The effect of fumigants on earthworms (*Eisenia andrei*) and soil microbial communities

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Dissertation submitted in fulfilment of the requirements for the degree *Magister Scientiae* in Environmental Sciences at the Potchefstroom Campus of the North-West University

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May 2015

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ACKNOWLEDGEMENTS

I would like to start by acknowledging my supervisors, Dr. Sarina Claassens and Prof. Mark Maboeta for their guidance, support and patience for the duration of my Masters project and especially Dr. Claassens for keeping in contact with me and always offering more support. You have been a great mentor through this process and I truly appreciate your support.

I am grateful that I had the opportunity to learn so many new scientific techniques in microbiology and biochemistry, which was not part of my undergraduate studies. I would like to single out Clarissa Willers for always being available to provide microbiology training, for all the additional literature she sent me and for her moral support. As an off campus student, it was challenging at times to have everything prepared for long hours of laboratory work and I owe my gratitude to Clarissa for always assisting me in planning and preparing solutions and equipment before my arrival. She also assisted me tremendously with the microbiology component of my laboratory work and I am forever grateful to her for offering so much of her time to assist me.

I would like to thank Dr. Patricks Voua Otomo for providing training and advice in the comet assay protocol as well as introducing me to and arranging training for the comet assay at the Biochemistry department. A special word of thank you to Dr. Etrisia van Dyk and Alicia Brümmer for the use of their laboratory and equipment as well as their assistance during my comet assay laboratory work.

Thank you to Dr. Jaco Bezuidenhout for his assistance and advice in the statistical data analysis.

I would like to acknowledge Javelin Seeds for their sponsorship of Bladrammenas and mustard seed and for allowing me the opportunity to visit a farm where biofumigation is practised. I would also like to thank Mr. Ken Reid for his willingness to sponsor earthworms for the use in the study.

To Dr. Claassens for arranging financial support in 2012 and 2013 and to the Unit of Environmental Sciences and Management (UESM) for funding in 2014, I thank you.

A special thank you to my mother for travelling with me and looking after my son while I was working in the laboratory.

Lastly I would like to thank my husband, Jacques Fouché, who is also a postgraduate science student, for his moral support, assisting me with long hours of laboratory work and for taking care of our son while I was writing. Thank you for always listening to me and giving advice when new research questions came up and for your tremendous help with difficult statistical analyses. I am so grateful that you are a scientist too and you have helped me greatly.

PREFACE

The experimental work done and discussed in this dissertation for the degree *Magister Scientiae* in Environmental Sciences was carried out in the Unit for Environmental Sciences and Management, North-West University, Potchefstroom Campus, Potchefstroom, South Africa. This study was conducted part-time during the period of January 2012 to November 2014, under the supervision of Dr. Sarina Claassens and Prof. Mark Maboeta.

The research done and presented in this dissertation signifies original work undertaken by the author and has not been submitted for degree purposes to any other university, before. Appropriate acknowledgements in the text have been made, where the use of work conducted by other researchers have been included.

Tanya Fouché
November 2014

SUMMARY

Biofumigation is an important crop protection practice that uses a plant's natural defence mechanisms to control agricultural crop pathogens and diseases. Glucosinolates are volatile compounds found in most *Brassica* species and when hydrolysed, it forms a range of natural toxins including isothiocyanates that act as biofumigants. Research suggests that biofumigation is a good alternative to chemical fumigants as it is effective in controlling plant pests but with lower health and environmental risks. Several studies have confirmed the effectiveness of the breakdown products, especially isothiocyanates, as fungicidal, bactericidal and nematicidal products against a series of plant pests. However, very little information is available on the effects of glucosinolates and its breakdown products on non-target and beneficial soil organisms. Negative effects on beneficial soil organisms can have serious negative impacts on soil quality especially when essential ecosystem functions such as nutrient cycling and soil bioturbation are affected.

Three biofumigants, broccoli, mustard and oilseed radish, and two chemical fumigants, metham sodium and cadusafos, were investigated for possible effects on non-target and essential soil organisms such as earthworms and the soil microbial community. Sublethal endpoints, including growth and reproductive success of the earthworms, were monitored. The genotoxicity of the biologically active compounds found in the fumigants, towards earthworms, was evaluated by means of the comet assay. The DNA damage was quantified by tail intensity parameters. Furthermore, the changes in the soil microbial community function and structure were evaluated by means of community level physiological profiling (CLPP) and phospholipid fatty acid (PLFA) analyses respectively. All exposures were done in artificial soil prepared according to the OECD standard guidelines.

In the biofumigant treated soils, results varied and different effects were observed on the non-target soil organisms. Broccoli reduced cocoon production and the number of hatchlings while mustard induced more DNA strand breaks in earthworm cells compared to the control. All the biofumigants stimulated microbial growth but broccoli and oilseed radish changed the microbial functional diversity. Mustard had no lasting effect on the functional diversity but altered the microbial community structure.

The chemical fumigants had a marked negative impact on the survival, growth, reproduction and the genotoxicity of the earthworms with metham sodium causing greater harm than cadusafos. The effects on the microbial community varied. Both chemicals had an inhibitory effect on the microbial growth in terms of the viable biomass determined by PLFA and the average well colour development in the Biolog™ Ecoplates. No lasting effects were

observed in the community structure. Overall, cadusafos had a more pronounced effect on the microbial community functional diversity than metham sodium.

Results indicated that each bioindicator species illustrates effects at their own level of organisation.

Key Terms: Biofumigation, Biolog™, Cadusafos, Comet assay, Earthworm biomarkers, Metham sodium, Microbial community, Phospholipid fatty acid.

TABLE OF CONTENTS:

LIST OF ABBREVIATIONS

AITC	allyl isothiocyanates
AChE	acetylcholinesterase
AM	arbuscualr mycorrhizal fungi
Amine_a	amino acids
ANOVA	analysis of variance
ARDRA	amplified ribosomal DNA restriction analysis
AWCD	average well colour development
B	soil amendments with broccoli
C	control soil samples
CA	chromosomal aberrations
Carb_a	carboxylic acids
Carb_h	carbohydrates
CAS	catalyse
CBD	Convention on Biological Diversity
CCA	canonical correspondence analysis
CLPP	community level physiological profiling
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
EC_{50}	effective concentration
EDTA	ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
EM	ectomycorrhizal fungi
EMS	ethyl methane sulfonate

EW	earthworm
FAME	fatty acid methylester
GSL	glucosinolates
IPM	integrated pest management
ITC	isothiocyanate
LC_{50}	lethal concentration 50
LD_{50}	lethal dose 50
M	soil amendments with mustard
MB	methyl bromide
MBSats	mid-chain branched saturated fatty acids
MITC	methyl isothiocyanate
MONOS	monounsaturated fatty acids
MS	metham sodium
NEMA	National Environmental Management Act 107 of 1998
NRF	National Research Foundation
NSats	normal saturated fatty acids
OECD	Organisation for Economic Cooperation and Development
PBS	phosphate buffered saline
PCA	principal component analysis
PCR	polymerase chain reaction
Phos_C	phosphorylated compounds
Poly	polymers
Polys	polyunsaturated fatty acids
PLFA	phospholipid fatty acid

Q-PCR	real-time PCR
R	soil amendment with oilseed radish
R10	soil amendment with RUGBY/cadusafos
RAPD-PCR	randomly amplified polymorphic DNA polymerase chain reaction
RDA	redundancy analysis
RISA	ribosomal intergenic spacer analysis
ROS	reactive oxygen species
SCE	sister chromatid exchange
SCGE	single cell gel electrophoresis
SD	standard deviation
SEM	standard error of the mean
SIR	substrate induced respiration
SOC	soil organic carbon
SOM	soil organic matter
TBSats	terminally branched saturated fatty acids
TGGE	temperature gradient gel electrophoresis
US EPA	Unites States Environmental Protection Agency

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1: INTRODUCTION

The suppression of soil-borne pathogens by the use of plants that contain special volatile compounds is known as biofumigation (Nova Science in the News, 2001). Biofumigation is an important crop protection practice for the commercial and emerging agricultural community because it can control agricultural crop pathogens and diseases without the health and environmental risks that chemical fumigants have (Brown and Morra, 1997). Crop residues of plant species such as *Brassica*, for example mustard (*B. juncea*) and broccoli (*B. oleracea),* are high in these volatile compounds namely glucosinolates (GSL), which once incorporated into the soil as green manure, acts as a biofumigant. When the GSLs come in contact with the enzyme myrosinase, also contained in the plant cell, it is hydrolysed and releases natural and biologically active compounds including isothiocyanates (ITC), thiocyanates and nitriles (Kirkegaard *et al.,* 2004). These compounds act as natural toxins against plant pests and are also effective nematicides and fungicides (van Ommen Kloeke, 2012).

Several studies have confirmed the effectiveness of these natural toxins, especially ITCs, as fungicidal, bacteriocidal and/or nematicidal products against a series of plant pests, for example crown rot, wilt and root-knot nematodes of corn and wheat crops (Fahey *et al.,* 2001; Kirkegaard *et al.,* 2004; Clark 2007; Henderson *et al.,* 2009). However, very little information is available on the effects of GSLs and its breakdown products on non-target and essential soil organisms such as earthworms and soil microorganisms. Non-target soil organisms can be exposed to the natural toxins (ITCs) through decomposition of crop litter (van Ommen Kloeke, 2012).

Earthworms and soil microbial communities are important members of all terrestrial ecosystems and play an important role in the productivity of agricultural systems (Girvan *et al.,* 2003). These organisms are critical for important biochemical processes such as the breakdown and transformation of organic matter in fertile soils. They control and maintain important soil functions such as carbon and nutrient flows, soil structure and soil aeration, decomposition and even pollutant degradation. Earthworms and soil microorganisms are also important biological indicators during risk assessments because they are sensitive to chemical and physical changes in soil and can detect negative effects on the soil ecosystem at an early stage. Any long-term interference with these biochemical processes could potentially affect nutrient cycling, which in turn could alter the soil fertility (OECD, 1984). Although the microbial communities responsible for these processes differ from soil to soil, the pathways of transformation are essentially the same. There is a need to better

understand the relationship between specific crop management practices such as biofumigation and the resultant changes in the soil ecology for the development of more efficient and sustainable crop production systems (Larkin and Honeycutt, 2005).

In this study, earthworms (*Eisenia andrei)* were exposed to three biofumigants and two chemical fumigants. Sublethal endpoints such as growth and reproductive success of the earthworms were monitored. Reproductive success was determined by the number of cocoons produced and the number of hatchlings after exposure to fumigants and comparing it to the control samples. In addition, the genotoxicity of the biologically active compounds found in the fumigants, was evaluated by means of the comet assay and compared. The comet assay is a rapid and sensitive technique to detect and quantify DNA damage in single cells and was used to determine the genotoxic effects of these substances. It has successfully been applied to a number of species already used in biomonitoring and toxicity testing and is a sensitive system for screening chemicals and complex mixtures for their genotoxicity (Cotelle and Ferard, 1999).

In support of the earthworm reproduction and genotoxicity study, the changes in the soil microbial community structure and function were evaluated by means of community level physiological profiling (CLPP) and phospholipid fatty acid (PLFA) analysis. Neither of these two approaches can provide a complete depiction of soil microbial characteristics on its own; however, each approach provides a somewhat different perspective. A more complete representation of the soil microbial characteristics can be achieved by the use of multiple approaches (Larkin and Honeycutt, 2005). Biolog™ EcoPlate analyses were used as a CLPP technique because it is an effective means of distinguishing spatial and temporal changes in microbial communities (Garland, 2007). Phospholipid fatty acid profiles offer rapid and reproducible measurement for characterising the numerically dominant portion of soil microbial communities and can identify the changes in the proportions of major functional groups of organisms in soil samples (Zelles, 1999). The Biolog™ EcoPlate analysis and PLFA analysis were used in conjunction to increase the complexity of the microbial community profiling.

With the information obtained from this research, a better understanding can be obtained of the effects of particularly the biofumigants on the non-target soil organisms and in turn the soil health and productivity when using biofumigation as a crop protection practice. Better informed decisions can be made when incorporating biofumigants as part of an integrated pest management (IPM) strategy for agricultural crops.

The aim of the study was to compare the survival, growth, reproduction and genotoxicity of bio- and chemical fumigants to earthworms (*Eisenia andrei*) as well as the effects on the soil microbial community function and structure. The specific objectives included:

- To assess the effect of the fumigants on the survival, growth and reproductive success of earthworms.
- To assess and compare, by means of the comet assay, the level of DNA damage in individual cells of earthworms exposed to soils treated with both types of fumigants.
- To assess, by means of PLFA analysis, the changes in soil microbial community structure and biomass after treatment of soil with bio- and chemical fumigants.
- To determine the effect of both types of fumigants on the functional response of soil microorganisms using the Biolog™ EcoPlate analysis.

Chapter 2 – In the *Literature Review* the relevant literature is discussed and the review includes different components. Firstly, the importance of soil as a natural resource and the management of our natural resources are discussed. The use of biofumigants as green manure, the use of chemical fumigants in agriculture, the essential role of soil organisms, especially earthworms and microbial communities are reviewed. Literature on the comet assay as a method used to test genotoxicity in earthworms as well as the Biolog™ EcoPlate and PLFA methods to assess microbial communities, are also discussed.

Chapter 3 – *Materials and Methods* gives a detailed description of the materials and methods used in this study. This includes the experimental outlay, details of treatments, methods used to investigate earthworms and microbial communities and statistical analysis methods that were used to analyse the data obtained.

Chapter 4 – The *Results and Discussion* gives the results of the in vitro experiments. It includes descriptive and graphical representations of the results obtained. A comprehensive discussion and interpretation of the results were included in this chapter.

Chapter 5 – The *Conclusions and Recommendations* provide a general conclusion for the study and recommendations for future studies based on the findings of the current investigation.

References - a complete list of references for all chapters are provided at the end of the dissertation.

CHAPTER 2: LITERATURE REVIEW

2.1 Importance of soil and soil management

Soil is the fundamental regulatory compartment for all terrestrial ecosystems and a valuable natural resource on which all life on earth depends. In an ecological context, soil is essential for important regulatory functions such as filtering of water, buffering functions, habitat functions and productive functions for example food production (Schüürman and Market, 1998). The core of a healthy ecosystem is soil quality, which is the "ability to sustain biological productivity, maintain environmental quality and promote plant and animal health" (Smith and Collins, 2007). However, the quality of soil worldwide, including South Africa, is deteriorating at an alarming rate. This is due to varying environmental impacts or disturbances on the soil resources especially due to human activities. In South Africa, several studies have focused on the threat of human activities on the soil environment. These include, but are not limited to, mining (Maboeta *et al.,* 2008), agricultural practises (Helling *et al.,* 2000; Schulz and Peall 2001; Vermeulen *et al.,* 2001; Reinecke and Reinecke 2007), urbanisation and industrialisation (Maboeta and Fouché, 2014). The availability and conservation of fertile and good quality soil remain major challenges in soil management.

In order for natural resources to be utilised in a sustainable way, the use and protection of these natural resources need to be managed. Studies investigating the effects of human activities on our natural resources such as different crop protection practises are important. Natural resource management (NRM) is defined as the responsible management of soil, water, and biological resources with the intention of sustained productivity and prevention of degradation (Barrow, 2006). Disturbances pose a threat to soil health when it has an adverse effect on the functioning of soil (Ashman and Puri, 2008). It is therefore imperative to manage the quality of our natural resources.

Natural resources are governed by a range of institutions, from international to local government bodies, which give rise to context-specific natural resource management regimes (Cousins *et al.,* 2007). The Convention on Biological Diversity (CBD) aims to bring about the sharing of the benefits due to the utilisation of our natural resources such as soil and water. This is implemented at a national level in the White Paper on the Conservation and the sustainable use of South Africa's biodiversity (Notice 1095 of 1997) through the integration of conservation and sustainable use of natural resources into all sectors. The Constitution of the Republic of South Africa, Act 108 of 1996, introduced a constitutional framework for post-1994 and has elevated environmental protection through the

entrenchment of the right to a non-harmful environment in the Bill of Rights. Legislation such as the principal National Environmental Management Act 107 of 1998 (NEMA) and its amendment acts, forms the cornerstone of environmental legislation in South Africa. Chapter 1 of this act provides fundamental environmental management principles for decision making to ensure that the environmental rights set out in section 24 of the Constitution are adhered to by all organs of state and private parties in South Africa (Van der Linde, 2006). One of the key features that make NEMA unique is to enforce measures, which will ensure the protection of the production potential of soil and the quality of the water resources of the country and agricultural land (Tainton, 1999).

2.2 Soil ecosystems

Soil ecosystems are highly complex and dynamic environments. It is made up of a huge diversity of biological communities and a range of physical and chemical components such as the soil, water and nutrients. Each ecosystem and the interactions within it are unique. The ecosystem services in soil environments depend on the natural and healthy functioning of the ecosystem as well as the structure of an ecosystem, which is determined by the kinds and combinations of species that make up the system. The physical characteristics of the environment such as the annual cycles of temperature and the rainfall of an area shape the structure and characteristics of the biological communities of the ecosystem (Muscolo *et al.,* 2014) When humans convert, for example, natural grassland to intensive agricultural land, it changes the species composition and functioning of the ecosystem, which in turn can alter the type and scale of ecosystem services that are provided.

Soil organic matter (SOM) is fundamental to the quality and functioning of soil ecosystems because it affects structure and stability. It provides the main binding sites for soil aggregates during decomposition and turnover and supplies most of the macronutrients for plant growth (Smith and Collins, 2007). The dynamic fraction of SOM consists of amino acids, proteins and carbohydrates. Intensive cropping is one of the most significant ecosystem disturbances that directly decrease SOM in agricultural soils (Smith and Collins, 2007). Two key features of soil ecosystem stability are resistance and resilience. Resilience is the soil ecosystem's ability to recover from disturbances in good time while resistance is the ability to resist invasions from exotic species and to maintain its regulatory functions despite disturbances. Resilience and resistance is directly dependent on the soil microbial communities that live in the soil and can be assessed by monitoring microbial activity (Nannipieri *et al.,* 2003; Allison and Martiny, 2008).

The quality of soil can be defined as its suitability for an intended purpose whether it is for domestic, agriculture, mining or industrial purposes (Ashman and Puri, 2008). It can also refer to its "ability to sustain biological productivity, maintain environmental quality and promote plant and animal health" (Smith and Collins, 2007). The quality of soil is determined by its chemical, physical and biological characteristics and by soil stability. All these factors can be affected by both natural processes and human activities. High quality soil is essential to maintain the integrity of the soil and terrestrial ecosystem (Muscolo *et al.,* 2014)

2.2.1 *Soil organisms*

Due to the immense biological complexity of soil organisms, they are generally grouped according to their size. Microbiota consists of bacteria, fungi, protozoa and algae. Mesobiota generally consist of nematodes, springtails, rotifers, mites and arthropods smaller than 10 mm. Macrobiota consist of invertebrate groups such as earthworms, enchytraeids and larger arthropod groups. This grouping however does not say much about the ecological role of each in the soil (Ashman and Puri, 2008). The activity, ecology and dynamics of soil organisms are affected by several environmental factors such as carbon and energy sources, mineral nutrients, water, temperature, pH, spatial relationships and the interactions of the organisms (Nannipieri *et al.,* 2003). In addition to soil factors, anthropogenic activities can also influence the activity, ecology and dynamics of soil organisms. These include but are not limited to the use of fertilisers and other chemicals, cultivation techniques, crop types, deforestation, harvesting, heavy metal contamination (Maboeta *et al.,* 1999; Lukkari *et a*l., 2005; Máthé-Gáspár and Anton*, 2005;* van Gestel *et al., 2009)* and veterinary medicines (Jensen *et al.,* 2007)*.*

Earthworms represent a large proportion of soil biomass and play a key role in soil organic matter dynamics and nutrient cycling (Edwards and Bohlen, 1996). Earthworms influence the availability of resources to other species and are accepted as ecosystem engineers because they have positive effects on soil structure (Pelosi *et al.,* 2013). It is also widely acknowledged that earthworms are very useful indicators of the condition of soil ecosystems (Cortet *et al.,* 1999; Bleeker and van Gestel*,* 2007; Maboeta *et al.,* 2008; Sizmur and Hodson 2009). Earthworms are widespread and sensitive enough to be used as indicators but they are also robust and resistant. They are in direct contact with the pore water and in other words with the bioavailable fraction of contaminants in the soil. An accurate estimation of their diversity and biomass is required to quantify their role in ecosystems and techniques to optimise their sampling from soil are important. Valckx *et al.* (2011), showed that chemical

expellants such as formalin and mustard suspensions are more efficient than the traditional hand sorting techniques.

