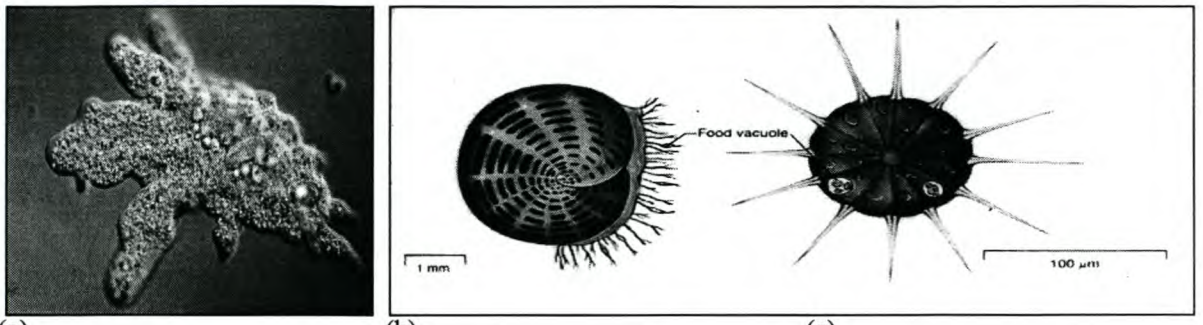


Figure 2. Universal Phylogenic Tree. (Olsen and Woese, 1993)

1.7.1. Protozoa

Protozoa can be defined as unicellular or acellular eukaryotic protists whose organelles have the functional role of organs and tissues in more complex forms (Prescott *et al.*, 1996). Protozoa vary greatly in size and shape (Figure 3), and these are often correlated with their ecological niche. Free-living protozoa in soils belong to bacterial-feeding or fungal-feeding groups depending on their food source, and they range in size from 2 to 100µm (Gupta & Yeates, 1997). Protozoa in water are often spherical; those that move in a particular direction are ellipsoidal; species that are attached to surfaces have radial symmetry; species that creep on the surface of objects are dorsoventrally flattened; and a few species are bilaterally symmetrical.

Protozoa are classified into a subkingdom with 7 phyla within the kingdom *Protista* (Levine *et al.,* 1980) based primarily on types of nuclei, mode of reproduction and mechanism of motility. The phylum *Sarcomastigophora* consists of flagellates and amoebae with a single type of nucleus. The phyla *Labyrinthomorpha, Apicomplexa, Microspora, Ascetospora* and *Myxospora* contain either saprozoic or parasitic species. The phylum *Ciliophora* contains ciliated protozoa with two types of nuclei.



(a) (b) (c)

Figure 3. Examples of various protozoa. (a) *Amoeba proteus* with blunt lobopodia. (b) A species of the foraminiferans (marine amoebae) feeding with a reticulopodial net. (c) *Actinophrys sol* with axopodia. (Prescott *et al.*, 1990)

Protozoan numbers in soil generally range from 10^4 to 10^5 organisms per gram and are usually found in greatest abundance within the top 15 cm from the soil surface (Atlas and Bartha, 1993). Many protozoa are capable of encystation when environmental conditions become unfavourable (Cowling, 1994). Cysts serve three major functions: (1) protection against adverse changes in the environment; (2) sites for nuclear reorganization and cell division (reproductive cysts); and (3) a means of transfer from one host to another in parasitic species. These organisms excyst when favourable conditions return.

Protozoa occupy a vast array of habitats and in addition to specialized organelles, have organelles similar to those found in other eukaryotic cells (Cowling, 1994). Only a few protozoa are non-motile. Most, however, can move by one of three major types of locomotory organelles: pseudopodia, flagella and cilia (Figure 3). Most protozoa are chemoheterotrophic which can either be holozoic (nutrients such as bacteria are acquired by phagocytosis and the subsequent formation of a food vacuole or phagosome) or saprozoic (nutrients such as amino acids and sugars cross the plasma membrane by pinocytosis, diffusion or carrier-mediated transport).

Protozoan involvement in nutrient flows within ecosystems stems from their feeding activities (Odum, 1971). Energy derived from food flows through protozoa according to the 'universal' model of ecological flow in which most of the ingested food is used

for reproduction, respiration and storage. Undigested material and excess nutrients are excreted and can later become re-available for ingestion by the same or other protozoa (Hekman *et al.*, 1992; Zwart & Darbyshire, 1992).

It is rare to find a soil without any protozoa, but terrestrial environments appear to impose exacting requirements on protozoa that invariably limit either their activity or ability to reproduce (Cowling, 1994). These restrictions are usually associated with fluctuations in the supply of food or moisture. Most protozoa reproduce asexually, although some also carry out sexual reproduction. The most common method of asexual reproduction is binary fission (Figure 4). Protozoa have been studied less frequently in soil than in aquatic environments (Griffiths, 1994). This is probably due to the fact that it is more difficult to observe and grow these microorganisms in soil. Protozoa can be considered as being active in the aquatic environment within the soil.

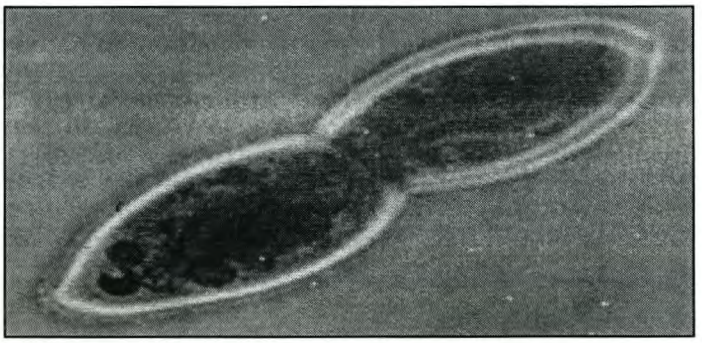


Figure 4. Binary fission in *Paramecium caudatum.* (Prescott *et al.*,1990)

1.7.1.1. Protozoa as bioindicators

Protozoa are increasingly being used as bioindicators in soil (Foisner, 1994). However, there are some factors that restrict the use of protozoa as bioindicators (Aescht & Foissner, 1992): (1) a large number of species; more than 1000 may occur in a square meter of forest soil; (2) enumeration of soil microbes is difficult and time-consuming; and (3) predatory organisms need other organisms for food. Thus, the constellation of factors is more complicated than in plants and bioindication often remains unspecific, *i.e.* different factors induce similar reactions.

Several unique features, however, favour the use of heterotrophic soil protozoa as bioindicators (Foissner, 1987): (1) protozoa are essential components of soil microcosms and changes in their dynamics and community structure will most probably influence the rate and kind of soil formation and fertility; (2) protozoa, with their rapid growth and delicate external membranes, can react more quickly to environmental changes than any other eukaryotic organism; (3) the eukaryotic genome of protozoa is similar to those of the metazoa and their reactions to environmental changes can thus be related to higher organisms more convincingly, than prokaryotes'; (4) protozoa inhabit and are particularly abundant in soil ecosystems that almost or entirely lack higher organisms due to extreme environmental conditions; and (5) many protozoa, but not all, are ubiquitous and are useful in comparing results from different regions.

1.7.2. Nematodes

Soil nematodes are vermiform animals ranging in size from 0.3 to 5.0 mm in length (Gupta & Yeates, 1997). Nematodes are bilaterally symmetrical "pseudocoelomates" (containing a body cavity) which are most often elongated, cylindrical and unsegmented, covered by a cuticle secreted by the underlying hypodermis (Maggenti, 1991b) (Figure 5). The exoskeleton of the nematode consists of the cuticle and the hypodermis which form a complex organ that protects the animal from detrimental external conditions. It also plays a critical role in maintaining the delicate internal chemical balance of the animal (Maggenti, 1991a). Nematodes can be found at densities up to 30 million/m^2, making them the most numerous multicellular organisms present in agro-ecosystems (Norton and Niblack, 1991).

Reproduction of nematodes are sexually and most commonly amphigonous (males and females separate), with the exception of a few, which are hermaphrodites (*e.g. Caenorhabditis*) (Maggenti, 1991a). In recent years the majority of workers in invertebrate zoology and nematology have treated nematodes as a separate phylum called Nemata (Nematoda) containing two classes: Adenophorea and Secernentea. Soil nematodes (free-living and parasitic) occupy an important position in the soil

detritus food web, *e.g.* they graze on bacteria and fungi, and are thus significant regulators of decomposition and nutrient mineralization (Pankhurst, 1997).

Like protozoa, nematodes are involved in a variety of ecosystem processes including decomposition/turnover of organic matter, nutrient mineralization, regulation of population densities of microflora including plant pathogenic organisms, and decomposition of agrochemicals (Stout & Heal, 1967; Freckman & Caswell, 1985; Yeates, 1981; Henkinet *et al.*, 1990; Gupta, 1993; Darbyshire, 1994).

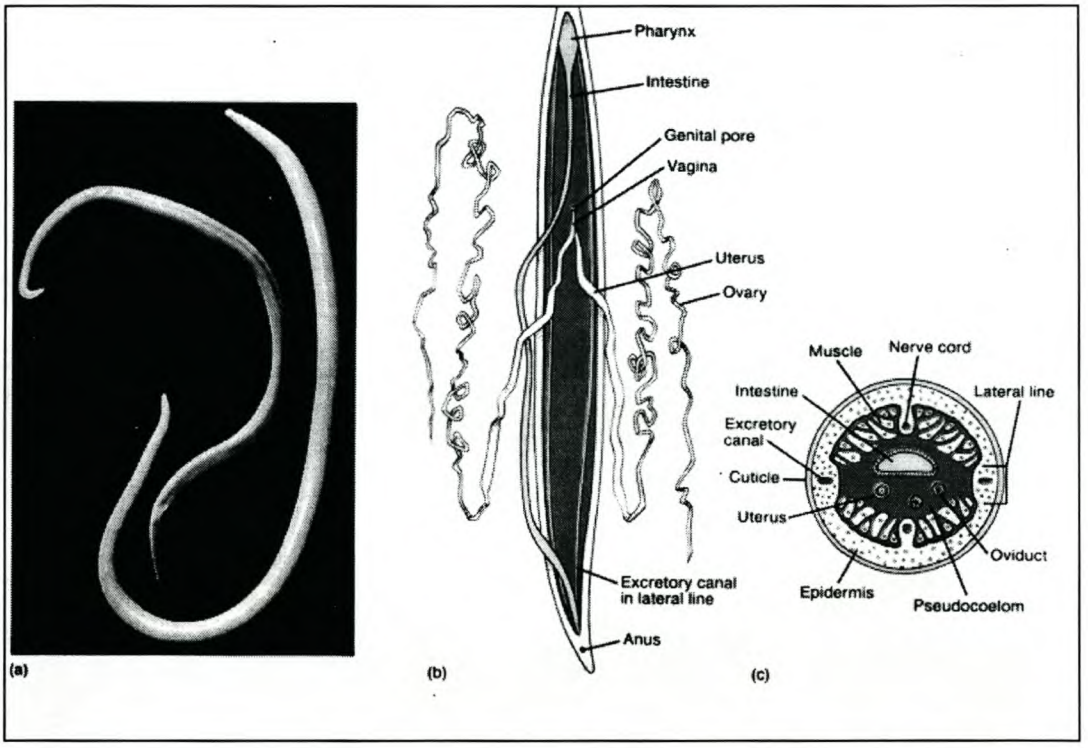


Figure 5. Nematode morphology. (a) Male and female *Ascaris* nematodes. The female is larger. (b) The internal anatomy of a female *Ascaris*. (c) Cross-sectional view of a female *Ascaris*. (Prescott *et al.*, 1990)

1.7.2.1. Nematodes as bioindicators

Productivity of the soil ecosystem as a whole may be indicated by the number of bacteriophagic nematodes (Freckman, 1988). Soil nematode communities have provided a focus for several studies where biodiversity indices have been used as indicators to document the impact of some perturbation on soil biological activity

(Freckman & Ettema, 1993). Functional groupings of nematodes or use of indices based upon species level comparisons may be useful as a qualitative indicator of the status of soil health.

It has been suggested that plant-feeding and predatory nematodes may be used as bioindicators of human impact on soil (Elliot, 1997). It was found that bacterial and fungal feeding forms increased as plant feeding and predacious forms decreased in ecosystems representing increasing human intervention. The community structure of selected groups of soil organisms is an indicator with considerable promise for distinguishing environmental conditions. As a great deal of knowledge exists on their taxonomy and feeding roles, soil nematodes may be the best group to use for community indicator analysis.

1.7.3. Actinomycetes

Actinomycetes are aerobic, gram-positive bacteria (Schaal, 1986). Most actinomycetes are non-motile but when motility is present, it is confined to flagellated spores. Actinomycetes closely resemble fungi in overall morphology, and this presumably results partly from adaptation to the same habitats. These bacteria are chemo-organotrophic organisms commonly forming filaments, often highly branched and sometimes tightly interwoven (Paul & Clark, 1996).

Actinomycetes were earlier designated as fungi because of their morphological appearance and the development of a true mycelium similar to fungi (Küster, 1967). Like fungi, actinomycetes have cells that develop into filamentous hyphae, but their mycelial threads break up into spores resembling bacterial cells (Tan, 1994). For this reason, they are sometimes referred to as thread bacteria (Brady, 1990). Actinomycete spores vary greatly in size and shape and develop by septal formation at filament tips, usually in response to nutrient deprivation (Prescott *et al.*, 1996) (Figure 6). Most are not particularly heat resistant, but do withstand desiccation well and thus have considerable adaptive value.

Actinomycetes are present in moist, well-drained soil and can degrade a variety of organic compounds (Brady, 1990). Actinomycetes are also extremely important in the mineralization of organic matter. These prokaryotes are very sensitive to changes in soil reactions and grow well at pH levels of 6.0 – 7.0, but will disappear in soils with a lower pH (Tan, 1994). Most actinomycetes are important in the decomposition of soil organic matter, which can be noticed from the musty smell in decomposing straw piles or freshly plowed land (Paul & Clark, 1996). Baecker & King (1978) showed that 20 different types of actinomycetes were able to break down cellulose when grown on a cellulose-containing medium. Most of the useful natural antibiotics are produced by actinomycetes. Although most actinomycetes are free-living microorganisms, a few are pathogens of humans, animals and some plants.

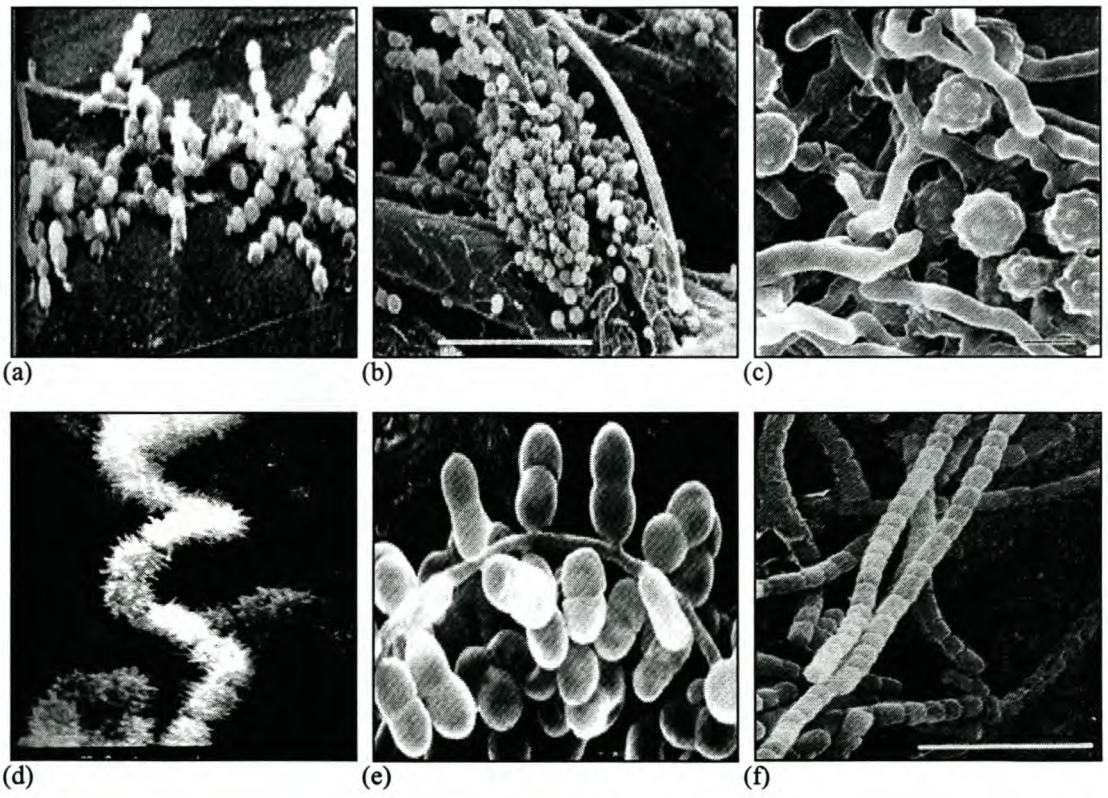


Figure 6. Examples of actinomycete spores as seen in Scanning Electron Microscopy. (a) Sporulating *Faenia* hyphae. (b) Sporangia of *Pilimelia columellifera* on mouse hair. (c) *Micromonospora echinospora.* (d) A chain of hairy streptomycete spores. (e) *Microbispora rosea*, paired spores on hyphae. (f) Arial spores of *Kitasatosporia setea.* (Prescott *et al.*, 1996)

According to Bergey's Manual (Holt *et al.*, 1994), actinomycetes can be classified into seven sections, primarily based on morphological properties such as cell wall

type, conidia arrangement and the presence or absence of a sporangium. The sections are (1) Nocardioform actinomycetes, (2) Actinomycetes with multilocular sporangia, (3) Actinoplanetes, (4) *Streptomyces* and related genera, (5) Maduromycetes, (6) *Thermomonospora* and related genera, and (7) Thermoactinomycetes. Phylogenetic analyses using a partial sequence of the 16S rRNA revealed a number of naturally related groups within the actinomyces (Logan, 1994) (Figure 7).

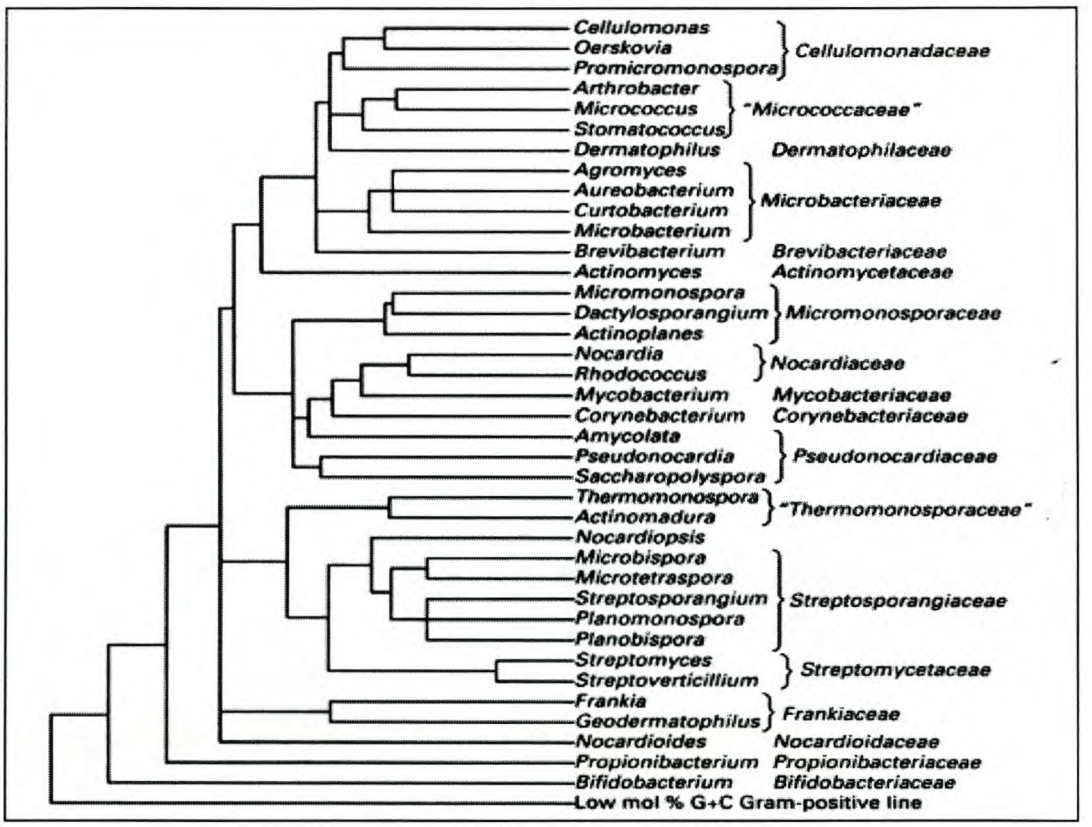


Figure 7: Phylogenetic analysis of actinomycetes based upon 16S r RNA partial sequencing (Logan, 1994).

1.7.3.1. Actinomycetes as bioindicators

Actinomycetes appear to be little affected by heavy metals (Hicks *et al.*, 1990). This may be due to the fact that the population density of predatory microorganisms, such as protozoa, are suppressed by heavy metals. Another factor might be that bacteria that are fed upon do not necessarily reflect total production if they are heavily predated (Freckman, 1988). Standing stocks of bacteria may be reduced under predation, but the turnover rate may be increased; therefore the yield may be the same in both cases.

1.7.4. Nitrifying Bacteria

Nitrifying bacteria are all aerobic, gram-negative organisms that do not produce endospores (Holt *et al.*, 1994). These bacteria are capable of obtaining energy from the oxidation of either ammonium (NH_4^+) or nitrite (NO_2^-). Nitrifying bacteria differ considerably in morphological properties. Nitrifiers may be rod-shaped, ellipsoidal, spherical, spirillar or lobate and may possess either polar or peritrichous flagella. They often have extensive membrane complexes embedded in their cytoplasm (Figure 8). Properties such as the preference for nitrite or ammonia, general shape and the nature of the cytomembranes, are primary criteria used in the identification of these bacteria.

Comparative sequencing studies based on 16S rRNA oligonucleotide analyses provided the first evidence for the phylogenetic diversity of nitrifying bacteria (Woese *et al.*, 1984; 1985). More recently, the phylogeny of ammonia-oxidizing bacteria has been studied using near-complete 16S rRNA sequences (Head *et al.*, 1993). The ammonia oxidizers include *Nitrosomonas* (at least ten species), *Nitrosococcus* (three species) and *Nitrosospira* (one recognized species and four other species as indicated by DNA homology studies). It also includes *Nitrosovibrio* (one described species and one additional species as indicated by DNA homology studies) and *Nitrosolobus* (one described species and one additional species as indicated by DNA homology studies). Nitrite-oxidizing genera have received less attention and few have been examined by comparative sequence analysis. The four recognized genera of nitrite-oxidizing bacteria include *Nitrobacter*, containing three species, and *Nitrospina, Nitrococcus* and *Nitrospira*, with one species each (Teske *et al.*, 1994).

Ecologically, nitrifying bacteria are very important and can be isolated from habitats such as soil, sewage disposal systems, as well as from freshwater and marine habitats (Holt *et al.*, 1994). The nitrite-oxidizing genera oxidize NO_2^- to nitrate (NO_3^-) while ammonia-oxidizing genera oxidize NH_4^+ to NO_2^- (Paul & Clark, 1996). When two genera such as *Nitrobacter* and *Nitrosomonas* occur together in a niche, NH_4^+ is converted to NO_3^-. This process is called nitrification. Although nitrification has

been typically associated with the chemoautotrophic bacteria, it is now recognized that heterotrophic nitrification also occurs.

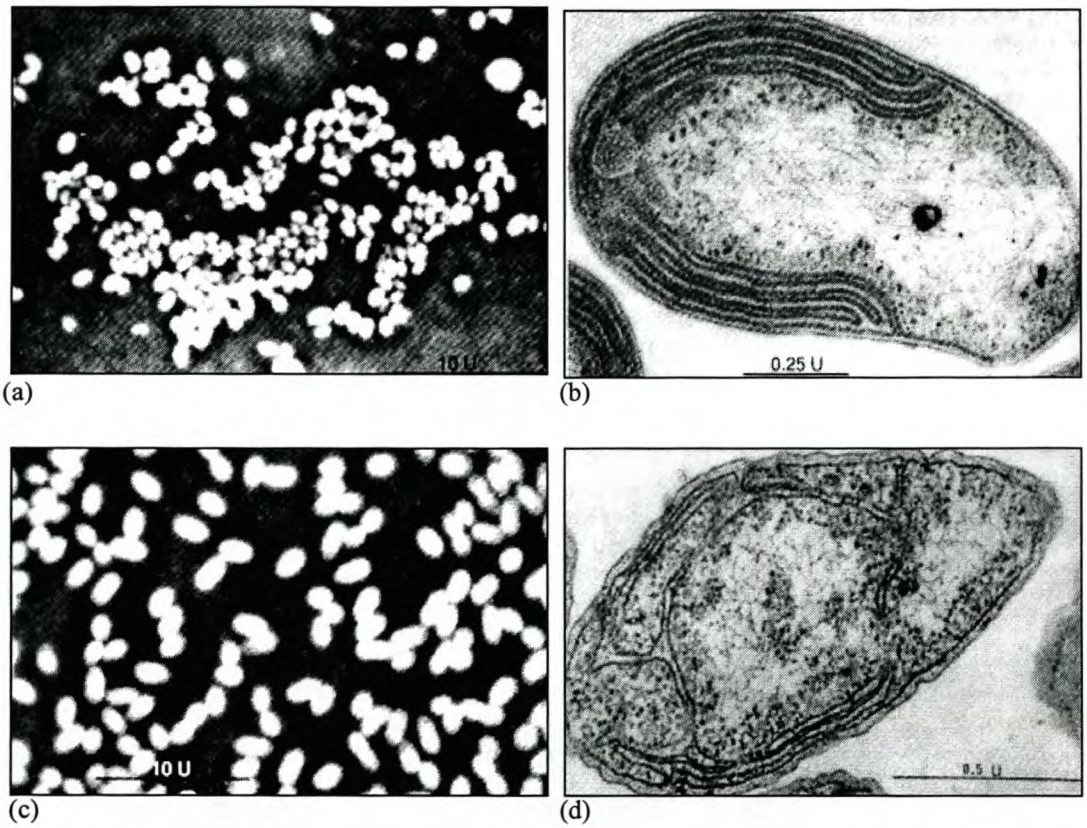


Figure 8. Examples of nitrifying bacteria. (a) *Nitrobacter winogradskyi;* phase contrast. (b) *N. winogradskyi.* Note the polar cap of cytomembranes. (c) *Nitrosomonas europaea;* phase contrast. (d) *N. europaea* with extensive cytoplasmic membranes. (Prescott *et al.*, 1996)

The oxidation of NH_4^+ in autotrophs are described as:

$$NH_4^+ + 2H + 2e^- + O_2 \xrightarrow{\text{ammonia mono-oxygenase}} NH_2OH + H_2O$$

Hydroxylamine is oxidized to NO_2^- as follows:

$$NH_2OH + H_2O \xrightarrow{NH_2OH\ \text{oxidoreductase}} HONO + 4e^- + 4H^+$$

The NO_2^- oxidizing bacteria catalyze the reaction:

$$HNO_2 + H_2O \xrightarrow{\text{nitrite dehydrogenase}} HNO_3^+ + 2H^+$$

(Paul & Clark, 1996)

Heterotrophic nitrifiers are known to be capable of producing NO_3^- from both inorganic and organic sources, but the amounts of nitrogen transformed are small compared with that attributed to *Nitrobacter* and *Nitrosomonas* (Alexander, 1977). Because of the small number of organisms involved, nitrification is very sensitive to environmental disturbances, i.e. if one organism is affected by a disturbance this has a significant effect on the overall process (Atlas, 1984). Nitrification is extremely sensitive to pH and proceeds very slowly in acid environments. As nitrifying organisms are obligate aerobes, nitrification is suppressed where wet conditions lead to anoxia (Alexander, 1977). Nitrification is usually measured by the disappearance of ammonium in soils dosed with ammonium-sulphate or organic forms of nitrogen, coupled with the appearance of nitrite and nitrate.

