Microbial community structure and nematode diversity in soybean-based cropping systems

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Our greatest weakness lies in giving up. The most certain way to succeed is always to try just one more time.

-Thomas A. Edison-

The true sign of intelligence is not knowledge but imagination.

-Albert Einstein-

Aim for the moon. If you miss, you may hit a star.

-William Clement Stone-

Table of Contents

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Preface.

The experimental work discussed in this dissertation for the degree *Magister Scientiae* in Environmental Sciences (M.Sc.Env) was carried out in the Unit for Environmental Sciences and Management, North-West University, Potchefstroom Campus, Potchefstroom, South Africa. This study was conducted full-time during the period of January 2013 to April 2014, under the supervision of Dr. Sarina Claassens and co-supervision of Prof. Driekie Fourie.

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

Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF.

Chantelle Jansen

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Summary.

Microbial community structure and nematode diversity in soybean-based cropping systems

Soil is an important ecosystem that supports a wide variety of organisms such as bacteria, fungi, arthropods and nematodes. This sensitive ecosystem may be influenced by various factors, including agricultural management practices. With the introduction of genetically modified (GM) glyphosate-tolerant (RoundUp ® Ready: RR) crops, herbicides such as glyphosate have been increasingly used. However, little is known about the effect of glyphosate on the biological communities in these herbicide-sprayed soils. With the intimate proximity that microorganisms and nematodes have with the roots of plants, these organisms can be used to assess changes that may occur in the soil surrounding roots of RR crops. The aim of this study was to determine microbial community structure and nematode diversity, with emphasis on that of non-parasitic nematodes, in soil samples from conventional soybean (CS) - and RR- soybean fields compared to that in adjacent natural veld (NV) areas.

Samples were collected from twenty three sites at six localities that are situated within the soybean-production areas of South Africa. These sites represented fields where RR and CS soybean grew, as well as surrounding NV. All RR fields have been treated with glyphosate for no less than five years. Microbial community structures of the twenty three sites in the RR, CS and NV ecosystems were determined by phospholipid fatty acid (PLFA) analyses. Nematode diversity was determined by extracting the nematodes from soil samples and conducting a faunal analysis. Soil physical and chemical properties were determined by an independent laboratory, Eco-Analytica (North West University, Potchefstroom) according to standard procedures.

Results from this study indicated differences in microbial community structure between the various localities. However, there were no significant ($p \leq 0.05$) differences in microbial community structures between RR- and CS ecosystems. Soils of both RR- and CS crops were primarily dominated by bacteria. Nematode identification and faunal analysis also indicated no significant ($p \leq 0.05$) differences between the different non-parasitic/beneficial nematodes that were present in soils of these two ecosystems during the time of sampling. Non-parasitic nematode communities were primarily dominated by bacterivores. A faunal analysis indicated that most of the sites contained enriched, but unstructured soil food-webs. However, four of the sites showed enriched and structured food webs due to the presence of non-parasitic nematodes with high coloniser-persister (cp) values. Relationships between

non-parasitic nematode – and microbial communities showed that there was a positive relationship between nematode functional groups and their corresponding microbial prey.

From the results obtained in this study, it can be concluded that the community structures of both non-parasitic nematodes and microorganisms shared similarities. These community structures showed no long-term detrimental effects of glyphosate application in the soils surrounding roots of RR soybean crops. Relationships existed between non-parasitic nematode and microbial communities in the rhizosphere of soybean crops and natural veld. For example, bacterivore nematodes had a strong positive relationship with gram-negative bacteria. Similar but weaker relationships also existed between carnivores, omnivores, plant-parasitic nematodes and gram-negative bacteria. A positive relationship also existed between fungivores and fungal fatty acids. This emphasises the value of these organisms as indicators of soil health and also the impact that agricultural practices can have on soils.

Keywords: Conventional soybean (CS), Faunal analysis, Genetically-modified, Glyphosate, Microbial community structure, Nematode diversity, Phospholipid fatty acid (PLFA) analyses, RoundUp ® Ready (RR) soybean.

Opsomming.