Microbial food webs in soil are vast, with each gram of soil containing an average of 10^9 bacterial organisms representing between 4000-10000 different species (Barton and Northup, 2011). Microorganisms live together in a defined physical area, abundance and distribution of coexisting populations to form a structured community (Barton and Northup, 2011). A structured community are made up of a specific species composition, diversity and abundance and include all the major microbial groups including bacteria, viruses, fungi, and archae. Due to the complex nature of the interactions between soil and the organisms that form part of its functioning, it is often difficult to study microbial communities independently.

Soil microorganisms form a critical part of soil environments and are involved in and affect many of the soil ecosystem functions. They also play an important role in the productivity of agricultural systems (Girvan *et al.,* 2003). One example is their crucial role in the decomposition and turnover of soil organic matter (SOM). Microbial communities control and maintain important soil functions and they affect the development and functioning of terrestrial ecosystems (Smith and Collins, 2007). The microorganisms in soil are essential to biogeochemical cycles and provide fundamental ecological services such as soil fertility (Whitman *et al.,* 1998), suppression of pathogens (Balvanera *et al.,* 2006), and promotion of nutrient availability to plants and degradation of pollutants. They affect the mobility of heavy metals, stabilise soil aggregates, improve the water retention and porosity of soil, and support higher organisms (Prescott *et al.,* 2008; Deng *et al.,* 2012.). Within the context of soil quality, microorganisms play a critical role in ecosystem health (Smith and Collins, 2007). Plant growth and health is directly dependent on the activity of soil microorganisms. Microorganisms form symbiotic associations with plants to assist in nutrient assimilation and are especially important in helping maintain a nontoxic soil environment by breaking down toxic compounds (Brady and Weil, 2008).

Due to the fact that microorganisms are very sensitive to disturbances in soil, the capacity of soil to recover after disturbances can be assessed by monitoring microbial activities (Nannipieri *et al.,* 2003). If microbial composition changes due to natural or anthropogenic disturbances, it can influence the rate of ecosystem processes and in turn ecosystem stability.

2.3 Soil and agriculture

Agriculture played an important role in the development of soil science. The role of soil factors such as the soil physical, chemical and biological condition for plant growth was only fully appreciated in the early 1800s when T de Saussure and J von Liebig published work on plant physiology coupled with scientific agricultural trials done in 1834 -1843 by English scientists. Today there is a general agreement that to optimise plant growth, we need to optimise these three factors (Ashman and Puri, 2008).

Only 13% percent of South Africa's land can be used for crop production and of this land, less than 20% are considered high-potential arable land (Mohamed, 2000). Cereals and grains are some of the more important crops in South Africa. Although water availability remains the greatest limiting factor in crop production, important factors such as the yield loss due to pests also present limitations on sustainable crop production. Crop protection practices play an important role in preserving the productivity of agricultural land against competition from pests, weeds, pathogens and viruses (Oerke and Dehne, 2004). In general, chemical compounds have become an essential part of pest control in agricultural land since the 1950s when the first organochlorine insecticides were introduced (Dent, 2000). Today, varying classes of chemical pesticides are used for crop protection and include herbicides, insecticides, nematicides, fungicides and soil fumigants. Pesticides represent a diverse group of inorganic and organic chemicals including organochlorines, organophosphates, carbamets, pyrethroids, insect growth regulators and several newer chemicals (Dent, 2000). The majority of these chemicals consist of an active ingredient and a number of additives to improve the efficacy when it is applied. These chemical substances are only efficient at controlling the specific pest if it is suitably toxic and reaches its intended target pest species.

2.3.1 *Soil fumigants*

Chemical fumigants are highly effective in controlling and reducing soil pests such as parasitic nematodes, weeds, fungi and insects to nearly undetectable levels. It is extensively used in agriculture to ensure the success of high value cash crops. In South Africa soil fumigation is used to control important pathogens for this region, including root knot nematodes (*Meloidogyne* spp) and ring nematodes (*Criconemoides* spp*).* Ideally, chemical fumigants should possess a high rate of pest elimination in as short a period as possible, low phytotoxicity and plant absorbed residue and a long residual activity in the soil (Dubey and Trivedi, 2001).

Methyl bromide (MB) is considered to be the most reliable soil fumigant for plant parasitic nematodes, fungi and weeds prior to planting and was extensively used for soil borne pathogens (Giannakou and Anastasiadis, 2005). However, it has a high toxicity in the environment and is considered to be a significant ozone depleting substance. Parties to the Montreal Protocol agreed to reduce its production and use and it was eventually phased out and finally banned by the United States Environmental Protection Agency (US EPA) in 2005 (US Environmental Protection Agency, 2011). Extensive research has been done to find effective and practical alternatives to replace MB. Several alternatives have been identified and some of the most common substances that are used for agricultural crops include cadusafos, chloropicrin, dazomet,1,3-dichloropropene, dimethyl, disulfide and metham sodium (MS) (US Environmental Protection Agency, 2011). Research shows that no single fumigant is as effective as MB, however, similar results can be obtained when combining two or more substances. Giannakou and Anastasiadis (2005), showed that MB treated plots resulted in the best nematode control and higher yields than any of the other treatments, however, the results were not statistically significant compared to a MS and cadusafos combination treatment.

Metham sodium (sodium N-methyldithiocarbamate) is a broad spectrum fumigant commonly used in the agricultural and horticultural environments for its ability to control a number of pests and diseases including nematodes, fungi, insects and weeds (Macalady *et al.,* 1998; Warton *et al.,* 2001). The US EPA (1997) considers MS to be a commercially viable fumigation alternative for MB for the purpose of vegetable and fruit crops. It is commonly used in integrated pest management systems because it can be used in conjunction with other treatments. Standard acute toxity testing studies by the US EPA (1994), to determine the LD_{50} or LC_{50}, have placed MS in Toxicity Category III, which is classified as slightly to moderately acutely toxic (Carlock and Dotson, 2001). However, these studies provide limited information regarding sublethal effects of the compound. Sublethal testing on rats and dogs show a definite dose-response effect by MS (Carlock and Dotson, 2001).

As soon as MS comes into contact with moist soil, it is largely converted to methyl isothiocyanates (MITC) and to a smaller extent to other degradates such as carbon disulfide (CS_2) and hydrogen sulphide (H_2S) (Carlock and Dotson, 2001). Methyl isothiocyanate is considered to be the active biocidal product due to its high reactivity with amines and thiols in biological molecules without enzymatic catalysis (Pruett *et al.,* 2010). Metham sodium decomposes to MITC within a few hours after application. The MITCs are sulphur containing molecules that can remain in the soil for twelve or more days. According to Carlock and

Dotson (2001), MS is not mutagenic, in other words it does not cause mutation but has been found to be cytotoxic to bacteria, fungi and mammals.

The database for toxicology studies on MS for risk assessment purposes are considered to be complete, however there is a lack of information available on the ecological impact of MITC to terrestrial organisms. Adverse effects on non-target species have been inferred from modelling studies while toxicity to aquatic organisms and adverse effects on soil microbes have been confirmed (Warton *et al.,* 2001; Pruett, *et al.,* 2010; Omirou *et al.,* 2010; Dubey and Trivedi, 2011). Studies testing the effect of MS on microbial diversity show that this fumigant decreases microbial biomass and leads to structural changes in the microbial community (Macalady *et al.,* 1998; Omirou *et al*., 2010). These effects remain active for up to 18 weeks after exposure (Macalady *et al.,* 1998; Pruett *et al.,* 2010). Metham sodium is susceptible to enhanced biodegradation. This refers to the accelarated rate at which a compound is degraded by soil microorganisms, usually bacteria, after several and regular applications of the same compound (Warton *et al.,* 2001). A study by Warton *et al. (*2001), showed that a normal dosage of MS produced less than half the MITC in soil that was extensively treated with MS in the past, due to microbial degradation. Soil bacteria that were isolated from soil exhibiting enhanced biodegradation, were all found to be Gram positive.

Cadusafos (S, S-di-sec-butyl 0-ethyl phosphorodithioate), is an organophosphate fumigant, specifically a phosphorodithioate, that controls nematodes and insects through acetylcholine esterase inhibition (Wu *et al.,* 2011). This compound is known by the commercial name RUGBY and it is available in the market in liquid form as RUGBY 100 ME/EW containing 100 g/L and in granular form as RUGBY 10 G containing 100 g/kg. It forms a colourless to yellow liquid, completely miscible with acetone, acetonitrile, dichloromethane, ethyl acetate, toluene, methanol, isopropanol and heptane. It is stable up to 50 °C and the biological activity lasts longer compared to other nematicides. Cadusafos has a broad spectrum of activity. It controls all nematodes and particularly the most dangerous genera such as *Meloidogyne*. Its uses include protection of crops such as tobacco, sugar cane, potatoes, maize, citrus and banana (Ungerer, 1996). In South Africa, cadusafos has been registered for citrus nematode control since 1992 (McClure and Schmidt, 1996). Biological activity of cadusafos lasts up to four to five months in medium to heavy textured soils while on sandy soils, the activity period lasts anything from eleven to sixty days (Elshafei *et al.,* 2009). Most organophosphorous pesticides are generally considered non-persistent in the environment and studies by Karpouzas *et al.* (2005), showed that cadusafos residues may persist in soil

at concentrations sufficient to control nematodes and insects for up to six weeks (ElShafei *et al.,* 2009).

According to the Material Safety Data Sheet, cadusafos is classified as a hazardous substance (Santa Cruz Biotechnology, 2010). Chemical or photochemical mechanisms may produce a leaving group, which is easily degraded. Breakdown products of cadusafos are composed of low-molecular weight, volatile molecules such as esters that are easily degraded by hydrolysis and can be utilised by microorganisms. In soil, the physical properties of the soil, water content and microbial communities can affect their persistence. Metal ions in soil may cause strong binding through hydrogen linkage, which makes them unavailable for biological decomposition.

According to the European Food Safety Authority (EFSA) Scientific Report (2006), cadusafos has no genotoxic potential and is not considered carcinogenic. Studies on rats showed that cadusafos does not produce mutations or chromosome aberrations in Chinese hamster ovary cells, hepatocytes and bone marrow cells. It also states that metabolism studies in potato crops to investigate residue levels, showed residues below 0.01 mg/kg. However, it is suggested that further supervised trials be carried out. In a first tier assessment in soil of potato crops, an acute and long term risk was identified for earthworm-eating birds and mammals. Cadusafos has the potential to bioaccumalate because it has an octanol/water partition coefficient (log P_{ow}) > 3. Considering the potential for bioaccumulation, residues in earthworms found in natural soils might be higher if the soil organic content is lower than that used in artificial soils prepared in the laboratory (EFSA Scientific report, 2006). Results from acute and reproduction studies with the formulation RUGBY 200CS, which is the capsule suspension containing 200 g/L cadusafos, shows that it is toxic to earthworms. No further information on acute toxicity testing is available. Studies to assess the risk to soil microorganisms were done in potato crops with the standard granular formulation and showed no statistically significant effects on non-target soil microorganisms (EFSA Scientific Report, 2006). It was suggested that further risk needs to be investigated with other crop types.

Several studies have documented the efficacy of cadusafos as a nematicidal fumigant. Dubey and Trivedi (2011), studied the effect of three nematicides for the control of *Meloidogyne* spp, including cadusafos. They confirmed the effectiveness of all three products, but in particular the cadusafos against nematode infections. In addition to controlling nematicides, this product was effective against other infestations like fungi and

weeds. Just like MS, cadusafos is susceptible to accelerated microbial degradation when certain soil microorganisms become adapted due to long term and frequent applications.

Since the introduction of chemical pest management practices, there have always been problems associated with the misuse of products and the initiation of more ecologically sound integrated pest management (IPM) approaches. Host plant resistance studies, genetic manipulation and biological control are just a few of the many available crop protection and pest management practices used today as control measures for agricultural land.

2.3.2 *Biofumigation*

Biofumigation is a form of biodynamic farming, which is a system of organic agriculture (Smith and Collins, 2007). Organic agriculture management promotes the maintenance of soil fertility and soil organic levels through practices such as providing plant nutrients through microbial decomposition of organic materials, the control of pests, disease, and weeds with crop rotations, cover crops or green manure and pest-resistant plant varieties. Cover crops or green manure is most commonly used for biofumigation. Biofumigation refers to a process in which certain plants' own protection function is used to control a range of soil pathogens such as fungi, nematodes, bacteria and certain weeds (Morra and Kirkegaard, 2002). The main aim of biofumigation is to promote the control of pests, diseases and weeds while maintaining the SOM for soil fertility. It can be achieved by incorporating fresh plant material (green manure), seed meals (a by-product of seed crushing for oil), or dried plant material into the soil (Flamini, 2000).

Biofumigation is an important alternative to synthetic chemical fumigants and has become an important crop protection practice for the commercial and emerging agricultural community (Brown and Morra, 1997). In addition to the volatile compounds that act as biofumigants, the plant material provides additional benefits to the soil. It improves soil fertility by recycling nutrients and returning organic matter to the soil and it improves soil structure. In general, research done in the first half of the 21st century with regards to biodynamically managed soils, shows that the soil tend to have a greater microbial biomass, a higher rate of nitrogen mineralisation, higher soil carbon levels and more active microbial respiration. Soil quality is generally improved due to enhanced microbial decomposition and stabilisation of SOM (Smith and Collins, 2007).

Plant species that act as biofumigants contain special volatile compounds that upon soil-incorporation act as biofumigants (Henderson *et al.,* 2009). The most important of these volatile compounds are glucosinolates (GSLs). Glucosinolates, β-thioglucoside and *N*-hydroxysulfates are stable water soluble molecules contained in plant cells. Upon damage to the plant cells by for example, mastication or freeze-thawing, the enzyme myrosinase (thioglucoside glucohydrolase E.C.3.2.3.1) (Brown and Morra, 1997; Kirkegaard *et al.,* 2000; Matthiessen and Kirkegaard, 2006), also contained in the plant, hydrolyses the GSLs to release a combination of natural and biologically active compounds which includes isothiocyanates (ITC), nitriles, thiocyanates and oxazolidine (Morra and Kirkegaard, 2002). These products are highly toxic to various microorganisms and the ITC have been found to be the most important for biofumigation purposes (Fahey *et al.,* 2001; Yulianti *et al.,* 2007). Of the one hundred and thirty two GSLs that have been identified, approximately thirty are present in the *Brassica* sp. (Bellostas *et al.,* 2007; Agerbirk and Olsen, 2012).

The concentration and type of GSL in the species vary greatly. Studies have shown that these differences are due to age and different environmental conditions (Bellostas *et al.,* 2007). This has encouraged new research to identify high GSL species as well as approaches to improve the biofumigation potential of *Brassica* amendments (Morra and Kirkegaard, 2002). Kirkegaard and Sarwar (1998), investigated the variation in GSL production in the roots and shoots of seventy six entries from thirteen *Brassica* species. Total plant GSL production on a ground area basis at mid-flowering stage, ranged from 0.8 - 45.3 $mmol.m^{-2}$. On average, the highest GSL concentration in the plant was found to be in the shoots, however, this could be contributed to the higher biomass in the shoots compared to the roots, which may have the same or even higher concentrations of GSL (Kirkegaard and Sarwar, 1998). Various factors determine the successful hydrolysis of GSLs to ITC including water availability, temperature fluctuations (Price *et al.,* 2005) and the GSL content (Matthiessen *et al.,* 2004). The amount of tissue disruption is also a major determining factor of how much ITC is released. Tissue disruption by freezing, drying or maceration can increase the contact between the enzyme and the GSLs and thus the concentration of ITC released (Morra and Kirkegaard 2002).

Isothiocyanates are reactive, electrophilic chemicals. Differences in ITC volatility are due to the variation in side chain structure (Agerbirk and Olsen, 2012). Isothiocyanates react with amine groups, sulph-hydryl groups and the disulphide bonds of proteins instigating the degradation of enzymes and the inhibition of microbial growth (Brown and Morra, 1997). Natural ITCs, also referred to as AITC, found in plants are different to MITCs generated by

the chemical fumigant MS because MITCs are structurally the simplest and the most volatile. Plants rich in aliphatic ITCs are more likely to have the potential to exert stronger ITC-based biofumigation effects than those similarly rich in aromatic ITC (Matthiessen and Shackleton, 2005). *Brassica* species are rich in aliphatic ITC such as 2-propenyl or 2-phenylethyl ITC (Morra and Kirkegaard, 2002). An in depth study was done by Morra and Kirkegaard (2002) to determine the amount and efficiency of ITC release by plant cells following soil incorporation. Results indicate that most of the ITC are released within the first four days after incorporating GSL containing plant material into the soil. This timing can vary greatly due to soil chemical and physical properties, temperature and moisture. The ITC release efficiency of two high glucosinolate containing plants namely *B. napa* and *B. juncea* was investigated and it was found that the release efficiency was very low compared to the potential available ITC. The potential ITC available from *B. juncea* after incorporation was 112 nmol.g^{-1} soil however, the measured ITC concentrations in the soil were near 1 nmol.g^{-1}, indicating a release efficiency of only 1 - 5 % of the potential ITCs present in the plant (Morra and Kirkegaard, 2002). However, even at these low levels, there was a success rate in controlling nematodes.

Table 1: ***Isolated allyl-isothiocyanates from oilseed radish, mustard and broccoli (Morra and Kirkegaard, 2002; Price et al., 2005; Matthiessen and Kirkegaard, 2006; Blazevic and Mastelic, 2009; van Ommen Kloeke, 2012)***

Broccoli (*Brassica oleracea)*	**Mustard (*Brassica juncea)***	**Oilseed radish (*Raphanus sativus*)**
3 butenyl ITC	2-propenyl ITC	4-(methylthio)butyl ITC (erucin)
4-(methylthio)butyl ITC (erucin)	2-pheneylethyl ITC	2-phenylethyl ITC
4(R)-methylsulfinylbutyl ITC	3 butenyl ITC	4-(methylthio)-3-butenyl ITC
		5-(methylthio)pentyl ITC

Research has been conducted on the effectiveness of natural ITC as nematicidal, biocidal and fungicidal agents. Many studies have proved it effective agents against plant parasitic nematodes such as root knot nematodes (*Meloidogyne* spp), lesion nematodes (*Pratylenchus neglectus)*, sugarbeet nematodes (*Heterodera schachtii)* (Potter *et al.,* 1998; Price *et al*., 2005; Wu *et al.,* 2011; Kruger *et al.,* 2013) and fungi (Fan *et al.,* 2008). Negative effects on invertebrate species such as wireworms and fruit flies have also been confirmed (Brown and Morra, 1997), while studies by Edmond (2003), show AITC to be an effective chemical expellant when sampling earthworms. Price *et al.* (2005), investigated the effects of *B. juncea* on *Pythium ultimum,* plant pathogens known for causing root rot and *Rhizoctonia*

solani, a plant pathogenic fungus, in closed jar experiments. The breakdown products of *B. juncea* completely suppressed the *P. ultimum* and reduced the growth of *R. solani* by more than 72 % (Price *et al.,* 2005). Wu *et al.,* 2011 determined the nematicidal efficacy of isothiocyanates against root knot nematodes. His findings showed that 0.5 – 2.0 ml ITC per kg of soil applied prior to planting was most effective in reducing root knot nematodes in soil and was equally effective as the chemical fumigant MS.

A study by Stevens *et al.* (2009), investigated the potential of GSL containing oilseed crops (*Limnanthes alba*) as herbicidal agents. In this study they investigated the possibility of converting the GSLs in the seed meal into herbicidal degradation products. Due to the lack of myrosinase, the enzyme that converts GSL to ITC, in seed meal, an alternative had to be found to convert the available GSLs to ITC. It was found that fermented seed meal had a potent and effective herbicidal activity with predictable activity for use in agriculture (Stevens *et al.,* 2009). However, some species of bacteria and fungi are known to be resistant to GSL (Fan *et al.,* 2008).

Although it is evident that biofumigation is effective against a large array of soil organisms, very little information is available on the effects of these agents against non-target soil organisms such as beneficial soil microorganisms and other soil macrofauna, for example earthworms. This highlights the need to investigate possible mechanisms leading to the disruptive effects on the non-target species. Henderson *et al.* (2009) did a study on the impacts of mustard biofumigation on non-target nematode species. The results indicated that mustard biofumigation with *B. carinata* seed meal disrupted the biological control of beneficial *Steinernema* spp in controlling root knot nematodes by disrupting their foraging efficiency. A noteworthy fact is that this disruption was detected thirty days after the incorporation of the biofumigants into the soil, which is longer than the expected toxic activity of the breakdown products as determined by Morra and Kirkegaard (2002).

In the USA, scientists have extracted ITCs from plant material to produce a product called Dazitol. However, this product is not readily available in South Africa and biofumigation is commonly applied as cover crops by growing *Brassica* crops such as mustard, oilseed radish and canola. Some of the plant cultivars with biofumigation potential that are commercially available in South Africa include *B. juncea* cv Caliente 199 (indian mustard), *B. napus* cv. AV Jade (canola), *Eruca sativa* cv Nemat (rocket) (Kruger *et al.,* 2013) and *Raphanus sativus* cv Bladrammenas Terranova and Doublet (oilseed radish). For this study,

two of the commercially available products, Doublet/Bladrammenas (oilseed radish) and Caliente 199 (indian mustard) were used together with a third species *B. oleracea* (broccoli).