1.7.4.1. Nitrifying bacteria as bioindicators

Measurement of nitrification was recommended as an important test for assessing changes in soil health as a consequence of agrochemical applications and soil degradation (Domsch *et al.*, 1983; Sims, 1990). Miller *et al.* (1991) enumerated populations of nitrifying bacteria in soils at three different pH values (5.6, 4.2 and 3.0) that was subjected to simulated acid rain. The authors concluded that nitrite-oxidizing bacteria could be used as experimental indicators of changes in soil microbial communities subjected to acid rain. They found significant decreases in the numbers of nitrite-oxidizing bacteria following acid rain, but the extent of the decrease depended on the crop and the season. During studies on the effects of long-term chemical pollution on soils, Remde & Hund (1994) monitored the activity of autotrophic nitrifying bacteria (as an indicator of the potential toxicity of the chemical on soil microorganisms), as well as microbial respiration (as an indicator of the bioavailability and degradability of the chemicals). The authors concluded that monitoring both parameters provided a comprehensive assessment of the influence of pollutants on soil microflora.

1.7.5. *Pseudomonas* species

Pseudomonas species are gram-negative straight or slightly curved rod shaped bacteria (Holt *et al.*, 1994). Species of the genus *Pseudomonas* are generally 0.5 - 1.0 μm by 1.5 - 5.0 μm in size and are rarely non-motile. *Pseudomonas* can move by means of one or several polar flagella and lack prosthecae or sheaths. *Pseudomonas* species are mainly aerobic chemohetrotrophs, having a strictly respiratory type of metabolism with oxygen as their terminal electron acceptor. In some cases nitrate can be utilized as an alternative electron acceptor, allowing growth to occur anaerobically. Most, if not all species do not grow under acidic conditions (pH 4.5). They are oxidase positive or negative, but all are catalase positive.

Ribosomal RNA (rRNA) cataloguing and later 16S rRNA sequencing of representative strains also contributed considerably to the present knowledge of the phylogenetic distribution of the pseudomonads within the rRNA framework of *Proteobacteria* (Woese, 1987). The most significant consequences of these rRNA studies was the classification of *Pseudomonas* species in the α-, β- and γ-subclasses of the *Proteobacteria* and the demonstration that some *Pseudomonas* species were closely related to other genera (*e.g. Escherichia, Halomonas, Xanthomonas, etc.*) (Kersters *et al.*, 1996). During the last decade polyphasic taxonomic studies have played a crucial role in improving the classification and gradually stabilizing the nomenclature of the pseudomonads. The classification of these bacteria into the major genera *Pseudomonas, Acidovorax, Brevundimonas, Burkholderia, Comamonas, Herbaspirillum, Hydrogenophaga, Ralstonia, Stenotrophomonas* and *Sphingomonas* reflects the phylogenetic relationships of the respective organisms. There are numerous validly described *Pseudomonas* species whose phylogenetic position remains undetermined, as well as validly named *Pseudomonas* species which, on the basis of rRNA relationships, do not belong to the genus *Pseudomonas* sensu stricto and which can therefore be expected to be transferred to existing or new genera in future.

The pseudomonads play an important part in ecosystem function (Atlas & Bartha, 1993). Many *Pseudomonas* species can utilize wide varieties of organic molecules

ranging from simple sugars to complex aromatic molecules. This has made them important components in the mineralisation process. *Pseudomonas* populations found in the rhizosphere have been reported to be capable of producing organic chemicals that stimulate growth of plants. Several species (*e.g., Pseudomonas aeruginosa*) are important experimental subjects (Prescott *et al.*, 1996), while other species are major animal and plant pathogens (*Pseudomonas aeruginosa* – infects people; *Pseudomonas syringae* and *Pseudomonas cepacia* – plant pathogens) (Atlas & Bartha, 1993). Some *Pseudomonas* species cause soft rots in plants and occur as saprophytes in the rhizospere.

Certain pseudomonads commonly produce diffusible fluorescent pigments containing siderophores that have great affinity for iron (Fe) (Paul & Clark, 1996). Some fluorescent species have been used for biological control of soil-borne phytopathogens. The fluorescent pseudomonads sequester Fe in alkaline soils of low Fe availability, depriving some microorganisms, including the pathogens, of this nutrient. Some *Pseudomonas* species produce growth stimulants, such as ethylene and indoleacetic acid. An interesting phenomenon among certain members of the genus *Pseudomonas* is the presence of a plasmid-borne copper resistance operon (Bender & Cooksey, 1986) which will be discussed in more detail in section 1.8.

1.7.5.1. Pseudomonas species as bioindicators

It was suggested that genetic modification can greatly enhance detection of microbial biosensor response, primarily through the use of reporter genes (*e.g. lux* genes) (Paton *et al.*, 1997). Consequently, biosensor detection of specific heavy metals has been achieved through transcriptional fusion of *lux* reporter genes to appropriate heavy metal resistance promotors (*i.e.* light output is switched on by the presence of the particular heavy metal). Paton *et al.* (1995) described a decline in bioluminescence with increasing concentrations of toxins for *lux*-marked *Pseudomonas fluorescens*. The order of toxicity (from most to least toxic) was Cu = Zn > Cd > Ni > Cr > 3,5-dichlorophenol for both the plasmid and chromosomal constructs. King *et al.* (1990) showed that *Pseudomonas fluorescens* (HK44), which contains the catabolic

naphthalene plasmid (pUTK21) and a naphthalene-*lux* construct (nah-*lux*), fluoresced in the presence of naphthalene and salicylic acid.

1.8. Microbial copper resistance

The appearance of Cu-resistant bacterial strains has reduced the effectiveness of copper sprays for the control of certain plant bacterial diseases (Cha & Cooksey, 1991). In the vicinity of where Cu compounds are being used as antimicrobial sprays on agricultural plants, several plant-pathogenic and saprophytic bacteria have been reported to carry plasmid-borne Cu resistance genes (Bender & Cooksey, 1986).

The *cop* operon (Figure 9) was first identified on a 35-kb plasmid (pPT23D) in strains of *Pseudomonas syringae* that infected tomato in southern California (Bender & Cooksey, 1986). Copper resistance genes were isolated on a 4.5-kb *PstI-PstI* fragment cloned from pPT23D (Bender & Cooksey, 1987). DNA sequence and deletion analysis demonstrated the involvement of four structural genes (*copABCD*) (Figure 9) for Cu resistance on plasmid pPT23D from *P. syringae* (Mellano & Cooksey, 1988). Two genes that encode a Cu-responsive signal transduction system were identified just downstream from the structural genes of the operon. These regulatory genes, *copR* and *copS,* are required for the Cu-inducible expression of *copABCD* but are themselves expressed constitutively from a separate promoter in front of *copR* (Mills *et al.*, 1993).

The four structural proteins that determine Cu resistance are the two periplasmic proteins, CopA and CopC, the outer membrane protein CopB, and the inner membrane protein CopD (Cooksey, 1994) (Figure 10). CopA (72 kDa) binds multiple Cu atoms – about 11 per polypeptide chain. This protein, and the smaller CopC (12 kDa) that binds one Cu atom per polypeptide, are both abundant in copper-induced cells of *P. syringae* (Cha & Cooksey, 1991), and colonies subsequently become blue with accumulated Cu (Cooksey, 1993). How CopB and CopD are involved in the movement of Cu across the membranes, is not yet understood. However, a mutant *cop* operon containing *copD* but lacking one or more of the other genes, confers hyper-

sensitivity and hyper-accumulation of cellular Cu, indicating a role for CopD in Cu uptake by the cell (Cha & Cooksey, 1993; Silver, 1996).

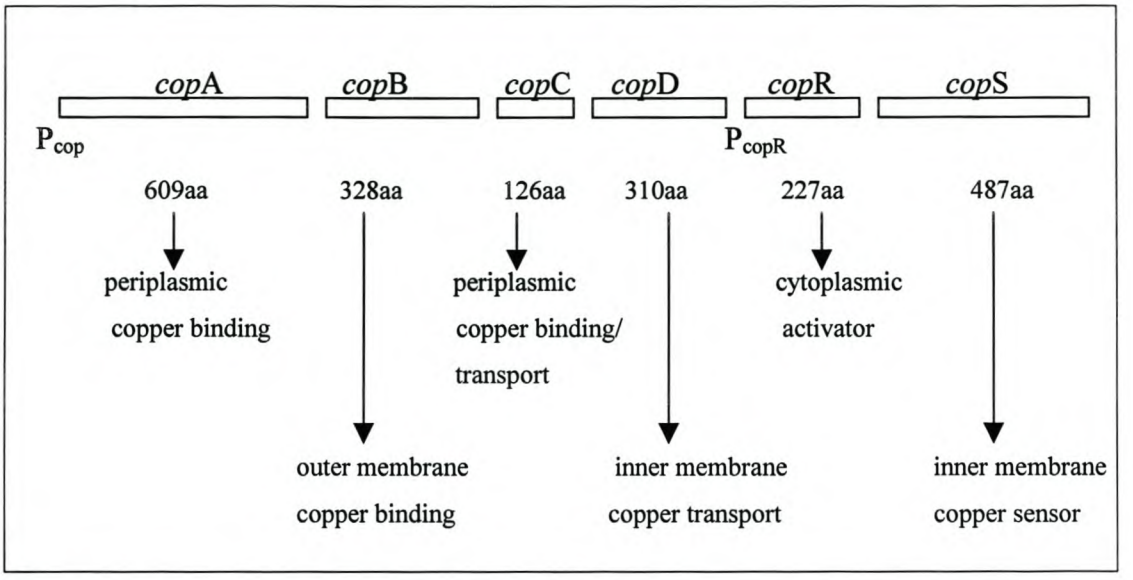


Figure 9. The *cop* operon of *Pseudomonas syringae* and probable locations and functions of its protein products. P_{cop} is the copper-inducible promoter that drives *cop*A-D, while P_{copR} is the constitutive promoter that drives *cop*RS. (Cooksey, 1994)

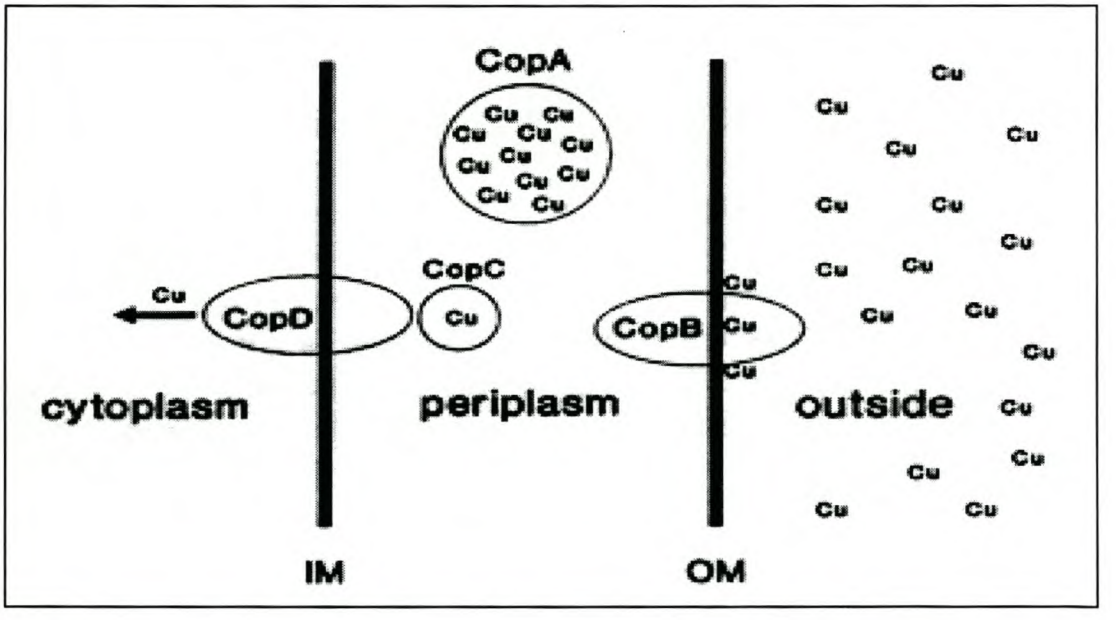


Figure 10. Model for the mechanism of Cu resistance in *P. syringae*. Copper is sequestered by the periplasmic proteins CopA and CopC and probably by CopB. CopC and CopD may also function in Cu transport into the cytoplasm. IM = inner membrane and OM = outer membrane. (Cooksey, 1993)

Although the Cu resistance genes in *E. coli (pco)* and *P. syringae* pathovar *tomato (cop)*, and many other *Pseudomonas* and *Xanthomonas* species have been located on plasmids, a few chromosomally located Cu resistance genes have also been found (Gilotora & Srivastava, 1996) (Figure 11). The chromosomal Cu resistance mechanism is dependent on a set of chromosomal (*cut*) genes (Cooksey, 1993). A model for the function of these genes proposed by Rouch *et al.* (1989), predicted their role in copper uptake (*cutA* and *cutB*), storage and intracellular transport (*cutE* and *cutF*), and efflux (*cutC* and *cutD*). The regulatory genes, *cutR* and *cutS,* appear to be coupled with the regulation of the plasmid-determined resistance genes. The distinction between plasmid-borne heavy metal resistance and resistance as a result of chromosomal genes has become blurred, because for some metals (notably mercury and arsenic), the plasmid and chromosomal determinants are basically the same (Silver & Phung, 1996). Other systems, such as Cu transport ATPases and metallothionein cation-binding proteins, are only known from chromosomal genes. The largest group of metal resistance systems function by energy dependent efflux of toxic ions. Some of the efflux systems are ATPases and others are chemiosmotic cation/proton transporters.

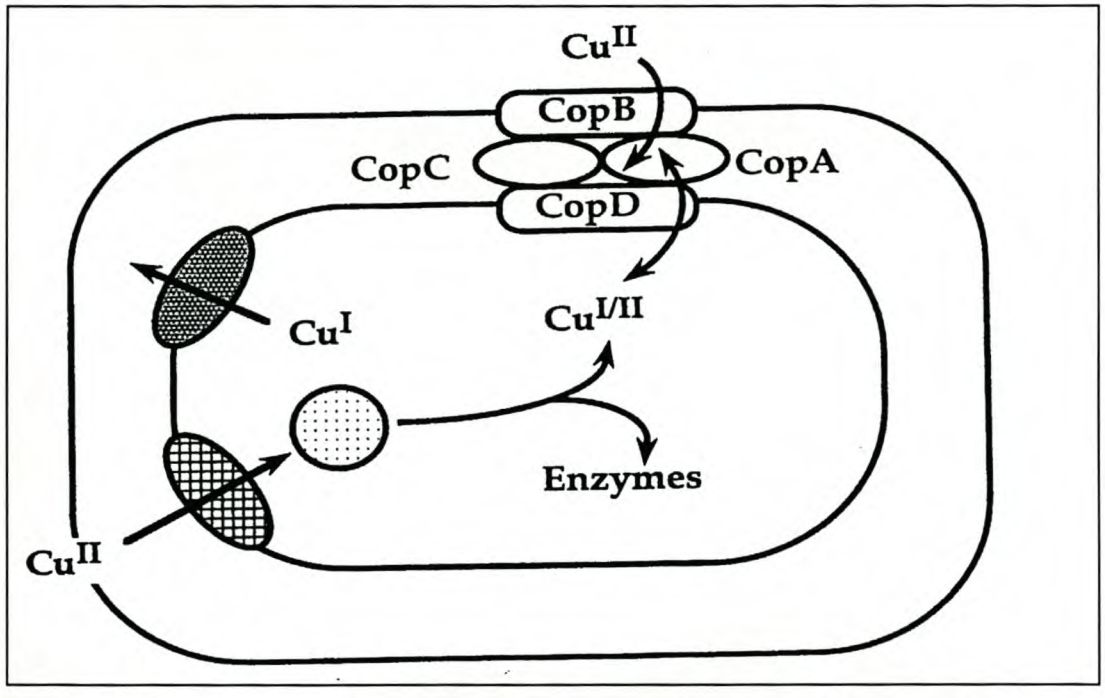


Figure 11. Copper transport and resistance in *Pseudomonas*. Hypothesized chromosomal uptake and efflux membrane transporters are shown on the left of the figure. The intracellular copper-binding protein and plasmid-encoded CopABCD are also illustrated in the figure. (Silver & Phung, 1996)

In general, microorganisms have adapted to the presence of both nutrient and nonessential metals by developing a range of resistance mechanisms (Bruins *et al.*, 2000). Six metal resistance mechanisms exist: exclusion by permeability barrier, intra- and extra-cellular sequestration, active transport efflux pumps, enzymatic detoxification, and reduction in the sensitivity of cellular targets to metal ions.

1.9. Effect of heavy metals on soil microbes

1.9.1. Effect on protozoa

The effects of heavy metals on aquatic protozoa have been studied extensively, but little is known of their effects on soil protozoa (Foissner, 1987). Brookes *et al.* (1984) found no effect of heavy metals on the total number of protozoa in liquid municipal sewage sludge treated soils containing Cd, Cr, Cu, Ni, Pb and Zn. In contrast, Forge *et al.* (1993) reported that the relative toxicity of heavy metals on the growth of the ciliate *Colpoda steinii* were Ni>Cd>Cu>Zn. Heavy metals caused growth abnormalities to both active and cystic forms of this ciliate, while it also reduced its rate of growth (Foissner, 1994).

1.9.2. Effect on nematodes

Nematodes generally appear to be little affected by heavy metals, and in some instances their numbers may even increase substantially (Weiss & Larink, 1991). However, Yeates *et al.* (1994) found that nematode community diversity declined with increasing levels of Cu contamination. They also found a shift in dominance from plant-feeding nematodes in uncontaminated soil to bacterial-feeding nematodes in highly contaminated soil, as well as a general increase in the proportion of predatory nematodes as the soils became increasingly contaminated.

Weiss & Larink (1991) found a similar increase in the abundance of predatory nematodes in soil following the addition of heavy metals. The changes in relative

abundance of different nematode trophic groups in metal-contaminated soils, appears to be largely related to changes in the amount of available nutrients in the soil. Similarly, it was found that soil contamination by industrial pollutants has varied effects on soil nematode populations, but consistently alters the relative proportions of trophic groups (Yeates & Bongers, 1997). For example, contamination of soils by Pb, Zn and Cu contained in exhaust fumes and fine powder of metallurgical plants and smelters, reduced the abundance and diversity of nematodes but increased the number of bacteriovores (Zullini & Peretti, 1986).

1.9.3. Effect on bacteria

Copper ions are essential for bacteria but can cause a number of toxic cellular effects if levels of free ions are not controlled (Cooksey, 1993). Bacteria were found to be more sensitive to heavy metals than fungi (Frostegård *et al.*, 1996). Bacteria can react with soluble heavy metals by binding and precipitating these metal ions on their surfaces, producing fine-grained minerals. Mineral precipitation reduces the cell surface area available for nutrient uptake and the mineralized cells then starve and die. Bacterial activity in high-Cu sludge amendment (8 g of Cu per kg) was much lower compared to that found in the unpolluted-sludge treatment (Bååth *et al.,* 1998).

1.9.4. Effect on fungi

A range of fungi from all major taxonomic groups may be found in metal-polluted habitats and the ability to survive and grow in the presence of potentially toxic concentrations is frequently encountered (Ross, 1975; Baldi *et al.*, 1990). General reductions in fungal numbers have, however, often been recorded in soils polluted with Cu, Cd, Pb, As and Zn (Babich & Stotzky, 1985). Along a steep gradient of Cu in soil towards a brass mill, fungal biomass decreased by *circa* 75% (Nordgren *et al.*, 1983).

Several reports suggest that some heavy metals, including Cu, damage the cytoplasmic membranes of fungal cells (Ross, 1993). In the presence of 0.06 mM Cu,

the ions of this metal caused a release of cellular potassium, resulting in a 90% loss of intracellular potassium (Kuypers & Roomans, 1979). Ohsumi *et al.* (1988) found that Cu at concentrations as low as 30 μM caused microscopically visible damage to yeast cells suspended in buffer. This effect was more severe if the cells were suspended in distilled water. These authors found that exposure to 100 μM cupric chloride led to loss of nucleotides and an immediate loss of 70% of cellular potassium. Amino acid leakage from cells treated with Cu also occurred.

1.10. Effect of heavy metals on microbial community structure

Using standard microbial culture techniques, it was found that heavy metal contamination does affect species composition of microbial communities in soil (Chander & Brooks, 1993; Berg *et al.*, 1991). However, it has become apparent that microbial communities can be enormously complex assemblages of populations (species) with diverse phylogenies and physiologies (Marsh, 1999). It is now common knowledge that only about 10% of the microbial species in soil can be cultivated in pure culture (Amann *et al.*, 1995; Hugenholtz *et al.*, 1998). Therefore, alternative methods are continually being explored to assess changes in microbial community structure.

1.10.1. Assessment of changes in microbial community structure by means of cultivation-dependent methods

It was found that changes in the composition of microbial communities can be assessed by cultivation-dependent methods such as the community–level catabolic profiling based on the commercially available Biolog™ substrate system (Lukow *et al.*, 2000). This rapid method for characterizing microbial communities based on inoculation of whole community samples into Biolog microtiter plates, was introduced ten years ago (Garland & Mills, 1991).

The Biolog EcoPlate™ contains growth media with 31 of the most useful carbon sources for soil community analysis (Figure 12) (Garland, 1997). On each of the microtitre plates, these 31 carbon-sources are repeated three times to give the scientist more replicates of the data (Figures 12 & 13).

A1 Water	A2 ß-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine
B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine
C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine
D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine
E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine
F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid
G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine
H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine

Figure 12. A schematic presentation of the carbon-sources present in the wells of a Biolog Eco MicroPlate™. The well labeled A1 is the blank that received no carbon-source (Garland, 1997).

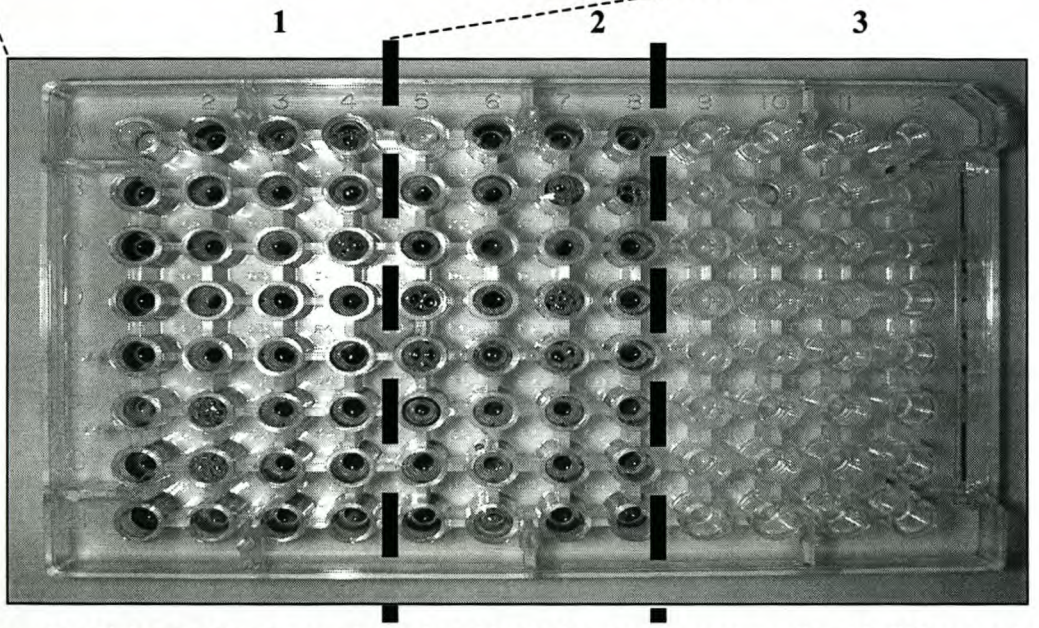


Figure 13. Biolog EcoPlate™ containing three identical series of growth media with different carbon-sources.

It was found that each community of organisms gives a characteristic reaction pattern called a metabolic fingerprint (Garland, 1997). This pattern is obtained by the formation of purple colours, which occur when the microbes utilize the carbon-source and begin to respire. The respiration of the cells in the community reduces the tetrazolium dye that is included with the carbon-source. If the carbon-source is not utilized, no colour reaction occurs within the wells.

1.10.2. Assessment of changes in microbial community structure by means of cultivation-independent molecular fingerprinting techniques

Cultivation-independent molecular fingerprinting techniques have gained popularity to address questions related to the diversity, structural composition and dynamics of microbial communities. Isolation of bacterial nucleic acids from natural environments has become a useful tool to detect bacteria that cannot be cultured (Liesack & Stackebrandt, 1992; Ward *et al.*, 1990).

The most common technique is denaturing gradient gel electrophoresis (DGGE) which is based on the electrophoretic separation of partial small-subunit (SSU) rDNA fragments of the same length, but different base pair composition within a linearly increasing gradient of denaturants (Lukow *et al.*, 2000). Similar separations can be obtained with temperature gradient gel electrophoresis (TGGE). A major pitfall of D/TGGE is that profiles generated for highly complex microbial communities are often characterized by a few dominating bands and a diffuse background caused by a large number of unresolved fragments (Heuer & Smalla, 1997). This hinders the correct qualitative and quantitative interpretation of the community fingerprint patterns obtained. The terminal-restriction fragment length polymorphism (T-RFLP) technique is a recent molecular approach that can assess subtle genetic differences between strains as well as provide insight into the structure and function of microbial communities (Marsh, 1999). The T-RFLP technique permits an automated quantification of the fluorescence signal intensities of the individual terminal restriction fragments (T-RFs) in a given community fingerprint pattern (Lukow *et al.*, 2000).

T-RFLP is a marriage of at least three technologies: (1) comparative genomics/RFLP; (2) polymerase chain reaction (PCR); and (3) nucleic acid electrophoresis (Marsh, 1999). Comparative genomics provides an insight into the design of primers for the amplification product (amplicon) of choice, and PCR amplifies the signal from a high background of unrelated markers. Subsequent digestion with judiciously selected restriction endonucleases produces terminal fragments appropriate for sizing on high resolution (± 1 base) sequencing gels. The latter is performed on automated systems such as the ABI gel or capillary electrophoresis systems that provide digital output. The use of a fluorescently tagged primer limits the analysis to only the terminal fragments of the digestion. Because size markers bearing a different fluorophore from the samples can be included in every lane, the sizing is extremely accurate. As the terminal fragment sizes can be compared to databases, the technique has potential in comparative community analysis.

Currently the number of investigations reported in the literature that have made use of T-RFLP is few (Marsh, 1999). The active interest, however, in the approach suggests that many investigations will follow. T-RFLP provides a high-resolution approach that many investigators can take advantage of. Because of its sensitivity and high throughput, it is an ideal technique for comparative community analyses. For example, Clement *et al.* (1998) were able to unambiguously distinguish between three different bacterial communities present in deer scat, sand and hydrocarbon contaminated sand respectively using T-RFLP. No tentative identifications of phylotypes were made but up to 25 different ribotypes, based on terminal fragment size, could be detected. Similarly Liu *et al.* (1997) were able to distinguish between four diverse bacterial communities including those from termite hind-gut, aquifer sediment and two different activated sludge samples. Again the sensitivity of the technique permitted the identification of up to 35 ribotypes in some communities.

Figure 14 presents an electropherogram from a T-RFLP analysis of a soil bacterial community (Marsh, 1999). Each peak is a terminal restriction fragment (T-RF) with a size calculated by the software on the basis of internal size markers. Numerous terminal fragments can be identified in such communities. The T-RFLP seems to represent the superior tool for the biomonitoring the highly diverse communities and enables a more objective comparison of community fingerprint patterns because of

the automated quantification of both size and relative abundance of individual T-RFs (Lukow *et al.*, 2000).