Mikrobiese gemeenskapstruktuur en nematooddiversiteit in sojaboon-gebaseerde ekosisteme

Grond is 'n belangrike ekosisteem en ondersteun 'n wye verskeidenheid van organismes soos bakterieë, swamme, geleedpotiges en nematode. Hierdie sensitiewe ekosisteem kan beïnvloed word deur verskeie faktore, insluitend landboubestuurspraktyke. Met die bekendstelling van geneties-gemodifiseerde (GM) glifosaatverdraagsame (RoundUp ® Ready: RR) gewasse, word onkruiddoders soos dié wat glifosaat as aktiewe bestanddeel bevat, toenemend gebruik. Min inligting is egter bekend rakende die effek van glifosaat op biologiese gemeenskappe in glifosaat-behandelde grond. Die noue assosiasie van mikroörganismes en nematode in die risosfeer van wortels van hierdie plante, maak hulle geskik om die moontlike veranderinge wat in die grond van RR gewasse mag plaasvind, te bepaal. Die doel van hierdie studie was om mikrobiese gemeenskapstruktuur en nematooddiversiteit, met die fokus op nie-parasitiese nematode, in grondmonsters van konvensionele (CS) - en RR- sojaboonlande te bepaal, en te vergelyk met aangrensende areas van natuurlike veld (NV).

Grondmonsters is versamel by drie-en-twintig punte wat geleë is in ses lokaliteite in die sojaboonproduksiegebiede van Suid-Afrika. Hierdie versamelpunte het RR en CS sojaboonlande asook omliggende NV ingesluit. Alle lokaliteite waar RR sojabone verbou is, is vir ten minste vyf jaar met glifosaat behandel. Mikrobiese gemeenskapstruktuur van die verskillende punte in die drie ecosisteme (RR, CS en NV) is bepaal deur fosfolipiedvetsuuranalises. Die diversiteit van die nematode is bepaal deur nematode uit grondmonsters te ekstraheer en aan ‘n fauna analise te onderwerp. Die fisiese en chemiese eienskappe van die grond is bepaal deur 'n onafhanklike laboratorium, Eco-Analytica (Noordwes-Universiteit, Potchefstroom) volgens standaard prosedures.

Resultate van hierdie studie het 'n verskil getoon in die mikrobiese gemeenskapstruktuur in gronde van die verskillende lokaliteite. Geen statisties betekenisvolle ($p \leq 0.05$) verskille was egter teenwoordig in mikrobiese gemeenskapstrukture tussen RR - en CS sojaboongrondmonsters nie. Gronde van beide RR - en CS sojaboonlande was primêr oorheers deur bakterieë. Nematoodidentifikasie en fauna analise het ook nie enige betekenisvolle ($p \leq 0.05$) verskille tussen hierdie twee ekosisteme aangedui ten opsigte van die voorkoms en diversiteit van nie-parasitiese nematode nie. Nie-parasitiese nematoodgemeenskappe is hoofsaaklik oorheers deur bakterievoeders en 'n fauna analise het aangedui dat die meeste punte ‘n verrykte maar nie-gestruktureerde voedselweb verteenwoordig het. Grond van vier van die punte het egter verrykte en gestruktureerde voedselwebbe verteenwoordig te danke aan die teenwoordigheid van nie-parasitiese

nematode met hoë cp- waardes. Verhoudings tussen nie-parasitiese nematood - en mikrobiese gemeenskappe het getoon dat 'n positiewe verwantskap tussen die funksionele nematoodgroepe en hul ooreenstemmende mikrobiese prooi bestaan het.

Resultate wat tydens hierdie studie verkry is, toon dat gemeenskapstrukture van beide nie-parasitiese nematode en mikroörganismes ooreenkomste deel. Hierdie gemeenskapstrukture het geen negatiewe langtermyn veranderinge, geassosieer met die toediening van glifosaat in die grond rondom die wortels van RR sojaboongewasse, getoon nie. Verwantskappe het bestaan tussen nie-parasitiese nematode en mikrobiese gemeenskappe in die risosfeer van sojaboongewasse en natuurlike veld. Byvoorbeeld, bakterievoededende nematode het ‘n sterk positiewe verwantskap gehad met gramnegatiewe bakterieë. Soortgelyke maar swakker verwantskappe het ook bestaan tussen karnivore, omnivore, plantparasitiese nematode en gramnegatiewe bakterieë. ‘n Positiewe verwantskap tussen fungivoedende nematode en fungi is ook gevind. Dit beklemtoon die waarde van hierdie organismes as indikatore van grondgesondheid asook die impak van landbouprаktyke.