Caliente 199 is a fast growing annual with high biomass production if it receives adequate soil moisture and sufficient nutrition (Kruger *et al.,* 2013). It was bred specificially for its high glucosinolate content and is primarily used in crop rotation programmes. It is effective in suppressing certain soil-borne diseases, weeds and some nematode species such as root knot nematodes (*Meloidogyne* spp). Bladrammenas/Doublet (oilseed radish) is used in crop rotation with cabbage due to its resistance towards beet cyst nematodes. It has a moderate level of glucosinolates and have been shown to be effective in nematode control of *Heterodera schachtii, Meloidogyne* spp *and Paratrichodorus teres (*Joordens Zaden, 2011).

2.4 Biomonitoring

Biomonitoring refers to the use of organisms (bioindicators) to monitor contamination and to understand the possible effects of contaminants on biota and humans. Structural and functional measurements of sublethal effects are important in risk assessment and ecotoxicological studies to evaluate the effects of chemical substances. Structural measurements assess total abundance or diversity on for example plant density, biomass and so forth, while functional measurements measure the rate processes such as growth rates, changes in physiological processes and reproductive success.

General methods of biological analysis through the application of bioindicators and biomarkers are important technologies used during risk assessments and ecotoxicological studies because it can assist in understanding the fate and effects of hazardous substances in the biosphere. Bioindicators and biomarkers indicate a sequence of events in the causal chain between exposure to a hazardous event and the related adverse effect (Grandjean, 1995). Therefore the aim of using bioindicators and biomarkers are to relate harmful chemical presence in an environment to the effects it has on living organisms (Walker *et al.,* 2006). A bioindicator provides information about the effect that environmental conditions has at the level of the organism through its behaviour (van Gestel and Brummelen, 1996). In past ecotoxicological evaluation procedures, acute toxicity testing such as lethal concentration (LC_{50}) or lethal dose (LD_{50}) were used to determine at what concentrations of contaminants, harmful responses were observed (Reinecke, 1992). However, LC_{50} or LD_{50} does not provide information on the sublethal effects that a substance might have on the organism or its habitat. A biomarker is another tool used in biomonitoring. Biomarkers are quantifiable biological parameters at the sub-organismic level for example biochemical, cellular or physiological variation that can be measured in the tissue or body fluids of organisms that

provides evidence of exposure and/or the effects of one or more chemical pollutants (Depledge *et al.,* 1995; van Gestel and Brummelen, 1996). In order for a biomarker to be considered useful in an ecotoxicological framework, it has to indicate exposure and effect of toxins (Peakall, 1992). Biochemical biomarkers are normally associated with indicating exposure before acute effects occur. It should where possible also be related to other sublethal effects of an organism for example, how it effects growth, reproductive output and energy utilisation in order to be extrapolated to higher levels of organisation (Chapman, 1995).

The use of sublethal endpoints of bioindicators and biomarkers has true potential in monitoring and assessment of risk because they can act as early warning systems and signal adverse ecological changes and effects before they occur (Depledge *et al.,* 1995). In the review article by Reinecke and Reinecke*,* (2007) a series of biomarker responses were correlated to changes at population levels and the conclusion was made that although there were not always mechanistic links between the biomarker responses and higher organisation levels, there was definite potential that biomarkers could serve as an early warning system. Depledge *et al.* (1995), state that the detection of early warning signals with biomarker responses is cause enough to instigate managerial action to prevent further damage to occur. There is no doubt that measuring stress responses in organisms at cellular level can indicate exposure and the effect of toxicity (Reinecke *et al.,* 2001).

2.5 Earthworm bioindicators and biomarkers

Earthworms have been used extensively as surrogates in ecotoxicological studies due to their ability to reflect trends in other species such as vertebrates. They have several characteristics that allow them to be considered as good bioindicators. These characteristics include the fact that they are ecologically significant due to the important role they play in soil ecosystems, they are common, resilient, widespread and genetically relatively uniform (Cortett *et al.,* 1999). Biomass, survival and reproductive success are useful measures to determine the functional capacity or state of the organism after exposure. Furthermore, molecular biomarkers allow the detection of alterations in the physiological status of the organisms and may be sensitive enough to indicate cellular stress before sublethal effects on growth and reproduction is detected (Velki and Hackenberger, 2013). Earthworm biomarkers have successfully been applied to investigate various factors in the soil environment including, toxicity of different insecticides (Jensen *et al*., 2007; Velki and Hackenberger, 2013), fungicides (Maboeta et al., 2004), the effects of metal pollution (Maboeta, 1999; van Gestel *et al.,* 2013), the genotoxicity of contaminated soils and

gaseous extracts mixed into soil (Cotelle and Ferard, 1999) as well as the monitoring of hazardous compounds in terrestrial ecosystems (Verschaeve and Gilles, 1995).

Biomarkers such as the neutral red retention assay (NRRT) is a technique derived from Svendsen *et al*. (1995), and is one of the easiest techniques to determine lysosomal membrane integrity, which is a marker for cellular stress. During cellular stress, the neutral red dye leaks into the lysosome cytosol after a distinct period of time (retention time), colouring it a pinkish colour (Harreus *et al.,* 1997). The NRRT assay is a cheap and time efficient tool that is capable of signifying the spatial distribution of biological effects that resulted after a pollution incident (Weeks, 1998).

Genotoxicity and mutagenicity are also used as ecotoxicological biomarkers. Genotoxic effects refer to the damage caused to cellular deoxyribonucleic acid (DNA) by environmental pollutants (Peakall, 1992). Chemical or physical injuries to the DNA structure refers to the genetic lesions such as DNA strand breaks, which are produced by the interaction of chromatin and a reactive oxygen species (ROS) such as a hydroxyl radical (Fairbairn *et al.,* 1995). These genetic lesions can promote changes (mutations) and/or damage that are evaluated by genotoxic studies. Genotoxins are called mutagens if it causes changes in the DNA sequence that is retained during cell division and carried forward in future generations. However, not all genotoxins are mutagens as they may not cause retained genetic alterations (Cestari, 2013). DNA repair mechanisms in invertebrate tissue are facilitated by enzymatic activity when the exogenous genotoxic substances cause oxidative stress in the form of ROS. However, when the ROS reaches very high levels, permanent DNA damage can occur. A variety of techniques exist to test for possible damage to genetic material by environmental pollutants, for example: the formation of adducts, chromosomal aberrations, breakage in the individual strands of DNA (comet assay) and the frequency of sister chromatid exchange (SCE) (Peakall 1992; Cestari, 2013).

2.6 Comet assay

The single cell gel electrophoresis test (SCGE) or comet assay is a rapid and inexpensive technique that can be used to assess DNA damage in any individual eukaryotic cell. Microgel electrophoresis was first introduced by Ostling and Johanson in 1984 to measure DNA strand breaks in individual mammalian cells (Cotelle and Ferard 1999). In 1988, Singh and his colleagues published a modified protocol in which they used alkaline conditions. The comet assay performed under alkaline conditions allows the detection of single strand breaks of DNA (Cestari, 2013). The process involves embedding individual cells in agarose on a microscope slide, placing the slides in a lysis solution for a specific time period and then

conducting a brief alkaline electrophoresis. The lysing procedure enables denaturation and unwinding of the DNA (Liu *et al.,* 2009). If the negatively charged DNA is damaged due to contamination, it will contain breaks. The electrophoresis allows the broken DNA fragments to move away from the damaged nuclei. Finally, an intercalating staining agent, for example ethidium bromide, is added. This allows for the unwinding of negative, super coiled, genetic fragments and visualisation under a fluorescence microscope (Collins, 2004). Under fluorescence microscopy the migration of the damaged DNA material can be visualised and gives the characteristic comet-like pattern (Reinecke and Reinecke, 2004).

DNA strand breaks may be caused by two main mechanisms including exogenous agents such as chemicals or pollutants or endogenous species. Direct mechanisms include chemicals like H_2O_2, while indirect mechanisms include by-products of organic xenobiotics like PAHs, metabolites, transition metals or oxidised free radicals in endogenous metabolism (Qiao *et al.,* 2007). Many investigators are interested in examining the DNA repair capacity of cells by measuring the decrease in damage as a function of time after exposure to a known genotoxic agent. For exposures over long periods, DNA damage is a measure of both induction and the level of DNA repair (Collins, 2004). Repair of DNA damage can be very rapid. Endogenous and some forms of exogenous strand breaks by ROS can be repaired with a half time of less than thirty minutes and as short as three minutes (Olive and Banáth, 2006). However, studies have shown that species that are exposed to contaminated soil are much slower to repair DNA and that these processes of DNA repair can be changed (Qiao *et al.,* 2007)

There are several different parameters used to assess the comet data to determine the extent of the DNA damage. This can be challenging and is seen as a disadvantage by some users due to the lack of standardisation (Olive and Banáth, 2006). The easiest method for measuring DNA damage is by calculating the percentage of comets relating to the percentage of damaged cells. With technological advancements, additional parameters are defined such as percentage of tail DNA or tail intensity, referring to the percentage of DNA that migrated away from the nucleus (Cotelle and Ferard, 1999). Another frequently used parameter is tail length (at low damage levels only), which can refer to the length of the displaced nuclear material from the edge of the nucleus to the end of the tail. Other literature refers to this parameter as displacement or distance of migration (Olive *et al.,* 1998). Yet another parameter described by Olive *et al.* (1994), is the tail moment. It is defined as the product of the percentage of DNA in the tail and the distance between the nucleus head and tail distributions. Of these parameters, it is suggested that relative tail intensity is the most

useful because it produces a linear relationship to break frequency and it allows for discrimination of damage over a wide range (Collins, 2004). Interpretation of comet results is complicated by the fact that there is no simple relationship between the amount of DNA damage caused by a specific chemical and the biological impact of that damage (Olive and Banáth, 2006). It is therefore advisable to compare comet assay results with other biological measures to understand the biological relevance of the damage (Olive and Banáth, 2006).

Despite the disadvantages mentioned, there are also a number of advantages of using this method. A significant advantage of SCGE is that it is relevant and can be used for any eukaryotic cell type including plant cells (Verschaeve and Gilles, 1994). Unlike other genotoxic tests, the comet assay is the only test that does not require proliferating cells for viability. Although the protocol was originally designed for human genetic toxicology applications, it has become an important tool in environmental toxicology or ecotoxicology. Numerous SCGE studies have been done using sentinel animal and plant species for genotoxicity screening tests (Di Marzio *et al.,* 2005, Olive and Banáth 2006, Liu *et al.,* 2009). Another important advantage is that environmentally complex mixtures can be tested with the SCGE protocol. Species that are frequently used in biomonitoring and toxicity tests have been proven to be sensitive in screening the genotoxicity of a range of chemicals. Earthworms have been used in screening the genotoxicity of contaminated soils (Verschaeve and Gilles, 1995; Reinecke and Reinecke, 2004; Fourie *et al.,* 2007). Other species such as molluscs, fish and amphibians have been used for in situ evaluation of genotoxicity of water (Cotelle and Ferard, 1999); fish are efficient in detecting in vitro genotoxicity of river sediments (Pandrangi *et al.,* 1995; Nacci *et al.,* 1996; Devaux *et al.,* 1997). Koppen and Verschaeve (1996) tested several chemicals (organic and metals) in plant cells by exposing seedling roots to the seven different chemicals. To avoid problems with plant cell membranes, which are not easily lysed, they first isolated nuclei from plant roots prior to applying the alkaline SCGE on the nuclear suspension. The results showed that six of the seven chemicals resulted in a significant migration of DNA. In another study by Gichner and Plewa in 1996, the effect of ethyl methane sulfonate (EMS) on tobacco plants were tested (Cotelle and Ferard, 1999). Once again, they isolated nuclear material from root cells. The authors compared the results of the nuclear isolated suspension in the standard comet protocol and a modified protocol in which the lysing stage were left out. It was found that there was no significant difference in the tail moment values with or without the lysing stage.

2.7 Microbial community assessment techniques

Our understanding of the structure and dynamics of soil microbial communities and their enormous diversity has improved drastically in recent decades (Bloem *et al.,* 2006). Traditional culturing techniques to study pure cultures are the oldest tools in microbiology, however it only allows a fraction of the microorganisms to be cultured and often a large proportion of the microbial community are left unaccounted for when using these techniques. The development and application of more modern culture-independent methods used to study soil microorganisms have revolutionised soil microbial ecology. This is largely due to the greater fraction of the soil microorganisms that can now be studied, including important factors such as the interactions between microorganisms and the factors influencing the microbial communities in the soil (Marschner, 2007). There have been ongoing advances in molecular techniques to study microbial ecology including metagenomics and other new DNA sequencing approaches (Imfeld and Vuilleumier, 2012).

A variety of parameters are measured in microbial ecological studies, including the nature and diversity of organisms, the metabolic pathways employed by the community of organisms, identification and measurement of the metabolic products, abiotic parameter measurement and their interactions with each other and their environment (Barton and Northup, 2011). These parameters are fundamental to determine the type of microorganism present, the function of the microorganisms and how the microbial activity relates to the ecosystem functions such as energy flow or nutrient cycling (Rastogi and Sani, 2011).

Due to the complex nature of the interactions between soil and the microorganisms that form part of its functioning, it is often difficult to study microbial communities independently. One example of studying microbial communities includes functional types. Microbial groups that perform a similar function or set of functions within an ecosystem can be defined as functional groups or functional types. Some have defined functional types according to how they use the same resources, mostly due to similar characteristics, whereas others have grouped functional types according to how they respond in a similar way to a number of environmental factors (Smith and Collins, 2007). Of importance however, is that a functional group is a non-phylogenetic classification and all functional groups are selected according to function although the sort of function is not the determining factor. Functional groups can be formed at any level of organisation. The use of functional types, instead of species, provide better opportunities to make comparisons and predictions about possible responses for different species as well as provide a pragmatic support to modelling changes at a much larger scale (Diaz *et al.,* 1998). Berg (2010) grouped soil decomposer organisms into

functional types according to their significance in nutrient mineralisation. The coexistence of such a large variety of microbes and fauna in the soil ecosystem raises questions as to whether all these species are important for the functioning of the ecosystem. There is an enormous variety in body size, feeding specialisation, life history strategy, spatial distribution and responses to abiotic factors. The functional groups were distinguished based on characteristics such as similar food types, predators, metabolic efficiencies and location in the soil. The chosen traits must be assigned and analysed for the most common species in terms of key functional traits, taking into consideration the relationship between the individual species and community structure, which could have consequences for the ecosystem functioning (Berg, 2010).

The profiling of soil microbial communities is generally based on their structural and functional characteristics and how community structure changes over time. Molecular, biochemical and physiological methods are commonly applied in combination for profiling soil microbial communities (Malik *et al.,* 2008).

2.7.1 Community structure

The analysis of microbial community structure involves the extraction of marker compounds such as DNA and components of cell membranes such as PLFAs (Leckie, 2005). The analysis of DNA uses molecular techniques, while PLFA and fatty acid methyl esters (FAME) analysis are biochemical methods used to study the structural diversity of microbial communities (Rastogi and Sani, 2011).

Some of the molecular techniques used to do DNA-based analysis include polymerase chain reaction (PCR), real-time PCR (Q-PCR), DNA microarrays and genetic fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE) to name a few. Polymerase chain reaction is based on the extraction and purification of nucleic acids from soil or other samples and amplification to give millions of copies of entire genes or portions of desired genes, which are then used as a template to characterise the microorganisms. Basically, the PCR product reflects the combination of microbial gene signatures that organisms are present (Rastogi and Sani, 2011). Polymerase chain reaction amplification of conserved genes such as 16S rRNA is extensively used in microbial ecological studies (Rastogi and Sani, 2011; Leckie, 2005). The highly conserved domains interspersed with variable regions provide comparative analysis of sequences and phylogenetic relatedness of species can be inferred (Leckie 2005).

In genetic fingerprinting techniques such as DGGE/TGGE, length or sequence polymorphism is commonly used to produce a community fingerprint (Rastogi and Sani, 2011). These techniques are often applied to complex communities to visualise (in the form of bands) the overall diversity of the given community at a given time and space (Barton and Northup, 2011). The principle of DGGE is the separation of amplified rDNA strands by their sequence composition along a linear gradient of a DNA denaturant for example urea and formamide (Rastogi and Sani, 2011; Barton and Northup, 2011). In TGGE the same principles applies, except that a temperature gradient is used instead of the chemical gradient as in DGGE. The number of bands produced is proportional to the number of dominant species in the community or sample (Malik *et al.,* 2008). Although DGGE and TGGE enables the monitoring of changes in communities over space and time, it may lack the particular data required for phylogenetic identification of some taxonomic groups and it might even produce more than one band for the same species, leading to overestimations of the diversity (Malik *et al.,* 2008). Denaturing gradient gel electrophoresis have been successfully applied to agricultural soils (Rastogi and Sani, 2011), and contaminated sites where the microbial diversity was largely unknown (Malik *et al.,* 2008). Various other PCR-based fingerprinting techniques exist, including amplified ribosomal DNA restriction analysis (ARDRA), randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) and ribosomal intergenic spacer analysis (RISA). Due to vast bacterial diversity in soil ecosystems in particular, the DNA-based methods still has some significant limitations such as the extraction of the DNA from the soil, which often produce limited reproducibility between replicates. Although new, improved methods are constantly being benchmarked for scientific use in microbiological studies, fatty acid marker compounds still provide a feasible established alternative to DNA-based methods in soil ecosystems (Imfeld and Vuilleumier, 2012). Phospholipid fatty acid patterns offer a rapid and reproducible measurement for characterising the numerically dominant portion of soil microbial communities and are a quantitative measure of the viable microbial community (Pryfogle, 2000; Zelles, 1999).

Phospholipids are important components of all living cell membranes and each species has its own characteristic fatty acid pattern. Fatty acid profiles are an important indicator of organism biomass in diverse bacterial monocultures and under a variety of conditions (White *et al.,* 1979). Fatty acids are identified and designated by the total number of carbon atoms, the degree of saturation and the type of branching in the fatty acid chain if present (Zelles, 1999). Fatty acids extracted from soil are derived from living plant residues, roots and soil organisms, however the fatty acids from soil microorganisms are shorter chains (predominantly $C<20$) compared to the longer fatty acid chains ($C>20$) from the remaining

soil biomass (Marschner, 2007). The fatty acids consist of polar and non-polar components and PLFA patterns are based on the polar portion of the fatty acids. After cell death, fatty acids decompose quickly in soil which means that the PLFA markers that are detected are derived from the living organisms, thus indicating the viable community (Pryfogle, 2000; White, 1993). There are between ten and fifteen PLFA markers for bacterial fatty acids (i15:0, a15:0, i16:0, 16:1*ω*9, 16:1*ω*7t, i17:0, a17:0, 17:0, cy 17:0, 18:1*ω*7 and cy 19:0) and only two to three marker fatty acids (18:2*ω*6) for fungi. Gram-negative bacteria are usually identified by the unique hydroxy acids (OH) in the lipid portions (Zelles, 1999), while the branched fatty acids are usually an indicator of Gram-positive bacterial biomass (Enami, *et al.,* 2001). The sum of PLFA's predominantly observed from bacteria, are generally used as an indicator for bacterial biomass (Claassens, *et al.,* 2011).

Microorganisms have the ability to change their PLFA patterns in response to stressful environmental conditions such as fluctuating temperatures and chemical stress (Frostegård *et al.,* 1996). Changes in the PLFA patterns can indicate rapid changes in the proportions of major groups of soil microorganisms, in other words, it describes the changes that occur in microbial community structure (Zelles, 1999). These changes in the PLFA patterns are a useful biomarker of the physiological state and community structure of the microbial community (Malik *et al.,* 2008) and have become a popular method used in soil ecotoxicology. Phospholipid fatty acid analysis has often been used to evaluate the effect of a range of factors on soil microbial communities including pesticides (Spyrou *et al.,* 2009), different land use systems (Enami *et al.,* 2001; Dong *et al.,* 2008), fertilizers (Marschner *et al.,* 2003) and the success of soil rehabilitation (Mummey *et al.,* 2002; Claassens, *et al.,* 2006; Claassens and Jansen van Rensburg, 2012). Due to the large variation of microbial communities, PLFA offers the advantage that a large number of samples can be processed at the same time and it includes the non-culturable organisms (Zelles, 1999). It is an effective method to describe the gross changes in microbial community structure; however, it does not permit the delineation down to species level (Malik *et al.,* 2008) and therefore does not provide any indication of changes in the populations of individual species. It is not as expensive as molecular techniques (Zelles, 1999).