Although it is now accepted that microbial diversity in soils is vast, 16S rDNA techniques have yet to fulfil their potential with respect to understanding and manipulating biological function in soils (O'Donnell & Görres, 1999). Such knowledge is important if we are to sustain agricultural productivity, to cope with changes in land use concomitant with global change or to remediate contaminated land.

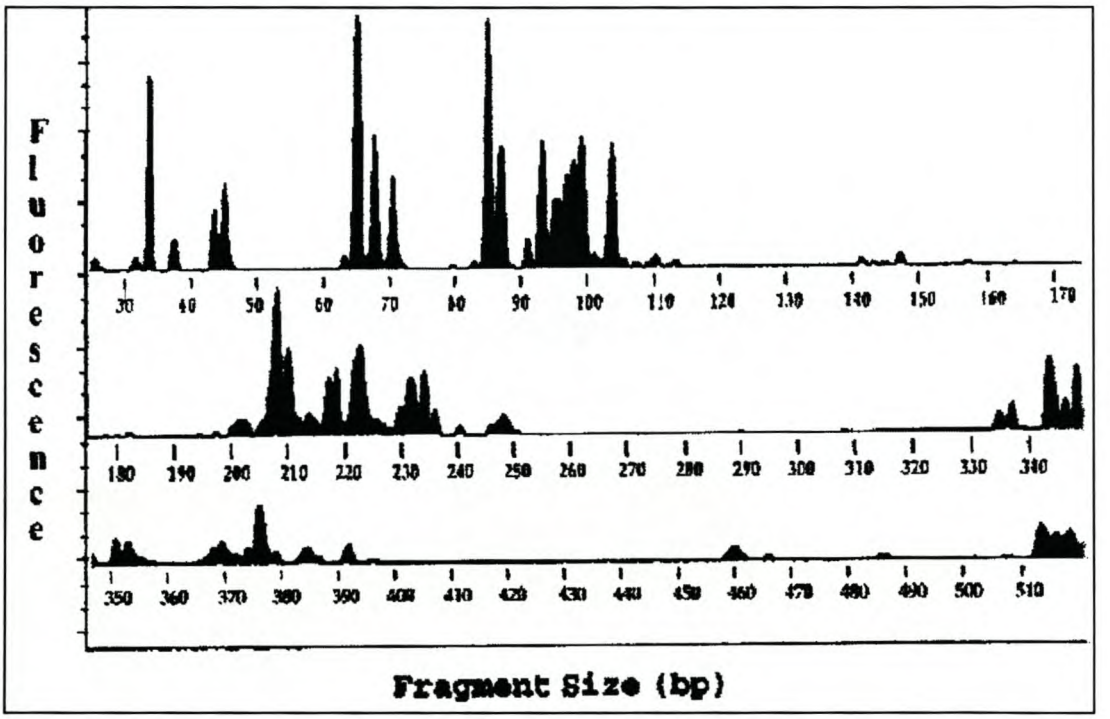


Figure 14. T-RFLP profile of a bacterial community from soil. The electropherogram can be accurately sized out to 600 basepairs with the standards used for this analysis. In this sample, over 60 terminal fragment sizes (ribotypes) can be detected. (Marsh, 1999)

1.11. Purpose of study

The objective this study was to identify potential bioindicators of Cu induced stress, by studying the responses of different soil microbial communities, indigenous to the Western Cape, to elevated levels of this heavy metal added to soil as a component of copper oxychloride. Consequently, the impact of copper oxychloride on a wide

diversity of soil microbial populations was investigated in pristine soil originating from Nietvoorbij experimental farm, Stellenbosch (Chapter 2). Potential bioindicators were again tested on experimentally contaminated soil from Koopmanskloof commercial farm, Stellenbosch and on potting soil from Nietvoorbij experimental farm (Chapter 3).

1.12. References

Adriano, D.C. (1986) *Trace elements in the Terrestrial Environment.* Springer-Verlag, New York.

Aescht, E. and Foissner, W. (1992) Effects of mineral and organic fertilizers on the microfauna in a high altitude afforestation trial. *Biology and Fertility of Soils* 13, 17-24.

Alexander, M. (1977) *Introduction to Soil Microbiology.* John Wiley and Sons, Inc., New York.

Alloway, B.J. (1995) *Heavy metals in soils.* Blackie Academic and Professional, Glasgow.

Amann, R.I., Ludwig, W. and Schleifer, K.H. (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiological Reviews* 59, 143-169.

Atlas, R.M. (1984) Use of microbial diversity measurements to asses environmental stress. In: Klug, M.J. and Reddy, C.A. (eds.), *Current perspectives in microbial ecology.* American Society for Microbiology, Washington, pp.540-545.

Atlas, R.M. and Bartha, R. (1993) *Microbial Ecology: Fundamentals and Applications.* Benjamin/Cummings Publishing Company, Inc., pp.526-528.

Bååth, E., Díaz-Raviña, M., Frostegård, Å. and Campbell, C.D. (1998) Effect of metal-rich sludge amendments on the soil microbial community. *Applied and Environmental Microbiology* 64, 238-245.

Babich, H. and Stotzky, G. (1985) Heavy metal toxicity to microbe-mediated ecologic processes: a review and potential application to regulatory policies. *Environmental Research* 36, 111-137.

Baecker, A.A.W. and King, B. (1978) Decay of wood by actinomycetes. In: Oxley, T.A., Becker, G. and Allsopp, D. (eds.), *Biodeterioration: The Proceedings of the Fourth International Biodeterioration Symposium, Berlin.* Pitman Publishing, Ltd., London, pp.53-57.

Baker, D.E. and Senft, J.P. (1995) Copper. In: Alloway, B.J. (ed.), *Heavy Metals in Soils*. Blackie Academic and Professional, Bishopbriggs, pp.179-205.

Baldi, F., Vaughan, A.M. and Olson, G.J. (1990) Chromium(vi)-resistant yeast isolated from a sewage treatment plant receiving tannery wastes. *Applied and Environmental Microbiology* 56, 913-918.

Bender, C.L. and Cooksey, D.A. (1986) Indigenous plasmids in *Pseudomonas syringae* pv. *tomato*: conjugative transfer and role in copper resistance. *Journal of Bacteriology* 165, 534-541.

Bender, C.L. and Cooksey, D.A. (1987) Molecular cloning of copper resistance genes from *Pseudomonas syringae* pv. *tomato. Journal of Bacteriology* 169, 470-474.

Berg, B., Ekbohn, G., Sonderstrom, B. and Staaf, H. (1991) Reduction of decomposition rates of Scots pine needle litter due to heavy-metal pollution. *Water, Air and Soil Pollution* 59, 165-177.

Brady, N.C. (1990) *The Nature and Properties of Soils, 10th edition.* Macmillan Publishing Co., New York.

Brookes, P.C., McGrath, S.P., Klein, D.A. and Elliot, E.T. (1984) Effects of heavy metals on microbial activity and biomass in fields treated with sewage sludge. In: *Environmental Contamination. International Conference, London*. CEP Consultants, Ltd., Edinburgh, pp.574-583.

Bruins, M.R., Kapil, S. and Oehme, F.W. (2000) Microbial resistance to metals in the environment. *Ecotoxicological and Environmetal Safety* 45, 198-207.

Cha, J.-S. and Cooksey, D.A. (1991) Copper resistance in *Pseudomonas syringae* mediated by periplasmic and outer membrane proteins. *Proceedings in National Academy of Science, USA* 88, 8915-8919.

Cha, J.-S. and Cooksey, D.A. (1993) Copper hypersensitivity and uptake in *Pseudomonas syringae* containing cloned components of the copper resistance operon. *Applied and Environmental Microbiology* 59, 1671-1674.

Chander, K. and Brooks, P.C. (1993) Residual effects of zinc, copper, and nickel in sewage sludge on microbial biomass in a sandy loam. *Soil Biology and Biochemistry* 25, 1231-1239.

Chaphekar, S.B. (1978) Biological indicators. The concept of new addition. *International Journal of Ecology and Environmental Science* 4, 45-52.

Clement, B.G., Kehl, L.E., DeBord, K.L. and Kitta, C.L. (1998) Terminal-restriction fragment length patterns (T-RFLPs), a rapid PCR-based method for the comparison of complex bacterial communities. *Journal of Microbiological Methods* 31, 135-142.

Conradie, W.J. (2001) Personal communication. Soil scientist, ARC-Infruitec/Nietvoorbij, Stellenbosch.

Cooksey, D.A. (1993) Copper uptake and resistance in bacteria. *Molecular Microbiology* 7, 1-5.

Cooksey, D.A. (1994) Molecular mechanism of copper resistance and accumulation in bacteria. *FEMS Microbiology Reviews* 14, 381-386.

Cowling, A.J. (1994) Protozoan distribution and adaptation. In: Darbyshire, J.F. (ed.), *Soil Protozoa.* CAB International, Wallingford, pp.65-91.

Darbyshire, J.F. (1994) *Soil Protozoa.* CAB International, Wallingford, p.209.

Doelman, P. (1985) Resistance of soil microbial communities to heavy metals. In: Jensen, V., Kjöler, A. and Sörensen, L.H. (eds.), *Microbial Communities in Soil.* Elsevier, London, pp.369-384.

Domsch, K.H., Jagnow, G. and Anderson, T.-H. (1983) An ecological concept for the assessment of side-effects of agrochemicals on soil microorganisms. *Residue Reviews* 86, 65-105.

Elliot, E.T. (1997) Rationale for developing bioindicators. In: Pankhurst, C.E., Doube, B.M. and Gupta, V.V.S.R. (eds.), *Biological indicators of soil health.* CAB International, Wallingford, pp.49-78.

Embley, T.M. and Stackebrandt, E. (1996) The use of 16S ribosomal RNA sequences in mirobial ecology. In: Pickup, R.W. and Saunders, J.R. (eds.), *Ecological approaches to Environmental Microbiology.* Prentice Hall, Oxford, pp.39-62.

Flores-Vélez, L.M., Ducaroir, J., Jaunet, A.M. and Robert, M. (1996) Study of the distribution of copper in an acidic vineyard soil by three different methods. *European Journal of Soil Science* 47, 523-532.

Foissner, W. (1987) Soil protozoa: fundamental problems, ecological significance, adaptations in ciliates and testaceans, bioindicators, and guide to literature. *Progress in Protistology* 2, 69-212.

Foissner, W. (1994) Soil protozoa as bioindicators in ecosystems under human influence. In: Darbyshire, J.F. (ed.), *Soil Protozoa.* CAB International, Wallingford, pp.147-194.

Forge, T.A., Berrow, M.L., Darbyshire, J.F. and Warren, A. (1993) Protozoan bioassays of soil ammended with sewage sludge and heavy metals, using the common soil ciliate *Colpoda steinii. Biology and Fertility of Soils* 16, 282-286.

Freckman, D.W. (1988) Bacteriovorous nematodes and organic matter decomposition. *Agriculture, Ecosystems and Environment* 24, 195-217.

Freckman, D.W. and Caswell, E.P. (1985) The ecology of nematodes in agroecosystems. *Annual review of Phytopathology* 23, 275-296.

Freckman, D.W. and Ettema, C.H. (1993) Assessing nematode communities in agroecosystems of varying human intervention. *Agriculture, Ecosystems and Environment* 45, 239-261.

Frostegård, Å., Tunlid, A. and Bååth, E. (1996) Changes in microbial community structure during long-term incubation in two soils experimentally contaminated with metals. *Soil Biology and Biochemistry* 28, 55-63.

Gadd, G.M. (1992) Metals and microorganisms: A problem of definition. *FEMS Microbiology Letters* 100, 197-204.

Garland, J.L. (1997) Analysis and interpretation of community-level physiological profiles in microbial ecology. *Federation of European Microbiological Societies, Microbiology Ecology* 24, 289-300.

Garland, J.L. and Mills, A.L. (1991) Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level-sole-carbon-source-utilization. *Applied and Environmental Microbiology* 57, 2351-2359.

Giller, K.E., Witter, E. and McGrath, S.P. (1998) Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils. A review. *Soil Biology and Biochemistry* 30, 1389-1414.

Gilotora, U. and Srivastava, S. (1996) Plasmid-encoded sequestration of copper by *Pseudomonas pickettii* strain US321. *Current Microbiology* 4, 378-381.

Griffiths, B.S. (1994) Soil nutrient flow. In: Darbyshire, J.F. (ed.), *Soil Protozoa.* CAB International, Wallingford, pp.65-91.

Gupta, V.V.S.R. (1993) The impact of soil and crop management practices on the dynamics of soil microfauna and mesofauna. In: Pankhurst, C.E., Doube, B.M., Gupta, V.V.S.R. and Grace, P.R. (eds.), *Soil Biota: Management of Sustainable Farming Systems.* CSIRO Press, Melbourne, pp.107-124.

Gupta, V.V.S.R. and Yeates, G.W. (1997) Soil microfauna as bioindicators of soil health. In: Pankhurst, C.E., Doube, B.M. and Gupta, V.V.S.R. (eds.), *Biological indicators of soil health.* CAB International, Wallingford, pp.201-233.

Harden, T., Joergensen, R.G., Meyer, B. and Wolters, V. (1993) Soil microbial biomass estimated by fumigation-extraction and substrate induced respiration in two pesticide-treated soils. *Soil Biology and Biochemistry* 25, 679-683.

Harris, J.A. and Birch, P. (1992) Land reclamation and restoration. In: Fry, J.C., Gadd, G.M., Herbert, R.A., Jones, C.W. and Watson-Craik, I.A. (eds.), *Microbial control of pollution.* Society of General Microbiology Symposium No. 48, Cambridge University Press, pp.269-291.

Hawksworth, D.L. and Mound, L.A. (1991) Biodiversity databases: the crucial significance of collections. In: Hawksworth, D.L. (ed.), *The biodiversity of microorganisms and invertebrates: Its role in sustainable agriculture.* CAB International, Wallingford, pp.17-29.

Head, I.M., Hiorns, W.D., Embley, T.M., McCarthy, A.J. and Saunders, J.R. (1993) The phylogeny of autotrophic ammonia-oxidizing bacteria as determined by analysis of 16S ribosomal RNA gene sequences. *Journal of General Microbiology* 139, 1147-1153.

Hekman, W.E., Van der Boogert, P.J.H.F. and Zwart, K.B. (1992) The physiology and ecology of a novel, obligate mycophagous flagellate. *FEMS Microbiology Ecology* 86, 255-265.

Henkinet, R., Couteaux, M.M., Billes, G., Bottner, P. and Palka, L. (1990) Acceleration of carbon turnover and stimulation of the priming effect by predation in forest humus. *Soil Biology and Biochemistry* 22, 555-561.

Heuer, H. and Smalla, K. (1997) Applications of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) for studying microbial communities. In: van Elsas, J.D., Trevors, J.T. and Wellington, E.M.H. (eds.), *Modern Soil Microbiology.* Marcell Decker, Inc., New York, pp.353-373.

Hicks, R.J., Stotzky, G. and Van Voris, P. (1990) Review and the evaluation of the effects of xenobiotic chemicals on microorganisms in soil. *Advances in Applied Microbiology* 35, 195-253.

Hiroki, M. (1992) Effects of heavy metal contamination on soil microbial population. *Soil Science and Plant Nutrition* 38, 141-147.

Holben, W.E. (1994) Isolation and purification of bacterial DNA from soil. In: Weaver, R.W., Angle, S., Bottomley, P., Bezdicek, D., Smith, S., Tabatabai, A. and Wollum, A. (eds.), *Methods in Soil Analysis. Part 2. Microbiological and Biochemical Properties.* Soil Science Society of America, Madison, Wisconsin, pp.727-751.

Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T. and Williams, S.T. (1994) *Bergey's Manual of determinative bacteriology, 9th edition.* Williams and Wilkins, Baltimore.

Hugenholtz, P., Goebel, B.M. and Pace, N.R. (1998) Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *Journal of Bacteriology* 180, 4765-4774.

Hung, I.H., Suzuki, M., Yamaguchi, Y., Yuan, D.S., Klausner, R.D. and Gitlin, J.D. (1997) Biochemical characterization of the Wilson disease protein and functional expression in the yeast *Saccharomyces cerevisiae. Journal of Biological Chemistry* 34, 21461-21466.

Kersters, K., Ludwig, W., Vancanneyt, M., De Vos, P., Gillis, M. and Schleifer, K.-H. (1996) Recent changes in the classification of the Pseudomonads: an overview. *Systematic and Applied Microbiology* 19, 465-477.

King, J.M.H., Digrazia, P.M., Applegate, B., Burlage, R., Sanseverino, J., Dunbar, P., Larimer, F. and Sayler, G.G. (1990) Rapid, sensitive bioluminescent reporter technology for naphthalene exposure and biodegradation. *Science* 249, 778-781.

Küster, E. (1967) The Actinomycetes. In: Burges, A. and Raw, F. (eds.), *Soil Biology*. Academic Press, New York, pp.111-127.

Kuypers, G.A.J. and Roomans, G.M. (1979) Mercury-induced loss of K^+ from yeast cells investigated by electron probe x-ray microanalysis. *Journal of General Microbiology* 115, 13.

Labuschagne, T. (2001) Personal communication. Management of Chempack cc., Paarl.

Levine, N.D., Corliss, J.O., Cox, F.E.G., Deroux, G., Grain, J., Honigberg, B.M., Leedale, G.F., Loeblich, A.R., Lom, J., Lynn, D., Meringeld, E.G., Page, F.C.,

Poljansky, G., Sprague, V., Varva, J. and Wallace, F.G. (1980) A newly revised classification of Protozoa. *Journal of Protozoology* 27, 37-58.

Liesack, W. and Stackebrandt, E. (1992) Occurrence of novel groups of the domain *Bacteria* as revealed by analysis of genetic material isolated from an Australian terrestrial environment. *Journal of Bacteriology* 174, 5072-5078.

Liu, W.T., Marsh, T.L., Cheng, H. and Forney, L.J. (1997) Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Applied and Environmental Microbiology* 63, 4516-4522.

Logan, N. A. (1994) *Bacterial Systematics.* Blackwell Scientific Publications, Oxford, p.217.

Lukow, T., Dunfield, P.F. and Liesack, W. (2000) Use of the T-RFLP technique to assess spatial and temporal changes in the bacterial community structure within an agricultural soil planted with transgenic and non-transgenic potato plants. *FEMS Microbiology Ecology* 32, 241-247.

Maggenti, A.R. (1991a) General nematode morphology. In: Nickle, W.R. (ed.), *Manual of Agricultural Nematology.* Marcell Decker, Inc., New York, pp.3-46.

Maggenti, A.R. (1991b) Nemata: Higher Classification. In: Nickle, W.R. (ed.), *Manual of Agricultural Nematology.* Marcell Decker, Inc., New York, pp.147-187.

Marsh, T.L. (1999) Terminal-restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplification products. *Current Opinion in Microbiology* 2, 323-327.

McBride, M.B. (1981) Forms and distribution of copper in solid and solution phases of soil. In: Loneragan, J.F., Robson, A.D. and Graham, R.D. (eds.), *Copper in Soils and Plants.* Academic Press, Sydney, pp.25-45.

McBride, M.B. (1994) *Environmental chemistry of soil.* Oxford University Press, Inc., New York.

Mejstrík, V. and Lepsová, A. (1993) Applicability of fungi to the monitoring of environmental pollution by heavy metals. In: Markert, B. (ed.), *Plants as biomonitors. Indicators for heavy metals in terrestrial environment.* VCH, Weinheim, pp.365-378.

Mellano, M.A. and Cooksey, D.A. (1988) Nucleotide sequence and organization of copper resistance genes from *Pseudomonas syringae* pv. *tomato*. *Journal of Bacteriology* 170, 2879-2883.

Mhatre, G.N. and Pankhurst, C.E. (1997) Bioindicators to detect contamination of soils with special reference to heavy metals. In: Pankhurst, C.E., Doube, B.M. and Gupta, V.V.S.R. (eds.), *Biological indicators of soil health.* CAB International, Wallingford, pp.349-368.

Miller, K.W., Cole, M.E. and Banwart, W.L. (1991) Microbial populations in an agronomically managed mollisol treated with simulated acid rain. *Journal of Environmental Quality* 20, 845-849.

Mills, S.D., Jasalavich, C.A. and Cooksey, D.A. (1993) A two-component regulatory system required for copper inducible expression of the copper resistance operon of *Pseudomonas syringae*. *Journal of Bacteriology* 175, 1656-1664.

Nordgren, A., Bååth, E. and Söderström, B. (1983) Microfungi and microactivity along a heavy metal gradient. *Applied and Environmental Microbiology* 45, 1829-1837.

Norton, D.C. and Niblack, T.L. (1991) Biology and ecology of nematodes. In: Nickle, W.R. (ed.), *Manual of Agricultural Nematology.* Marcell Decker, Inc., New York, pp.47-72.

Oades, J.M. and Waters, A.G. (1991) Aggregate hierarchy in soils. *Australian Journal of Soil Research* 29, 815-828.

Ochiai, E.I. (1987) *General Principles of Biochemistry of the Elements*. Plenum Press, New York.

O'Donnell, A.G. and Görres, H.E. (1999) 16S rDNA methods in soil microbiology. *Current opinion in Biotechnology* 10, 225-229.

Odum, E.P. (1971) *Fundementals of Ecology, 3rd edition*. W.B. Saunders Company, Philadelphia.

Ohsumi, Y., Kitamoto, K. and Anraku, Y. (1988) Changes induced in the permeability barrier of the yeast plasma membrane by cupric ions. *Jounal of Bacteriology* 170, 2676.

Olsen, G.J. and Woese, C.R. (1993) Ribosomal RNA: a key to phylogeny. *The FASEB Journal* 7, 113-123.

Pankhurst, C.E. (1997) Biodiversity of soil organisms as an indicator of soil health. In: Pankhurst, C.E., Doube, B.M. and Gupta, V.V.S.R. (eds.), *Biological indicators of soil health*. CAB International, Wallingford, pp.297-324.

Pankhurst, C.E., Doube, B.M. and Gupta, V.V.S.R. (1997) Biological indicators of soil health: Synthesis. In: Pankhurst, C.E., Doube, B.M. and Gupta, V.V.S.R. (eds.), *Biological indicators of soil health*. CAB International, Wallingford, pp.419-435.

Parr, J.F., Papendick, R.I., Hornick, S.B. and Meyers, R.E. (1992) Soil quality: attributes and relationship to alternative and sustainable agriculture. *American Journal of Alternative Agriculture* 7, 5-11.

Paton, G.I., Campbell, C.D., Glover, L.A. and Killham, K. (1995) A novel method to assess ecotoxicity using a *lux*-modified strain of *Pseudomonas fluorescence*. *FEMS Letters in Microbiology* 20, 52-57.

Paton, G.I., Rattray, E.A.S., Campbell, C.D., Cresser, M.S., Glover, L.A., Meeussen, J.C.L. and Killham, K. (1997) Use of genetically modified microbial biosensors for soil ecotoxicity testing. In: Pankhurst, C.E., Doube, B.M. and Gupta, V.V.S.R. (eds.), *Biological indicators of soil health.* CAB International, Wallingford, pp.397-418.

Paul, E.A. and Clark, F.E. (1996) *Soil Microbiology and Biochemistry, 2nd edition.* Academic Press, Inc., San Diego.

Prescott, L.M., Harley, J.P. and Klein, D.A. (1990) *Microbiology.* Wm. C. Brown Publishers, Dubuque.

Prescott, L.M., Harley, J.P. and Klein, D.A. (1996) *Microbiology 3rd edition.* Wm. C. Brown Publishers, Dubuque.

Remde, A. and Hund, K. (1994) Response of soil autotrophic nitrification and respiration to chemical pollution in long-term experiments. *Chemosphere* 29, 391-404.

Ross, I.S. (1975) Some effects of heavy metals on fungal cells. *Transactions of the British Mycological Society* 64, 175-193.

Ross, I.S. (1993) Membrane transport processes and response to exposure to heavy metals. In: Jennings, D.H. (ed.), *Stress Tolerance of Fungi.* Marcel Dekker, Inc., New York, pp.97-125.

Rouch, D., Camakaris, J. and Lee, B.T.O. (1989) Copper transport in *Escherichia coli.* In: Hamer, D.H. and Winge, D.R. (eds.), *Metal ion homeostasis: molecular biology and chemistry.* Alan R. Liss, Inc., New York, pp.469-477.

Rowell, M.J. and Florence, L.Z. (1993) Characteristics associated with differences between undisturbed and industrially-disturbed soils. *Soil Biology and Biochemistry* 25, 1499-1511.

Sandaa, R.-A., Torsvik, V., Enger, Ø., Daae, F.L., Castberg, T. and Hahn, D. (1999) Analysis of bacterial communities in heavy metal-contaminated soils at different levels of resolution. *FEMS Microbiology Ecology* 30, 237-251.

Schaal, K. P. (1986) Genus Actinomyces. In: Sneath, P. H. A. (ed.), *Bergey's Manual of Systematic Bacteriology. Volume 2.* Williams and Wilkins, Baltimore, pp.1383.

Silver, S. (1996) Bacterial resistance to toxic metal ions – a review. *Gene* 179, 9-19.

Silver, S. and Phung, L.T. (1996) Bacterial heavy metal resistance: new surprises. *Annual Review of Microbiology* 50, 753-789.

Sims, G.K. (1990) Biological degradation of soil. *Advances in Soil Science* 11, 289-330.

Stout, J.D. and Heal, O.W. (1967) Protozoa. In: Burges, A. and Raw, F. (eds.), *Soil Biology.* Academic Press. Inc., New York, pp.149-195.

Tan, K.H. (1994) *Environmental Soil Science.* Marcel Dekker, Inc., New York.

Teske, A., Alm, E., Regan, J.M., Toze, S., Rittmann, B.E. and Stahl, D.A. (1994) Evolutionary relationships among ammonia-and nitrite-oxidizing bacteria. *Journal of Bacteriology* 176, 6623-6630.

Tessier, A. and Campbell, P.G.C. (1988) Partitioning of trace metals in sediments. In: Kramer, J.R. and Allen, H.E. (eds.), *Metal Speciation: Theory Analysis and Application.* Lewis Publishers, Chelsea, Michigan, pp.183-199.

Turco, R.F., Kennedy, A.C. and Jawson, M.D. (1994) Microbial indicators of soil quality. In: Doran, J.W., Coleman, D.C., Bezdicek, D.F. and Stewart, B.A. (eds.), *Defining soil quality for a sustainable environment.* Soil Science society of America Special Publication No. 35, Madison, Wisconsin, pp.73-90.

Ward, D.M., Weller, R. and Bateson, M.M. (1990) 16S rRNA sequences reveal numerous uncultured inhabitants in a natural community. *Nature* 345, 63-65.

Waters, A.G. and Oades, J.M. (1991) Organic matter in water-stable aggregates. In: Wilson, W.S. (eds.), *Advances in Soil Organic Matter Research: The Impact on Agriculture and the Environment.* Royal Society of Chemistry, Cambridge.

Weiss, B. and Larink, O. (1991) Influence of sewage sludge and heavy metals on nematodes in an arable soil. *Biology and Fertility of Soils* 12, 5-9.

West, E.G. (1982) *Copper and its alloys*. Ellis Horwood Limited Publishers, Chichester, pp.12-42.

Wittig, R. (1993) General aspects of biomonitoring heavy metals by plants. In: Market, B. (ed.), *Plants as Biomonitors*. VCH, Weinheim, pp.3445-363.

Woese, C.R. (1987) Bacterial evolution. *Microbiological Reviews* 51, 221-271.

Woese, C.R., Stackebrandt, E., Weisburg, W.G., Paster, B.J., Madigan, M.T., Fowler, V.J., Hahn, C.M., Blanz, P., Gupta, R., Nealson, K.H. and Fox, G.E. (1984) The phylogeny of purple bacteria: the alpha subdivision. *Systematic and Applied Microbiology* 5, 315-326.