Sleutelterme: Fauna analises, Fosfolipidvetsuur (PLFA) analise, Geneties-gemodifiseerde, Glifosaat, Konvensionele sojaboon (CS), Mikrobiese gemeenskapstruktuur, Nematooddiversiteit, RoundUp ® Ready (RR) sojaboon.

List of Abbreviations.

a.i.	Active ingredient
AMF	Arbuscular mychorrhizal fungi
AMPA	Aminomethylphosphonic acid
ANOVA	Analyses of variance
Ba	Bacterivore
Bsat	Base saturation
Ca	Carnivore
CEC	Cation exchange capacity
CI	Channel Index
CLPP	Community-level physiological profiling
C-P lyase	Carbon-Phosphorus lyase
cp-value	Coloniser-persister value
CS	Conventional soybean
EC	Electrical conductivity
EI	Enrichment Index
EPN	Entomopathogenic nematodes
EPSPS	5-enolpyruvylshikimic acid-3-phosphate synthase
F / B ratio	Fungal to bacterial ratio

FAMEs	Fatty acid methyl esters
FISH	Fluorescent *in situ* hybridisation
Fu	Fungivore
GC-MS	Gas chromatography-mass spectrometry
GM	Genetically modified
GOX	Glyphosate oxidoreductase
Gram + / total ratio	Gram–positive to total PLFA ratio
HR	Herbicide-resistant
Iso / anteiso ratio	Iso to anteiso PLFA ratio
MBsats	Mid-chain branched saturated fatty acids
MI	Maturity Index
Mole%	Mole percentage
NCR	Nematode channel ratio
Nsats	Normal saturated fatty acids
NV	Natural veld
Om	Omnivore
P	Predator
PCA	Principal Component Analysis

PCN	Potato cyst nematode
PCR	Polymerase chain reaction
PLFA	Phospholipid fatty acid
Polys	Polyunsaturated fatty acids
PPI	Plant-Parasitic Index
PPN	Plant-parasitic nematode(s)
RDA	Redundancy Analysis
rDNA	ribosomal Deoxyribonucleic acid
RNA	Ribonucleic acid
RR	RoundUp ® Ready
rRNA	ribosomal Ribonucleic acid
sat	Saturated to monounsaturated PLFA ratio
sat:unsat	Saturated to unsaturated
SEM	Standard error of mean
SI	Structure Index
TBsats	Terminally branched saturated fatty acids
Trans / *cis* ratio	*Trans* to *cis*-monoenoic unsaturated fatty acids
TSFAME	Total soil fatty acid methyl ester

Tukey's HSD	Tukey's Honest Significant Difference

List of figures.

List of tables.

Chapter 1: Introduction.

1.1. Microorganisms and non-parasitic nematodes as biological indicators of soil quality.

Soil is an important ecosystem that supports a wide variety of organisms such as bacteria, fungi, algae, arthropods and nematodes (Neher, 2001). These organisms play important roles in the soil ecosystem in a variety of processes including energy flow and nutrient transfer (Altieri, 1999). According to Sharma *et al.* (2011), soil quality has been defined as "the capacity of soil to function within ecosystem boundaries to sustain plant-animal productivity, maintain or enhance water and air quality, and support human health and habitation". Recently, healthy soil has been described as a "stable soil system with high levels of biological diversity and activity, internal nutrient cycling and resilience to disturbance" (Sharma *et al.,* 2011). Therefore microorganisms and nematodes, non-parasitic nematodes in particular, can be used to assess changes that take place in the soil environment.

Soil quality can be influenced by various factors including chemical contamination and agricultural practices/management (Sochová *et al.,* 2006). With the introduction of genetically modified (GM) glyphosate-tolerant (RoundUp ® Ready: RR) crops, herbicides such as glyphosate has been increasingly used (Bonini *et al*., 2009; Hart *et al*., 2009; Zobiole *et al*., 2010a). Herbicide use is one of the factors that can influence soil quality and for this reason it is important to determine the effects of this factor on soil quality. This can be done by using biological indicators of soil quality such as non-parasitic nematodes and other microorganisms (Sochová *et al.,* 2006). Non-parasitic nematodes and other microorganisms are well suited to evaluate changes in the soil environment as they respond rapidly to environmental changes and play crucial roles in ecological processes (Doran and Zeiss, 2000).