2.7.2 Functional diversity

The determination of functional diversity is critical when analysing microbial communities and ecosystem functioning. An important aspect in functional diversity studies is functional redundancy, primarily due to its relationship to ecosystem stability, in other words, the ability to resist and be resilient to environmental disturbances. Generally, as the species richness

increase, the numbers of ecological functions that are present also increase. However, when it reaches a certain point, the addition of new species no longer add novel capacities but instead add to redundancy of specific functions. It is generally accepted that a functionally redundant system is more likely to retain overall function after environmental stress (Kanopka, 2009).

An important goal of soil microbial ecology is to link community structure and function. The assessment of microbial community structure must be combined with functional diversity techniques such as assays of enzymatic activity and real-time PCR of functional genes (Marschner, 2007). These methods are important in monitoring and assessment of overall functioning of soil microbial communities in terms of, for example, the flux of energy and change in resources through the system and how it is influenced by natural and anthropogenic changes (Bloem *et al.,* 2006).

Community-level physiological profiling is an easily applied method that provides information about mixed microbial community function and functional adaptations over space and time. Biolog™ microplates and substrate induced respiration (SIR) are two techniques commonly used for CLPP. Both of these methods assess sole-carbon source utilisation to determine the physiological capacity of microbial communities. It provides insight into the community structure and functional diversity based on the ability of different organisms within the community to utilise different carbon sources at different rates (Barton and Northup, 2011).

Substrate induced respiration focuses on active populations while Biolog™ microplates focus on fast growing cultivable organisms (Leckie *et al.,* 2005). Biolog™ manufactures different types of microplates with EcoPlate, Gram-negative (GN2) and Gram-positive (GP2) being the most popular. Biolog™ EcoPlate analysis is an effective means of distinguishing spatial and temporal changes in soil microbial communities (Garland, 2007). The carbon sources used in the EcoPlate were selected specifically for environmental studies. The EcoPlate contains a blank and thirty one different carbon sources in triplicate. Triplication affords for increased confidence in statistical analysis. It is an important tool in pollution induced community tolerance of polluted soils and therefore useful in environmental risk assessments (Stefanowicz, 2006). Importantly, fungi do not metabolise the tetrazolium dye used in the plates and therefore do not contribute to the CLPP on these plates (Stefanowicz, 2006).

Although CLPP can be an effective technique, it has several limitations including long incubation times, indirect measurement of microbial activity, and challenging data analysis

(Weber and Legge, 2010). In addition, it involves incubation and cultivation that can lead to biases in the responding organisms, which in turn can affect results (Barton and Northup, 2011). Even with these limitations, the EcoPlate analysis has been used extensively and successfully in numerous microbial community studies and has proven to be an economical tool with reproducible results. When used in conjunction with other tools such as DGGE or PLFA analysis, it can produce valuable results. All three these approaches produce fingerprint-type data.

Gomez *et al.* (2006), did a study on the response of microbial functional diversity to organic soil amendments. The study also looked at the relationship between microbial functional diversity and soil carbon availability. Vermicompost from different types of manure and household waste were added to soil. Eleven and eighteen months later, samples were used to inoculate Biolog™ EcoPlates for CLPPs. Significant differences in functional diversity were found among the different samples that were treated with different types of vermicompost. A principal component analysis (PCA) indicated that soils with different amendments were significantly different from one another whilst soils with the same amendment were significantly similar. The differences were confirmed with soil organic carbon (SOC) analysis (Gomez, *et al.,* 2006).

2.7.3 Molecular techniques

Another molecular method that provides insight into functionality of microbial communities is metagenomics. Metagenomics uses a culture independent method to sample all the genes present in a community of microorganisms and not just the ribosomal genes (Barton and Northup, 2011) thus allowing the analysis of entire genetic composition of microbial communities. Metagenomics is a means of studying the physiology and ecology of environmental microorganisms by either taking a phylogenetic or functional approach. It involves the construction of a metagenomics library through several steps. This includes the extraction and cloning of DNA into a vector that is transformed into a host bacterium, for example Escherichia coli, after which it is screened for positive clones (Barton and Northup, 2011; and Rastogi and Sani, 2011). When screening for a functional approach for example, enzyme activity is often included. Metagenomics approaches have been successfully conducted in a number of environments such as soil, marine ecosystems, the phyllosphere and acid mine drainage (Rastogi and Sani, 2011).

CHAPTER 3: MATERIALS AND METHODS

3.1 Experimental design

A laboratory experiment was conducted at the North-West University to test the possible effects of biofumigants and chemical fumigants in soil on the reproductive output and DNA structure of the earthworm species *Eisenia andrei* and the structure and function of soil microorganisms.

In this study, all exposures were done in artificial soil prepared according to the standard guidelines set out by the Organisation for Economic Co-operation and Development (OECD, 1984). Toxicological studies are often done using artificial soil that is prepared according to standard guidelines. Although some variables of real ecosystems that can affect bioavailability are excluded, it does have practical advantages as well. The use of artificial soil is a means of manipulating important parameters such as soil organic matter (SOM) and simplifying soil functioning in order to test specific ecological factors on microbial communities (Guenet *et al.,* 2011). In addition, by using artificial soil, the variability in adsorptive properties of different soil types can be overcome. The artificial soil was prepared seven days prior to the start of the experiment and consisted of (based on dry weight):

1. 10 % sphagnum-peat (Mystics).
2. 69 % quartz sand with a grain size of 50 - 200 µm.
3. 20 % kaolinite clay.
4. Chemically pure calcium carbonate ($\leq$ 1 %) was added to obtain a pH of 6.

The water holding capacity (WHC) of the artificial soil was determined using a Sartorius moisture analyser. Four tubes were each filled with dry artificial soil. The bottom of each tube was sealed with filter paper, placed into a glass beaker with water and left for three hours. After this period, the tubes were removed and placed in a beaker with silicate sand for a further two hours in order for the soil in the tubes to reach a 100 % WHC. After two hours, two replicate samples of each tube were analysed using the Sartorius moisture analyser. Readings obtained were used to calculate the 60 % and 100 % WHC.

$$\text{WHC (in \% of dry mass)} = \frac{S-D}{D} \times 100$$

Where S is H_2O saturated soil and D is dry soil (OECD, 2004).The arithmetic mean of three soil samples indicated 31.07 % moisture content (Annexure A).

3.2 Test substances

3.2.1 *Chemical fumigants*

The chemical fumigants used in this experiment were metham sodium (MS) 510 g/L and cadusafos/ RUGBY (R10) 100 g/L. The recommended field application dose of MS is between 0.3 - 10 mg/kg of soil (Omirou *et al.,* 2010; Wu *et al.,* 2011). Cadusafos was applied at a rate of 4 µg/g of soil (McClure and Schmidt, 1996; Karpouzas *et al.,* 2005). The recommended field dosage of 10 mg MS/kg soil resulted in 100 % mortality in the earthworm genotoxicity assay and a subsequent treatment at one tenth of the recommended dosage was also included.

3.2.2 *Biofumigants*

Three biofumigants were used for this study. Oilseed Radish (R) (*Raphanus sativu*) *seed* also known as Bladrammenas was used. Fresh plant material and seed was obtained from Javelin seeds, a company providing various services to South African farmers including advice on biofumigation crops. It was planted in commercial potting soil in 45 cm pots. Broccoli (B) (*Brassica oleracea)* and the leafy vegetable Asian mustard (M) (*Brassica juncea)* seedlings were bought from a commercial nursery and all three plant species were grown for 80 days before the start of the experiment. Four plants of each species were grown to provide enough uniform leaves and stem material for use in the experiment.

Studies have shown that the recommended dosage of biofumigant plant material to be incorporated into the soil is 15 g of plant material per kg of soil (McLeod *et al.,* 1995; Omirou *et al.,* 2010). Fresh plant material was incorporated into the test containers consisting of roots, leaves and stems of the biofumigant plants. The plant material was cut into smaller pieces prior to incorporating it into the soil to ensure proper cell disruption for glucosinolate conversion to isothiocyanates (ITC). The soil was moistened with de-ionised water to obtain a 50 % of the total WHC for the artificial soil. The earthworms were added to the test containers in the case of the comet assay analysis.

The soil moisture content for the test substrate was 50 % of its WHC. A solution of the chemicals and de-ionised water (equal to 50 % WHC) was prepared for each of the chemical fumigants. The solutions were mixed with the soil substrate before introducing it into the containers. The biofumigants were fresh plant material consisting of leaves, stems and roots. A study by Morra and Kirkegaard (2002) on the release efficacy of ITC showed that fresh *B. juncea* leaf tissue added to soil produced extractable ITC concentrations of 0.032 - 0.068 % of the potential ITC. This compared to 14 % and 16 % efficacy rate when frozen plant

material was used. For this study only fresh plant material was used in order to replicate real situations of crop biofumigation by farmers.

Eighteen 350 ml containers were each filled with 200 g of artificial soil for the microorganism study and eighteen 1 L containers were each filled with 600 g of artificial soil for the earthworm study. The chemical and biofumigants were mixed into the soil immediately prior to the start of the experiment. The five treatments and a control treatment were done in triplicate.

1. Control: Moistened artificial soil
2. Soil containing biofumigant 1 - Oilseed Radish (Bladrammenas) (*R. sativus*)
3. Soil containing biofumigant 2 - Asian mustard (*B. juncea*)
4. Soil containing biofumigant 3 - Broccoli (*B. oleracea)*
5. Soil containing chemical fumigant 1 - MS
6. Soil containing chemical fumigant 2 - Cadusafos

Exposures were performed for a period of twenty eight days in a climate controlled room maintaining the temperature between 20 and 22 °C.

Research by Gimsing and Kirkegaard (2006) showed that *Brassica* green manures persist in the soil for up to 8 -14 days after incorporation, whereas chemical fumigants persist longer in the soil, depending on the product used. Based on this information, sampling for microbial analysis took place on days 0, 14 and 28.

3.3 Earthworm survival, growth and reproduction test

Earthworms of the species *Eisenia andrei* were used in this experiment. The worms selected were all adult worms of similar weight (individual weight of 350 – 550 mg) and from the same source. Ten individuals were used in each replicate. The earthworms were acclimatised in the artificial soil 72 hours prior to the start of the experiment and then briefly rinsed in clean water and placed on absorbent paper. The starting weights of the worms were determined by weighing them individually before placing them in the test containers. A food substrate of horse manure was added to each of the test containers at the start of the experiment and equal amounts of food substrate were added on a weekly basis (OECD, 1984). After 28 days, the adult worms were removed from the containers, weighed individually and counted to determine their survival rate. They were also observed for any unusual behaviour and morphology.

After removal of the adult earthworms, the test containers containing the cocoons were incubated for an additional 32 days to assess the reproductive success of the earthworms in the first incubation period. The containers were kept under the same test conditions except that food substrate was only added in the beginning of the second incubation period. At the end of the second incubation period, the numbers of cocoons were counted, as well as the number of juveniles that hatched. The juveniles were collected from the test containers by using the hand sorting technique (OECD, 2004). The operation was done in triplicate on all samples to ensure that all juveniles were removed. The number of cocoons per earthworm per week was calculated as well as the juveniles that hatched per cocoon.

3.4 Comet assay

The sampling for the comet assay was done at the end of the 28 day exposure. The comet assay was carried out according to a modified protocol of Singh *et al*. (1988), by Reinecke and Reinecke (2004) in order to assess the extent of cellular DNA strand breaks in earthworm coelomic cells. Three earthworms from each sample (n = 9 per treatment) were used for the comet assay to assess cellular DNA damage. Earthworms were removed from the exposure containers, rinsed and placed on moist paper the day prior to the test to allow them to empty their gut content so that no soil contamination occurred while preparing the suspension cell samples. All the comet assay solutions (lysis solution, phosphate buffered saline, alkaline solution and alkaline electrophoresis solution) were prepared one day prior to the start of the test and left in a refrigerator to chill to 4 °C overnight, with the exception of the extrusion fluid, which was prepared on the morning of the test and chilled for at least two hours prior to the start of the test.

Coelomic cells were harvested from the earthworms by a non-invasive technique using an extrusion fluid, originally described by Eyambe (1991). Each earthworm was first rinsed in clean phosphate buffered saline (PBS) and placed on moist absorbent paper to rid of excess fluid. They were exposed separately to 1 ml of the chilled extrusion fluid (95 % PBS, 5 % absolute ethanol, 10 mg/ml guaiacol glycerol ether and 2.5 mg/ml EDTA) in Eppendorf tubes for a period of three minutes. The earthworms were removed after three minutes, rinsed in clean PBS and returned to clean soil.

The Eppendorf tubes containing the extrusion fluid and cell suspensions were centrifuged at 700 x 2 for fifteen minutes at 4 °C. The supernatant was carefully removed with a pipette and resuspended in 0.5 ml PBS to wash the cells. The Eppendorf tubes were then centrifuged again at the same speed for a period of three minutes. The washing of cells was

repeated twice to allow the removal of excess mucous in the coelomic fluid and finally resuspended in 0.5 ml PBS solution (Voua Otoma and Reinecke, 2010). Afterwards, 10 µl of the cell suspension was mixed with 90 µl of low melting point agarose and heated to 40 °C. Of this mixture, 75 µl was then spread on a precoated agarose microscope slide so that the cell suspension was embedded in agarose to immobilise the cellular DNA.

Cell proteins were lysed and DNA exposed by placing the slides in a high salt lysis solution for one hour at 4 °C and in the dark. After lysis, the slides were removed from the lysis solution and placed in a horizontal electrophoresis chamber and filled with ice cold alkaline electrophoresis solution so that the slides were fully covered and left for twenty five minutes in the dark. Electrophoresis was conducted for fifteen minutes by applying 25 V (0.8 V/cm), 300 mA in the same solution to allow for denaturation and migration of the broken ends from the nucleus (Voua Otoma and Reinecke, 2010). After electrophoresis, the slides were rinsed twice in ice cold distilled water and then removed, allowed to dry and stored in darkness until staining and analysis.

For analysis of the comets, the slides were rehydrated in pre-chilled de-ionised water for at least five to ten minutes after which they were stained with a fluorescent dye, ethidium bromide, (20 µl/ml) for an hour. After staining, the slides were briefly rinsed and kept on moist paper in order to view the comet images and remaining nuclear DNA using a Leitz Diaplan fluorescence microscope.

The Comet 4 image analysis system was used to determine the quantitative and qualitative extent of DNA damage in the cells by measuring several comet parameters. Parameters included head and tail length, head and tail intensity (as percentages) and tail moment. The tail moment is the product of the distance between the centre of gravity of the head and the centre of gravity of the tail and the percentage tail DNA or tail intensity. A minimum of fifty randomly selected cells were analysed per sample (Collins, 2004). The parameter selected to quantify the extent of DNA damage was tail intensity (measured as percentage tail DNA). The percentage tail DNA in the tail is measured by the intensity of the pixels located in the comet tail.

3.5 Phospholipid fatty acid analysis

The microbial biomass was estimated from the total extractable phospholipid fatty acids (PLFAs) and the community structure was determined from the relative concentrations (mole %) of the signature fatty acids (McKinley *et al.,* 2005).

All glassware used for the PLFA analysis were washed prior to the start of the experiment with phosphate-free soap and rinsed five times with hot water, distilled water, nano-pure water and baked at 450 °C in a muffle furnace for a minimum of four hours to prevent any lipid contamination.

Phospholipid fatty acid extraction was performed using a modified Bligh and Dyer (1959) extraction protocol (White *et al.,* 1979). The three main steps of the process consisted of extracting total lipids, fractionation of the total lipids into phospholipids, glycolipids and neutral lipids and finally preparing the fatty acid methyl esters (FAMEs). Lipids were extracted from 5 g freeze dried soil using a single-phase chloroform-methanol-aqueous buffer system in the ratio 1:2:0.8 (v:v:v) (5 ml:10 ml:4 ml) (White *et al.,* 1979). Nano-pure water (5 ml) and chloroform (5 ml) were added to each sample and centrifuged to separate the two phases. The bottom chloroform phase, containing the lipid fractions, was recovered and transferred into clean tubes and dried using a nitrogen dryer to evaporate the chloroform.

Silicic acid column chromatography was used to fractionate the total lipids extracts into neutral lipids, glycolipids and polar lipids. Chloroform (5 ml) was used to separate the neutral fatty acid fraction, acetone (5 ml) to separate the glycolipid fraction and finally, methanol (5 ml) to separate the polar fraction. The polar fraction, containing the phospholipids, was subjected to mild alkaline methanolysis to form the FAMEs (McKinley *et al.,* 2005). The lipids were resuspended in methanol toluene to which freshly prepared 0.2 N methanolic KOH was added. The mixture was incubated for 30 min at 37 °C, neutralised with acetic acid, 2 ml chloroform and nano-pure water added and mixed. The mixture was then centrifuged in order for the FAMEs and phospholipids to be recovered from the organic phase (White *et al.,* 1979).

The FAMEs were analysed by capillary gas chromatography with flame ionisation detection on an Agilent 7890A series chromatograph using a 60 m SPB-1 column. The injector and detector temperature was maintained at 270 °C and 290 °C, respectively. Hydrogen was used as the carrier gas and the gas flow was maintained at a constant pressure of 300 kPa while 1 µl of each sample was injected into columns. The column temperature was programmed to start at 60 °C for 2 minutes, increased at a rate of 10 °C / minute to 150 °C and then increased at 3 °C / minute to 320 °C.

Peak identification was made for representative samples by gas chromatography using an Agilent 6890 series II gas chromatograph interphased with an Agilent 5973 mass selective

detector. Mass spectra were determined by electron impact at 70eV and fatty acids were quantified by calibrating against an internal standard, methyl nonadecanonate (19:0) (McKinley *et al.,* 2005).

Standard fatty acid nomenclature was used for analysis (Frostegård *et al.,* 1996). Fatty acids are designated by the total number of carbon atoms followed by the number of double bonds, with the position of the double bond indicated from the methyl end (ω) of the molecule. The configuration of the double bonds are described by "c" (*cis*) and "t" (*trans*) while branched fatty acids are described by "a" and "i", which refer to anteiso- and iso-branching, respectively (Zelles, 1999; Pryfogle, 2000). Methyl branching at undetermined positions is indicated by the prefix "br". Cyclopropyl fatty acids are indicated by the prefix "cy", followed by the number of carbon atoms (Pryfogle, 2000).

3.6 Community level physiological profiling

Soil suspensions were prepared from 10 g of soil and 90 ml of sterilised water. The suspensions were shaken in a shaking incubator at 200 rpm and 25 ˚C for one hour and left to stand for another hour to allow particles to settle. The supernatant was diluted by mixing a 1 ml aliquot of the soil suspension to 2 ml of sterilised water. 0.1 ml was mixed with 9.9 ml sterilised water. Lastly, 2 ml of this suspension was added to 18 ml sterilised water to give a suspension with a final dilution of 1:3000 (Campbell *et al.,* 1997). Each well of the Biolog™ EcoPlate was inoculated with 150 µl of the bacteria containing suspension. The reduction of the colourless tetrazolium dye to a violet coloured formazan in each well was measured spectrophotometrically using a microplate reader at a wavelength of 590 nm. The first reading was taken immediately after inoculation and recorded as the time 0 reading. Thereafter, the plates were incubated for a predetermined period and readings were taken at eight and sixteen hour intervals until the average well colour development (AWCD) of 0.25 – 0.30 absorbance units were reached (± 5 - 7 days). A value of 0.25 – 0.30 AWCD was selected to eliminate weak false positive responses (Garland, 1997). Samples were analysed on day 0, 14 and 28 of exposure.

3.7 Statistical analysis

Statistical analyses were performed and graphs generated using Statistica 11 (Statsoft Inc., Tulsa, Oklahoma, USA) and Canoco for Windows 4.5 (Biometris – Plant Research International, Wageningen, The Netherlands; Ter Braak and Šmilauer, 1998). A significance level of $p \leq 0.05$ was used in all analyses. Standard error of the mean (SEM) was determined where analysis of variance (ANOVA) was determined from averages and standard deviation (SD) was determined for ANOVA of all the data.

The Biolog™ EcoPlate readings were corrected for background absorbance by subtracting the absorbance value of the control well (H_2O) from the individual substrate wells in order to obtain net absorbance values (Stefanowicz, 2006; Weber *et al.,* 2007).

$$\overline{A_k} = \frac{A_k - A_0}{\frac{1}{31}\sum_{i=1}^{31}(A_i - A_0)}$$

Where $\bar{A}k$ is the average well colour development (AWCD), Ai is the absorbance reading of the well i and A_0 is the absorbance reading of the blank well, which has been inoculated but does not contain a carbon source. Further, normalisation of the CLPP data was done by dividing individual well responses by the average well colour (Garland, 1997). Negative values of normalised absorbance were coded as zeros (Garland and Mills, 1991; Weber, *et al.,* 2007). Standard error of the mean was calculated for the nine replicates per treatment. Statistica 11 (StatSoft, Inc.©) was used to do analysis of variance (ANOVA) as well as a Post Hoc Tukey's Honest Significant Difference (HSD) test at 5 % probability level.