Woese, C.R., Weisburg, W.G., Hahn, C.M., Paster, B.J., Zablen, L.B., Lewis, B.J., Macke, T.J., Ludwig, W. and Stackebrandt, E. (1985) The phylogeny of purple bacteria: the gamma subdivision. *Systematic and Applied Microbiology* 6, 25-33.

Wondratschek, I. and Röder, U. (1993) Monitoring of heavy metals in soils by higher fungi. In: Markert, B. (ed.), *Plants as biomonitors. Indicators for heavy metals in terrestrial environment.* VCH, Weinheim, pp.345-363.

Yeates, G.W. (1981) Nematode populations in relation to soil environmental factors: a review. *Pedobiologia* 22, 312-338.

Yeates, G.W. and Bongers, T. (1997) Nematode diversity in agro-ecosystems. In: Paoletti, M.G. (ed.), *Biodiversity in Agro-ecosystems – Role for sustainability and Bioindication.* Lewis Publishing, Boca Raton.

Yeates, G.W., Orchard, V.A., Speir, T.W., Hunt, J.L. and Hermans, M.C.C. (1994) Reduction in soil biological activity following pasture contamination by copper, chromium, arsenic timber preservative. *Biology and Fertility of Soils* 18, 200-208.

Zullini, A. and Peretti, E. (1986) Lead pollution and moss-inhabiting nematodes of an industrial area. *Water, Air and Soil Pollution* 27, 403-410.

Zwart, K.B. and Darbyshire, J.F. (1992) Growth and nitrogenous excretion of a common soil flagellate *Spumella* sp. – a laboratory experiment. *Journal of Soil Science* 43, 145-157.

CHAPTER 2

THE IMPACT OF COPPER OXYCHLORIDE ON EUKARYOTIC AND PROKARYOTIC MICROBIAL POPULATIONS IN SOIL

2.1. Introduction

Copper (Cu) containing compounds have widely been used in agricultural practices as fertilizers (Baker & Senft, 1995) and fungicides (Flores-Vélez *et al.*, 1996). This resulted in the accumulation of Cu in soils (Flores-Vélez *et al.*, 1996). However, the accumulation of heavy metals, including Cu, may generate stress on the environment, resulting in organisms and communities of organisms displaying injury symptoms or shifts in community composition (Chaphekar, 1978). Since differences in sensitivity towards environmental stress exist between taxa (Atlas, 1984), this shift in community composition results in a change in microbial diversity.

Changes in soil microbial diversity may impact on ecosystem processes and hence the capacity of agricultural land to remain productive (Naeem *et al.*, 1995). Although the soil on which the vineyards of the Western Cape were planted, originally contained a relatively low Cu concentration (Conradie, 2001), about 160 tons of copper oxychloride are annually being applied on these soils as a fungicide (Labuschagne, 2001). In general, the soils in the Western Cape are slightly acidic, making Cu more mobile and bio-available compared to Cu in alkaline soils (McBride, 1994). It is therefore important to estimate how elevated Cu concentrations, as a result of spraying with Cu containing compounds such as copper oxychloride, will impact on the diversity of indigenous soil microbes in the Western Cape.

Sandaa *et al.* (1999) found that the biodiversity of actinomycetes and other bacteria are sensitive to elevated Cu levels in soil and that it may therefore serve as an indicator for the effects of metal contamination. Filamentous fungi were found to be less sensitive than these prokaryotes (Doelman, 1985; Hiroki, 1992). However, other eukaryotes, such as protozoa were found to be sensitive to environmental stress, because of their rapid growth and delicate external membranes (Foissner, 1994). Changes in microbial

diversity as a result of environmental stress was measured by some authors (e.g. Zak *et al.*, 1994; Grayston *et al.*, 1994), by analyzing the function of the whole community on the basis of the ability to utilize a series of substrates (Biolog^{TM} systems). Other authors compared the structure of soil communities by approaches based on DNA technology (Pankhurst, 1997). DNA-based detection of bacterial populations helps to overcome a major limitation of microbial ecology and soil biology, since one can now specifically monitor an individual population of microbes in an environment, and in the presence of almost the entire microbial community (Holben, 1994).

With the above as background, it became the aim of this study to determine the impact of elevated soil Cu levels, as a result of the addition of copper oxychloride, on selected eukaryotic and prokaryotic microbial populations in pristine soil originating from Nietvoorbij experimental farm, Stellenbosch.

2.2. Materials and Methods

2.2.1. Collection and preparation of soil. Soil was collected from Nietvoorbij experimental farm, Stellenbosch. After the organic matter at the surface was removed, the top 30 cm of soil was collected. The soil was a sandy loamy soil of the Clovelly form derived from an orthic A-horizon, a yellow-brown apedal B-horizon and a sapprolite C-horizon (Fry, 1987; Soil Classification Working Group, 1991). *Circa* 600 kg of soil was allowed to dry for 2 weeks at 30 °C, whereafter it was sieved (pore size 2 mm). The main physical and chemical properties of the sieved soil are listed in Table 1.

2.2.2. Preparation of soil microcosms. A series of soil microcosms was prepared by adding various concentrations of copper oxychloride ($CuCl_2 \cdot 2CuO \cdot 4H_2O_4$) to each of eight aliquots of soil. The series consisted of *circa* 2 kg aliquots of soil, contained in plastic soil bags, that received the following estimated amounts of Cu: 0 (control microcosm), 10, 20, 30, 40, 50, 100, 500 and 1000 ppm (Figure 1). The final concentration of bio-available Cu in each microcosm was subsequently determined and is listed in Table 2. All experiments were conducted in triplicate and were labeled A, B and C.

Table 1. Characteristics of the soil that was used in the experimentation.

Physical characteristics	
[1]**Texture**	
Stone % (Particle diameter > 2.0 mm)	0.54
Rough sand % (Particle diameter 0.5 - 2.0 mm)	30.90
Medium sand % (Particle diameter 0.2 - 0.5 mm)	13.10
Fine sand % (Particle diameter 0.02 - 0.2)	24.50
Silt % (Particle diameter 0.002 - 0.02)	22.80
Clay % (Particle diameter < 0.002)	5.70
Chemical characteristics	
[2]Organic carbon %	3.54
[3]Total nitrogen %	0.20
[4]Ammonium (ppm)	5.40
[5]Nitrate (ppm)	3.20
[6]Phosphorus (ppm)	29.00
[7]Copper (ppm)	1.37
[8]Zinc (ppm)	6.80
[9]Manganese (ppm)	4.20
[10]Boron (ppm)	0.86
[11]Exchangeable cations (cmol/kg)	
Calsium	4.70
Magnesium	3.25
Potassium	0.63
Sodium	0.11
[12]pH of a suspension containing 1 part soil and 2.5 parts 1M KCl.	6.10

[1] Determined by Bemlab CC** using the hydrometer method (Van der Watt, 1966).

[2] Determined by Bemlab CC using the Walkey-Black method (Nelson & Sommers, 1982).

[3] Determined by Bemlab CC through digestion in a LECO FP-528 nitrogen analyser.

[4-5] Determined in a 1M KCl extract by Bemlab CC (Bremner, 1965).

[6] Determined in a Bray-2 extract by Bemlab CC (Thomas & Peaslee, 1973).

[7-9] Determined in a di-ammonium EDTA extract by Bemlab CC (Beyers & Coetzer, 1971).

[10] Determined in a hot water extract by Bemlab CC according to the methods of the Fertilizer Society of South Africa (1974).

[11] Determined in a 1M ammonium acetate extract by Bemlab CC (Doll & Lucas, 1973).

[12] Determined by Bemlab CC according to the method of McClean (1982).

** Bemlab CC, P.O. Box 12457, Die Boord, Stellenbosch, 7613, South Africa.

Table 2. Bio-available copper concentrations in the soil microcosms used in the experimentation.

Microcosms no. in triplicate set	* Cu	** Cu (mg/kg)	*** Cu (mg/l)	% saturation of soil extract
0A; 0B; 0C (Control)	0 ppm	1.89 ± 0.04	0.004 ± 0.0015	46.8 ± 0.9
10A; 10B; 10C	10 ppm	12.18 ± 0.88	0.026 ± 0.0014	46.4 ± 1.5
20A; 20B; 20C	20 ppm	22.61 ± 0.70	0.036 ± 0.0025	45.7 ± 1.1
30A; 30B; 30C	30 ppm	33.79 ± 4.50	0.045 ± 0.0046	45.2 ± 0.4
40A; 40B; 40C	40 ppm	42.14 ± 3.35	0.056 ± 0.0026	43.8 ± 0.3
50A; 50B; 50C	50 ppm	59.46 ± 3.38	0.067 ± 0.0060	43.9 ± 1.2
100A; 100B; 100C	100 ppm	125.69 ± 2.90	0.105 ± 0.0038	47.4 ± 1.2
500A; 500B; 500C	500 ppm	516.12 ± 6.70	0.353 ± 0.0213	43.9 ± 1.1
1000A; 1000B; 1000C	1000 ppm	1112.40 ± 52.17	0.947 ± 0.0719	49.8 ± 2.3

* Estimated concentration of copper as a component of copper oxychloride, added to each microcosm.

** Determined in a di-ammonium EDTA extract by Bemlab CC according to the methods of Beyers and Coetzer (1971). Values represent the means and standard deviations of three repetitions.

*** Determined in the water phase of a saturated soil extract prepared according to the methods of Longenecker and Lyerly (1964). Values represent the means and standard deviations of three repetitions.

2.2.3. Monitoring of microbial communities in soil microcosms. The experiment was initiated by adding distilled water to a final concentration of 15% (v/w) to each microcosm. The microcosms were subsequently incubated at 22 °C for 245 days. Soil dilution plates were prepared periodically (day 1, 3, 7, 14, 28, 70 and day 245) to enumerate specific culturable microbial populations. General microbial counts, actinomycete counts and fluorescent bacterial counts were performed by inoculating each of a series of plates containing different media with 100 µl of a 10^{-3} soil dilution.

To obtain general microbial counts, tryptone soy agar (TSA) (Biolab™) was used as enumeration medium. Actinomycetes were enumerated by using sodium caseinate agar. This medium (pH 6.7) consisted of 0.2 g/l sodium caseinate, 0.5 g/l K_2HPO_4, 0.2 g/l $MgSO_4$, 0.01 g/l $FeCl_3$ and 15 g/l agar. Fluorescent bacterial counts were performed on King's medium B (KMB) (Biolab™) and *Pseudomonas* strains were enumerated by using *Pseudomonas* CFC medium (Oxoid™). All plates, except the actinomycete enumeration plates, were incubated at 30 °C for 24 to 48 h. The actinomycete

enumeration plates were incubated at 22 °C for 5 to 7 days. All experiments were conducted in triplicate.

Figure 1. Microcosms containing various amounts of Cu.

2.2.4. Measurement of metabolic potential of the whole microbial community. After 21 and 245 days of incubation, 10 g of soil from each of the microcosms that received 0, *ca.* 30, *ca.* 100 and *ca.* 1000 ppm Cu, were transferred to 90 ml sterile distilled water. Each suspension was shaken for 10 minutes and allowed to settle for 2 h, whereafter the supernatant was used to inoculate a duplicate set of wells on a Biolog™ Eco microplate, each containing a series of growth media with different carbon sources. After each well was inoculated with 150 µl of the supernatant, the plates were incubated at 22 °C for 24 h to 48 h. Utilization of the different carbon sources in the wells were recorded by observing colour changes in the wells and comparing it to a control well with no carbon source. The results were analyzed with Statistica computer software (StatSoft®).

2.2.5. Enumeration of protozoa and nematodes. The procedure of Griffiths and Ritz (1988) was followed, whereby 5.0 g of soil from each microcosm that received 0, *ca.* 30, *ca.* 100 and *ca.* 1000 ppm Cu, was added to 50 ml of Tris-HCl buffer (pH 8.0) and shaken for 10 min on a wrist shaker. After shaking, the samples were allowed to settle for 1 min, after which a 1 ml aliquot was removed from 5 cm below the meniscus in each tube. This was then added to 0.1 ml of 0.4% (w/v) iodonitrotetrazolium (INT) and incubated at 25°C for 4 h. After fixing the cells with 0.1 ml 25% (v/v) glutaraldehyde, each aliquot was loaded onto a 5 ml Percoll phosphate column in sterile 15 ml polypropionate centrifuge tubes. The column was allowed to settle for 30 min after which it was centrifuged at 3000 × g for 2 h. The supernatant was decanted and stained with 1 ml of 5 µg/ml diamidinophenyl indole (DAPI). This suspension was subsequently filtered with black filters (Osmonics^{TM}, 47 mm diameter, 0.8 µm pore size), using gentle suction. Each filter was placed on top of an inverted Petri dish, and the protozoa and nematodes, present on the entire filter, were enumerated using epifluorescence microscopy.

2.2.6. Enrichment for nitrifying bacteria. This procedure was conducted according to the method of Alef (1995). After 245 days of incubation, two enrichment media were used to determine the presence of ammonium and nitrite oxidizing bacteria in all the microcosms. The enrichment media for ammonium-oxidizing bacteria (pH 7.6) consisted of the following: 1.0 g/l $[NH_4]_2SO_4$, 0.5 g/l K_2HPO_4 , 2.0 g/l NaCl, 0.2 g/l $MgSO_4 \cdot 7H_2O$, 0.05 g/l $FeSO_4 \cdot 7H_2O$ and 6.0 g/l $CaCO_3$ as well as 10 ml trace element solution. The trace element solution consisted of the following (g/500ml distilled water): 0.25 H_3BO_3, 0.02 $CuSO_4 \cdot 5H_2O$, 0.05 KI, 0.10 $FeCl_3 \cdot 6H_2O$, 0.20 $MnSO_4 \cdot H_2O$, 0.10 $Na_2MoO_4 \cdot H_2O$, 0.2 $ZnSO_4 \cdot 7H_2O$, 0.5 NaCl, 0.05 $CoSO_4$, 0.05 $CaCl_2$ and 0.005 $AlK(SO_4)_2 \cdot 12H_2O$.

The enrichment media for nitrite oxidizing bacteria (pH 7.6) consisted of the following: 0.5 g/l K_2HPO_4, 6.0 g/l $CaCO_3$, 0.5 g/l NaCl, 0.1 g/l $NaNO_2$, 0.2 g/l $MgSO_4 \cdot 7H_2O$, and 0.005 g/l $FeSO_4 \cdot 7H_2O$. Both media were dispersed into 125 ml conical flasks, each receiving 50 ml of a respective medium, and autoclaved at 121°C for 20 min. After cooling, *circa* 0.2 g of soil from each microcosm was used to inoculate each flask. The

inoculated media were incubated at 30°C for 3 weeks in darkness on a shaker (125 rpm; 2 cm throw). Test sticks (Quantofix^{R}) were used to semi-quantitatively determine the concentrations of nitrate (10-500 mg/l) and nitrite (1-80 mg/l).

2.2.7. DNA Extraction and Purification. After 245 days of incubation, extraction of DNA from soil microcosms challenged with 0, *ca.* 30, *ca.* 100 and *ca.* 1000 ppm Cu, was performed according the method of Zhou *et al.* (1996). Each sample consisting of 0.375 g soil, was mixed with 1.0 ml of DNA extraction buffer at pH 8.0 (100 mM Tris-HCl, 100 mM EDTA, 100 mM sodium phosphate, 1.5 M NaCl, 1% (w/v) cetyltrimethylammonium bromide [CTAB]). The preparations were frozen at –70°C for 15 min and thawed at 60°C for 5 min. This step was repeated three times. After the freeze-thaw treatment, 5 µl of proteinase K (20 mg/ml) was added to each sample, and it was incubated on a rotary shaker at 225 rpm for 30 min at 37°C. Subsequently, 150 µl of 20% (w/v) SDS was added. The samples were then incubated at 65°C in a water bath for 2 h with gentle end-over-end inversions every 15 to 20 min.

After centrifugation at 6000 × g for 10 min, the supernatant of each sample was collected and transferred to a 15 ml polypropylene tube. The remaining soil pellet was re-extracted twice in the following manner: a solution containing 0.5 ml of extraction buffer and 50 µl of 20% SDS (w/v), was added to the pellet. The resulting suspension was mixed with a vortex for 10 s, incubated for 10 min at 65°C, and centrifuged (6000 × g; 10 min). Supernatants from the three cycles of extractions were combined and mixed with an equal volume of chloroform:isoamyl alcohol (24:1, vol/vol). The nucleic acids were precipitated from the aqueous phase by adding 0.6 volume of isopropanol. After 1 h of incubation on ice, a crude nucleic acid preparation was obtained by centrifugation (16000 × g; 20 min; room temperature). The precipitate was washed with cold 70% ethanol.

The resulting DNA samples were dried in a speed evaporator and dissolved in 200 µl Tris EDTA (TE) buffer (pH 8.0). The DNA extracts were subjected to electrophoresis on 0.8% (w/v) agarose gels containing ethidium bromide (10mg/ml). *Lambda* DNA digested with *Pst1* and *Hind111* (Amersham Pharmacia Biotech Inc.) respectively, were

used as molecular size markers. The DNA was purified by running the extracts through two MicroSpin™ S-300 HR columns (Amersham Pharmacia Biotech Inc.).

2.2.8. Polymerase Chain Reaction (PCR). The purified DNA was labeled with the oligonucleotide primers FAM63F (3'-CAGGCCTAACACATGCAAGTC-5') and HEX1389R (5'-ACGGGCGGTGTGTACAAG-3') specific for eubacteria (Osborn *et al.*, 2000). These primers were previously labeled at the 5' end with the phosphoramidite dyes 6-FAM and HEX respectively. Each PCR reaction, with a final volume of 50 µl, contained 300 ng template DNA, 5 µl thermophillic DNA 10 × buffer (Promega), 2µM $MgCl_2$, 0.25 µM of each primer, 200 µM each of dNTP's and 1 U *Taq* DNA Polymerase (Promega). The reactions were carried out in a Perkin-Elmer, Gene Amp 2400 thermocycler with an initial denaturation at 94°C for 2 min, followed by 30 cycles of amplification (1 min at 94°C, 1 min at 57°C and 2 min at 72°C). This was followed by a final extension step of 72°C for 10 min. Each PCR product (5µl) was visualized on a 0.8% (w/v) agarose gel, containing 1 µl ethidium bromide. The PCR products were subsequently purified using Nucleospin® Extract columns (Macherey-Nagel).

2.2.9. Terminal-Restriction Fragment Length Polymorphism (T-RFLP) analysis. Each PCR product was split into two equal volumes to produce two series of amplified DNA. The restriction enzymes *Hha1* and *Msp1* (Amersham Pharmacia Biotech Inc.) were used to digest the two series respectively. Each 30 µl digestion reaction mixture consisted of 20 µl of PCR product, 1 µl (10 U) of *Hha1* or *Msp1*, 3 µl restriction digest buffer and 6 µl sterile distilled water. The mixtures were incubated at 37°C for 10 hours. Placing the digestion reaction mixtures at 70°C for 15 min terminated the digestion reaction. The digested DNA was mixed with the DNA size standard, ROX 500 (Applied Biosystems), itself labeled with a distinct dye, and the DNA fragments were analyzed on an ABI 3100 genetic analyzer (Applied Biosystems).

2.3. Results

2.3.1. Concentrations of copper in soil. The concentration of Cu added to the soil as a component of copper oxychloride and the resulting concentrations of bio-available Cu are given in Table 2. As the Cu added to the soil was increased from 0 ppm to *circa* 1000 ppm, the concentration of Cu in the water phase of a saturated soil extract increased from 0.004 ppm to 0.947 ppm (*circa* 240 times). However, the concentration of exchangeable Cu, as measured in the di-ammonium EDTA extract (Beyers & Coetzer, 1971), increased from 2 ppm to 1100 ppm (*circa* 590 times). Subsequent comparisons were made using the Cu concentrations in the di-ammonium EDTA extract of the soil.

2.3.2. Enumeration of culturable bacteria. In all of the soil microcosms, except those that were challenged with 1112 ppm exchangeable Cu, the heavy metal had no significant lasting impact on the general microbial numbers as enumerated on TSA plates. There was an increase in the general microbial numbers during the initial 14 days of incubation, after which it more or less reached a plateau (Figure 2). However, compared to the control microcosms, the microbial numbers were lower in the microcosms that received *circa* 1000 ppm Cu. The numbers of fluorescent bacteria in the microcosms (Figure 3) generally peaked after 14 to 28 days of incubation.

Interestingly, in the presence of moderate amounts of Cu (34 and 126 ppm exchangeable Cu), the *Pseudomonas* numbers peaked at day 7 (Figure 4), whereas it peaked at day 3 in the presence of 2 and 1112 ppm exchangeable Cu. In the presence of 1112 ppm exchangeable Cu, the *Pseudomonas* numbers remained lower than the control throughout the monitoring period. A temporary decrease in actinomycete numbers after 28 days of incubation was noted in all the microcosms (Figure 5). However, the numbers of these organisms in the microcosms that received Cu, remained similar to that in the control microcosms.

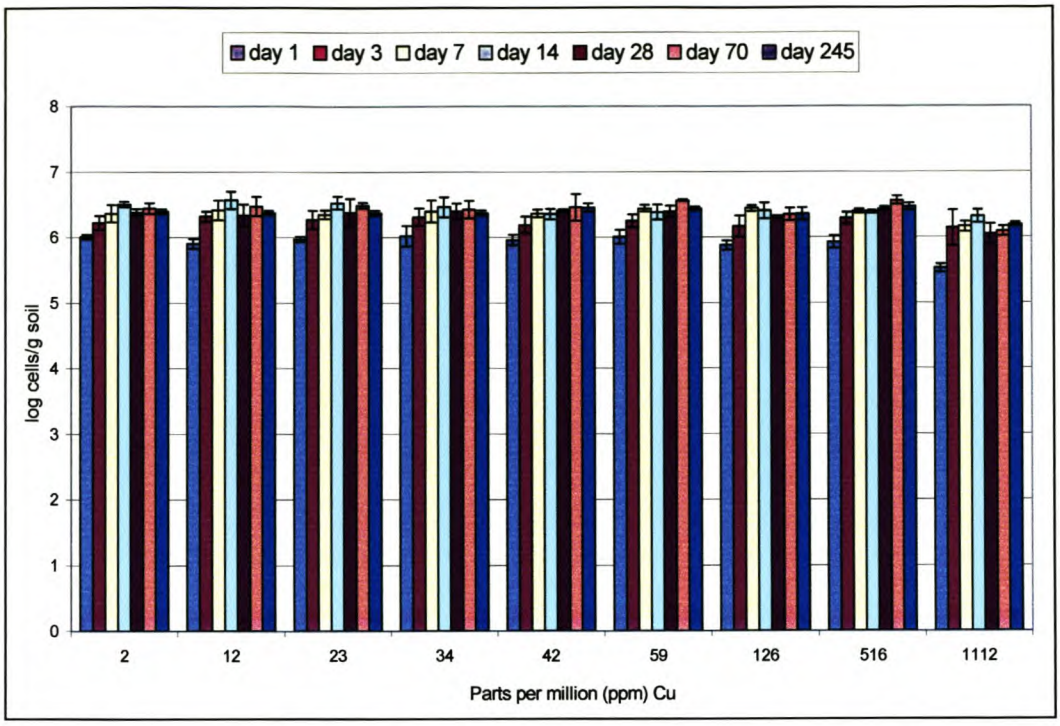


Figure 2. Total microbial counts of the soil microcosms. The y-axis represents log of the viable bacteria as enumerated on tryptone soy agar (each bar represents the mean of three repetitions). The x-axis represents the Cu concentration in a di-ammonium EDTA extract of the soil. The incubation periods after which the counts were made are depicted at the top of the graph.

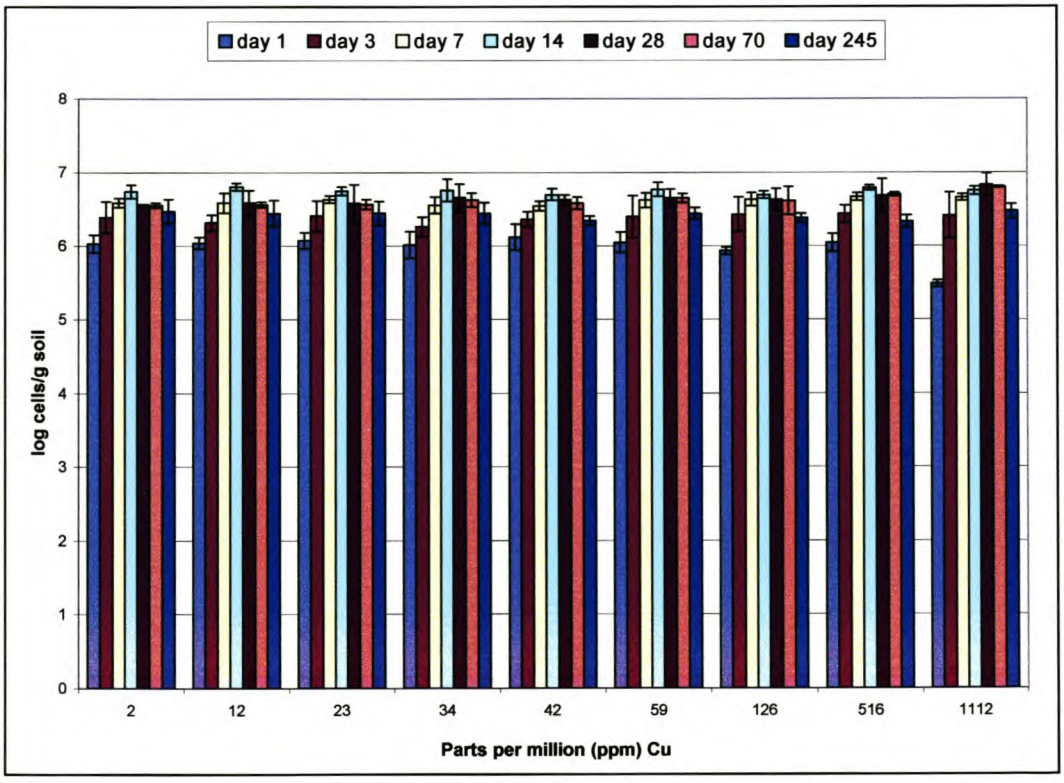


Figure 3. Fluorescent bacterial counts of the soil microcosms obtained on King's B agar. The y-axis represents log of the viable number of bacteria (each bar represents the mean of three repetitions). The x-axis represents the Cu concentration in a di-ammonium EDTA extract of the soil. The incubation periods after which the counts were made are depicted at the top of the graph.

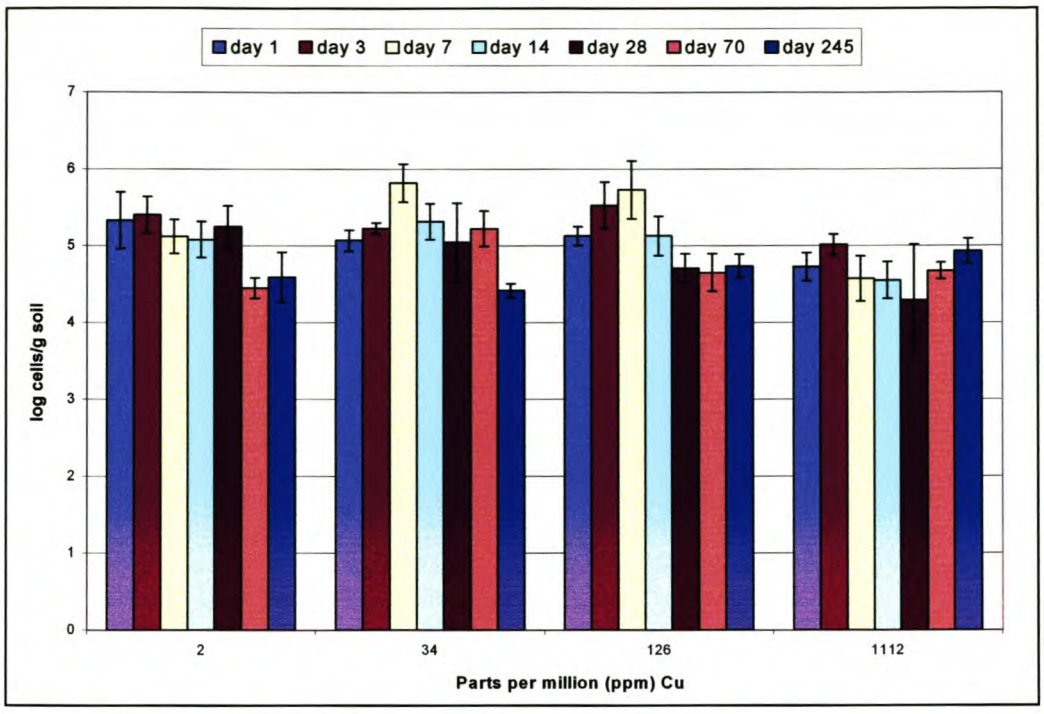


Figure 4. *Pseudomonas* counts of soil microcosms obtained on *Pseudomonas* CFC agar. The y-axis represents log of the viable bacterial counts (each bar represents the mean of three repetitions). The x-axis represents the Cu concentration in a di-ammonium EDTA extract of soil. The incubation periods after which the counts were made are depicted at the top of the graph.