Principal component analysis (PCA) was conducted on the multidimensional data obtained from the Biolog™ EcoPlate (Garland, 1997). Multivariate analysis methods such as PCA allow for the distinction between composite profiles while canonical correspondence analysis (CCA) relates community composition to environmental factors (Marschner, 2007). Redundancy analysis (RDA) is similar to CCA but differ in that it uses a linear model to relate community composition to environmental factors. It is used when data do not conform to a unimodal distribution as used in CCA (McKinley *et al.,* 2005). The well colour development of the thirty-one carbon sources was first standardised and then a time point representing an AWCD closest to 0.25 was selected as the metric (Weber and Legge, 2009). The mean of the nine replicates of each carbon source for the different treatments were calculated. The carbon sources were grouped into seven main carbon groups (Table 2) and each group's mean was calculated using Canoco for Windows 4.5 (Biometris-Plant Research

International, Wageningen, the Netherlands) the data was reduced to a smaller number of interpretable principal components that characterise a subset of the original variables.

Table 2: ***The 31 carbon substrates of the Biolog™ EcoPlates divided into the seven functional groups.***

Amines (amine)	**Carboxylic acids (carb_A)**	**Carbohydrates (carb_h)**
Phenylethylamine	D-Galactonic acid γ-lactone)	ß-Methyl-D-Glucoside
Putrescine	D-Galacturonic acid	D-Xylose
	2-Hydroxy Benzoic acid	I-Erythritol
Amino acids (amin_A)	4-Hydroxy Benzoic acid	D-Mannitol
L-Arginine	γ-Hydroxybutyric acid	N-Acetyl-D-Glucosamine
L-Asparagine	D-Glucosaminic acid	D-Cellobiose
L-Phenylalanine	Itaconic acid	α-D-Lactose
L-Serine	α-Ketobutyric acid	
L-Threonine	D-Mallic acid	**Polymers (poly)**
Glycyl-L-Glutamic Acid		Tween 40
	Phosphorylated compounds (phos_C)	Tween 80
Esters (ester)	Glucose-1-Phosphate	α-Cyclodextrin
Pyruvic acid methyl ester	D,L,-α-Glycerol phosphate	Glycogen

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Earthworm survival, growth and reproductive success

Survival of the earthworms was recorded at the end of the 28 day test period. In the biofumigant treatments, Brocolli (B) had 97 % survival and although this is lower than the control samples, it was not statistically significant ($P > 0.05$) and it could not be concluded whether this was due to the soil amendments or due to natural mortality (Table 3). There was no mortality in the control (C) and any of the other biofumigant (mustard (M) and oilseed radish (R)) treatments. In the metham sodium (MS) treatment at the recommended field dosage (10 mg/kg soil) (Omirou *et al.,* 2010; Wu *et al.,* 2011), there was 100 % mortality of the earthworms. It was noted that the earthworms tried to escape immediately after placing them into the test containers with the MS treated soil. A subsequent MS treatment at one tenth (1 mg/kg of soil) of the recommended dosage resulted in an 87 % survival rate and no pathological symptoms were observed (Table 3). The cadusafos (R10) amended soil had a significantly lower survival rate of 80 %. Mortality is a frequently used parameter to assess chemical toxicity in earthworms; however, it is not considered the most sensitive from an ecotoxicological point of view and only provides information about acute toxicity (Yasmin and D'Souza, 2007). Sublethal endpoints such as growth and reproduction are more sensitive measures in earthworm ecotoxicology (Reinecke *et al.,* 2001) and have become regulatory test methods in ecotoxicological and risk assessments.

Growth and reproduction are important measures of an individual's fitness after exposure to a possible contaminant and valuable parameters in ecotoxicology (van Gestel and van Brummelen, 1996). Growth was measured as the percentage change in mean bodyweight of the earthworms over time after the exposures and results are summarised in Table 2. Results indicated that the biofumigant treatments (B, M and R) resulted in an increase in mean bodyweight but were statistically similar to the control group. Earthworms in the chemical fumigant treatments (MS and R10) had a significant ($P < 0.001$) decrease in bodyweight (Table 3). All the treatments received equal amounts of horse manure for feeding however, it needs to be considered that in the biofumigant treatments the plant material could have provided additional food substrate for the earthworms. Valckx *et al.* (2011), demonstrated that earthworms readily consume fresh and partially decomposed plant material from *Brassica* cover crops.

Table 3: ***Earthworm survival and change in mean (± SEM) bodyweight (g) represented as mean weight change (%) over 28 days. Significant differences (P < 0.05, Tukey's HSD) are indicated with different letters.***

	Survival (%)	Mean bodyweight (g) Day 1	Mean bodyweight (g) Day 28	Average weight change (%)
C	100[a]	0.42 ± 0.006	0.47 ± 0.003[a]	10.65[a]
B	97[a]	0.41 ± 0.001	0.44 ± 0.014[a]	8.40[a]
M	100[a]	0.42 ± 0.018	0.45 ± 0.012[a]	7.70[a]
R	100[a]	0.39 ± 0.006	0.43 ± 0.009[a]	10.53[a]
MS	87[b]	0.40 ± 0.011	0.29 ± 0.010[b]	-28.39[c]
R10	80[b]	0.40 ± 0.010	0.35 ± 0.009[b]	-12.27[b]

Treatments: C (control); B (broccoli); M (mustard); R (oilseed radish); MS (metham sodium) and R10 (cadusafos).

Reproductive success was measured by looking at the number of cocoons and the number of juveniles that hatched during the exposure period. The number of juveniles that hatched from the total number of cocoons was determined 60 days after the start of the test. Cocoon production in the biofumigant exposure groups (B, M and R) was statistically similar to that of the control treatment (Figure 1). It was assumed that all hatchlings survived the post exposure incubation. The control treatment had the largest number of juveniles with an average of 111 hatchlings. A Tukey's HSD test was performed to determine if these changes were significantly different to the C group. Results show that the number of juveniles that hatched in the B treatment was statistically less than the control treatments ($P < 0.05$). However, the overall reproductive potential determined by the cocoons per earthworm per week and juveniles per cocoon, was not statistically ($P > 0.05$) different to the control or the other biofumigant treatments (Table 4) which could be interpreted as no difference in the overall reproductive success. Studies by van Ommen Kloeke *et al.* (2012), found earthworms of *E. andrei* to be tolerant to the breakdown products of three different *B. oleracea* genotypes in terms of survival, but it did have varying effects on reproduction.

No reproduction occurred in the chemical fumigant treatments. Although there was a higher survival rate, 80 % in R10 and 87 % in the second MS exposure treatments, none of the surviving earthworms produced cocoons. This could be ascribed to the fact that physiological processes of the earthworms, such as growth and reproduction, are placed under stress when the earthworm body uses extra resources to assist with the excretion of

toxic chemicals. A sublethal effect that results in the inability of an individual to reproduce could be considered a lethal effect because its fitness to contribute to the next generation is the same as that of a dead individual (Newman, 2010).

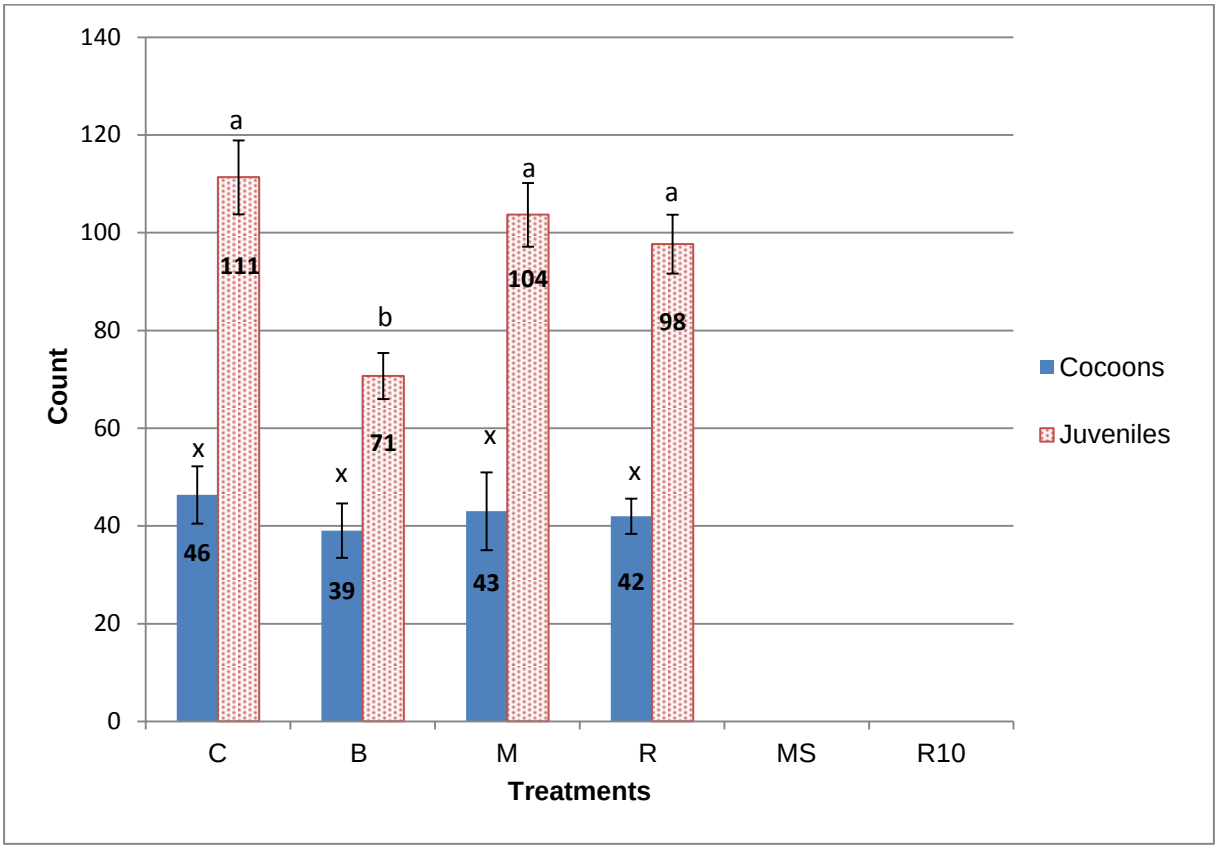

Figure 1*: Earthworm reproduction represented as the mean number (± SD) of cocoons and the mean number (± SD) of juveniles from three replicates. Significant differences (P < 0.05) are indicated by different letters. Treatments: C (control); B (broccoli); M (mustard); R (oilseed radish); MS (metham sodium) and R10 (cadusafos).*

Table 4: ***Reproductive success of E. andrei represented as cocoons per earthworm per week and number of hatchlings per cocoon (mean ± SD). No statistical difference (P > 0.05) were observed between treatments.***

	C	B	M	R
Cocoons/earthworm/week	1.16 ± 0.15[a]	0.98 ± 0.14[a]	1.08 ± 0.20[a]	1.05 ± 0.09[a]
Juveniles/cocoon	2.40 ± 0.35[a]	1.81 ± 0.18[a]	2.41 ± 0.35[a]	2.35 ± 0.24[a]

Treatments: C (control); B (broccoli); M (mustard); R (oilseed radish); MS (metham sodium) and R10 (cadusafos).

4.2 Comet assay

In total, 450 earthworm cells (n = 9 x 50) in each treatment were analysed for comets. The results showed significant heterogeneity in the DNA damage because it produced values ranging from 0 – 100 % tail DNA in the same sample. Dominant ratios of strand breaks could be observed when looking at the 75th percentile data. Previous studies found that 75th percentile data and median provide meaningful statistics for comparing stress conditions when using the % tail DNA parameter and it can demonstrate a genotoxic effect (Duez *et al.,* 2003). The box plot of the comet assay results (Figure 2) gives the minimum and maximum values but importantly indicates the 75 percentile data for each treatment. Comet assay data was tested for normality and a Tukey's HSD test was done to determine significant differences between treatments.

In the control treatment (C), 84 % of the cells that were analysed resulted in less than 20 % tail DNA (Figure 2 and Table 6, Annexure C). These low levels of tail intensity were assumed to be the background values derived from endogenous and natural exogenous sources (Qiao *et al.,* 2007). Results from two of the biofumigant treatments broccoli (B) and radish (R) showed 78 % and 82 % of the total cells observed were below 20 % tail intensity respectively (Table 6, Annexure C) and were not statistically different from the control group. However, samples from the mustard (M) exposure treatments were found to be significantly different ($P < 0.02$) to the control group and other biofumigants. This indicates that in this exposure, the treatment substance was the exogenous genotoxic cause for the higher genetic damage, suggesting that the breakdown products, ITC, in mustard caused the effect. Literature shows that of the three biofumigants used in this study, *B. juncea* (M) has the highest GSL content in the plant material and thus the highest potential ITC availability (Morra and Kirkegaard, 2002; Bellostas *et al.,* 2007). Although M exposure resulted in significantly higher DNA tail intensity, the level of genotoxicity is still considered moderate because 71 % of the cells had less than 20 % tail DNA and an average of 15.9 % tail DNA (Table 6, Annexure C).

Very little information is available on the genotoxicity of natural toxins to earthworms, and no information was found on the effect of the incorporation of fresh plant material containing natural toxins, to earthworms. Kassie *et al.* (1999), tested the genotoxic effect of the natural toxin, benzyl ITC, on laboratory rodents and found that it was a strong mutagen in vitro but only had moderate genotoxicity in vivo. Studies done by van Ommen Kloeke, (2012), tested the effects of a 99% pure liquid form of the natural toxin ITC on the life history and gene expression of *E. andrei*. The liquid solution, 2-phenylethyl ITC, produced as a secondary

metabolite by almost all *Brassica* sp*.,* was used in the study. Results showed a dose dependant reduction in survival and the reproductive success in earthworms at an effective concentration (EC_{50}) of 556 nmol/g soil.

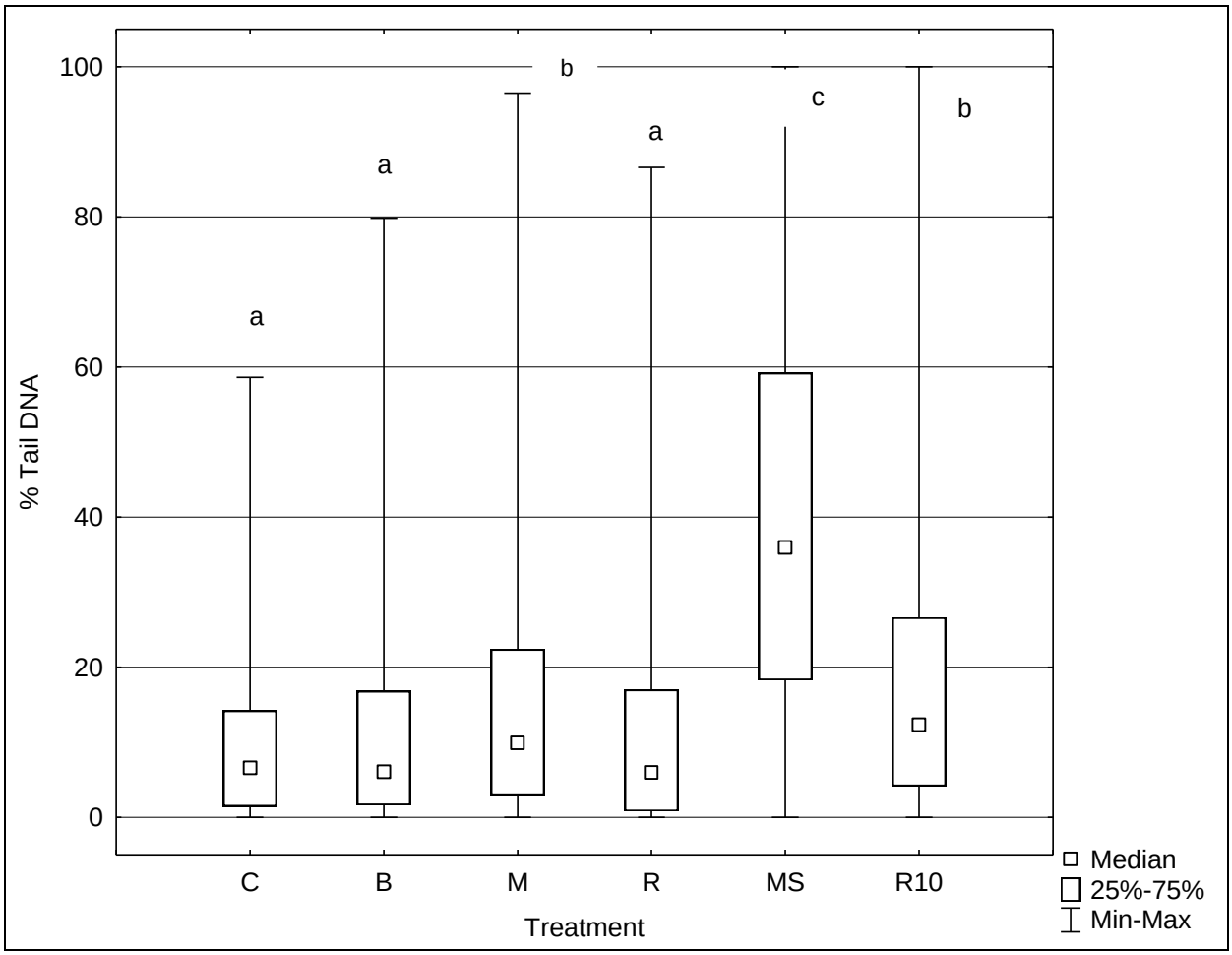

Figure 2: ***Comet assay results of DNA strand breaks measured as percentage tail DNA. The box area indicates the 75 percentile data for each treatment. Significant differences (P < 0.05, Tukey's HSD) are indicated by different letters. Treatments: C (control); B (broccoli); M (mustard); R (oilseed radish); MS (metham sodium) and R10 (cadusafos).***

Research suggests that the ITC formation from fresh incorporated *Brassica* plant material is less than 5 % of the potential ITC available in the plant (Morra and Kirkegaard, 2002). Even in specially selected biofumigant crops high in biomass and GSL content *(B. juncea and B. napus*), the actual total concentration of ITC released is never more than 1.2 nmol/g soil. This suggests a release efficiency of only 1.2 % of the potential ITC per plant. Wu *et al*. (2011), showed that as little as 0.5 - 2.0 ml ITC per kg of soil applied prior to planting was effective in reducing root knot nematodes in soil and was equally effective as the chemical fumigant MS. In the present study, fresh plant material was used to imitate application of biofumigant crops as utilised by farmers.

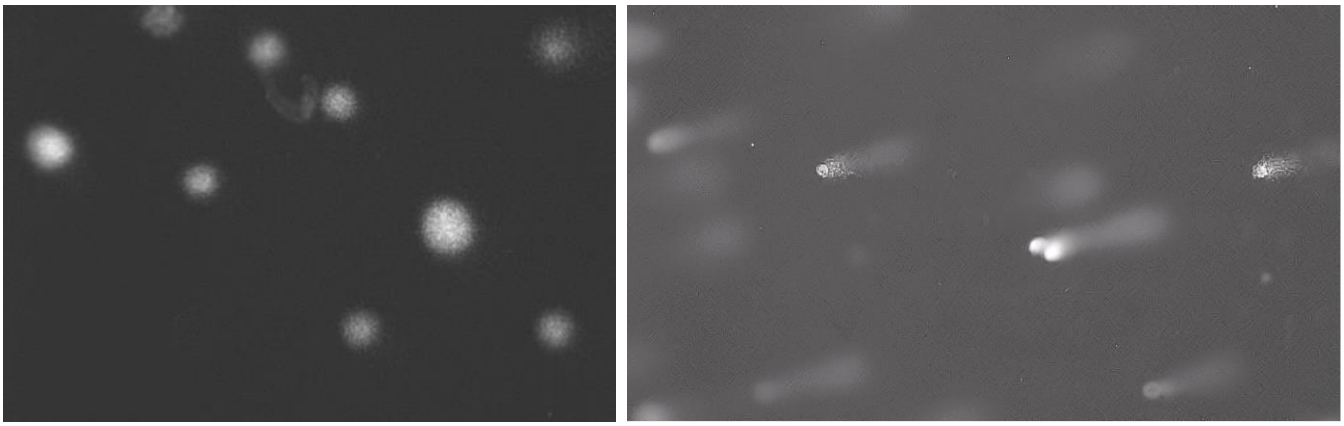

Figure 2: ***Fluorescence microscope images of earthworm cells observed in the comet assay. a) Left panel shows intact DNA in earthworm coelomic cells of mustard treatments b) Right panel shows damaged DNA from earthworm cells treated with metham sodium, forming the characteristic "comets"***

The scope of this study did not allow for the measurement of ITC in the soil following incorporation of fresh plant material. Research findings from Morra and Kirkegaard (2002), suggest that the actual exposure concentrations in this study, never reached the effective concentration (EC_{50}) as suggested by van Ommen Kloeke (2012). From the fact that M showed significantly higher DNA damage at these low ITC levels, it could be speculated that the comet assay was able to detect early signs of cellular stress even before it reached the EC_{50} given in the study by van Ommen Kloeke (2012).

Comet assay results from the two chemical fumigant treatments were different. The R10 treatments resulted in significantly ($P < 0.05$) higher DNA damage compared to C with an average of 19 % tail DNA. Genotoxicity of the MS treatment at the recommended field dosage (10 mg/kg soil) could not be tested as it resulted in 100 % mortality. The MS treatment at one tenth of the dosage (1 mg/kg soil) resulted in DNA strand breaks significantly different to all other treatments ($P < 0.0002$). The coelomic cells tested had an average of 40.37 % tail DNA indicating that the MS had a much more significant genotoxic effect on earthworms (Table 6, Annexure C). Figure 3 (right panel) shows the formation of the comets in MS treated cells and the migration of damaged DNA after electrophoresis.

Several studies have confirmed the toxicity of organophosphate and carbamate type pesticides to earthworms (Stenersen *et al.,* 1973; Reinecke and Reinecke, 2007; Chakra Reddy and Venkateswara Rao, 2008; Das Gupta *et al.,* 2010; Wang *et al.,* 2012). However,

little information is available on the genotoxicity of specifically organophosphate and carbamate type pesticides to earthworms. Ribera *et al.,* 2001, tested the activities of several enzymes including catalase (CAT) which is a marker of oxidative stress and acetylcholinesterase (AChE), a marker of neurotoxicity. After exposing *E. andrei* to increasing concentrations of the carbamate type pesticide, carbaryl, results indicated that carbaryl mainly targeted cholinesterase activity but did not induce significant oxidative stress. Genotoxicity has also been tested for neonicotinoid (Zang *et al.,* 2000), triazine type herbicides (Song *et al.,* 2009) and organochlorine type pesticides (Liu *et al.,* 2009). It is understood that DNA repair enzymes especially CAT and glutathione-related enzymes are able to deal with and facilitate basal level of DNA repair activities caused by natural or endogenous substances such as reactive oxygen species (ROS) (Ching *et al.,* 2001; Qiao *et al.,* 2007; *Song et al.,* 2009). Further DNA repair mechanisms, in invertebrate tissue by enzymatic species, are activated only when the exogenous genotoxic substances causing the DNA damage, have accumulated and reached a threshold level. However, when the ROS reaches very high levels, permanent DNA damage can occur. In the study by Song *et al*., 2009, the herbicide atrazine caused oxidative stress and DNA damage.

Further, little information is available on the mechanisms involved in MS and cadusafos toxicity in soil invertebrates although it is understood that these effects are due to the release of the breakdown products, specifically methyl-isothiocyanates (MITC) from MS (Kassie *et al.,* 2001). The DNA-damaging properties of MITCs are reduced by non-enzymatic protein binding (Kassie *et al.,* 2001). This suggests that with additional enzymatic repair mechanisms, the DNA damage might not be permanent. However, the exact mechanism involved causing the genotoxic effect in earthworms is unknown. Repair of DNA damage can be very rapid with reports showing that strand breaks by ROS can be repaired with a half time of less than thirty minutes and as short as three minutes (Olive and Banáth, 2006). In this study the high levels of DNA damage was still observed 28 days after exposure suggesting a more permanent level of DNA damage.

Most chemical toxins including MS and R10, have the potential to bioaccumalate. The effects of the MITC released by MS are likely enhanced by the fact that it has a tendency to become trapped in soil moisture which leads to a relatively slow release of the volatile substances (Pruett *et al.,* 2010). Bioaccumulation of chemicals is often higher in natural soil with low SOM and lower in soil containing a higher SOM content such as artificial soil prepared in the laboratory (EFSA Scientific report, 2006). This would suggest that in natural

soil these effects could have been accentuated if bioaccumulation of the chemicals occurred due to soil adsorption.

The results from the sublethal growth and reproduction measurements and the comet assay were not always in agreement. The M treatment had no significant difference in the survival, growth or reproduction success compared to the control treatment; in fact the reproductive success for M was the closest to the results of the control treatment. In the comet assay, however, the results from the M treatment showed a significant difference in the genotoxicity results compared to C and the other biofumigants. In order for responses of individual organisms to be related to higher levels within an ecosystem, a variety of end-points need to be selected so that findings can be translated (Chapman, 1995). Although no negative effects were detected in the growth and reproduction success of M treatments, the higher level of DNA damage can be related to a higher impact at the population level (Vasseur and Bonnard, 2014). The control and other biofumigant treatments had similar results for both the sublethal measurements and the comet assay.

Overall, the chemical fumigants had a much more significant effect at the individual level which will result in severe changes at population level and in turn at an ecological level. Edwards and Bohlen (1992), stated that the majority of chemical fumigants and nematicides are highly toxic to earthworms. Very little further information is available on the toxicity of chemical fumigants and in particular MS and cadusafos on earthworms. The limited information that is available date back to the 1980's and only state that insecticides and nematicides with fumigant action are very toxic to earthworms (Edwards, 1984). Results from this study confirm this statement as both the chemical fumigants had significant effects on the survival, growth, reproduction and genotoxicity of earthworms.

4.3 Phospholipid fatty acids

Phospholipid fatty acid (PLFA) profiling was used to determine if there were any changes in the soil microbial community structure (Frostegård and Bååth, 1996) due to the various fumigant treatments. In this study the estimated viable biomass, community structure and fungal to bacterial (F:B) ratio were investigated. Previous studies confirm that there is generally a good correlation between the total amount of PLFAs detected and the viable microbial biomass (Zelles, 1999). The sum of PLFAs detected from these soil samples, ranging from C14 to C19, were used as an indicator for the estimated viable biomass (Zelles, 1999; Claassens *et al.,* 2011).

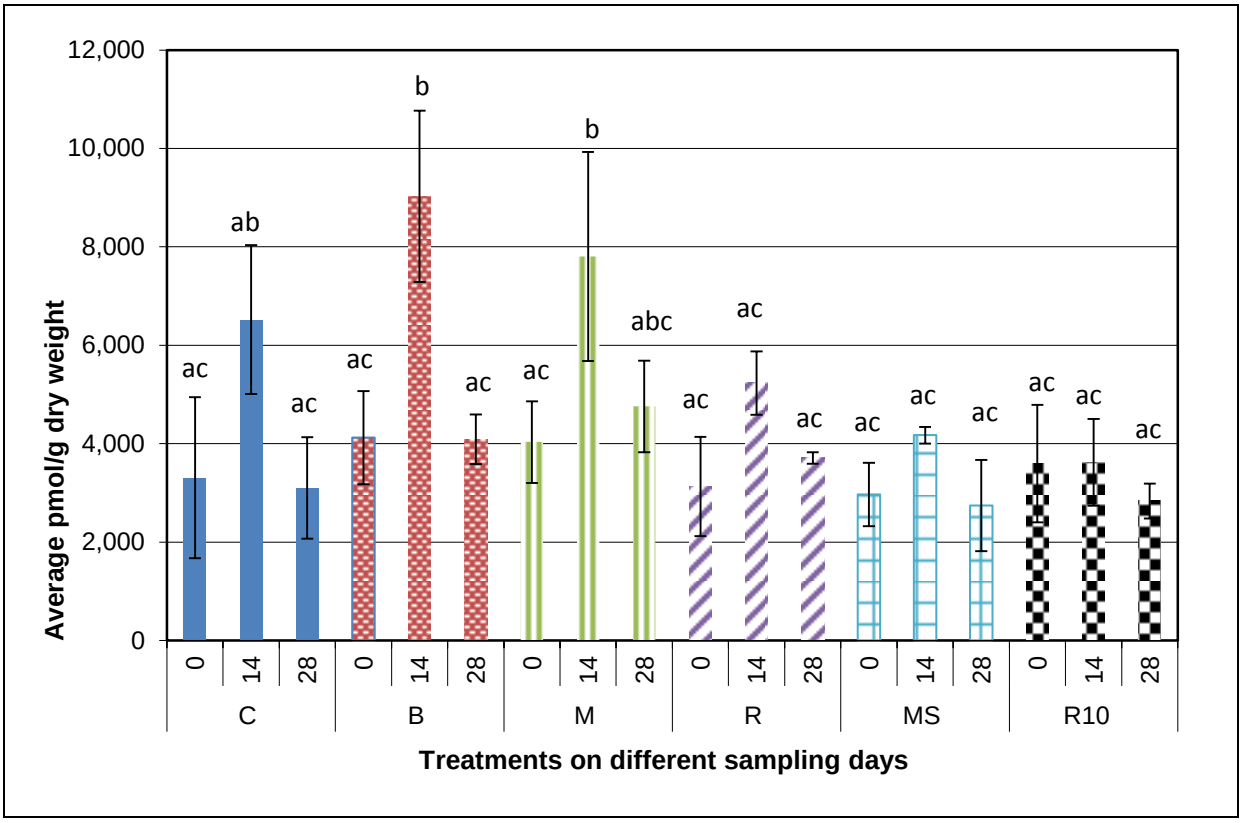

Figure 3: ***The change in viable microbial biomass (average mole % of total PLFAs) over time (day 0, 14 and 28). Error bars indicate standard error of the mean (SEM). Significantly (P < 0.05) different results are denoted by different letters. Treatments: C (control); B (broccoli); M (mustard); R (oilseed radish); MS (metham sodium) and R10 (cadusafos).***

The results of the average mole percentage of the total PLFAs were used to determine the microbial biomass and are given in Figure 3. Results show that there was an initial increase (control (C), broccoli (B) and mustard (M) were significant, $P < 0.05$) in total PLFAs at day 14 in all the treatments. The highest biomass was recorded for the B and M treatments, 14 days after application, and the lowest biomass was observed in the chemical fumigant treatments metham sodium and cadusafos (MS and R10) 28 days after application. These changes were only temporary and the total PLFAs, for all samples, decreased at day 28 to a value statistically similar to the initial values. Only M and R had a higher biomass at day 28 but was not statistically significant. The C, B and both the chemical fumigants resulted in a lower biomass at day 28 than its initial values. The increase in biomass that were observed on day 14 in the C, biofumigant treatments and MS, are most likely due to the disturbance of the soil environment after placing it into the containers and the addition of the moisture, rather than the addition of the plant material. The R10 treatments, however, did not have the same increase and continued to decrease in biomass at day 28 which would suggest that the fumigant was responsible for the negative effect on the viable microbial biomass.

Specific signature fatty acids are markers for different microbial groups (Zelles, 1999; Kozdrój and van Elsas, 2001). The analysis of the PLFA patterns allows for the classification of the PLFA markers into six major structural groups (Pryfogle, 2000). The proportions of each group can be used in the description of the total community structure (Waldrop *et al.,* 2000). Changes in the proportions of the PLFA markers are interpreted as shifts in the major microbial groups that make up the community structure (Zelles, 1999). The specific PLFA markers detected in the soil samples of this study indicated the presence of five major microbial groups as shown in Table 5. Phospholipid fatty acid markers for branched monounsaturated fatty acids (Bmonos), indicative of obligate anaerobes, were not detected in these soil samples.

Table 5: ***Phospholipid fatty acid markers detected in this study associated with different structural subgroups (Frostegård et al., 1996; Olsson, 1999; Pryfogle, 2000.***

PLFA structural subgroup	**PLFA markers detected**	**Classification of microorganisms**
Normal Saturated (NSats)	14:0 15:0 16:0 17:0 18:0 21:0 22:0	Universal straight chained markers found in most *prokaryotic and eukaryotic* organisms.
Mid-chain branched saturated (MBSats)	12Me16:0 10Me17:0 12Me18:0	Primarily *Actinomycetes* and some Gram-positive *bacteria*
Terminally Branched Saturated (TBSats)	i15:0 a15:0 i16:0 i17:0 a17:0	Mainly *Gram-positive bacteria*
Monounsaturated (Monos)	15:1 16:1ω7c 16:1ω7t 17:1ω8 cy17:0 18:1ω7c 18:1ω7t 19:1ω12 cy19:0	*Gram-negative bacteria* (fast growing and able to adapt quickly to a variety of environments)
Polyunsaturated (Polys)	18:2ω6 20:2ω6 20:5ω3	Representative of *fungi*

The specific values for each PLFA structural group, per treatment, are summarised in Table 7 (Annexure D). Normal saturated fatty acids (NSats) are straight chained markers found in most prokaryotic and eukaryotic organisms. The NSats formed the dominant portion of the microbial community in this study (Figure 5). A high proportion of NSats is generally an indication of a lower diversity within the microbial community (Pryfogle, 2000; Claassens *et al.,* 2006) and might be due to the use of artificial soil. Although there were changes in the NSats over time, Tukey's HSD showed that these changes were not significant ($P > 0.05$) and the NSats remained relatively constant as the dominant group throughout the experiment.

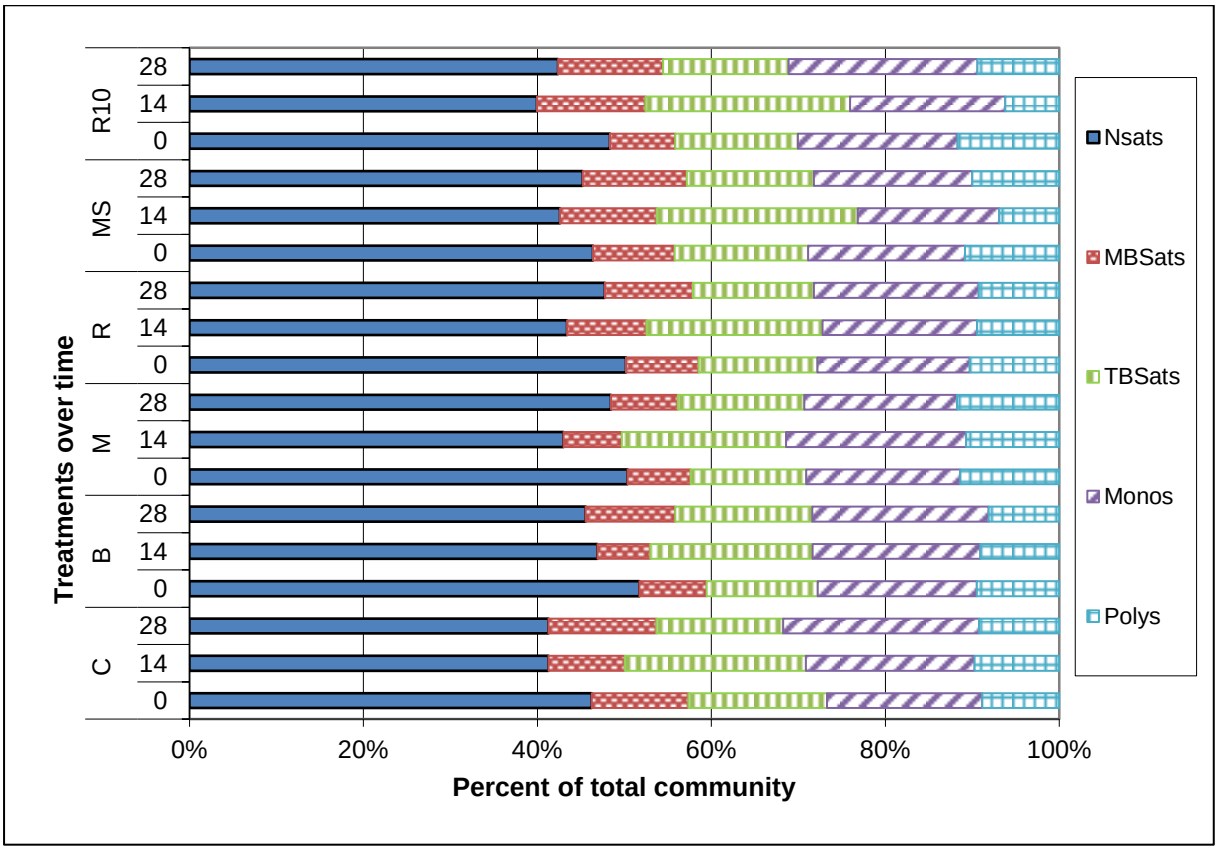

Figure 4: ***Change in community structure indicated by the change in major PLFA structural groups (NSats, MBSats, TBSats, Monos and Polys). Treatments: C (control); B (broccoli); M (mustard); R (oilseed radish); MS (metham sodium) and R10 (cadusafos).***

Mid-chain branched saturated fatty acids (MBSats) are indicative of Actinomycetes that are primarily soil inhabitants. They are of ecological importance due to the important role they play in decomposition and mineralisation of organic matter (Prescott *et al.,* 2008). Analysis of variance was done of MBSats on the different sampling days to show significant differences. In the C and biofumigant treated soil, Actinomycetes decreased within the first 14 days. The

decrease in the biofumigant treatments was to a lesser extent than in C. This is similar to findings by Potgieter *et al.* (2013), who found that Actinomycetes decreased after biofumigant (mustard and canola) soil amendments. On sampling day 28, the MBSats increased again to values higher than its original values, however, in the M treatments the MBSats increased at a slower rate and was statistically ($P < 0.05$) less than the C sample but not the other biofumigants. The chemical fumigant soil amendments (MS and R10) had an opposite effect and stimulated growth of the Actinomycetes. Cadusafos resulted in a significant increase ($P = 0.002$) in MBSats within the first 14 days but on day 28 it was not statistically different to C. Macalady (1998), also found that Actinomycetes increased after fumigation with the soil fumigant, MS.

The terminally branched fatty acids (TBSats) are commonly associated with Gram-positive bacteria that are considered to be tolerant to stress and are able to metabolise complex carbon substrates (Waldrop *et al.,* 2000). Results showed an increase in TBSats for all the samples on day 14. The increase in both the biofumigant and chemical fumigant amended soil was statistically significant ($P < 0.01$ and $P < 0.002$ respectively). The significantly higher values of TBSats in the fumigant treatments, suggests that the breakdown products of the biofumigants and chemical fumigants stimulated growth of the Gram-positive bacteria. The chemical fumigants had a greater stimulatory effect on the TBSats than the biofumigants. The changes observed were not lasting and the TBSats in all the samples returned to their initial values at sample day 28.

Monounsaturated (Monos) PLFA markers are associated with fast growing Gram-negative bacteria that are able to utilise many different carbon sources. They grow faster than Gram-positive bacteria and adapt better in stress situations (Peacock, 2001). Results for this PLFA group were inconsistent between treatments. The control and all the biofumigant treatments had an increase in Monos, suggesting an increase in the Gram-negative bacteria. The M treatment resulted in the highest proportional increase in the abundance of Monos on sample day 14. However, after the initial increase, there was a significant decrease ($P < 0.05$) in this group for the M treatment, on sample day 28, to values lower than its original values and the C samples (Table 7, Annexure D). This is in contrast to the control and other biofumigants (B and R) that continued to increase. In the chemical fumigant treatments, the Monos also increased after 14 days but returned to its original values by day 28. Interestingly, the Monos for R10 was associated with the C samples, which show that this treatment had the smallest effect on the Gram-negative bacteria.

Three PLFA markers were used to determine the polyunsaturated (Polys) structural group (Table 5). The signature lipid, 18:2ω6 (linoleic acid) is considered the main PLFA marker for fungi (Zelles, 1999), however in this study 20:5ω3 was the major contributing PLFA to the Polys group. Both these markers can occur in both fungi and plants (McKinley *et al.,* 2005; Zelles, 1999). The PLFA marker 20:5ω3 is considered a good indicator of arbuscular mycorrhizal (AM) fungi (Olsson *et al.,* 1995). The marker 20:5ω3 was found in all the samples including the C and chemical fumigant samples that did not contain additional plant material, which means that this marker could have been from the peat used in the artificial soil. The two markers responded differently to the soil amendments. The 18:2ω6 marker increased significantly due to biofumigant amendments, in some cases more than 1000 % increase (data not showed) within the initial 14 days. In the chemical fumigant treatments, it also initially showed increases in this marker but 2 - 3 times less than the C samples. The 20:5ω3 marker, in contrast, decreased between 0.5 – 51 % for all the soils except for the M and R10 soil amendments which increased 24 % and 55 % respectively. The control samples had a continual and steady growth over the experimental period. Overall the results of the Polys group indicated that both the biofumigants and the chemical fumigants inhibited growth of the fungal population, but not significantly different to the C group. However, the initial stimulating effect of the biofumigants on the signature fungal marker (18:2ω6) are obscured by the changes in the 20:5ω3 marker.