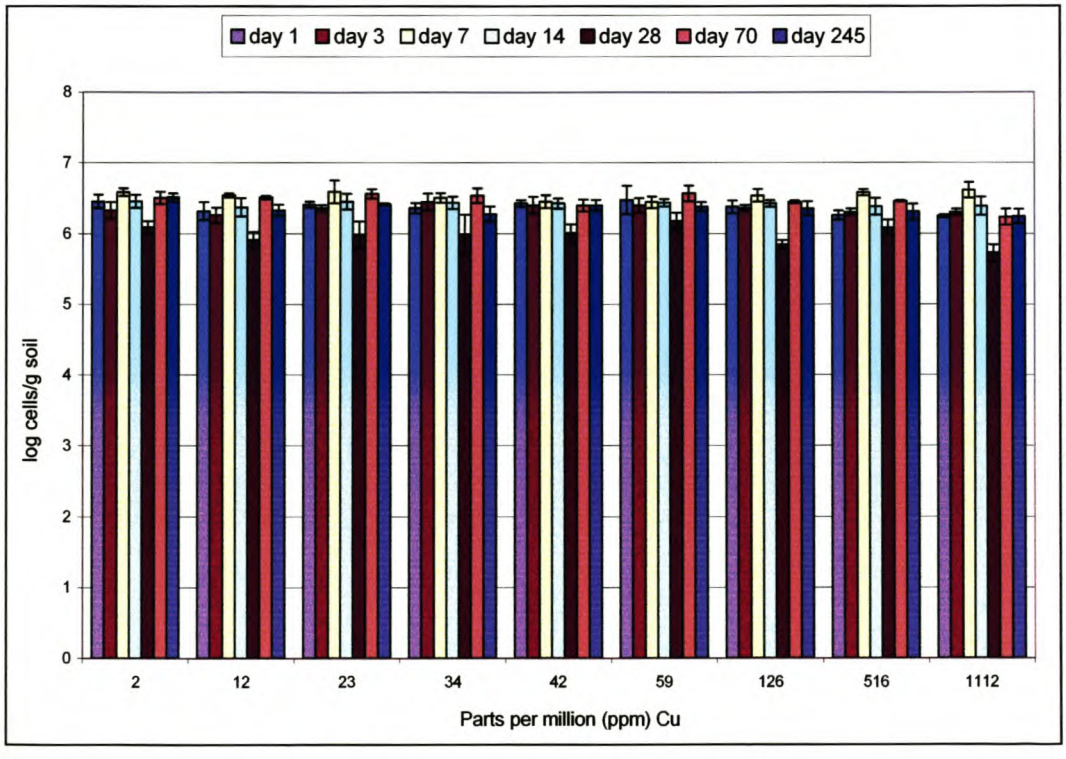


Figure 5. Actinomycete counts of soil microcosms obtained on sodium caseinate agar. The y-axis represents log of the viable number of actinomycetes (each bar represents the mean of three repetitions). The x-axis represents the Cu concentration in a di-ammonium EDTA extract of soil. The incubation periods after which the counts were made are depicted at the top of the graph.

2.3.3. Measurement of metabolic potential of the soil microbial community. The ability of the soil microbes to utilize the series of carbon compounds in the Biolog™ Eco microplates, were notably reduced at higher Cu concentrations in the microcosms (Tables 3 & 4). When these carbon utilization profiles were compared by Statistica computer software (StatSoft®) using cluster analysis, changes in the soil metabolic potential with increased Cu concentrations became apparent (Figure 6).

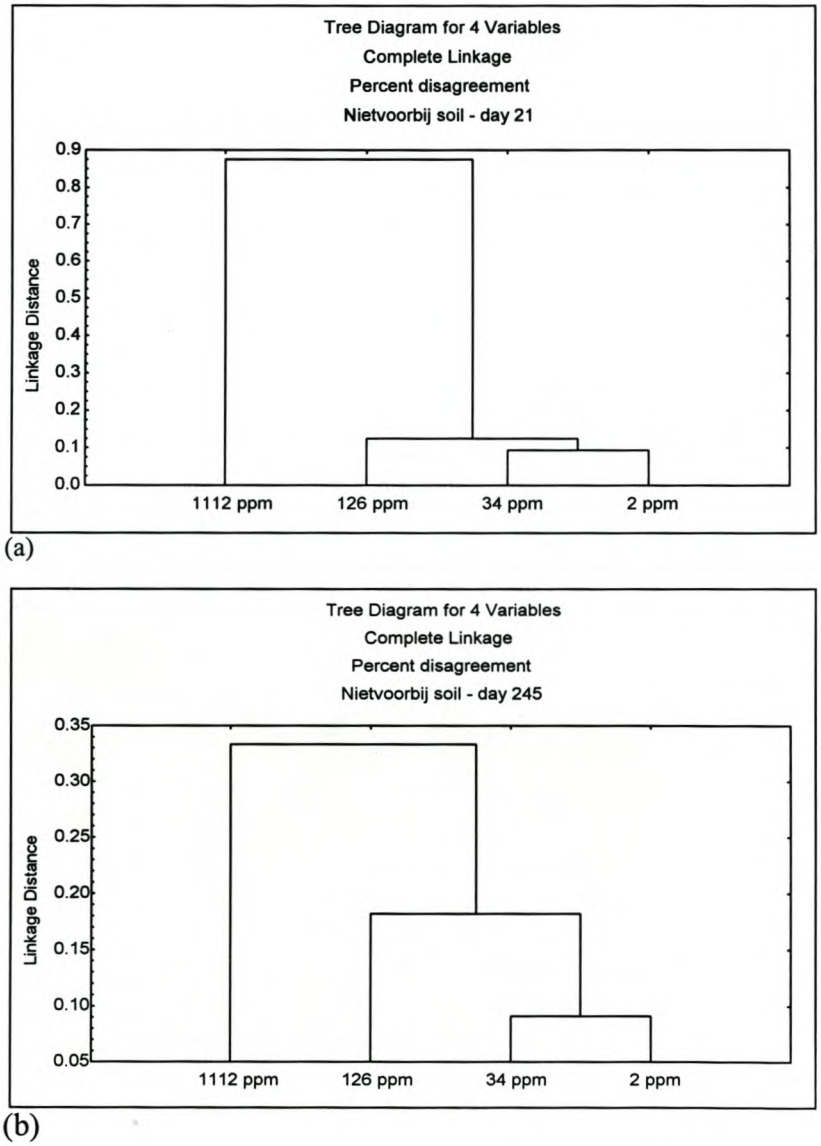


Figure 6. Dendrograms illustrating shifts in metabolic profiles after incubating the microcosms, containing different copper concentrations, for (a) 21 days and (b) 245 days. In each dendogram, the y-axis represents the linkage distance, while the x-axis represents the concentration of Cu as determined in the di-ammonium EDTA extract of the soil.

Table 3. The ability of the microbial communities in the microcosms, after 21 days of incubation, to utilize a series of carbon sources on Biolog™ Eco microplates.

2	34	126	1112	Cu (ppm) in di-ammonium EDTA extract
-	-	-	-	Water (No carbon source)
+	-	-	-	ß-Methyl-D- Glucoside
+	+	-	-	D-Galactonic Acid γ-Lactone
+	+	-	-	L-Arginine
+	+	-	-	Pyruvic Acid Methyl Ester
+	+	-	-	D-Xylose
+	+	-	-	D-Galacturonic Acid
+	+	+	-	L-Asparagine
+	+	-	-	Tween 40
+	-	-	-	i-Erythritol
+	+	-	-	2-Hydroxy Benzoic Acid
+	+	-	-	L-Phenylalanine
+	+	-	-	Tween 80
+	+	-	-	D-Mannitol
+	+	-	-	4-Hydroxy Benzoic Acid
+	+	-	-	L-Serine
+	+	-	-	α-Cyclodextrin
+	+	-	-	N-Acetyl-D-Glucosamine
+	+	-	-	γ-Hydroxybutyric Acid
+	+	+	+	L-Threonine
+	+	-	-	Glycogen
+	-	-	-	D-Glucosaminic Acid
+	+	-	-	Itaconic Acid
+	+	-	-	Glycyl-L-Glutamic Acid
+	+	-	-	D-Cellobiose
+	-	-	-	Glucose-1-Phosphate
-	-	-	-	α-Ketobutyric Acid
+	+	+	-	Phenylethyl-amine
+	+	-	-	α-D-Lactose
+	+	-	-	D,L-α-Glycerol Phosphate
+	+	+	-	D-Malic Acid
+	+	+	-	Putrescine
30	27	27	2	Total no. of C-sources utilized

+ : Carbon-source utilized; - : Carbon-source not utilized

Table 4. The ability of the microbial communities in the microcosms, after 245 days of incubation, to utilize a series of carbon sources on Biolog™ Eco microplates.

2	34	126	1112	Cu (ppm) in di-ammonium EDTA extract
-	-	-	-	Water (No carbon source)
+	+	-	-	ß-Methyl-D- Glucoside
+	+	+	+	D-Galactonic Acid γ-Lactone
+	+	+	+	L-Arginine
+	+	+	-	Pyruvic Acid Methyl Ester
+	+	+	+	D-Xylose
+	+	+	+	D-Galacturonic Acid
+	+	+	+	L-Asparagine
+	+	+	+	Tween 40
+	-	-	-	i-Erythritol
'	+	+	-	2-Hydroxy Benzoic Acid
+	+	+	-	L-Phenylalanine
+	+	+	+	Tween 80
+	+	+	-	D-Mannitol
+	+	+	+	4-Hydroxy Benzoic Acid
+	+	+	+	L-Serine
+	+	+	+	α-Cyclodextrin
+	+	+	+	N-Acetyl-D-Glucosamine
+	+	+	+	γ-Hydroxybutyric Acid
+	-	-	-	L-Threonine
+	+	+	-	Glycogen
+	+	+	+	D-Glucosaminic Acid
+	+	+	+	Itaconic Acid
+	+	+	-	Glycyl-L-Glutamic Acid
+	+	+	+	D-Cellobiose
+	+	+	-	Glucose-1-Phosphate
-	-	-	-	α-Ketobutyric Acid
+	-	+	+	Phenylethyl-amine
+	+	-	-	α-D-Lactose
+	+	-	-	D,L-α-Glycerol Phosphate
+	+	+	+	D-Malic Acid
+	+	+	+	Putrescine
29	28	25	19	Total no. of C-sources utilized

+ : Carbon-source utilized; - : Carbon-source not utilized

2.3.4. Enumeration of protozoa and nematodes. The numbers of protozoa in the soil microcosms were significantly reduced by the addition of Cu (Figures 7 & 8). However, no significant reduction in the number of nematodes was observed.

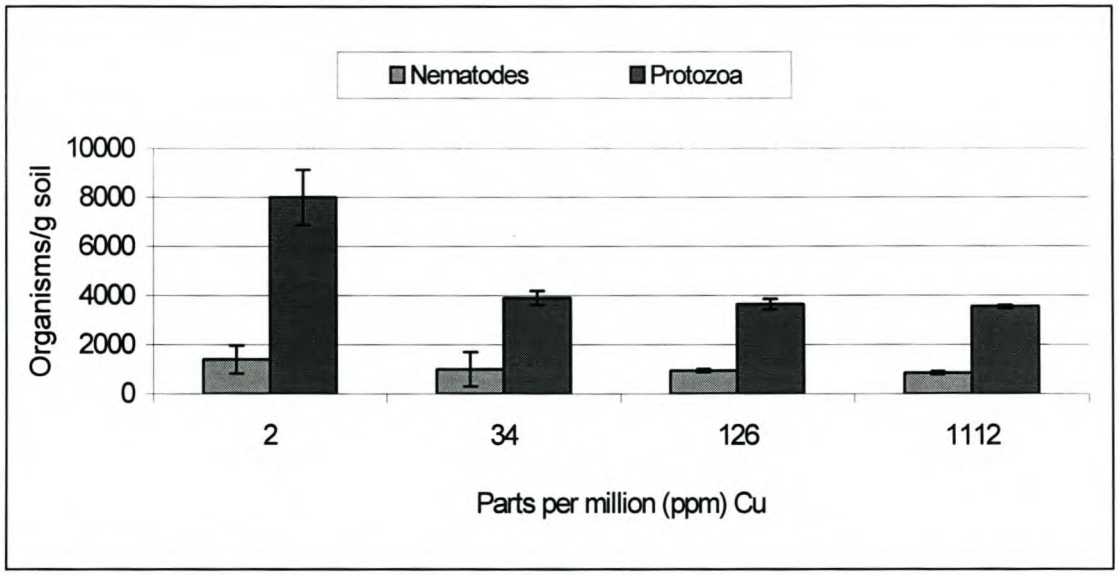


Figure 7. The numbers of protozoa and nematodes in selected microcosms after 70 days of incubation. The y-axis represents the viable numbers of protozoa and nematodes (each bar represents the mean of three repetitions). The x-axis represents the Cu concentration in a di-ammonium EDTA extract of soil.

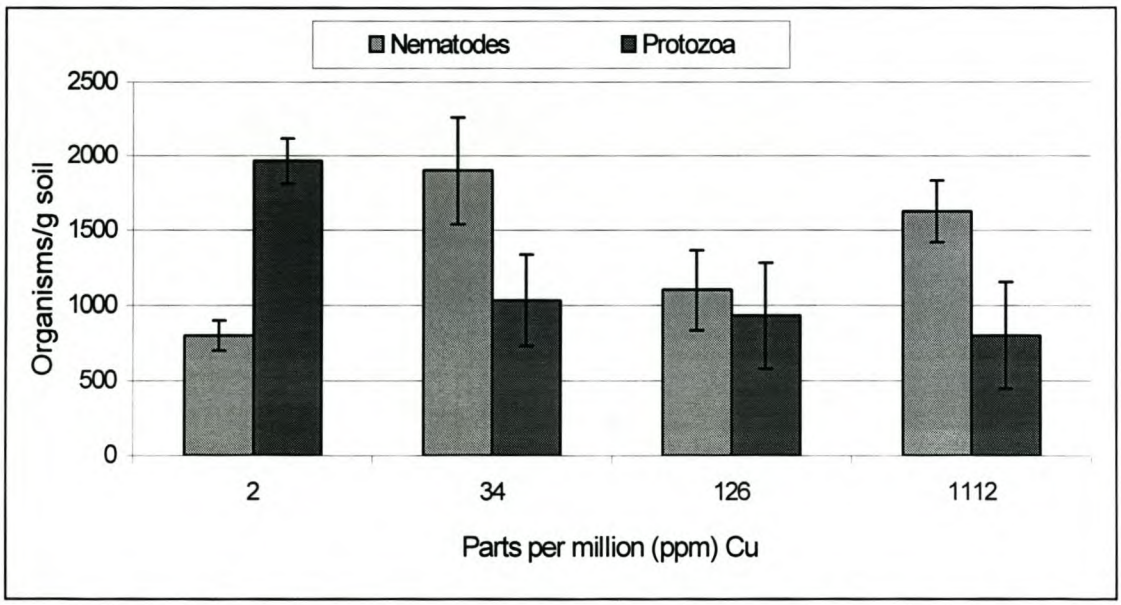


Figure 8. The numbers of protozoa and nematodes in selected microcosms after 245 days of incubation. The y-axis represents the viable numbers of protozoa and nematodes (each bar represents the mean of three repetitions) The x-axis represents the Cu concentration in a di-ammonium EDTA extract of soil.

2.3.5. Enrichment for nitrifying bacteria. The nitrifying bacteria survived the addition of Cu in all the microcosms (Tables 5, 6, 7 & 8). While significant quantities of residual nitrite remained in most enrichment cultures for nitrite-oxidizing bacteria, nitrate formation occurred in all enrichment cultures for ammonium-oxidizing bacteria.

2.3.6. T-RFLP analysis. All the T-RFLP patterns of the PCR products digested with *Hha1*, except those originating from microcosms B30, B100, A1000 and B1000, generated a terminal-restriction fragment (T-RF) at 332 base pairs (bp) (Table 9; also see Appendix; Figures 1, 2, 3 & 4). A 168-bp T-RF was generated when the PCR products originating from microcosms C0, A1000, B1000 and C1000 were digested with *Hha1*. The electropherograms of the PCR products originating from microcosms B100, C100, A1000 and C1000 showed the presence of a 283-bp T-RF that was absent in all the other samples digested with *Hha1*. When *Msp1* was used as restriction enzyme to digest the PCR products, no difference was observed between the T-RFLP patterns of PCR products of different bacterial communities challenged with different Cu concentrations. All the T-RFLP patterns of the PCR products digested with *Msp1* generated a T-RF at 452 bp (Table 9; also see Appendix; Figures 5, 6, 7 & 8).

2.4. Discussion

The microbial numbers within the microcosms during the monitoring period, as determined using plate counts, were not static. The increase in microbial numbers during the initial 28 days, in all the microcosms (Figures 2 & 3), may be attributed to the fact that the soil was re-wetted after it was dried for 2 weeks prior to initiation of the experiment. This phenomenon was also illustrated by Franzleubbers *et al.* (2000). The temporary decrease that occurred in the actinomycete numbers after 28 days of incubation (Figure 5) may be attributed to an experimental error since it also occurred in the control microcosms.

Table 5. The concentrations of *nitrate* determined after using a soil dilution as inoculum for a *selective medium for ammonium-oxidizing bacteria.*

	Parts per million of Cu in di-ammonium EDTA extract of soil.								
	2	12	23	34	42	59	126	516	1112
A	+++	+++	+++	+++	+++	+++	+	+	++
B	+++	+	+++	+++	++	++	++	+	++
C	+++	+++	+++	+++	+++	+++	+	+	+

+++ indicates that ≥ 500 mg/l nitrate was formed
++ indicates that 100 ≤ 499 mg/l nitrate was formed
+ indicates that 1 ≤ 99 mg/l nitrate was formed
A, B & C represent triplicate microcosms.

Table 6. The concentrations of *nitrite* determined after using a soil dilution as inoculum for a *selective medium for ammonium-oxidizing bacteria.*

	Parts per million of Cu in di-ammonium EDTA extract of soil.								
	2	12	23	34	42	59	126	516	1112
A	+++	+++	+++	+++	+++	+++	+	+	++
B	+++	+	+++	+++	++	++	++	+	++
C	+++	+++	+++	+++	+++	+++	+	+	+

+++ indicates that ≥ 80 mg/l of nitrite was formed
++ indicates that 20 ≤ 79 mg/l of nitrite was formed
+ indicates that 1 ≤ 19 mg/l of nitrite was formed
A, B & C represent triplicate microcosms.

Table 7. The concentrations of *nitrate* determined after using a soil dilution as inoculum for a *selective medium for nitrite-oxidizing bacteria.*

	Parts per million of Cu in di-ammonium EDTA extract of soil.								
	2	12	23	34	42	59	126	516	1112
A	+	+++	+++	++	+++	+++	++	++	++
B	+++	+++	++	++	++	++	++	++	+++
C	++	++	+++	++	++	+	+++	++	+++

+++ indicates that ≥ 500 mg/l nitrate was formed
++ indicates that 100 ≤ 499 mg/l nitrate was formed
+ indicates that 1 ≤ 99 mg/l nitrate was formed
A, B & C represent triplicate microcosms.

Table 8. The concentrations of *nitrite* determined after using a soil dilution as inoculum for a *selective medium for nitrite-oxidizing bacteria.*

	Parts per million of Cu in di-ammonium EDTA extract of soil.								
	2	12	23	34	42	59	126	516	1112
A	+	++	++	++	+++	++	++	++	++
B	++	++	++	++	++	++	+++	++	++
C	++	++	+++	++	++	-	++	++	++

+++ indicates that ≥ 80 mg/l of nitrite was still present in the medium
++ indicates that 20 ≤ 79 mg/l of nitrite was still present in the medium
+ indicates that 1 ≤ 19 mg/l of nitrite was still present in the medium
- indicates that no nitrite was present in the medium
A, B & C represent triplicate microcosms.

Table 9. Representation of the T-RFLP community fingerprint patterns obtained from the different microcosms.

Microcosm	Cu concentration in a di-ammonium EDTA extract (mg/kg)	*Hha1* Fragment size			*Msp1* Fragment size
		168 bp	283 bp	332 bp	452 bp
		Intensity (relative fluorescent units - RFU)			
A 0		0	0	106	95
B 0	1.89 ± 0.04	0	0	23	60
C 0		13	0	22	25
A 30		0	0	108	62
B 30	33.79 ± 4.50	0	0	0	30
C 30		0	0	15	no result
A 100		0	0	20	14
B 100	125.69 ± 2.90	0	50	0	16
C 100		0	10	76	80
A 1000		24	59	0	9
B 1000	1112.40 ± 52.17	22	0	0	25
C 1000		168	20	198	128

A, B & C represent triplicate microcosms

Culturable populations of microorganisms, either total populations or specific bacterial groups, have been used to monitor changes in soil biota in response to land management and, therefore, as indicators of the biological status of the soil (Maltby, 1975; Kale & Raghu, 1989). Although plate counts correlate poorly with microbial biomass and enzymatic measurements of microbial growth (Frankenberg & Dick, 1983), there is evidence that plate count techniques are useful in comparative studies of specific microbial populations (Harris & Birch, 1992). A study conducted by Smit *et al.* (1997) showed no differences from direct counting, but did find a lower diversity of isolates in soils contaminated with 750 kg/ha added Cu compared to uncontaminated soil. In this study, Cu had little affect on fluorescent bacterial and actinomycete numbers in the soil and only slightly affected general microbial and *Pseudomonas* numbers in the microcosms challenged with *circa* 1000 ppm Cu. Subsequently, Biolog™ analyses were performed to examine the possibility of shifts occurring in those microbial communities challenged with additional Cu.

Characterization of community-level substrate utilization via incubation of environmental samples in Biolog™ microtiter plates has been used as a sensitive and

reproducible tool for characterizing microbial communities (Garland, 2000). In this study, the Biolog™ analysis revealed a decrease in carbon-source utilization by the soil microbial communities as well as a shift in the carbon sources that could be utilized, as the Cu concentration was increased (Tables 3 & 4). This indicated that shifts in the soil microbial community did indeed take place. These shifts may be ascribed to some microbial species being killed by the increased Cu concentration, resulting in other species with different nutritional needs taking its place within the soil. The results also revealed that more carbon-sources were utilized after 245 days than after 21 days of incubation. This may be due to certain species, previously present in such low numbers that it could not be detected by the Biolog™ Eco microplate, increasing in abundance as other species were killed by the increasing Cu concentration. However, to confirm these observations, T-RFLP fingerprint patterns of the different soil microcosms were analyzed.

According to Lukow *et al.* (2000), the T-RFLP technique generates community fingerprint patterns in a format that provides a means for the estimation of similarities and dissimilarities between bacterial diversity profiles obtained by this technique. Compared to the results of Liu *et al.* (1997), who frequently found peaks in excess of 1000 relative fluorescent units (RFU), the peak intensities of the T-RFs obtained in this study were low (less than 200 RFU). This may indicate that the particular bacteria selected for by the specific primers and restriction enzymes in this technique were present in low numbers. However, in this study the variation in peak intensities between different microcosms and even within triplicate microcosms was disregarded, because it may reflect some inconsistencies in the method of sampling. Nevertheless, the T-RFLP fingerprint pattern varied between microcosms, even within some triplicate microcosms, indicating that the microbial populations of the different microcosms differed from each other.

The T-RFLP community fingerprint for the PCR products digested with *Hha1* showed that as the Cu concentrations increased, shifts occurred in the community composition of some microcosms (Table 9). As a general rule, a single species will contribute a uniquely sized terminal fragment although several species may have terminal fragments of identical size (Marsh *et al.*, 2000). The bacterium represented by the 168-bp T-RF

could not be identified using the TAP-TRFLP database (http://rdp.cme.msu.edu/html/TAP-trflp.html). A 283-bp T-RF representing an unknown eubacterium was only detected in the microcosms challenged with 126 or 1112 ppm exchangeable Cu. Higher Cu concentrations in the soil therefore seemed to be advantageous for this bacterium. Another bacterium represented by a 332 bp T-RF in the *Hha1* digestions occurred in a number of microcosms, irrespective of the Cu concentrations. This bacterium was later tentatively identified, using the TAP-TRFLP database, as either from the genus *Pseudomonas* or *Pseudoalteromonas.* Interestingly, it is known that members of the genus *Pseudomonas* are resistant to Cu-containing compounds since they carry plasmid-borne copper resistance genes (Bender & Cooksey, 1986).

In contrast, the PCR products digested with *Msp1* did not show notable shifts in community composition as the Cu concentration increased (Table 9). For example, the results obtained with *Msp1* digestions showed that the presence of the specific organism, represented by the 452-bp T-RF, was not affected by an increase in copper concentration. This bacterium was tentatively identified to be either from the genus *Pseudoalteromonas* or *Pseudomonas* using the TAP-TRFLP database. To summarize our findings on the T-RFLP analysis, the results support our observations on the changes that occurred in the metabolic potential of the microbial communities as the Cu concentration was increased in the soil. It must, however, be noted that other combinations of labeled primers and restriction enzymes may have generated T-RFs with different sizes and intensities.

Population shifts may also have occurred within the soil nematode communities (Elliot, 1997). However, the nematode numbers in our study were not significantly reduced with increased Cu concentrations (Figures 7 & 8), instead it rather increased in the presence of higher Cu concentrations toward the end of the incubation period (Figure 8). Studies of several authors on nematode trophic group structure in metal contaminated soils revealed that some groups, such as the predatory nematodes and the bacterial feeders, may increase in abundance at higher Cu concentrations whilst other groups such as the plant parasitic nematodes and omnivores may show little change or decrease in numbers (Weiss & Larink, 1991; Yeats *et al.*, 1994). This could explain why in this study, nematode numbers generally appear to be little affected by heavy metals and in

some instances their numbers may even increase substantially with increase in heavy metal concentration (Weiss & Larink, 1991).

Measurement of nitrification is recommended as a key test for assessing changes in soil health as a consequence of agrochemical application (Domsch *et al.*, 1983). These authors stated that a decrease in the formation of nitrate and nitrite is coupled to a decrease in the number of nitrifying bacteria. However, since we employed an enrichment procedure for nitrifying bacteria surviving in the soil, we were unable to directly determine the viable numbers of these bacteria in the soil microcosms. We were only able to determine that ammonium-oxidizing and nitrate-producing bacteria survived the additional Cu in all the microcosms (Table 5, 6, 7 & 8).

Our direct counts of soil protozoa, however, revealed that the addition of Cu to the soil impacted negatively on the numbers of these eukaryotes (Figures 7 & 8). The decrease in protozoan numbers, with the addition of Cu, may be attributed to their delicate external membranes (Foissner, 1994). Importantly, it is known that a decrease in protozoan numbers may have a negative impact on the flow of nutrients in soil and subsequently on plants and other microorganisms (Griffiths, 1994).