To better understand the changes in the fungal group, the ratio of fungal to bacterial biomass (F:B) ratio was assessed and results are given in Figure 6. The signature lipid, 18:2ω6 (linoleic acid) is considered the main PLFA marker for fungi (Zelles, 1999) and 20:5ω3 was not used in the F:B ratio. The F:B ratio was determined by dividing the mole percentage of the 18:2ω6, by the sum of the mole percentages of the bacterial PLFA markers (15:0, i15:0, a15:0, i16:0, 16:1ω7c, 16:1ω9t, i17:0, a17:0, 17:0, cy17:0, 18:1ω7c and cy19:0) (Frostegård *et al.,* 1996).

The dynamics of fungi and bacteria relative to changes in the soil environment is often used as an indicator of soil quality and soil fertility. This is because fungi and bacteria have different abilities to store carbon and therefore contribute differently in the regulation of soil organic carbon (Frostegård *et al.,* 1996; Bailey *et al.,* 2002; Leckie, 2005). Leckie (2005) stated that soils with a lower fertility and nitrogen availability tend to have a greater proportion of fungal biomass compared to bacterial biomass. According to Bailey *et al.* (2002), high fungal biomass allow for more storage of carbon, while bacterial biomass is able to mediate more labile carbon. A fungal to bacterial ratio of 1.0 would indicate that the fungal

and bacterial populations contribute equally to the microbial activity in the soil, consequently F:B ratios are usually less than 1.0 in populations where one group dominates over the other (Bailey *et al.,* 2002). Results show that the bacterial biomass always formed the greater proportion of the lipids suggesting bacterial populations dominated over fungi. However, 18:2ω6 increased at a much higher rate than the bacterial PLFA markers, changing the F:B ratio. The same trend in the F:B ratio was observed for the C and biofumigant samples. It increased on sample day 14 and then decreased on day 28. Analysis of variance showed that the increase in 18:2ω6, significantly ($P < 0.05$) increased the F:B ratios in all three the biofumigant treatments (Figure 6). The availability of the additional carbon substrate due to the addition of plant material was most likely the contributing factor to the significant increase in the signature fungal communities and in turn the increased F:B ratios. Mustard treatments had the highest F:B ratio of 0.23 at sample day 14. Although the F:B ratios for the biofumigant treatments decreased on sample day 28, these values were still considerably higher than the C samples. The addition of the chemical fumigants also increased the F:B ratio over the 28 days but it was not statistically significant ($P > 0.5$) as in the biofumigant treatments.

Literature suggests that most of the ITCs will be released within the first four days after soil incorporation of the plant material, after which ITC concentrations decrease rapidly (Morra and Kirkegaard, 2002). The PLFA results (Figure 4 and Figure 5) are consistent with these findings as most of the significant stimulatory or inhibitory effects occurred within the first 14 days after tissue incorporation. In general, the biofumigant treated soil (B, M and R) resulted in a higher average microbial biomass (Figure 4) than the C sample. This stimulating effect had a greater impact on the fungal growth, specifically the 18:2ω6 marker, than the bacterial growth when looking at the F:B results (Figure 6). The changes in the biomass in the biofumigant treatments are more related to the additional carbon substrate availability than the ITC breakdown products. Similar results have been observed in other studies where microbial growth was stimulated due to the additional nutrition availability from the green manure cover crops (Omirou *et al*., 2010; Omirou *et al.,* 2013; Potgieter *et al.,* 2013). Studies by Price *et al.* (2005), showed that microbial communities readily use natural ITC, including allyl-isothiocyanates (AITC) as a carbon source in their metabolic pathways, although the specific effects on the dynamics of the microbial community is not clear.

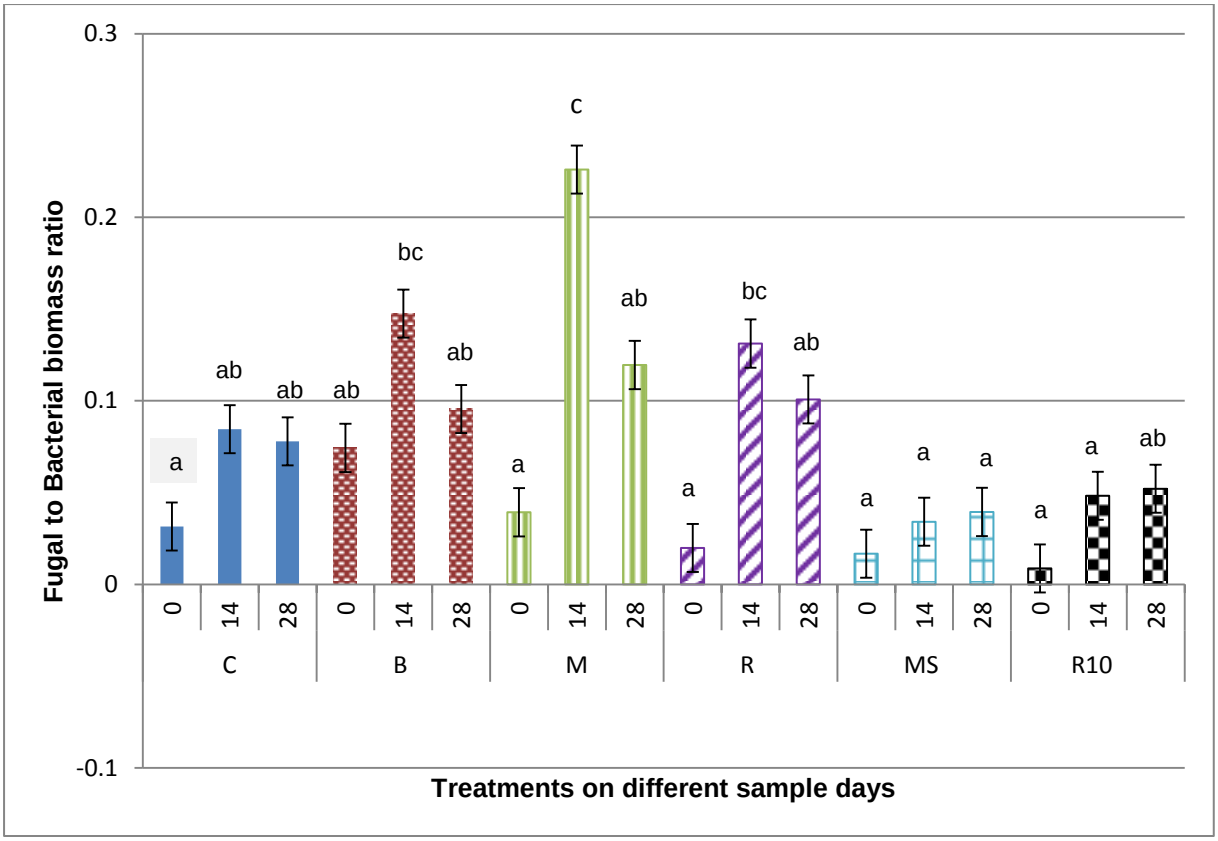

Figure 5*: Fungal to Bacterial biomass ratio of the PLFA markers for the various treatments over time (± SEM). Significant differences (P < 0.05) for the same treatment over time are denoted by different letter. Treatments: C (control); B (broccoli); M (mustard); R (oilseed radish); MS (metham sodium) and R10 (cadusafos).*

In contrast, the chemical fumigant treatments resulted in lower average biomass than the C sample suggesting an inhibiting effect of the microbial community. Studies by Toyota *et al.* (1999), found that fumigated soil reduced the total biomass to almost half within the first 15 days but then recovered to former levels after 28 days. In this study, the biomass did not recover after 28 days but it was not statistically different to C samples. Even though MS and R10 have different breakdown products, results suggest that they had a similar effect on the community structure. Most of the effects observed within the first 14 days were non-persistent and the overall microbial community composition returned to the initial state. The only lasting changes in the community structure, compared to the C, were observed in the M treatment, which inhibited growth of the MBSats and Monos groups. This suggests that the breakdown products in M, could have resulted in the inhibitory effect on the Actinomycetes and Gram-negative bacteria.

4.4 Community level physiological profiles

Biolog™ EcoPlate analysis was used as a community level physiological profiling (CLPP) technique to distinguish between the different treatments. Average well colour development (AWCD) is indicative of the density of microorganisms (Weber and Legge, 2009) (Figure 6) while the principal component analysis (PCA) of the grouped carbon substrate utilisation patterns provides an overview of the differences in the functional potential of the soil microbial communities (Figure 7). The AWCD results are summarised in Table 8 (Annexure E). Due to the variance in inoculum density, a reference AWCD closest to 0.25 was selected (Chen *et al.,* 2007). Absorbance data from the 64 hour time point was chosen for AWCD because this is where equivalent reference AWCD of 0.25 occurred for the different plates (Weber and Legge, 2009).

Initial AWCD results (Figure 7) show that soil amendments with M and R10 had significant but contrasting effects on the colour development. Mustard resulted in a significantly ($P < 0.05$) higher AWCD whereas R10 had significantly ($P < 0.002$) low colour development (Table 8, Annexure E). At sample day 14, results show that there was no significant difference in the AWCD between C and the biofumigant treatments but both the chemical fumigants (MS and R10) had lower colour development than the C. At sample day 28, R10 remained significantly lower ($P < 0.005$) than all the other groups while the biofumigant treatments resulted in higher AWCD values, although not statistically different compared to the control group. Metham sodium amended soil was statistically similar compared to the C.

Over the test period, a similar pattern was observed in the AWCD change within the treatments. The results show that the addition of the carbon substrate in the form of the fumigants caused an initial stimulatory effect and an increase in the AWCD at day 14. Several authors report an increase in AWCD after the incorporation of organic matter in the form of green manure to the soil (Giannakou *et al.*, 2004; Gomez *et al.,* 2006; Potgieter *et al.,* 2013). The observed increases on day 14 were not lasting and at day 28 AWCD all the treatments returned to values similar to that of day 0 (Table 8, Annexure E). At all three sample days, the biofumigants always resulted in higher AWCD and chemical fumigants in lower AWCD. The AWCD (Figure 7) value of fumigant amended soil, with the exception of R10, does not show a significant variance in the microbial community density over time. However, the PCA reveal a differential effect upon the microbial functional diversity (Figure 8).

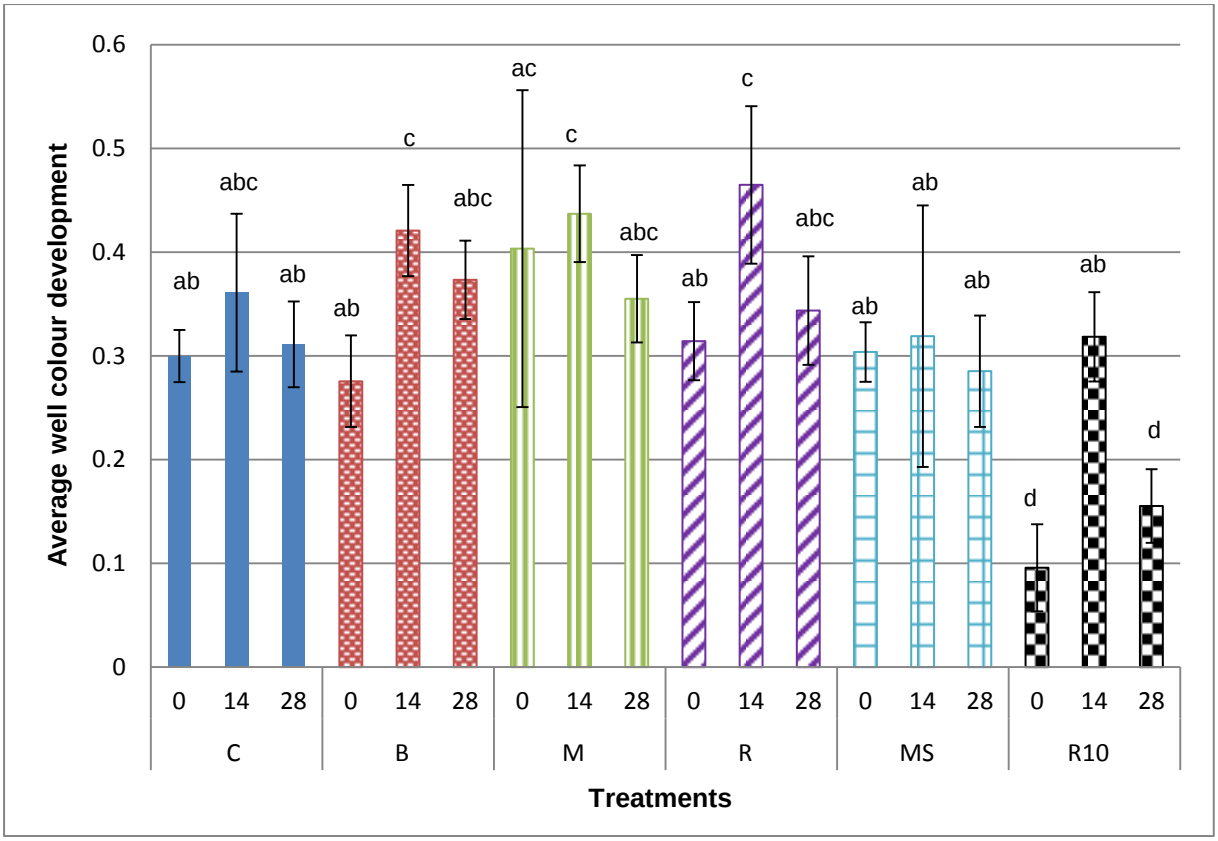

Figure 6*: Average well colour development (± SD) for treatments on different sampling days as an indicator of microbial density. Treatments: C (control); B (broccoli); M (mustard); R (oilseed radish); MS (metham sodium) and R10 (cadusafos).*

Principal component analysis (PCA) was done to show the relationship between the grouped carbon substrates (Table 1) and the change in the functional diversity of the microbial community over time. Carbon substrate utilisation patterns were used to determine the functional response. Total variability in the first principal component (PC1) was 55.2 % and 25.0 % in the second principal component (PC2). The control did not statistically change throughout the test period and grouped centrally on the PCA, indicating no specific functional association with any of the major substrate groups. In the fumigant amended soil, the biofumigants, B and R as well as the chemical fumigant MS, were similarly grouped compared to C at day 0 while M and R10 had different functional groups. On sample day 14, the functional groups in all the fumigant treated soil changed while the C changed very little. This suggests a change in functional response due to the fumigant soil treatments. Mustard and R showed similar carbon substrate associations. Brocolli and MS, however, had different and opposite utilisation patterns. Research suggest that ITCs react with amine

groups, sulph-hydryl groups and the disulphide bonds of proteins instigating the degradation of enzymes (Brown and Morra, 1997). Several studies confirm the inhibitory effect of AITC and MITC on enzymatic processes (Brown and Morra, 1997; Macalady, 1998). This could explain why the substrate utilisation patterns for all the biofumigant and MS amended soil were less associated with amine and amin_a on sample day 14.

The microbial communities exposed to R10 grouped to the right of the PCA and away from all the other treatments. It showed a different pattern of substrate utilisation compared to all the other treatments at all three sampling times. Cadusafos do not form ITCs as breakdown products but instead produce low-molecular weight, volatile molecules such as esters that are easily degraded by hydrolysis and can be utilised by microorganisms as a carbon and energy source (Santa Cruz Biotechnology, 2010). This could explain why R10 had a significantly different functional response to the other treatments. After 14 days the functional groups changed and results show that the microbial communities were more closely associated with phosphorylated compounds (phos_c). On sample day 28, the substrate utilisation profiles for the M and R10 returned to similar profiles than on sampling day 0, while the utilisation patterns for two of the biofumigants (B and R) and MS remained different. This would suggest that in M and R10 the microbial communities showed signs of resilience while the B, R and MS treatments had the greatest lasting effect on the substrate utilisation patterns and in turn the function of the microbial community. Toyota *et al.* (1999), found that the carbon utilisation profiles of microbial communities fumigated with MS, remained different from the control samples 105 days after fumigation. Studies by Floch *et al.* (2011), found that the microbial functional diversity in soils treated with several pesticides, including carbamate type pesticides, were resilient after 6 – 12 months of incubation.

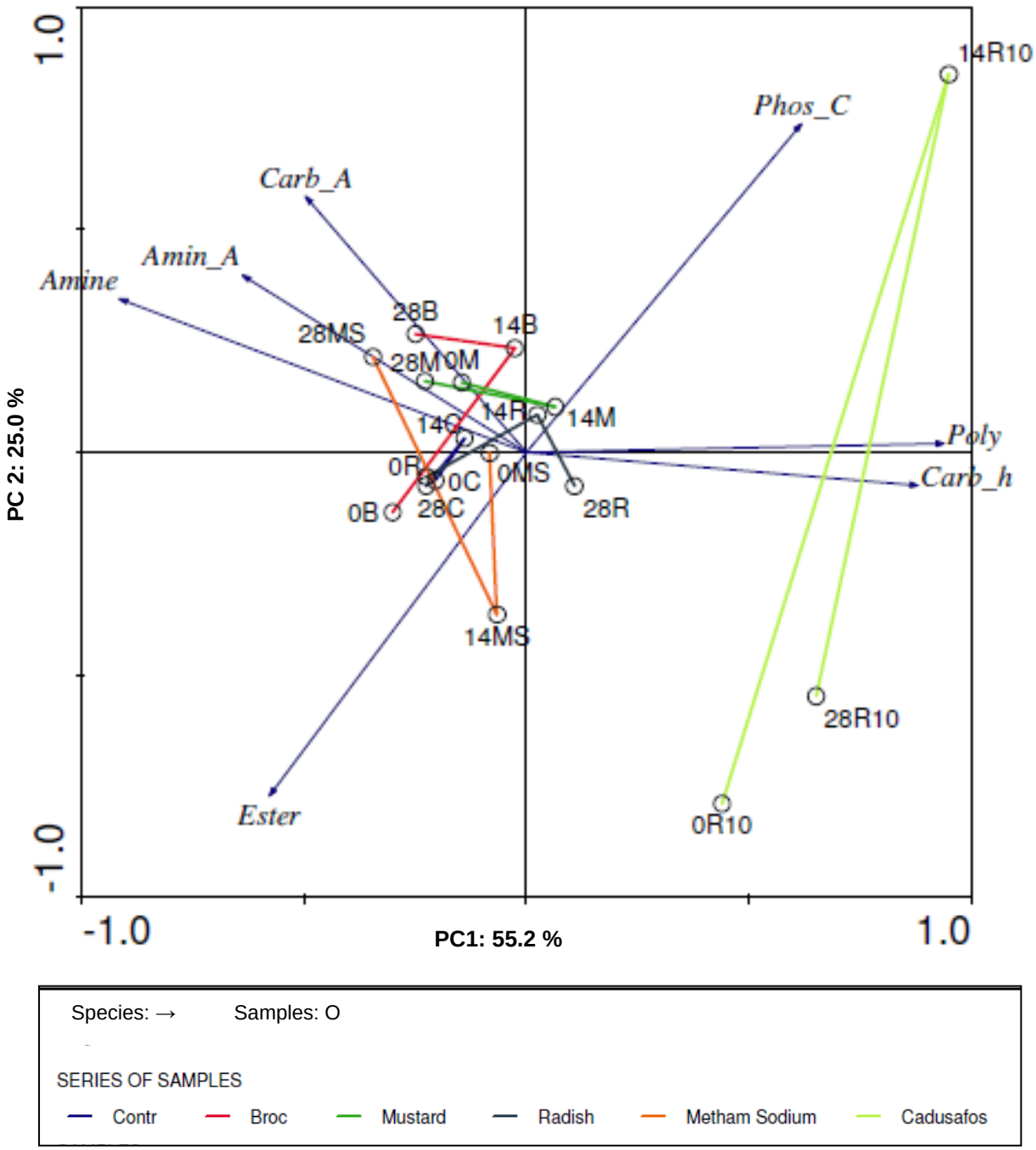

Figure 7: ***Principal component analysis of the relationship between the change in functional diversity of microbial groups (circles) and the grouped carbon substrates (arrows) over time.***
Treatments: C (control); B (broccoli); M (mustard); R (oilseed radish); MS (metham sodium) and R10 (cadusafos).

4.5 Pearson's correlation

Similar trends were observed in the average mole percentage of total PLFAs, used to determine the viable microbial biomass, and the Biolog™ AWCD results, used to determine the microbial density. Correlation analyses were conducted using Pearson's product-moment correlation coefficients to determine if there was a correlation between the changes over time in the two sets of data. Each method analyses a different aspect of the soil microbial community and previous studies have found that there is not always a correlation in the trends of the respective methods (Xue *et al.,* 2008). In this study, however, a weak positive correlation (correlation coefficient r = 0.63) was found between the PLFA and Biolog™ results.