2.5. Conclusions

General microbial and fluorescent bacterial numbers as well as actinomycete and *Pseudomonas* numbers, were little affected by the addition of copper oxychloride to the soil. However, analysis of T-RFLP community fingerprints and especially analysis of the whole community metabolic profiles using Biolog™ Eco microplates, revealed that shifts in the soil microbial communities took place as the Cu concentration increased. The metabolic potential of the soil decreased as the Cu concentration increased. Also, it was found that a significant decrease in protozoan numbers occurred when the Cu concentration in a di-ammonium EDTA extract reached or exceeded *circa* 30 ppm. This may impact negatively on the flow of nutrients in the soil ecosystem. However, our observations were based on the impact of copper oxychloride on the ecosystem present in only one type of soil. Therefore, since protozoa enumeration and whole community

metabolic profiling gave the most significant results, these two potential bioindicators were subsequently used in the following chapter to study the impact of this Cu-containing compound on three additional soil ecosystems.

2.6. References

Alef, K. (1995) Enrichment of physiological groups. In: Alef, K. and Nannipieri, P. (eds.), *Methods in Applied Soil Microbiology and Biochemistry.* Academic Press, London, p.132.

Atlas, R.M. (1984) Use of microbial diversity measurements to asses environmental stress. In: Klug, M.J. and Reddy, C.A. (eds.), *Current perspectives in microbial ecology.* American Society for Microbiology, Washington, pp.540-545.

Baker, D.E. and Senft, J.P. (1995) Copper. In: Alloway, B.J. (ed.), *Heavy Metals in Soils*. Blackie Academic and Professional, Bishopbriggs, pp.179-205.

Bender, C.L. and Cooksey, D.A. (1986) Indigenous plasmids in *Pseudomonas syringae* pv. *tomato*: conjugative transfer and role in copper resistance. *Journal of Bacteriology* 165, 534-541.

Beyers, C.P. and Coetzer, F.J. (1971) Effect of concentration, pH and time on the properties of di-ammonium EDTA as a multiple soil extractant. *Agrochemophysica* 3, 49-54.

Bremner, J.M. (1965) Inorganic forms of nitrogen. In: Black, C.A. (ed.), *Methods of soil analysis. Part 2.* American Society of Agronomy, Madison, Wisconsin.

Chaphekar, S.B. (1978) Biological indicators. The concept of new addition. *International Journal of Ecology and Environmental Science* 4, 45-52.

Conradie, W. J. (2001) Personal communication. Soil scientist, ARC-Infruitec/Nietvoorbij, Stellenbosch.

Doelman, P. (1985) Resistance of soil microbial communities to heavy metals. In: Jensen, V., Kjöller, A. and Sörensen, L.H. (eds.), *Microbial communities in soil.* Elsevier, London, pp.369-384.

Domsch, K.H., Jagnow, G. and Anderson, T.-H. (1983) An ecological concept for the assessment of side-effects of agrochemicals on soil microorganisms. *Residue Reviews* 86, 65-105.

Doll, E.C. and Lucas, R.E. (1973) Testing soils for potassium, calcium and magnesium. In: Walsh, L.M. and Beatons, J.D. (eds.), *Soil testing and plant analysis.* Soil Science Society of America, Madison, Wisconsin, pp.133-152.

Elliot, E.T. (1997) Rationale for developing bioindicators. In: Pankhurst, C.E., Doube, B.M. and Gupta, V.V.S.R. (eds.), *Biological indicators of soil health.* CAB International, Wallingford, pp.49-78.

Fertilizer Society of South Africa. (1974) Manual of soil analysis methods. *FSSA Publication No. 37.*

Flores-Vélez, L.M., Ducaroir, J., Jaunet, A.M. and Robert, M. (1996) Study of the distribution of copper in an acidic vineyard soil by three different methods. *European Journal of Soil Science* 47, 523-532.

Foissner, W. (1994) Soil protozoa as bioindicators in ecosystems under human influence. In: Darbyshire, J.F. (ed.), *Soil Protozoa.* CAB International, Wallingford, pp.147-194.

Frankenberg, W.T. and Dick, W.A. (1983) Relationship between enzyme activity and microbial growth and activity indices in soil. *Soil Science Society of America Journal* 47, 945-951.

Franzluebbers, A.J., Haney, R.L., Honeycutt, C.W., Schomberg, H.H. and Hons, F.M. (2000) Flush of carbon dioxide following rewetting of dried soil relates to active organic pools. *Soil Science Society of America Journal* 64, 613-623.

Fry, M. St. L. (1987) *A detailed characterization of soils under different fynbos-climate-geology combinations in the southwestern Cape.* M.Sc. Thesis. University of Stellenbosch, Stellenbosch, South Africa.

Garland, J.L. (2000) Potential and limitations of BIOLOG for microbial community analysis. In: Bell, C.R., Brylinsky, M. and Johnson-Greene, P. (eds.), *Proceedings of the 8th International Symposium on Microbial Ecology.* Atlantic Canada Society for Microbial Ecology, Halifax, pp.521-527.

Grayston, S.J., Campbell, C.D. and Vaughan, D. (1994) Microbial diversity in the rhizospheres of different tree species. In: Pankhurst, C.E. (ed.), *Soil Biota: Management and Sustainable Farming Systems*, Poster Papers. CSIRO Press, Melbourne, pp.155-157.

Griffiths, B.S. (1994) Soil nutrient flow. In: Darbyshire, J.F. (ed.), *Soil Protozoa.* CAB International, Wallingford, pp.65-91.

Griffiths, B.F. and Ritz, K. (1988) A technique to extract, enumerate and measure protozoa from mineral soils. *Soil Biology and Biochemistry* 20, 163-173.

Harris, J.A. and Birch, P. (1992) Land reclamation and restoration. In: Fry, J.C., Gadd, G.M., Herbert, R.A., Jones, C.W. and Watson-Craik, I.A. (eds.), *Microbial control of pollution.* Society of General Microbiology Symposium No. 48, Cambridge University Press, pp.269-291.

Hiroki, M. (1992) Effects of heavy metal contamination on soil microbial population. *Soil Science and Plant Nutrition* 38, 141-147.

Holben, W.E. (1994) Isolation and purification of bacterial DNA from soil. In: Weaver, R.W., Angle, S., Bottomley, P., Bezdicek, D., Smith, S., Tabatabai, A. and Wollum, A.

(eds.), *Methods in Soil Analysis. Part 2. Microbiological and Biochemical Properties.* Soil Science Society of America, Madison, Wisconsin, pp.727-751.

Kale, S.P. and Raghu, K. (1989) Relationship between microbial numbers and other microbial indices in soil. *Bulletin of Environmental Contamination and Toxicology* 43, 941-945.

Labuschagne, T. (2001) Personal communication. Management of Chempack cc., Paarl.

Liu, W.T., Marsh, T.L., Cheng, H. and Forney, L.J. (1997) Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Applied and Environmental Microbiology* 63, 4516-4522.

Longenecker, D.E. and Lyerly, P.J. (1964) Making soil pastes for salinity analysis. A reproducible capillary procedure. *Soil Science* 97, 268-275.

Lukow, T., Dunfield, P.F. and Liesack, W. (2000) Use of the T-RFLP technique to assess spatial and temporal changes in the bacterial community structure within an agricultural soil planted with transgenic and non-transgenic potato plants. *FEMS Microbiology Ecology* 32, 241-247.

Maltby, E. (1975) Numbers of soil microorganisms as ecological indicators of changes resulting from moorland reclamation on Exmoor: UK. *Journal of Biogeography* 2, 117-136.

Marsh, T.L., Saxman, P., Cole, J. and Tiedje, J. (2000) Terminal restriction fragment length polymorphism analysis program, a web-based research tool for microbial community analysis. *Applied and Environmental Microbiology* 66, 3616-3620.

McBride, M.B. (1994) *Environmental chemistry of soil.* Oxford University Press, Inc., New York.

McClean, E.O. (1982) Soil pH and lime requirement. In: Page, A.L. (ed.), *Methods of soil analysis. Part 2.* American Society of Agronomy, Madison, Wisconsin, pp.570-571.

Naeem, S., Thompson, L.J., Lawler, S.P., Lawton, J.H. and Woodfin, R.M. (1995) Declining biodiversity can alter the performance of ecosystems. *Nature* 368, 734-737.

Nelson, D.W. and Sommers, L.E. (1982) Total carbon, organic carbon and organic matter. In: Page, A.L. (ed.), *Methods of soil analysis. Part 2.* American Society of Agronomy, Madison, Wisconsin, p.199.

Osborn, A.M., Moore, R.B. and Timmins, K.N. (2000) An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. *Environmental Microbiology* 2, 39-50.

Pankhurst, C.E. (1997) Biodiversity of soil organisms as indicators of soil health: Synthesis. In: Pankhurst, C.E., Doube, B.M. and Gupta, V.V.S.R. (eds.), *Biological indicators of soil health.* CAB International, Wallingford, pp.297-324.

Sandaa, R.-A., Torsvik, V., Enger, Ø., Daae, F.L., Castberg, T. and Hahn, D. (1999) Analysis of bacterial communities in heavy metal-contaminated soils at different levels of resolution. *FEMS Microbiology Ecology* 30, 237-251.

Smit, E., Leeflang, P. and Wernars, K. (1997) Detection of shifts in microbial community structure and diversity in soil caused by copper contamination using amplified ribosomal DNA restriction analysis. *FEMS Microbiology Ecology* 23, 249-261.

Soil classification working group (1991) Soil classification. A taxonomic system for South Africa. *Memoirs on the Agricultural Natural Resources of South Africa No 15.* Department of Agricultural Development, Pretoria, South Africa.

Thomas, G.W. and Peaslee, D.E. (1973) Testing for soil phosphorus. In: Walsh, L.M. and Beatons, J.D. (eds.), *Soil testing and plant analysis.* Soil Science Society of America, Madison, Wisconsin, pp.15-122.

Van der Watt, H.V.H. (1966) Improved tables and a simplified procedure for soil particle size analysis by the hydrometer method. *South African Journal of Agricultural Science* 9, 911-916.

Weiss, B. and Larink, O. (1991) Influence of sewage sludge and heavy metals on nematodes in an arable soil. *Biology and Fertility of Soils* 12, 5-9.

Yeates, G.W., Orchard, V.A., Speir, T.W., Hunt, J.L. and Hermans, M.C.C. (1994) Reduction in soil biological activity following pasture contamination by copper, chromium, arsenic timber preservative. *Biology and Fertility of Soils* 18, 200-208.

Zak, J.C., Willig, M.R., Moorhead, D.L. and Widham, H.G. (1994) Functional diversity of microbial communities: a quantitative approach. *Soil Biology and Biochemistry* 26, 1101-1108.

Zhou, J., Bruns, M.A. and Tiedje, J.M. (1996) DNA recovery from soils of diverse composition. *Applied and Environmental Microbiology* 62, 316-322.

CHAPTER 3

THE USE WHOLE COMMUNITY METABOLIC PROFILES AND PROTOZOA NUMBERS TO DETERMINE THE IMPACT OF COPPER OXYCHLORIDE ON SOIL ECOSYSTEMS

3.1. Introduction

Characterization of community-level substrate utilization by means of Biolog™ microplates has been increasingly applied in many areas of microbial ecology (Garland, 2000). This multivariate profile allows for intensive spatial and temporal analysis of microbial communities, and was found to be a sensitive and reproducible tool for comparing microbial communities. However, since shifts in whole community metabolic profiles can not be related to changes in the abundance of specific phylogenetic or functional groups, results can not be used as the sole basis for defining mechanistic models of the factors affecting community stability.

Protozoa are increasingly being used as bioindicators in soil because changes in their dynamics and community structure influence the rate and kind of soil formation and fertility (Foisner, 1994; Foisner, 1987). Protozoa are at the base of the heterotrophic eukaryotic food web and an essential component in soil ecosystems because they consume a significant portion of the bacterial productivity, thereby enhancing nutrient cycles and energy flows to the benefit of microorganisms, plants and animals (Foissner, 1999).

When the impact of copper oxychloride added to soil was determined on the microcosms present in uncultivated agricultural soil, it was found that the general microbial counts, as well as the numbers of actinomycetes and pseudomonads were not affected much (Chapter 2). The nematode numbers increased slightly towards the end of the monitoring period. However, the most significant impact was on the metabolic potential and protozoan numbers in the soil. To confirm these findings it was decided to test the impact of copper oxychloride on the latter two bioindicators in other soil ecosystems. Consequently, whole community metabolic profiling was used in conjunction with protozoa enumeration to determine the impact of increased copper concentrations on

potting soil obtained from Nietvoorbij experimental farm, as well as fynbos and vineyard soil originating from Koopmanskloof wine farm in the Stellenbosch region.

3.2. Materials and Methods

3.2.1. Collection and preparation of soil. Soil was collected from Nietvoorbij experimental farm and Koopmanskloof wine farm in the Stellenbosch region. The soil from Nietvoorbij was prepared for potting experiments and was classified as a sandy soil. The soils from the wine farm were fynbos and vineyard soils that were sandy loamy soils. Fynbos, a unique collection of hard leafed evergreen shrubs, adapted for the Mediterranean climate of the south-western Cape Province, is an indigenous vegetation type to southern Africa (Versfeld et al. 1992). The two soils were prepared for experimentation in the following manner: after the organic matter at the surface was removed, the top 30 cm of soil was collected. *Circa* 600 kg of soil from each of the respective farms was allowed to dry for 2 weeks at 30°C, whereafter it was sieved (pore size 2 mm). The main physical and chemical properties of the sieved soils are listed in Table 1.

3.2.2. Preparation of soil microcosms. For each soil type, a series of soil microcosms was prepared by adding various concentrations of copper oxychloride ($CuCl_2 \bullet 2CuO \bullet 4H_2O_4$) to the soil. The series consisted of *ca.* 2 kg aliquots of soil, contained in plastic soil bags, that received the following estimated amounts of copper: 0 (control microcosm), 30, 100 and 1000 ppm. The final concentration of bio-available Cu in each microcosm was subsequently determined and is listed in Table 2. The experiment was initiated by adding distilled water to a final concentration of 15% (v/w) to each microcosm.

3.2.3. Measurement of metabolic potential of the whole microbial community. Seventy days after initiating the experiment, 10 g of soil from each of the microcosms were transferred to 90 ml sterile distilled water. Each suspension was shaken for 10 minutes. The suspension was allowed to settle for 2 h, where after the supernatant was used to inoculate a duplicate set of wells on a Biolog^{TM} Eco microplate. After each well was inoculated with 150 µl of supernatant, the plates were incubated at 22 °C for 24 h.

Table 1. Characteristics of the soil used in the experimentation.

Characteristics	Koopmanskloof (vineyard soil)	Koopmanskloof (fynbos soil)	Potting soil (obtained from Dr. W.J. Conradie, Nietvoorbij)
Classification	Sandy Loamy	Sandy Loamy	Sandy
Physical Characteristics			
[1]**Texture**			
Stone (%)	0	0	0
Rough Sand (%)	18.20	22.50	33.50
Medium Sand (%)	18.70	20.80	52.50
Fine Sand (%)	38.10	36.20	7.00
Silt (%)	16.10	15.40	3.20
Clay (%)	8.90	5.10	3.80
Chemical Characteristics			
[2]Organic Carbon (%)	1.61	1.71	0.75
[3]Total Nitrogen (%)	0.16	0.13	0.05
[4]Ammonium (ppm)	0.35	4.57	1.53
[5]Nitrate and Nitrite (ppm)	27.74	45.88	5.51
[6]Phosphorous (ppm)	43.00	10.00	22.00
[7]Copper (ppm)	3.14	1.63	0.83
[8]Zinc (ppm)	2.63	1.87	6.60
[9]Manganese (ppm)	47.37	34.93	17.50
[10]Boron (ppm)	0.64	0.14	0.07
[11]**Exchangeable cations**			
Calcium (cmol/kg)	2.24	2.50	1.40
Potassium (cmol/kg)	0.33	0.35	0.10
Potassium (ppm)	128.00	135.00	39.00
Sodium (cmol/kg)	0.16	0.18	0.03
Magnesium (cmol/kg)	1.06	1.17	0.33
[12]pH (KCl).	4.30	4.70	5.10

[1] Determined by Bemlab CC** using the hydrometer method (Van der Watt, 1966).

[2] Determined by Bemlab CC using the Walkey-Black method (Nelson & Sommers, 1982).

[3] Determined by Bemlab CC through digestion in a LECO FP-528 nitrogen analyser.

[4-5] Determined in a 1M KCl extract by Bemlab CC (Bremner, 1965).

[6] Determined in a Bray-2 extract by Bemlab CC (Thomas & Peaslee, 1973).

[7-9] Determined in a di-ammonium EDTA extract by Bemlab CC (Beyers & Coetzer, 1971).

[10] Determined in a hot water extract by Bemlab CC according to the methods of the Fertilizer Society of South Africa (1974).

[11] Determined in a 1M ammonium acetate extract by Bemlab CC (Doll & Lucas, 1973).

[12] Determined by Bemlab CC according to the method of McClean (1982).

** Bemlab CC, P.O. Box 12457, Die Boord, Stellenbosch, 7613, South Africa.

Table 2. Copper concentrations in the EDTA, and saturated soil extract of the soil microcosms

*Cu (ppm)	[1]Koopmanskloof (vineyard soil)			[2]Koopmanskloof (Fynbos soil)			[3]Potting soil (obtained from Dr. W.J. Conradie, IFT-NVB)		
	** Cu mg/kg	*** Cu mg/l	% saturation	**Cu mg/kg	*** Cu mg/l	% saturation	** Cu mg/kg	*** Cu mg/l	% saturation
0	**3.14** ± 0.31	0.43 ± 0.11	43.23 ± 1.22	**1.63** ± 0.23	0.76 ± 0.11	68.29 ± 25.69	**0.83** ± 0.23	0.14 ± 0.02	35.26 ± 2.15
30	**19.06** ± 1.04	0.10 ± 0.02	43.74 ± 1.09	**15.34** ± 1.01	0.35 ± 0.05	41.41 ± 2.53	**29.47** ± 1.96	0.85 ± 1.14	33.51 ± 0.39
100	**60.14** ± 4.74	0.31 ± 0.09	42.17 ± 2.19	**56.75** ± 1.02	0.42 ± 0.01	45.46 ± 2.40	**101.89** ± 9.40	0.84 ± 0.12	32.83 ± 0.76
1000	**608.17** ± 26.02	30.57 ± 2.09	41.31 ± 2.78	**652.49** ± 25.74	9.83 ± 0.39	49.91 ± 5.60	**1008.15** ± 41.81	2.04 ± 0.73	33.67 ± 3.39

[1]Vineyard soil obtained from the farm Koopmanskloof.

[2]Fynbos soil obtained from the farm Koopmanskloof.

[3]Potting soil to be used to study the impact of copper on young vines.

* The estimated concentration of copper as a component of copper oxychloride, added to each microcosm.

** Determined in a di-ammonium EDTA extract by Bemlab CC according to the methods of Beyers and Coetzer (1971). Values represent the means and standard deviations of three repetitions.

*** Determined by Bemlab CC in the water phase of a saturated soil extract according to the methods of Longenecker and Lyerly (1964). Values represent the means and standard deviations of three repetitions.

Utilization of the different carbon sources in the wells were recorded by observing colour changes in the wells and comparing them to a control well with no carbon source. The results were analyzed with Statistica computer software (StatSoft®).

3.2.4. Enumeration of protozoa. Seventy days after initiating the experiment, direct counts were made of the viable protozoa in the microcosms. The procedure of Griffiths & Ritz (1988) was followed, whereby 5.0 g of soil from each microcosm was added to 50 ml of Tris-HCl buffer (pH 8.0) and shaken for 10 min on a wrist shaker. After shaking, the samples were allowed to settle for 1 min after which a 1 ml aliquot was removed from 5 cm below the meniscus in each tube. This was then added to 0.1 ml of 0.4% (w/v) iodonitrotetrazolium (INT) and incubated at 25°C for 4 h. After fixing the cells with 0.1 ml 25% (v/v) glutaraldehyde, each aliquot was loaded onto a 5 ml Percoll phosphate column in sterile 15 ml polypropionate centrifuge tubes. The column was allowed to settle for 30 min after which it was centrifuged at 3000 × g for 2 h. The supernatant was decanted and stained with 1 ml of 5 µg/ml diamidinophenyl indole (DAPI). This was filtered using black filters (Osmonics™, 47 mm diameter, 0.8 µm pore size), using gentle suction. The filters were placed on top of an inverted Petri dish, and the protozoa on the entire filter were enumerated using epifluorescence microscopy.

3.3. Results

The results obtained on the determination of the metabolic potential of the microbial communities in the microcosms are depicted in Tables 3, 4 and 5. It can be seen that the ability of the microbes to utilize the number and type of carbon compounds in the Biolog™ Eco microplates, were reduced at higher Cu concentrations in the microcosms. Changes in the soil metabolic potential with increased Cu concentrations became apparent when these carbon utilization profiles were compared using cluster analysis (Statistica computer software, StatSoft®) (Figure 1). Similarly, an increase in the bio-available Cu in the microcosms, measured as the concentration of Cu in a di-ammonium EDTA extract of the soil, seem to cause a reduction in the numbers of viable protozoa (Figures 2, 3 & 4).

Table 3. The ability of the microbial communities in the microcosms prepared from Nietvoorbij potting soil, after 70 days of incubation, to utilize a series of carbon sources on Biolog™ Eco microplates.

Cu (ppm) in di-ammonium EDTA extract	1	29	102	1008
Water (No carbon source)	-	-	-	-
ß-Methyl-D- Glucoside	-	+	+	-
D-Galactonic Acid γ-Lactone	+	+	+	-
L-Arginine	+	-	+	-
Pyruvic Acid Methyl Ester	+	+	+	-
D-Xylose	+	-	-	-
D-Galacturonic Acid	+	+	+	-
L-Asparagine	+	+	+	+
Tween 40	+	+	+	-
i-Erythritol	+	-	-	-
2-Hydroxy Benzoic Acid	-	-	-	-
L-Phenylalanine	+	-	+	-
Tween 80	+	+	+	-
D-Mannitol	+	+	+	-
4-Hydroxy Benzoic Acid	+	+	+	-
L-Serine	+	+	+	-
α-Cyclodextrin	+	-	-	-
N-Acetyl-D-Glucosamine	+	-	-	-
γ-Hydroxybutyric Acid	+	-	-	-
L-Threonine	+	+	-	-
Glycogen	+	+	+	-
D-Glucosaminic Acid	+	+	-	-
Itaconic Acid	+	+	+	-
Glycyl-L-Glutamic Acid	+	+	+	-
D-Cellobiose	+	+	+	-
Glucose-1-Phosphate	+	+	+	-
α-Ketobutyric Acid	+	-	+	-
Phenylethyl-amine	-	-	-	-
α-D-Lactose	-	-	-	-
D,L-α-Glycerol Phosphate	+	-	+	-
D-Malic Acid	+	+	+	-
Putrescine	+	-	+	-
Total no. of C-sources utilized	27	19	20	1

+ : Carbon-source utilized; - : Carbon-source not utilized

Table 4. The ability of the microbial communities in the microcosms prepared from Koopmanskloof vineyard soil, after 70 days of incubation, to utilize a series of carbon sources on Biolog™ Eco microplates.

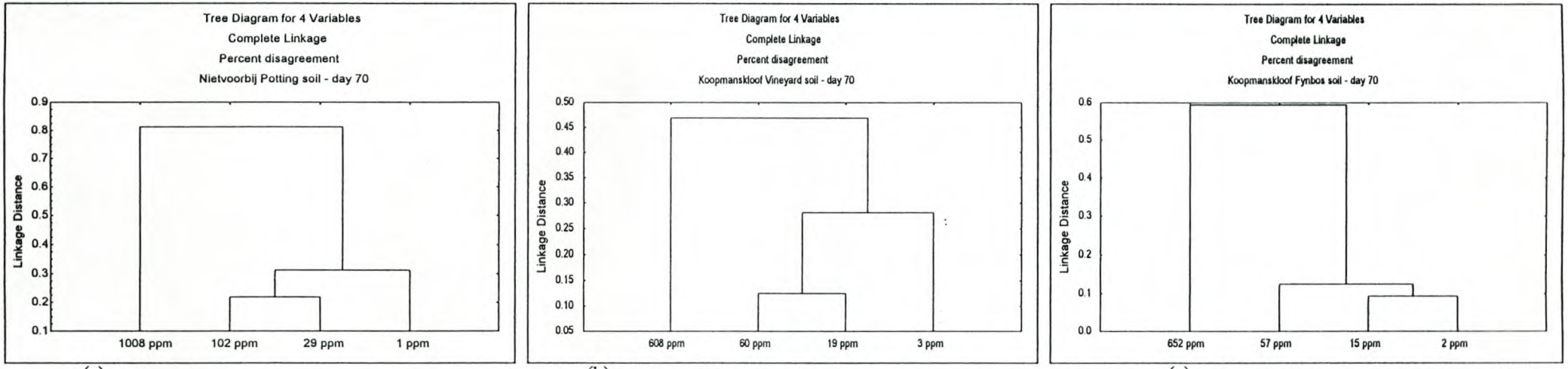
Cu (ppm) in di-ammonium EDTA extract	3	19	60	608
Water (No carbon source)	-	-	-	-
ß-Methyl-D- Glucoside	+	+	+	-
D-Galactonic Acid γ-Lactone	+	+	+	-
L-Arginine	+	+	+	-
Pyruvic Acid Methyl Ester	+	+	+	+
D-Xylose	+	+	-	-
D-Galacturonic Acid	+	-	+	+
L-Asparagine	+	+	+	+
Tween 40	+	+	+	+
i-Erythritol	+	-	-	-
2-Hydroxy Benzoic Acid	-	-	-	-
L-Phenylalanine	+	+	+	+
Tween 80	+	+	+	+
D-Mannitol	+	+	+	-
4-Hydroxy Benzoic Acid	+	+	+	-
L-Serine	+	+	+	-
α-Cyclodextrin	-	+	+	+
N-Acetyl-D-Glucosamine	+	+	+	-
γ-Hydroxybutyric Acid	+	+	+	-
L-Threonine	+	-	-	-
Glycogen	+	+	+	+
D-Glucosaminic Acid	+	+	+	-
Itaconic Acid	-	+	-	-
Glycyl-L-Glutamic Acid	+	+	+	+
D-Cellobiose	+	+	+	+
Glucose-1-Phosphate	-	+	+	-
α-Ketobutyric Acid	+	-	-	-
Phenylethyl-amine	-	-	-	-
α-D-Lactose	+	-	-	-
D,L-α-Glycerol Phosphate	+	-	-	-
D-Malic Acid	+	+	+	+
Putrescine	+	+	+	+
Total no. of C-sources utilized	26	23	23	13

+ : Carbon-source utilized; - : Carbon-source not utilized

Table 5. The ability of the microbial communities in the microcosms prepared from Koopmanskloof fynbos soil, after 70 days of incubation, to utilize a series of carbon sources on Biolog™ Eco microplates.

Cu (ppm) in di-ammonium EDTA extract	Water (No carbon source)	ß-Methyl-D- Glucoside	D-Galactonic Acid γ-Lactone	L-Arginine	Pyruvic Acid Methyl Ester	D-Xylose	D-Galacturonic Acid	L-Asparagine	Tween 40	i-Erythritol	2-Hydroxy Benzoic Acid	L-Phenylalanine	Tween 80	D-Mannitol	4-Hydroxy Benzoic Acid	L-Serine	α-Cyclodextrin	N-Acetyl-D-Glucosamine	γ-Hydroxybutyric Acid	L-Threonine	Glycogen	D-Glucosaminic Acid	Itaconic Acid	Glycyl-L-Glutamic Acid	D-Cellobiose	Glucose-1-Phosphate	α-Ketobutyric Acid	Phenylethyl-amine	α-D-Lactose	D,L-α-Glycerol Phosphate	D-Malic Acid	Putrescine	Total no. of C-sources utilized
2	-	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+	27
15	-	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	26
57	-	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	-	+	+	+	-	+	-	-	+	+	+	+	-	24
652	-	-	-	-	+	-	-	+	+	-	-	+	+	-	-	-	-	+	+	+	-	-	-	+	-	-	-	-	-	-	+	-	10

+ : Carbon-source utilized; - : Carbon-source not utilized



(a) (b) (c)

Figure 1. Dendrograms illustrating shifts in metabolic profiles after incubating the microcosms, containing different copper concentrations, for 70 days. In each dendogram, the y-axis represents the linkage distance, while the x-axis represents the concentration of Cu as determined in a di-ammonium extract of the soil. (a) Nietvoorbij potting soil. (b) Koopmanskloof vineyard soil. (c) Koopmanskloof fynbos soil.

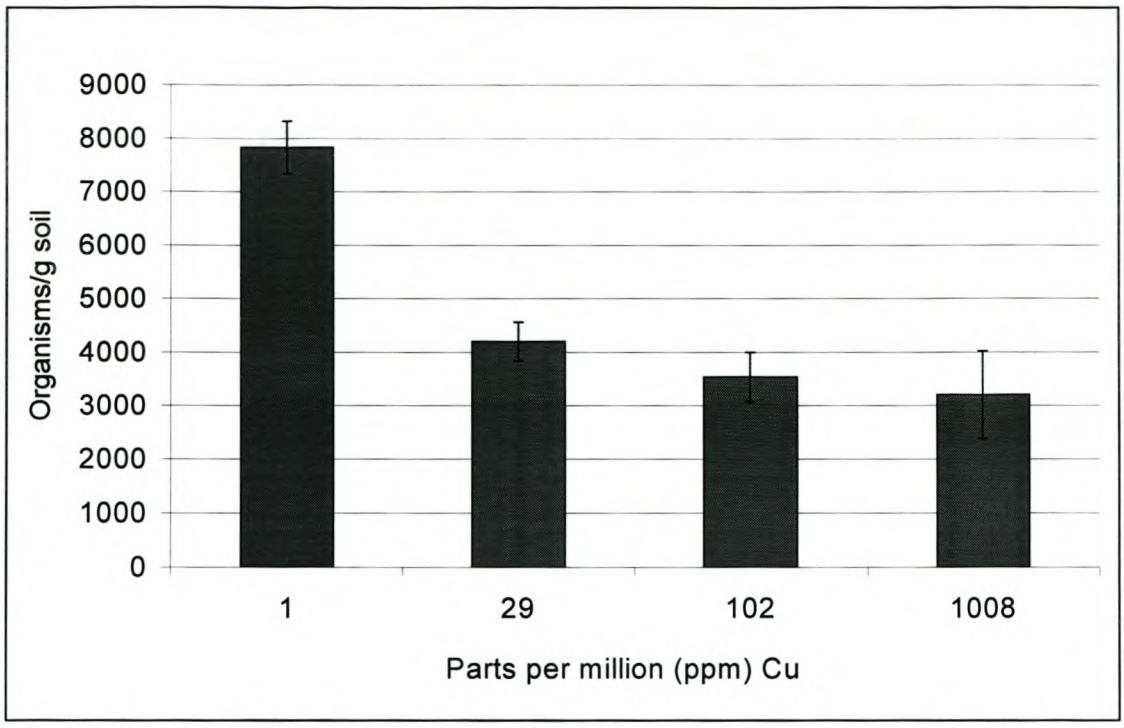


Figure 2. The numbers of protozoa in Nietvoorbij potting soil after incubating microcosms, containing different copper concentrations, for 70 days. The y-axis represents the viable numbers of protozoa (each bar represents the mean of three repetitions). The x-axis represents the Cu concentration in a di-ammonium EDTA extract of soil.

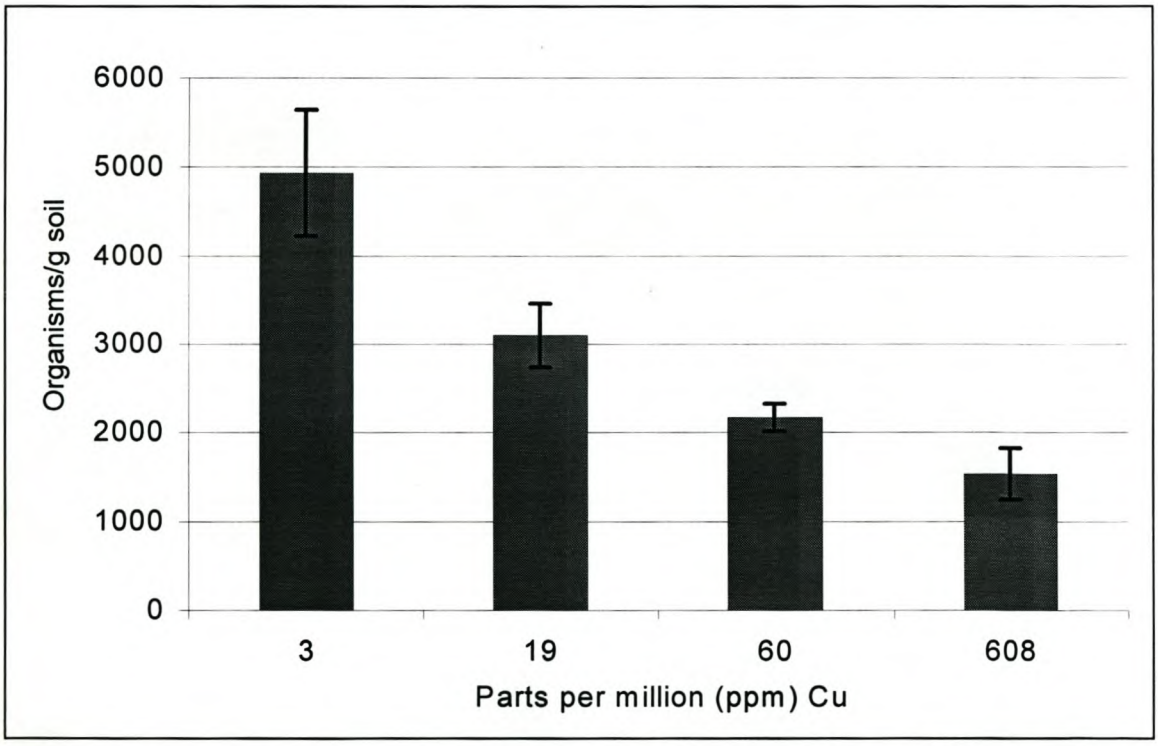


Figure 3. The numbers of protozoa in Koopmanskloof vineyard soil after incubating microcosms, containing different copper concentrations, for 70 days. The y-axis represents the viable numbers of protozoa (each bar represents the mean of three repetitions). The x-axis represents the Cu concentration in a di-ammonium EDTA extract of soil.

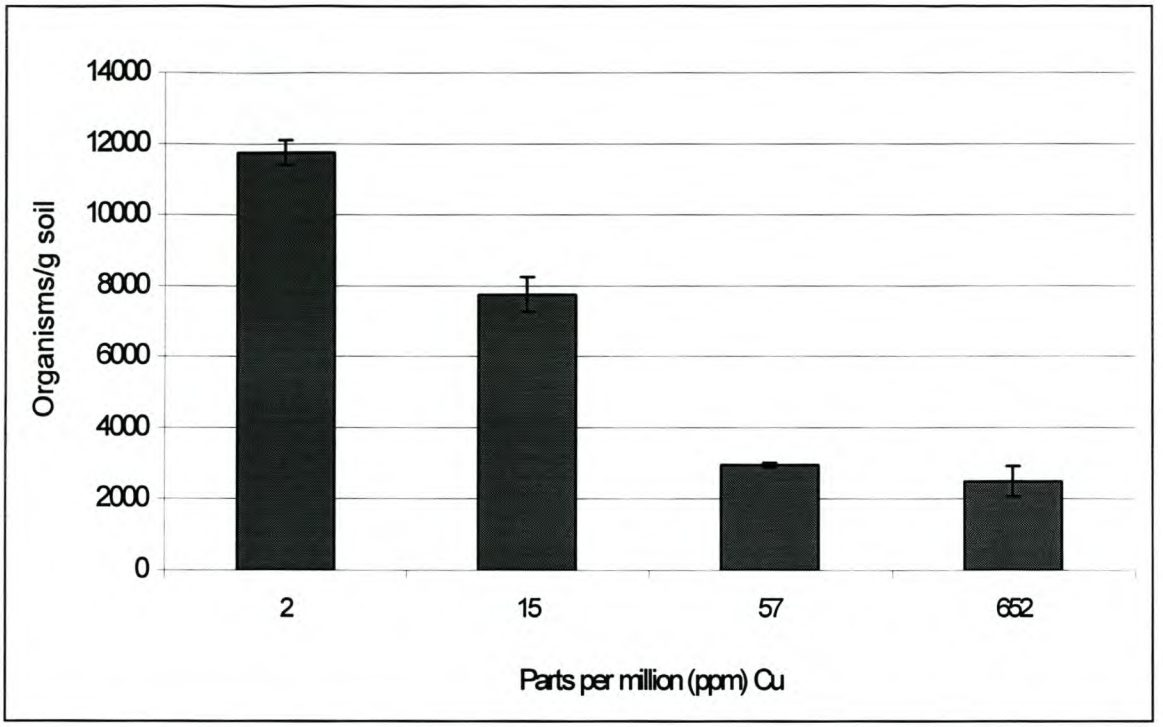


Figure 4. The numbers of protozoa in Koopmanskloof fynbos soil after incubating microcosms, containing different copper concentrations, for 70 days. The y-axis represents the viable numbers of protozoa (each bar represents the mean of three repetitions). The x-axis represents the Cu concentration in a di-ammonium EDTA extract of soil.

3.4. Discussion

The total bio-available Cu concentrations as determined in di-ammonium EDTA extracts of the Koopmanskloof soils, were low compared to the estimated amounts of Cu, as a component of copper oxychloride added to each microcosm (Table 2). This may be due to the chemical properties of the soils or an error that may have occurred when the copper oxychloride was added to the soil. Nevertheless, the results obtained in this section of the study confirmed our findings as presented in Chapter 2, that elevated levels of copper as a result of the addition of copper oxychloride may impact negatively on the metabolic potential as well as on the protozoan numbers in soil. With the sensitivity to respond to an increase in soil Cu concentrations (Figures 2, 3, & 4) and the fact that these organisms play a pivotal role in soil ecosystems, protozoa appear to possess the general features required of bioindicators (Elliot, 1997). Also, these eukaryotes are ubiquitous in nature and are present in numbers that can easily be determined.

Soil pH is known to affect the adsorption of Cu to soil particles and organic material (Mcbride, 1994). Consequently, it was decided to investigate the affect of soil pH and exchangeable Cu on protozoan numbers as found in this study. Figure 5 shows that when the protozoan numbers, recorded in both components of this study (Chapters 2 and 3) were compared with the concentration of exchangeable Cu and soil pH, the numbers of soil protozoa not only decreased as a result of increasing exchangeable Cu concentrations, but also as a result of a decreasing soil pH. This phenomenon may be ascribed to increased concentrations of available Cu at lower soil pH, as a result of the Cu being desorbed from mineral surfaces and organic material (Mcbride, 1994). Evidence supporting this is presented in Table 2 of this chapter. The concentration of soluble Cu in the water phase of a saturated soil extract, of soil previously challenged with *circa* 1000 ppm Cu, was notably higher in the soil with the lowest pH (Koopmanskloof vineyard soil, 31 ppm Cu), than in the soil with the highest pH (Nietvoorbij potting medium, 2 ppm Cu).

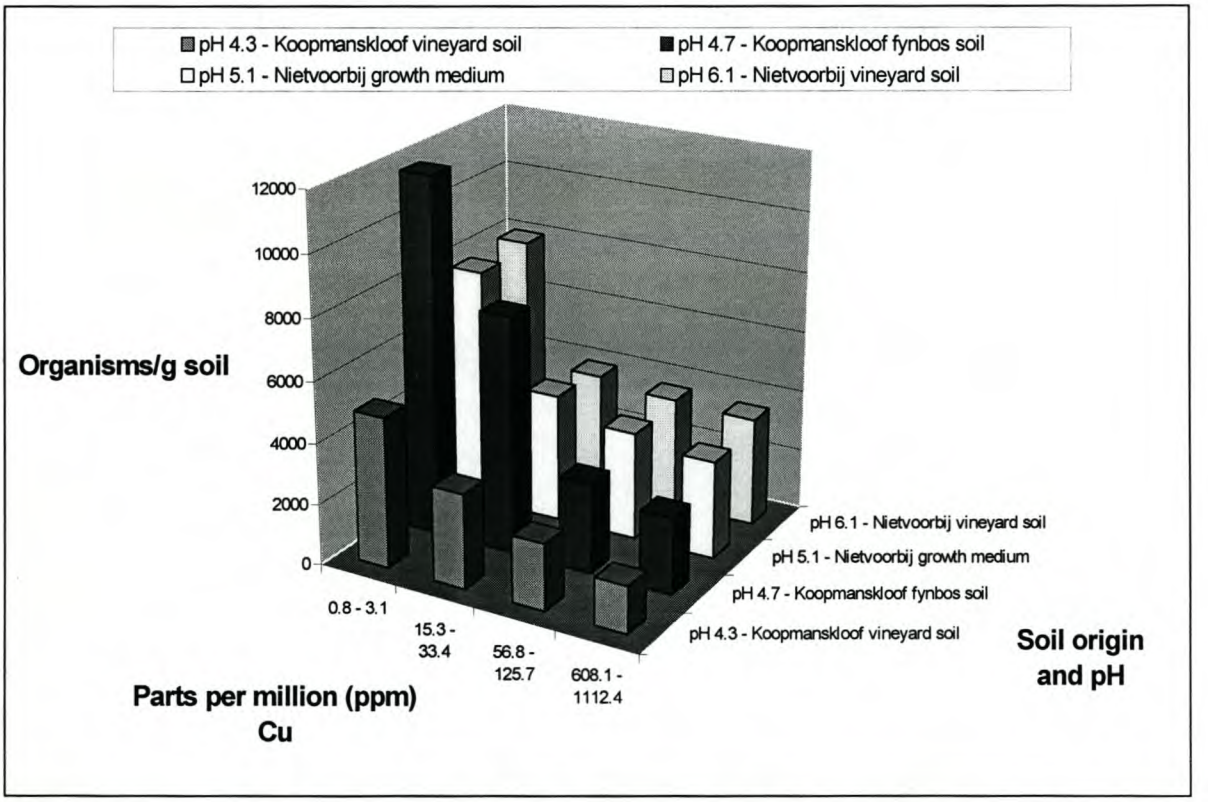


Figure 5. The numbers of protozoa in different ecosystems after incubating microcosms, containing different copper concentrations, for 70 days. The y-axis represents the soil origin and pH. The x-axis represents the Cu concentration in a di-ammonium EDTA extract of soil. The z-axis represents the viable numbers of protozoa (each bar represents the mean of three repetitions).

When the impact of elevated Cu concentrations on the metabolic potential of soil microbial communities, was investigated after 70 days of incubation, it was found that the ability to utilize several carbon sources was lost during this period in all soils (Table 6). This indicated that the determination of metabolic potential might be used as a bioindicator of copper induced stress in these soils. However, it is known that metabolic profiling can not be used as the sole basis for defining mechanistic models of the factors affecting community stability (Garland, 2000). Subsequently, a dendrogram was constructed with Statistica computer software (StatSoft®), using the carbon source utilization profiles (see Appendix; Table 1) in combination with protozoa enumeration (see Appendix; Table2) of all the microcosms challenged with 0 ppm, *ca.* 30 ppm, *ca.* 100 ppm and *ca.* 1000 ppm Cu in the entire study (Figure 6). It was found that the microcosms grouped in two distinct clusters. The first cluster contained those microcosms with 600 to 1100 ppm exchangeable Cu in the soil. The other cluster contained microcosms with less exchangeable Cu, the concentration of which ranged from 1 ppm to 130 ppm. This latter cluster also formed two sub-clusters, one containing microcosms with 20 to 130 ppm exchangable Cu. The other sub-cluster contained microcosms with the least amount of exchangeable Cu, ranging from 1 to 15 ppm.

Table 6. Carbon-sources of which the utilization was lost after 70 days of incubation in all 3 soil types.

C-sources	Cu (ppm) in di-ammonium EDTA extract			
	< 3.1	15.3 – 29.5	56.8 – 101.9	608.2 - 1008.2
(2) ß-Methyl-D- Glucoside	V	+	+	-
(10) i-Erythritol	V	-	-	-
(14) D-Mannitol	+	+	+	-
(26) Glucose-1-Phosphate	V	+	V	-
(27) α-Ketobutyric Acid	V	V	-	-
(30) D,L-α-Glycerol Phosphate	+	V	V	-

+ = indicates that utilization occurred throughout monitoring period

- = indicates that ability to utilize C-sources was lost

V = indicates that in some microcosms utilization varied

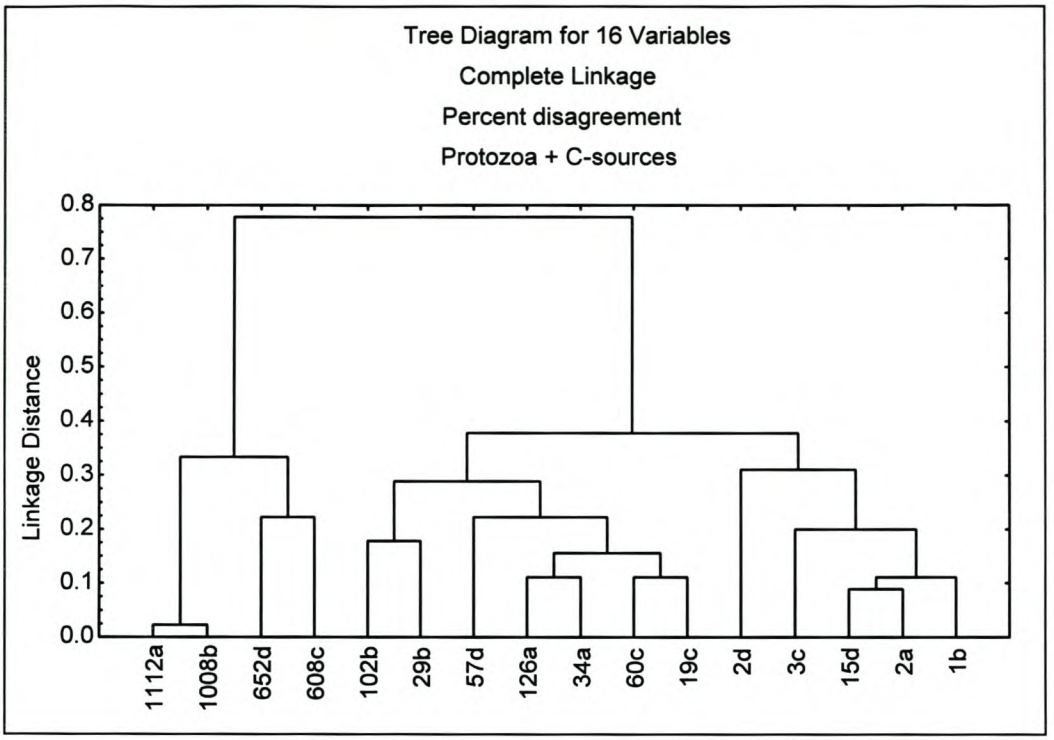


Figure 6. Dendrogram illustrating relationships between different soil microcosms using a combination of metabolic profiles and protozoa numbers. The y-axis represents the linkage distance, while the x-axis represents origin of the sample as well as the concentration of Cu (ppm) as determined in a di-ammonium extract of the soil. (a) Nietvoorbij vineyard soil, (b) Nietvoorbij potting soil, (c) Koopmanskloof vineyard soil, and (d) Koopmanskloof fynbos soil.

3.5. Conclusion

The results indicate that the numbers of soil protozoa was particularly susceptible to Cu-induced stress in soils with a low pH. It also indicates that soil metabolic profiles of the whole microbial community in conjunction with protozoan numbers may be used to estimate the concentration of exchangeable Cu in soil as a result of the addition of copper oxychloride.

4.2. References

Beyers, C.P. and Coetzer, F.J. (1971) Effect of concentration, pH and time on the properties of di-ammonium EDTA as a multiple soil extractant. *Agrochemophysica* 3, 49-54.

Bremner, J.M. (1965) Inorganic forms of nitrogen. In: Black, C.A. (ed.), *Methods of soil analysis. Part 2.* American Society of Agronomy, Madison, Wisconsin.

Conradie, W. J. (2001) Personal communication. Soil scientist, ARC-Infruitec/Nietvoorbij, Stellenbosch.

Doll, E.C. and Lucas, R.E. (1973) Testing soils for potassium, calcium and magnesium. In : Walsh, L.M. and Beatons, J.D. (eds.), *Soil testing and plant analysis.* Soil Science Society of America, Madison, Wisconsin, pp.133-152.

Elliot, E.T. (1997) Rationale for developing bioindicators. In: Pankhurst, C.E., Doube, B.M. and Gupta, V.V.S.R. (eds.), *Biological indicators of soil health.* CAB International, Wallingford, pp.49-78.

Fertilizer Society of South Africa. (1974) Manual of soil analysis methods. *FSSA Publication No. 37.*

Foissner, W. (1987) Soil protozoa: fundamental problems, ecological significance, adaptations in ciliates and testaceans, bioindicators, and guide to literature. *Progress in Protistology* 2, 69-212.

Foissner, W. (1994) Soil protozoa as bioindicators in ecosystems under human influence. In: Darbyshire, J.F. (ed.), *Soil Protozoa.* CAB International, Wallingford, pp.147-194.

Foissner, W. (1999) Soil protozoa as bioindicators: pros and cons, methods, diversity, representitive examples. *Agriculture ecosystems and environment* 74, 95-112.

Garland, J.L. (2000) Potential and limitations of BIOLOG for microbial community analysis. In: Bell, C.R., Brylinsky, M. and Johnson-Greene, P. (eds.), *Proceedings of the 8th International Symposium on Microbial Ecology.* Atlantic Canada Society for Microbial Ecology, Halifax, pp.521-527.

Griffiths, B.F. and Ritz, K. (1988) A technique to extract, enumerate and measure protozoa from mineral soils. *Soil Biology and Biochemistry* 20, 163-173.

Longenecker, D.E. and Lyerly, P.J. (1964) Making soil pastes for salinity analysis. A reproducible capillary procedure. *Soil Science* 97, 268-275.

McBride, M.B. (1994) *Environmental chemistry of soil.* Oxford University Press, Inc., New York.

McClean, E.O. (1982) Soil pH and lime requirement. In: Page, A.L. (ed.), *Methods of soil analysis. Part 2.* American Society of Agronomy, Madison, Wisconsin, pp.570-571.

Nelson, D.W. and Sommers, L.E. (1982) Total carbon, organic carbon and organic matter. In: Page, A.L. (ed.), *Methods of soil analysis. Part 2.* American Society of Agronomy, Madison, Wisconsin, p.199.

Thomas, G.W. and Peaslee, D.E. (1973) Testing for soil phosphorus. In: Walsh, L.M. and Beatons, J.D. (eds.), *Soil testing and plant analysis.* Soil Science Society of America, Madison, Wisconsin, pp.15-122.

Van der Watt, H.V.H. (1966) Improved tables and a simplified procedure for soil particle size analysis by the hydrometer method. *South African Journal of Agricultural Science* 9, 911-916.

Versfeld, D.B., Richardson, D.M., van Wilgen, B.W., Chapman, R.A. and Forsyth, G.G. (1992) The climate of Swartboskoof. In: van Wilgen, B.W., Richardson, D.M., Kruger, F.J. and van Hensbergen, H.J. (eds.), *Ecological Studies 93, Fire in South African Mountain Fynbos.* Springer-Verlag, Berlin, pp.21-36.

CHAPTER 4

GENERAL CONCLUSIONS

The use of Cu-containing compounds in agricultural practices as fertilizers and fungicides has resulted in the accumulation of Cu in soils (Baker & Senft, 1995; Flores-Vélez *et al.*, 1996). This accumulation of Cu may generate stress on the environment, resulting in organisms and communities of organisms displaying injury symptoms or shifts in community composition (Chaphekar, 1978). The goal of this study was to evaluate the potential of selected eukaryotic and prokaryotic microbial populations as bioindicators of soil ecosystem health. Specifically, the following observations were made:

1) Addition of Cu as a component of copper oxychloride had an effect on various microbial parameters over an extended time period (245 days). These parameters included the enumeration of protozoa and whole community metabolic analysis. It was found that culturable microbial numbers did not provide a reliable indication of the effect of Cu, as a component of copper oxychloride, on community integrity.

2) The combination of Cu concentration and pH had an interesting effect on the protozoan numbers. It was found that the numbers of soil protozoa not only decreased as a result of increasing Cu concentrations, but also as a result of a decreasing soil pH. This phenomenon may be ascribed to increased concentrations of available Cu at lower soil pH, because at low pH, H^+ associates with phenolic groups which suppresses Cu^{2+} adsorption to organic matter and soil particles (McBride, 1994).

3) Enumeration of soil protozoan numbers provided a valuable addition to whole community metabolic analyses by increasing the resolution of these analyses that resulted in finer separation of clusters.

4.1. Future research

This study focused on the impact of elevated Cu concentrations, as a component of copper oxychloride, on selected soil microorganisms. It would, however, be important to evaluate the impact of other Cu containing compounds, heavy metals and pollutants on soil ecosystems situated in the same topographical area. It can subsequently be determined whether the potential bioindicators of this study can be used as bioindicators of Cu-induced stress in other soils from the Western Cape. However, it will be important to acquire information about the community structures within different ecosystems from this region so that one can be able to compare results of metal toxicology tests to a 'healthy' ecosystem.

The ability to utilize certain carbon sources was lost over the monitoring period. Therefore, further studies may lead to the development of a system to evaluate the impact of Cu on the metabolic potential of a microbial community, based on the utilization of these specific carbon sources.

4.2. References

Baker, D.E. and Senft, J.P. (1995) Copper. In: Alloway, B.J. (ed.), *Heavy Metals in Soils*. Blackie Academic and Professional, Bishopbriggs, pp.179-205.

Chaphekar, S.B. (1978) Biological indicators. The concept of new addition. *International Journal of Ecology and Environmental Science* 4, 45-52.

Flores-Vélez, L.M., Ducaroir, J., Jaunet, A.M. and Robert, M. (1996) Study of the distribution of copper in an acidic vineyard soil by three different methods. *European Journal of Soil Science* 47, 523-532.

McBride, M.B. (1994) *Environmental chemistry of soil.* Oxford University Press, Inc., New York.