4.6 Summary of microbial analysis

Throughout this study it was found that the effects exerted on the microbial community by the biofumigants and chemical fumigant metham sodium (MS) were the highest within the first 14 days after application. In most cases it was not persistent, most probably due to the short half-lives of the breakdown products formed from these chemicals. The biofumigants and MS both form ITCs as breakdown products. Glucosinolates in the biofumigant plant material break down in the presence of the enzyme myrosinase to form AITC, while MS releases MITC, which is structurally different and more volatile than AITC. The natural toxins, AITC, in biofumigants have short half-lives, reported between 20 – 60 hours (Morra and Kirkegaard, 2002). Similarly, chemical fumigant type pesticides are considered non-persistent in the environment and have short half-lives ranging from hours to several weeks and sometimes months, depending on various environmental factors such as temperature and pH (Dent, 2000; Santa Cruz Biotechnology, 2010). The amount and efficiency of ITC release by the different species following soil incorporation also vary greatly, which can be seen from the differential responses by microbial communities in the different biofumigants and MS treatments

Results indicate that of the biofumigants, the mustard (M) treatment caused the most significant changes in the microbial community. It significantly changed the community structure (Figure 5) and resulted in a different functional diversity (Figure 7). In addition it also increased the microbial biomass (Figure 4) and density on day 0 at the 64 hour incubation time (Figure 7) and had the highest change in F:B ratio on day 14. The stimulating effects on the microbial biomass and density can be ascribed to the additional carbon substrate from the plant material. However, the inhibitory effect of the M in the

community structure negatively affected the growth pattern of Actinomycetes and Monos (Gram-negative bacteria) and was probably caused by the ITCs. This suggests a lower resilience of these two groups to the breakdown products.

Soil microorganisms contribute significantly in the dissipation of carbamates and organophosphates from the soil because they are able to hydrolyse the compounds and use the breakdown products in their metabolic pathways. Carbamates and organophosphates have a similar mode of action in that it inhibits enzyme activity (Dent, 2000). Metham sodium (carbamates) and cadusafos (organophosphate) are fumigant type pesticides. Literature suggests that fumigants commonly cause long-lasting effects on soil microbial communities in contrast to synthetic and non-fumigant pesticides that do not have any long-lasting effects (Macalady *et al.,* 1998; Ibekwe, *et al.,* 2001). Microbial soil communities are sensitive to disturbances and their resistance and resilience to disturbances determine microbial ecosystem stability (Allison and Martiny, 2008). Resistance is the microbial community's ability to resist change over time despite disturbances. Resilience is the soil ecosystem's ability to recover from disturbances in good time. However, soil microorganisms differ in their ability to survive and recover after a disturbance (Taylor *et al.,* 1996; Macalady *et al.,* 1998). The microbial communities showed signs of resilience to the breakdown products of the chemical fumigation because there were very few lasting shifts in the overall microbial structure (Figure 5). Analysis of the PLFA data from MS, suggest that very little variability was observed in the overall community structure after 28 days and no meaningful differences in the microbial community composition were observed. Different results were obtained in the study by Macalady *et al.* (1998), which showed that fumigation with MS at recommended field applications resulted in more persistent changes in the Gram-negative bacteria and fungi. This significantly altered the microbial community structure for up to 18 weeks after application. Application dosages used by Macalady et al. (1998), was between 90 – 320 mg/kg soil, which is much higher than the field application dosages that were used in this study as suggested by Omirou *et al.* (2010) and Wu *et al.* (2011). Collins *et al.* (2006), also suggest significant changes in the soil fungal populations and free living nematodes when fumigated with chemical fumigants like MS compared to cover crop treatments. The application of cadusafos imposed significant changes on the microbial community, particularly the viable microbial biomass (Figure 4) and the substrate utilisation patterns (Figure 7). After 28 days the microorganisms treated with chemical fumigants recovered in terms of the density of microorganisms as shown by the AWCD (Table 4) and the community structure (Figure 5) shown by the PLFA results whereas the functionality of the microbial groups remained significantly different (Figure 7).

Research by Warton *et al.* (2001) and Karpouzas *et al.* (2005), showed that soils that receive frequent or repeated applications of MS and cadusafos, can develop enhanced biodegradation. Enhanced biodegradation refers to the enhanced removal of the chemical due to the activity of specialised soil microorganisms that results in the reduction of the chemical to perform its intended purpose (Karpouzas, *et al.,* 2005). The enhanced biodegradation is due to soil microorganisms that have adapted to use these breakdown products in their metabolic pathways. In MS treated soil, the AITC from biofumigants run the risk of also being dissipated at an accelerated rate even if AITC have never been applied before due to the similarities of the AITCs and MITCs (Warton *et al.,* 2001). The soil microorganisms that most commonly adapted to catabolise this product were Gram-positive and Actinomycete type bacteria (Warton *et al.,* 2001, Karpouzas *et al.,* 2005). In this study, the application of both these fumigants resulted in a significant ($P < 0.05$) increase in the TBSats, which are indicative of Gram-positive bacteria. This suggests that both these chemicals can have serious effects of the microbial community structure if it is used regularly.

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

5.1 General conclusions

The effects of the fumigants were assessed on the survival, growth and reproduction of earthworms. Overall, findings showed that biofumigants (fresh plant material) applied at the prescribed amendment rates, had limited effects on these earthworm parameters. None of the biofumigants had any effect on survival or growth. Reproductive success was negatively impacted by the broccoli treatment in terms of juveniles hatching. The chemical fumigants had a marked negative impact on all three parameters, survival, growth, and reproduction, of the *E. andrei* with metham sodium causing greater harm than cadusafos. As a result, it can be concluded that both these chemical fumigants can possibly cause adverse ecological effects based on earthworm responses. This is because the negative effects on the earthworms, could impact their ecosystem functions for example bioturbation and nutrient cycling.

Genotoxic effect of fumigants was assessed by means of the comet assay. In the biofumigant exposure groups, mustard was the only substance that showed a genotoxic effect. This could indicate a possible early response to the ITCs found in mustard, which is consistent with similar studies that used higher dosages of a pure liquid form of the same ITC. The chemical fumigants, and in particular metham sodium, showed a more pronounced genotoxic effect on earthworms. These high levels of DNA strand breaks measured in the chemical fumigant exposures are an indicator of cellular stress and indicate detrimental effects in exposed individuals. Comet assays of earthworm coelomic cells could therefore be used as an early warning system for fumigant exposure.

Changes in the soil microbial community biomass and structure were evaluated by means of PLFA analysis. Results demonstrate that the application of biofumigants increased the size, structure and activity of the soil microbial biomass (Figure 5). The observed increase in the microbial biomass in the biofumigant treatments is most likely more related to the additional carbon source provided by the plant material, than the natural toxins produced by the breakdown of the plant material. The M treatment was the only biofumigant exposure group that resulted in shifts in the community structure that lasted for the duration of the experiment. These shifts were only observed in two of the five major PLFA structural groups, namely the MBSats (Actinomycetes) and Monos (Gram-negative bacteria). Statistically, the chemical fumigants resulted in the most prominent changes in the microbial biomass (Figure 4) but had no lasting effect on the community structure.

Biolog™ results of the community level physiological profiles showed differences in the functional response of the microbial community to the fumigant treatments (Figure 7). The broccoli, oilseed radish, metham sodium and cadusafos resulted in changes that could be observed for the duration of the experiment. Overall, cadusafos had the most noticeable effects on the microbial community because it resulted in lower viable biomass, a limiting effect on microbial density and a significant and lasting effect on the functional response of the microbial community (Figure 7). The lasting changes in the utilisation patterns suggest that the fumigants, and in particular the breakdown products of the fumigants, could have a negative impact on the rates of ecosystem processes and the overall functioning of the soil ecosystem.

This study demonstrates the complexity of the factors controlling soil health and productivity. When comparing the effects of the fumigants on the earthworms and the microbial community, it was found that at each level of organisation there were different effects. Each bioindicator species illustrates effects at their own level of organisation and results need to be interpreted as such. The results from the biofumigant treatments varied and resulted in different effects. Although the broccoli and oilseed radish treatments had no significant effects on any of the earthworm bioassays, it did have more lasting effects on the microbial functional response (Figure 4). However, mustard had a more significant negative effect on both these non-target species, suggesting that the natural toxins (ITCs) produced by mustard could affect soil functioning even at low concentrations. The chemical fumigants also showed different results for the two groups of organisms. Earthworms were more sensitive to metham sodium than cadusafos, whereas in the microbial community, cadusafos had a more pronounced effect than metham sodium. The varying effects of one treatment on different organisms, illustrates the importance of using different bioindicators to get a better understanding of the overall effects on the soil ecosystem.

Results from this study found that the use of artificial soil did not present any limiting factors for analyses of the earthworm bioassays or microbial analysis in terms of assessing the effects of fumigants. Studies using artificial soil are often questioned due to many variables of real ecosystems that are excluded. However, the use of artificial soil is a means of manipulating important parameters such as soil organic matter (SOM) and simplifying soil functioning in order to test specific ecological factors on soil organisms including microbial communities (Guenet *et al.,* 2011). The data obtained indicate that the comet assay is a highly sensitive technique that detected low levels of DNA damage induced by the natural

toxin ITC and it proved to be more sensitive than the other sublethal tests such as growth and reproductive success. The comet assay remains an important biomarker for environmental purposes. Furthermore, the use of the PLFA and Biolog™ analyses proved to be useful in measuring the degree of disturbance within the microbial community when exposed to natural and chemical toxins.

Finally, from the results obtained, it can be concluded that biofumigants had a less significant impact on non-target species such as earthworms and microbial communities. With the additional benefits of suppressing soil borne disease, nematodes and weeds, it is a good alternative to chemical fumigants or as part of an IPM strategy for agricultural crops in South Africa. Care should, however, be taken when using different products that form AITCs and MITCs as it could result in enhanced biodegradation that can cause a reduction in the ability of the chemical to perform its intended purpose (Karpouzas *et al.,* 2005).

5.2 Recommendations for future research

After conducting this research, several new questions arose and it is recommended that further studies are done to investigate some of these questions.

- Further research is required on the implications of biofumigants on soil biology under more realistic agricultural conditions to get a clearer picture of the interactions between crop protection practices and the soil biology. The use of artificial soil could have been the cause of the lower microbial diversity as indicated by the high proportion of NSats found in the PLFA results. It is recommended that in future studies natural soils are also utilised, especially for microbial community studies so that important ecosystem variables such at native microbial communities, climatic changes, moisture and SOM are taken into consideration. This will facilitate the assessment of not only the direct, but also the indirect effects of fumigants.
- Due to the variability in the concentration of the GSL breakdown products it is recommended that the ITCs in the soil are measured at several intervals after soil incorporation. The concentrations of the ITCs measured in the soil, should be related to the effects observed in the non-target soil organisms. From this information the EC_{50} of the ITCs in the biofumigants can be determined. In this study the effect of mustard on the earthworm DNA strand breaks and the shifts in the microbial community structure could not be related to a specific concentration of ITC produced by mustard.

- Due to the general lack of information with regards to the effects of fumigants on soil organisms, it is recommended that it be evaluated using standardised ecotoxicological test methods. This could include LC_{50} followed by sublethal tests such as growth and reproduction using different concentrations. Due to the low breakdown efficiency of GSL to ITC, it can be assumed that the actual concentrations of natural ITC was much lower than the concentrations of the chemical fumigants at the recommended field dosages. It is therefore advised that further studies are conducted to compare the effect of the natural toxins in biofumigants and toxins in the chemical fumigants at similar concentrations.
- In terms of the comet assay, it is recommended that long term studies are done to investigate the temporal nature of DNA strand breaks and if DNA repair will occur after long term exposure to both the biofumigants and chemical fumigants. The scope of this study only analysed DNA damage as a measure of induction but the kinetic repair of DNA is an important factor that needs to be taken into consideration before genotoxicity results are interpreted in terms of populational effects (Vasseur and Bonnard, 2014). Furthermore, it is recommended that genotoxicity of individuals are linked to population growth rates over several generations.

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ANNEXURE A

Calculation of water holding capacity (WHC) of artificial soil.

$$\text{WHC (in \% of dry mass)} = \frac{S-D}{D} \times 100$$

Where S = water-saturated soil and D = dry soil.

Sample 1: S = 1.328g and D = 0.993 g $\therefore \frac{1.328 - 0.993}{0.993} \times 100 = 33.76\ \%$

Sample 2: S = 1.387 g and D = 1.062 g $\therefore \frac{1.387 - 1.062}{1.062} \times 100 = 30.60\ \%$

Sample 3: S = 1.810 and D = 1.407 g $\therefore \frac{1.810 - 1.407}{1.407} \times 100 = 28.85\ \%$

- $\therefore$ the mean WHC in % dry mass is $(33.76\% \times 30.60\% \times 28.85\%) \div 3 = 31.07\ \%$

ANNEXURE B

Calculation of liquid formulas of the chemical fumigants, metham sodium (MS) and Cadusafos (R10).

1. Metham sodium 510 g/L. Application rate is 10 mg/ kg soil (Wu et al., 2011; Omirou et al., 2010).

    - 510 g/L = 510 mg/mL
    - $\frac{1\ \text{ml}}{510\text{mg}} \times 10\ \text{mg}$ = 0.019607 ml MS
    - = 19.61 µL MS / kg soil

2. RUGBY (cadusafos) 100 g/L. Application rate is 4 µg/g of soil (McClure and Schmidt, 1996; Karpouzas, 2004).

    - 100 g/L = 100 mg/ml
    - 4 µg = 0.004 mg/g soil or 4 mg/ kg soil
    - $\frac{1\ \text{ml}}{100\ \text{mg}} \times 4\ \text{mg cadusafos}$ = 0.04 ml cadusafos
    - = 40 µL cadusafos / kg soil

ANNEXURE C

Table 6: ***Comet assay results of earthworm coelomic cells (n = 450) in each treatment.***

Treatment	Percent (%) tail DNA	Percent (%) cells in each class				
		Class selection according to percent (%) tail DNA				
		Class 1	Class 2	Class 3	Class 4	Class 5
	Mean ± SEM	< 20 %	20 - 40 %	40 - 60 %	60 - 80 %	> 80 %
C	9.85 ± 1.12[a]	84.48	13.08	2.44	0.00	0.00
B	11.64 ± 1.33[a]	78.27	16.85	3.99	0.89	0.00
M	15.98 ± 1.47[b]	70.95	19.29	6.65	2.22	0.89
R	12.10 ± 1.48[ab]	81.60	10.20	6.43	1.33	0.44
MS	40.37 ± 2.67[c]	27.78	28.67	19.56	12.67	11.33
R10	19.28 ± 1.80[b]	68.22	17.56	7.56	4.67	2.00

Treatments: C (control); B (broccoli); M (mustard); R (oilseed radish); MS (metham sodium) and R10 (cadusafos).

ANNEXURE D

Table 7*: Phospholipid fatty acid groups (total mole %) ± SEM for different treatments over time. Different letters indicate statistically significant differences (P < 0.05, Tukey HSD). (Lowercase letters indicate significant differences between treatments on the same sample day. Different capital letters indicate significant changes for the same groups per treatment over time.*

Day 0	**C**	**B**	**M**	**R**	**MS**	**R10**
NSats	44.26 ± 2.44$^{a(A)}$	50.44 ± 0.16$^{a(A)}$	48.67 ± 0.73$^{a(A)}$	48.66 ± 1.27$^{a(A)}$	43.92 ± 2.76$^{a(A)}$	46.75 ± 0.19$^{aA)}$
MBSats	10.61 ± 2.19$^{a(A)}$	7.53 ± 1.22$^{a(A)}$	7.01 ± 0.58$^{a(A)}$	8.06 ± 0.32$^{a(A)}$	8.88 ± 0.85$^{a(A)}$	7.25 ± 0.62$^{a(A)}$
TBSats	15.25 ± 1.09$^{a(A)}$	12.45 ± 1.64$^{a(A)}$	12.83 ± 0.81$^{a(A)}$	13.22 ± 0.86$^{a(A)}$	14.52 ± 0.96$^{a(A)}$	13.64 ± 1.02$^{a(A)}$
Monos	17.07 ± 1.06$^{a(A)}$	17.81 ± 2.19$^{a(A)}$	17.07 ± 2.24$^{a(A)}$	16.99 ± 2.43$^{a(A)}$	17.06 ± 1.67$^{a(A)}$	17.75 ± 0.41$^{a(A)}$
Polys	8.43 ± 1.25$^{a(A)}$	9.23 ± 1.00$^{a(A)}$	10.98 ± 0.83$^{a(A)}$	9.94 ± 0.33$^{a(A)}$	10.25 ± 0.64$^{a(A)}$	11.27 ± 1.81 $^{a(A)}$
Day 14	**C**	**B**	**M**	**R**	**MS**	**R10**
NSats	39.47 ± 0.78$^{a(A)}$	46.07 ± 1.31$^{b(A)}$	42.25 ± 1.75$^{ab(A)}$	42.34 ± 0.48$^{ab(A)}$	41.26 ± 1.$^{17ab(A)}$	38.85 ± 0.19$^{a(B)}$
MBSats	8.34 ± 0.46$^{a(A)}$	5.93 ± 0.60$^{a(A)}$	6.58 ± 1.07$^{a(A)}$	8.90 ± 0.14^{ab}	10.78 ± 0.53$^{ab(A)}$	12.11 ± 0.62$^{b(B)}$
TBSats	19.90 ± 1.45$^{a(A)}$	18.32 ± 0.38$^{a(B)}$	18.53 ± 1.65$^{a(B)}$	19.74 ± 0.77$^{a(B)}$	22.37 ± 0.55$^{a(B)}$	22.83 ± 1.02$^{a(B)}$
Monos	18.51 ± 2.21$^{a(A)}$	18.92 ± 0.74$^{a(A)}$	20.34 ± 1.76$^{a(A)}$	17.32 ± 0.29$^{a(A)}$	15.72 ± 0.75$^{a(A)}$	17.32 ± 0.41$^{a(A)}$
Polys	9.28 ± 1.30$^{a(A)}$	8.87 ± 0.21$^{a(A)}$	10.49 ± 0.54$^{a(A)}$	9.18 ± 0.29$^{a(A)}$	6.67 ± 0.58$^{a(A)}$	6.01 ± 1.81 $^{a(B)}$
Day 28	**C**	**B**	**M**	**R**	**MS**	**R10**
NSats	39.86 ± 1.38$^{a(A)}$	44.27 ± 1.38$^{a(A)}$	46.29 ± 1.99$^{a(A)}$	46.51 ± 1.99$^{a(A)}$	43.63 ± 0.68$^{a(A)}$	40.95 ± 0.60$^{a(A)}$
MBSats	11.95 ± 1.12$^{a(A)}$	9.99 ± 0.11$^{ab(A)}$	7.33 ± 0.33$^{b(A)}$	9.84 ± 0.54$^{ab(A)}$	11.51 ± 0.43$^{a(A)}$	11.65 ± 0.36$^{a(B)}$
TBSats	14.07 ± 0.35$^{a(A)}$	15.30 ± 0.52$^{a(A)}$	13.87 ± 0.41$^{a(A)}$	13.53 ± 0.42$^{a(A)}$	14.07 ± 0.67$^{a(A)}$	13.93 ± 0.59$^{a(A)}$
Monos	21.73 ± 0.68$^{a(A)}$	19.71 ± 1.57$^{ab(A)}$	16.77 ± 0.84$^{b(A)}$	18.46 ± 1.22$^{ab(A)}$	17.56 ± 0.26$^{ab(A)}$	20.95 ± 0.63$^{ab(A)}$
Polys	8.86 ± 0.46$^{a(A)}$	7.84 ± 0.74$^{a(A)}$	11.19 ± 0.98$^{a(A)}$	8.98 ± 0.69$^{a(A)}$	9.62 ± 0.67$^{a(A)}$	9.06 ± 0.62$^{a(A)}$

Treatments: C (control); B (broccoli); M (mustard); R (oilseed radish); MS (metham sodium) and R10 (cadusafos).

ANNEXURE E

Table 8*: Average well colour development ± SEM at the 64 hour incubation time point for 0, 14 and 28 days after soil amendments. Different letters indicate significant differences (P < 0.05) (Tukey's HSD) at the same time period.*

Treatment	Day 0	Day 14	Day 28
C	0.30 ± 0.02 ^{a}	0.36 ± 0.08 ^{xy}	0.31 ± 0.04^{A}
B	0.28 ± 0.04 ^{a}	0.42 ± 0.04 ^{x}	0.37 ± 0.04 ^{B}
M	0.40 ± 0.12 ^{b}	0.44 ± 0.05 ^{x}	0.36 ± 0.04 ^{AB}
R	0.31 ± 0.04 ^{ab}	0.46 ± 0.07 ^{x}	0.34 ± 0.05 ^{AB}
MS	0.30 ± 0.03 ^{a}	0.32 ± 0.13 ^{y}	0.29 ± 0.05 ^{A}
R10	0.10 ± 0.04^{c}	0.32 ± 0.04 ^{y}	0.16 ± 0.03 ^{C}

Treatments: C (control); B (broccoli); M (mustard); R (oilseed radish); MS (metham sodium) and R10 (cadusafos).