Appendix

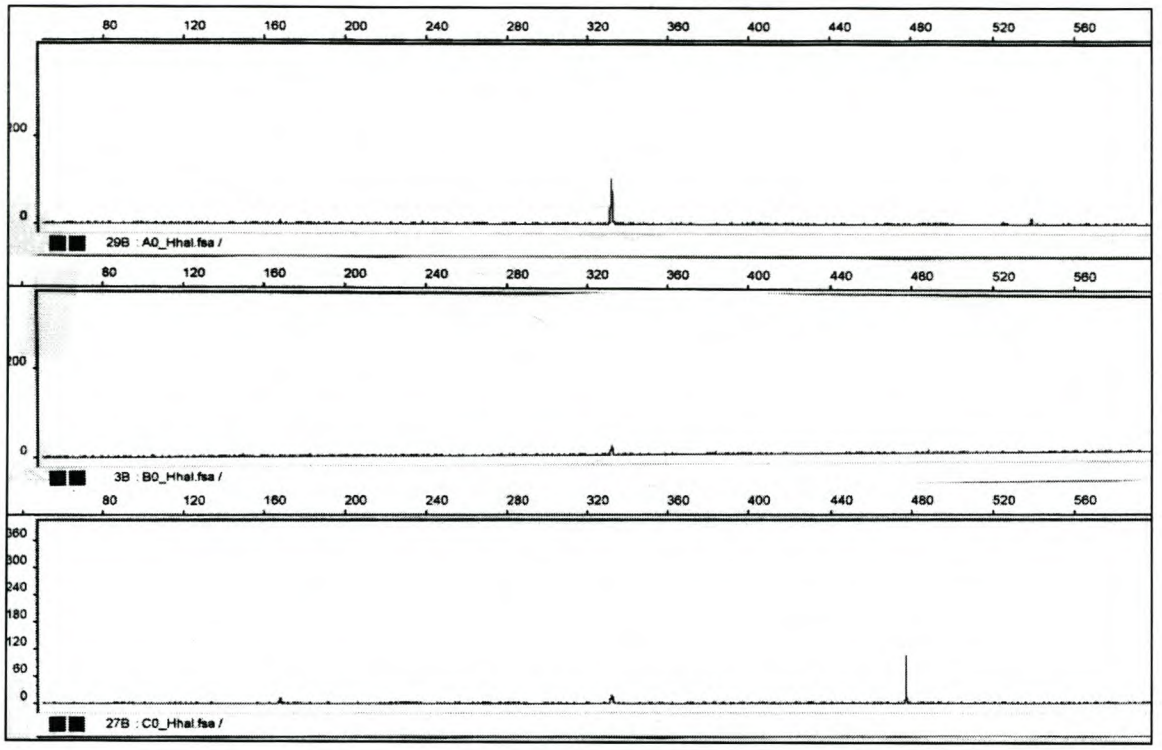


Figure 1. T-RFLP community fingerprint patterns of PCR products, digested with *Hha1*, originating from the microcosms that received 0 ppm Cu.

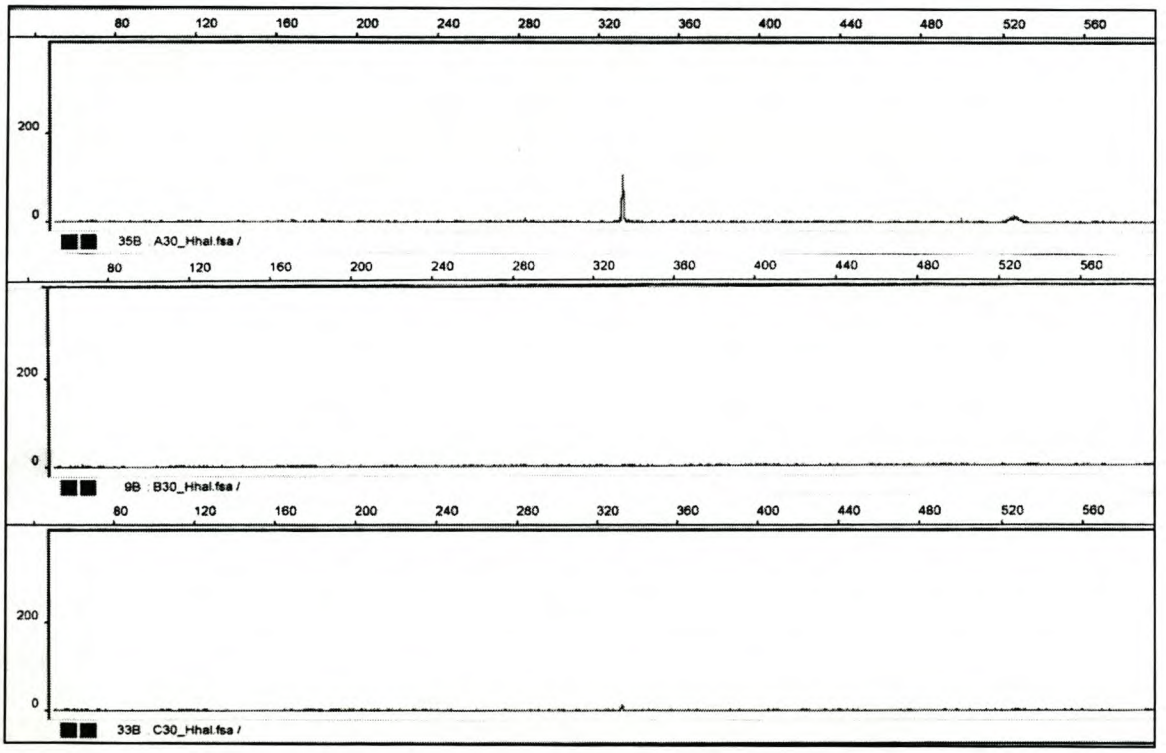


Figure 2. T-RFLP community fingerprint patterns of PCR products, digested with *Hha1*, originating from the microcosms challenged with 34 ppm exchangeable Cu.

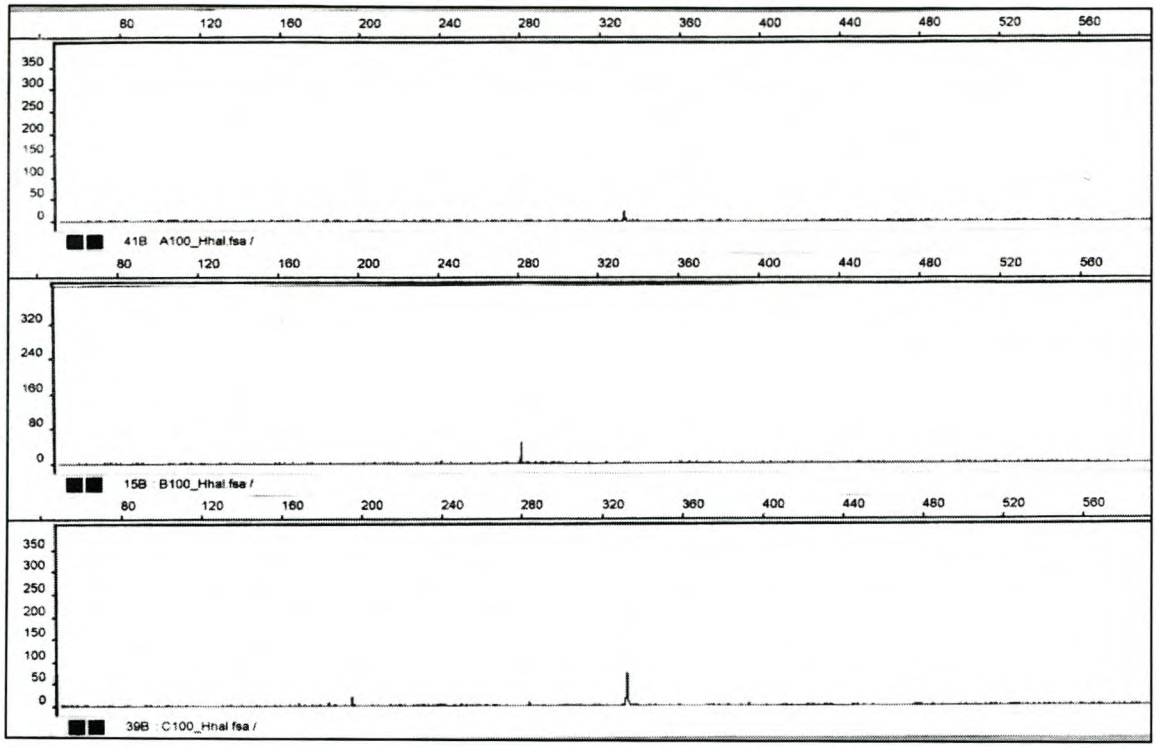


Figure 3. T-RFLP community fingerprint patterns of PCR products, digested with *Hha1*, originating from the microcosms challenged with 126 ppm exchangeable Cu.

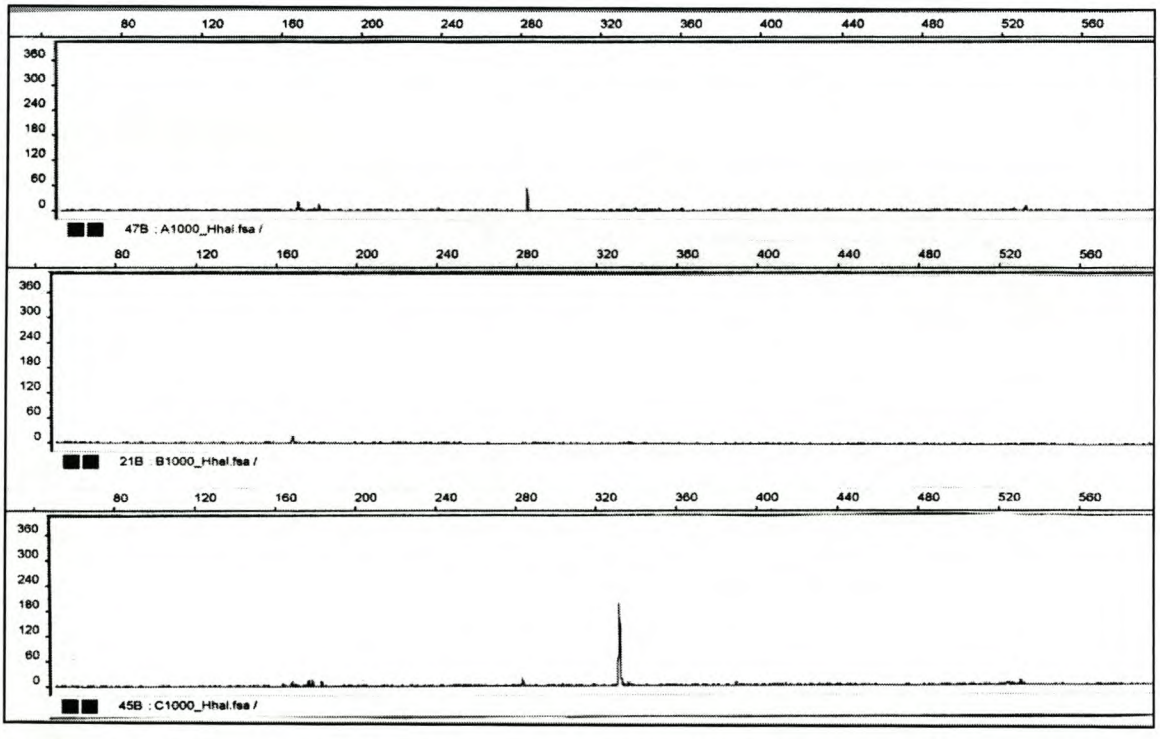


Figure 4. T-RFLP community fingerprint patterns of PCR products, digested with *Hha1*, originating from the microcosms challenged with 1112 ppm exchangeable Cu.

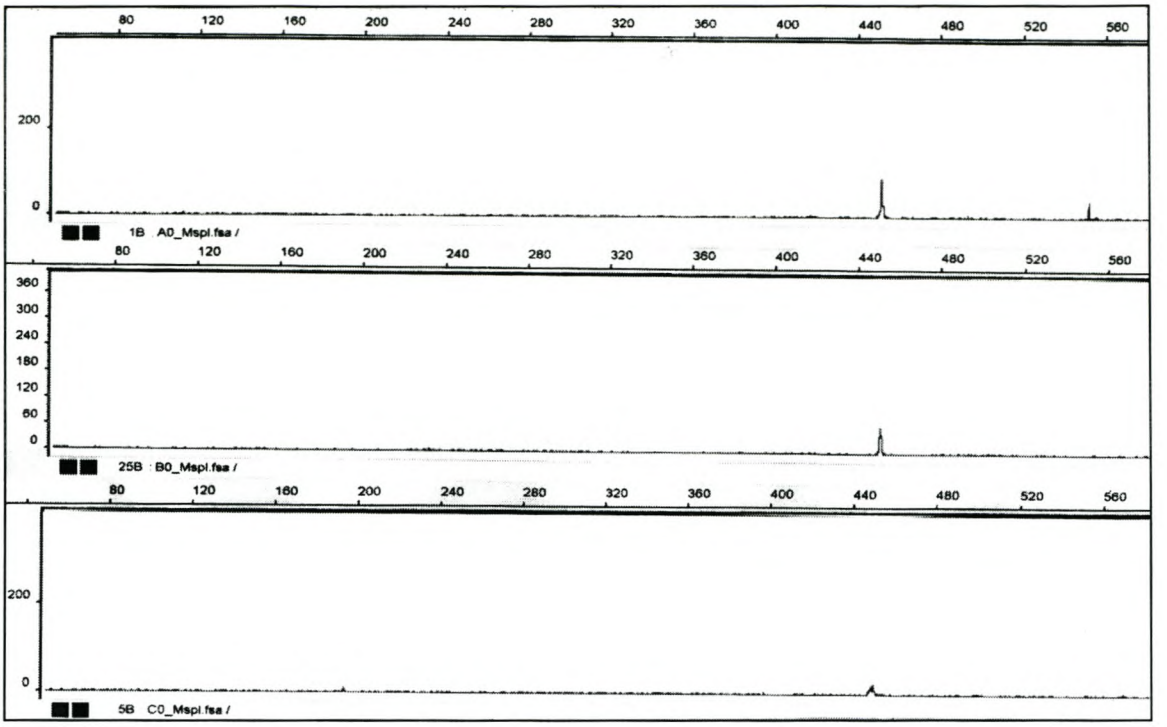


Figure 5. T-RFLP community fingerprint patterns of PCR products, digested with *Msp1*, originating from the microcosms that received 0 ppm Cu.

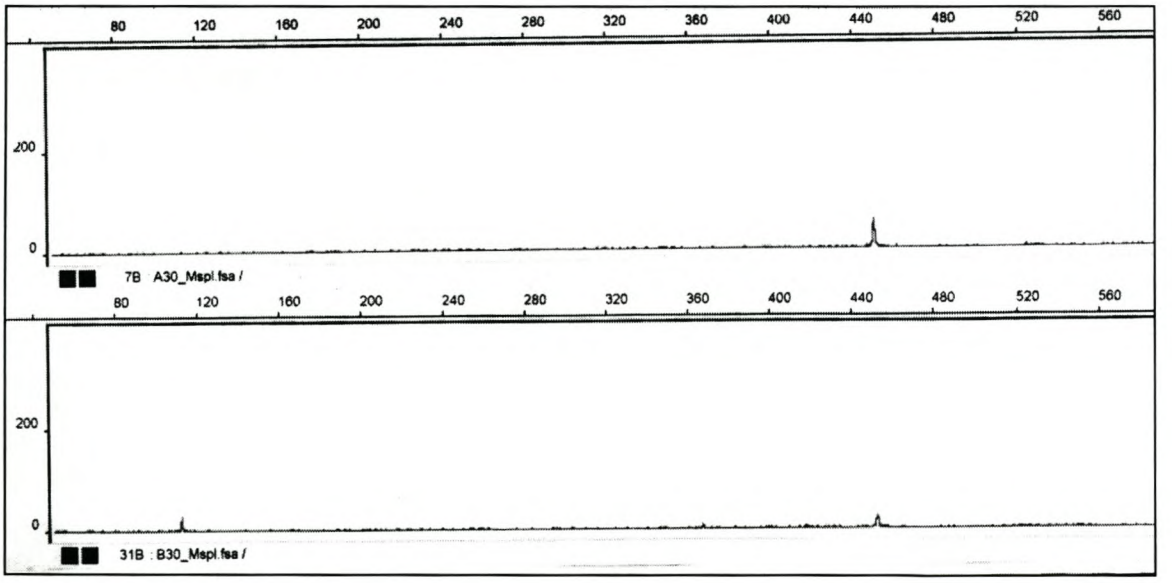


Figure 6. T-RFLP community fingerprint patterns of PCR products, digested with *Msp1*, originating from the microcosms challenged with 34 ppm exchangeable Cu.

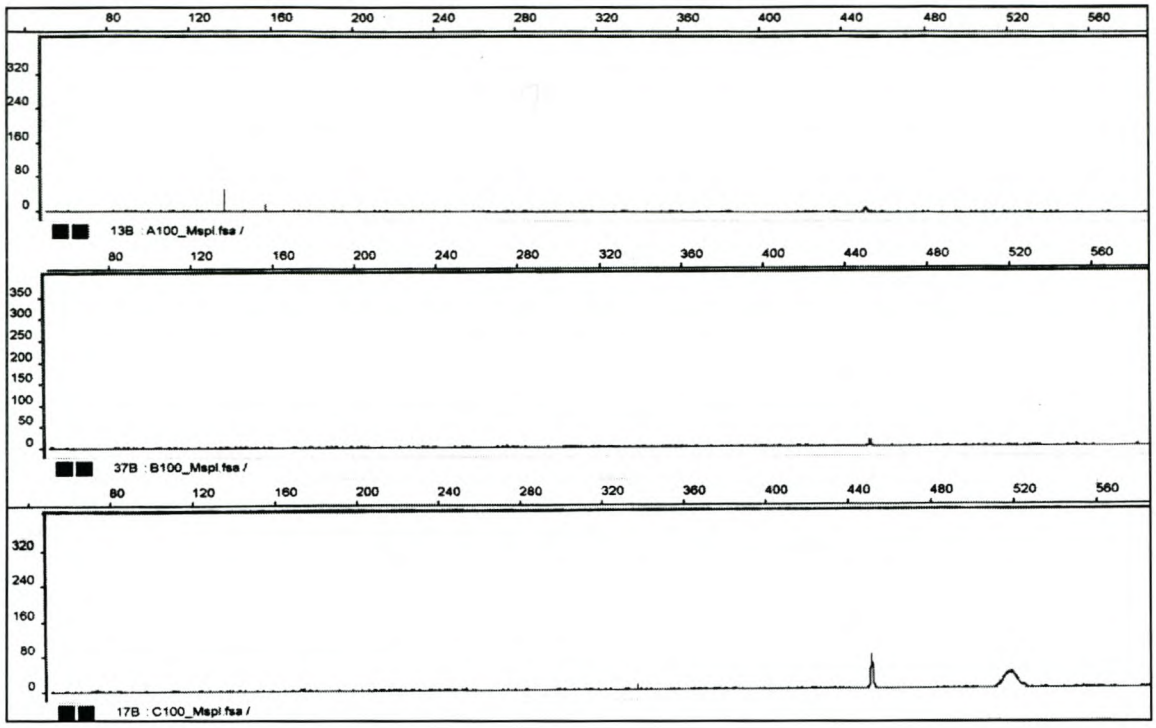


Figure 7. T-RFLP community fingerprint patterns of PCR products, digested with *Msp1*, originating from the microcosms challenged with 126 ppm exchangeable Cu.

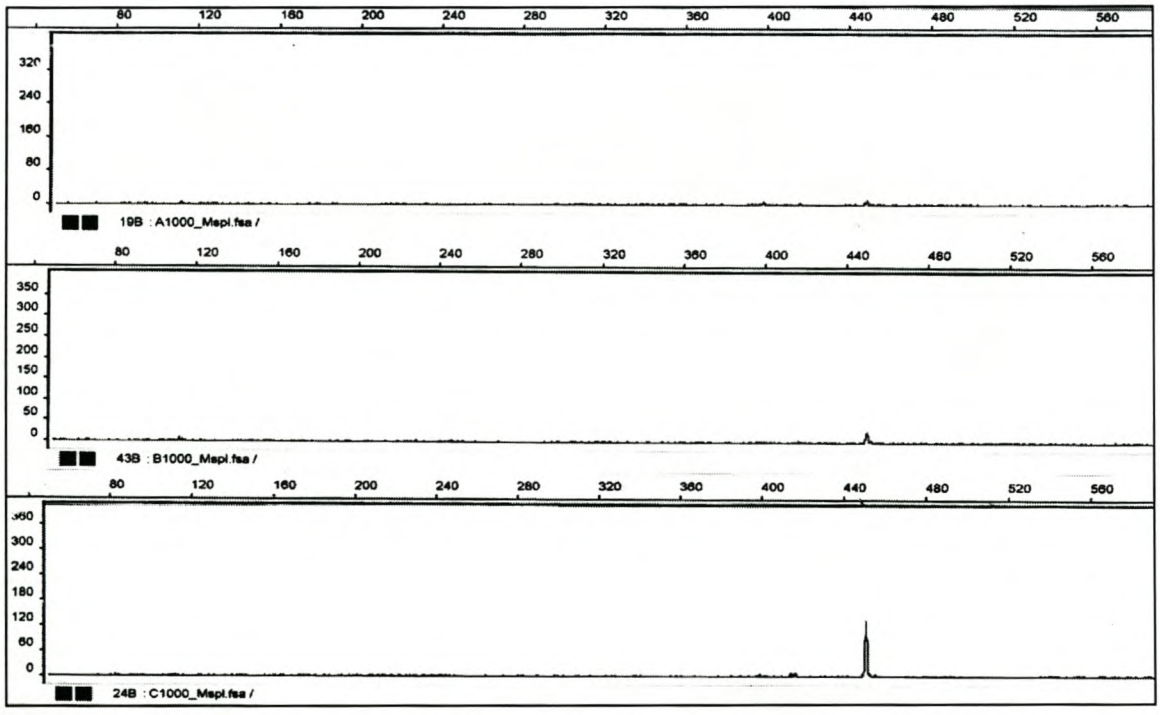


Figure 8. T-RFLP community fingerprint patterns of PCR products, digested with *Msp1*, originating from the microcosms challenged with 1112 ppm exchangeable Cu.

Table 1. Binary system illustrating the ability of the microbial communities in the microcosms, after 70 days of incubation, to utilize a series of carbon sources on Biolog™ Eco microplates.

Carbon-source	Cu concentration (ppm) and Origin of soil															
	1-b	2-a	2-d	3-c	15-d	19-c	29-b	34-a	57-d	60-c	102 -b	126 -a	608 -c	652 -d	1008 -b	1112 -a
Water	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ß-Methyl-D- Glucoside	0	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0
D-Galactonic Acid γ-Lactone	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0
L-Arginine	1	1	1	1	1	1	0	1	1	1	1	1	0	0	0	0
Pyruvic Acid Methyl Ester	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
D-Xylose	1	1	1	1	1	1	0	1	1	0	0	1	0	0	0	0
D-Galacturonic Acid	1	1	1	1	1	0	1	1	1	1	1	1	1	0	0	0
L-Asparagine	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Tween 40	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
i-Erythritol	1	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0
2-Hydroxy Benzoic Acid	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0
L-Phenylalanine	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	0
Tween 80	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
D-Mannitol	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0
4-Hydroxy Benzoic Acid	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0
L-Serine	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0
α-Cyclodextrin	1	1	1	0	1	1	0	1	1	1	0	1	1	0	0	0
N-Acetyl-D-Glucosamine	1	1	1	1	1	1	0	1	1	1	0	1	0	1	0	0
γ-Hydroxybutyric Acid	1	1	1	1	1	1	0	1	1	1	0	1	0	1	0	0
L-Threonine	1	1	0	1	1	0	0	0	0	0	1	0	0	1	0	1
Glycogen	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0
D-Glucosaminic Acid	1	1	1	1	1	1	0	1	1	1	1	1	0	0	0	0
Itaconic Acid	1	1	1	0	1	0	1	1	1	1	1	1	0	0	0	0
Glycyl-L-Glutamic Acid	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	0
D-Cellobiose	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0
Glucose-1-Phosphate	1	1	1	0	1	1	1	1	0	1	1	1	0	0	0	0
α-Ketobutyric Acid	1	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0
Phenylethyl-amine	0	1	1	0	0	0	0	0	1	0	0	1	0	0	0	0
α-D-Lactose	0	1	1	1	1	0	0	1	1	0	0	0	1	0	0	0
D,L-α-Glycerol Phosphate	1	1	1	1	1	0	1	1	1	0	0	0	0	0	0	0
D-Malic Acid	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
Putrescine	1	1	1	1	0	1	0	1	0	1	1	1	1	0	0	0

a – Nietvoorbij vineyard soil
b – Nietvoorbij potting soil
c – Koopmanskloof vineyard soil
d - Koopmanskloof fynbos soil
0 – Carbon-source utilized
1 – Carbon-source not utilized

Table 2. An illustration of the conversion of protozoan counts to a binary system.

Protozoan numbers	Cu concentration (ppm) and Origin of soil															
	1-b	2-a	2-d	3-c	15-d	19-c	29-b	34-a	57-d	60-c	102 -b	126 -a	608 -c	652 -d	1008 -b	1112 -a
12001 – 13000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11001 – 12000	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
10001 – 11000	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
9001 – 10000	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
8001 – 9000	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
7001 – 8000	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0
6001 – 7000	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0
5001 – 6000	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0
4001 – 5000	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0	0
3001 – 4000	1	1	1	1	1	1	1	1	0	0	1	1	0	0	1	1
2001 – 3000	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
1001 – 2000	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
0 – 1000	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

a – Nietvoorbij vineyard soil
b – Nietvoorbij potting soil
c – Koopmanskloof vineyard soil
d – Koopmanskloof fynbos soil
0 – The numbers of protozoa per gram soil were less than the indicated range
1 – The numbers of protozoa per gram soil were between 0 and the top of the indicated range

Table 3. A combination of the binary systems of whole metabolic profiles and protozoan counts in the different soil microcosms..

	Cu concentration (ppm) and Origin of soil															
	1-b	2-a	2-d	3-c	15-d	19-c	29-b	34-a	57-d	60-c	102 -b	126 -a	608 -c	652 -d	1008 -b	1112 -a
Water	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ß-Methyl-D- Glucoside	0	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0
D-Galactonic Acid γ-Lactone	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0
L-Arginine	1	1	1	1	1	1	0	1	1	1	1	1	0	0	0	0
Pyruvic Acid Methyl Ester	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
D-Xylose	1	1	1	1	1	1	0	1	1	0	0	1	0	0	0	0
D-Galacturonic Acid	1	1	1	1	1	0	1	1	1	1	1	1	1	0	0	0
L-Asparagine	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Tween 40	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
i-Erythritol	1	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0
2-Hydroxy Benzoic Acid	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0
L-Phenylalanine	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	0
Tween 80	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
D-Mannitol	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0
4-Hydroxy Benzoic Acid	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0
L-Serine	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0
α-Cyclodextrin	1	1	1	0	1	1	0	1	1	1	0	1	1	0	0	0
N-Acetyl-D-Glucosamine	1	1	1	1	1	1	0	1	1	1	0	1	0	1	0	0
γ-Hydroxybutyric Acid	1	1	1	1	1	1	0	1	1	1	0	1	0	1	0	0
L-Threonine	1	1	0	1	1	0	0	0	0	0	1	0	0	1	0	1
Glycogen	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0
D-Glucosaminic Acid	1	1	1	1	1	1	0	1	1	1	1	1	0	0	0	0
Itaconic Acid	1	1	1	0	1	0	1	1	1	1	1	1	0	0	0	0
Glycyl-L-Glutamic Acid	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	0
D-Cellobiose	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0
Glucose-1-Phosphate	1	1	1	0	1	1	1	1	0	1	1	1	0	0	0	0
α-Ketobutyric Acid	1	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0
Phenylethyl-amine	0	1	1	0	0	0	0	0	1	0	0	1	0	0	0	0
α-D-Lactose	0	1	1	1	1	0	0	1	1	0	0	0	1	0	0	0
D,L-α-Glycerol Phosphate	1	1	1	1	1	0	1	1	1	0	0	0	0	0	0	0
D-Malic Acid	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
Putrescine	1	1	1	1	0	1	0	1	0	1	1	1	1	0	0	0
12001 – 13000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11001 – 12000	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
10001 – 11000	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
9001 – 10000	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
8001 – 9000	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
7001 – 8000	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0
6001 – 7000	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0
5001 – 6000	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0
4001 – 5000	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0	0
3001 – 4000	1	1	1	1	1	1	1	1	0	0	1	1	0	0	1	1
2001 – 3000	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
1001 – 2000	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
0 – 1000	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

a – Nietvoorbij vineyard soil
b – Nietvoorbij potting soil
c – Koopmanskloof vineyard soil
d – Koopmanskloof fynbos soil