Microorganisms are used as biological indicators of soil quality due to the relationships between ecosystem sustainability, microbial diversity, and soil and plant quality (Anderson, 2003; Drenovsky *et al*., 2004; Hill *et al*., 2000). Microbial community structure is commonly used as an indicator of soil quality and possible contamination because any environmental changes may lead to changes in microbial community structure (Neher, 2001). The latter may be affected by agricultural practices, including herbicide use (Frostegård *et al*., 1996; Hill *et al*., 2000) and have been evaluated in several studies using phospholipid fatty acid (PLFA) analyses (Drenovsky *et al.,* 2004; Frostegård *et al.,* 1996; Hill *et al.,* 2000).

Therefore, in this study, PLFA analyses were used to determine the effect of glyphosate use in RR soybean crops on soil microbial community structures.

Nematodes are an abundant and diverse group of organisms found in the soil environment and can be influenced by several factors, including agricultural practices such as soil fumigation and herbicide use (Ferris *et al.,* 2012). For these reasons, non-parasitic nematodes are frequently used as biological indicators of soil quality. Nematode communities are usually large and they are sensitive to environmental changes (Sánchez-Moreno *et al.,* 2006; Yeates, 2003). Moreover, nematode communities are divided into functional groups based on their feeding strategies and include plant feeders (parasites), bacterial feeders (bacterivores); fungal feeders (fungivores), substrate ingesters, animal predators, unicellular eukaryote feeders, omnivores, and infective stages of animal parasites (Bongers and Bongers, 1998; Neher, 2001; Sochová *et al.,* 2006). Each functional group demonstrates varying sensitivity to environmental changes. Nematode diversity, with the emphasis on non-parasitic nematodes, and community structure were thus also investigated in this study to determine the effect of glyphosate application in soybean crops.

1.2. Problem statement.

Currently RR soybean cultivars dominate the local soybean market (De Beer, 2013). Since soybean is an important protein source for human and animal use, the use of RR cultivars has increased over time (Zobiole *et al*., 2010a). Although there are some advantages to growing RR crops, the intensive cultivation of these crops and extensive use of glyphosate could lead to detrimental ecological effects such as alterations of soil microbial communities (Liphadzi *et al*., 2005) and ultimately declining soil quality. However, little is known about the effect of glyphosate in terms of its effect on nematode diversity and microbial community structure in soybean production areas. Limited and fragmented research has also been done in this regard in other world countries including studies done by the following authors: Barriuso and Mellado (2012) in Spain; Dewar *et al.* (2000) in the United Kingdom (UK); and Johal and Huber (2009); Kremer and Means (2009); Lane *et al*. (2012); Liphadzi *et al.* (2005); Ratcliff *et al.* (2006) in the United States of America (USA).

Glyphosate absorbed in the leaves of RR plants, can alter root exudation by inhibiting the 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSPS) enzyme which is important in the shikimate pathway in plants (Barriuso and Mellado, 2012). For example, glyphosate inhibits the Through the inhibition of this enzyme, the production of antifungal compounds such as phytoalexins that is usually produced by plants through the shikimate pathway, is shut down and can lead to increased infection by pathogenic fungal species (Kremer *et al.,* 2005). For these reasons, changes to root exudation may lead to changes in the microbial community

structure associated with the rhizosphere of such plants (Dick *et al.,* 2010). It has been well documented that soil-applied herbicides can alter populations of certain microbes (Liphadzi *et al.,* 2005). Some research has suggested that glyphosate is toxic to some bacteria and fungi (Hart *et al*., 2009) and may lead to changes in the environment, altering the soil nematode community structure (Liphadzi *et al*., 2005). This indicates that long-term and extensive use of glyphosate could lead to changes in soil microbial communities (Dick *et al*., 2010).

Research on the short and long term effects of glyphosate on soils in which soybean and other GM crops are planted is inconclusive (Dick *et al*., 2010). Some studies have suggested that in the short term, some microbial communities are robust to changes in their environment that might have been caused by glyphosate (Hart *et al*., 2009).

Due to the importance of soil quality in agriculture, determining the effect of glyphosate on soil organisms may lead to a better understanding of its long term use on soil quality. In this study, soil microorganisms and non-parasitic nematodes were used as biological indicators to determine whether glyphosate has an impact on soil quality. Microbial community structure and non-parasitic nematode structure and diversity were determined in RR and conventional soybean (CS) fields as well as in surrounding natural veld (NV) areas. The RR soybean fields used in this study have been treated with glyphosate for no less than five years, therefore the long-term effect of glyphosate was evaluated. Microbial community structures were investigated through PLFA, while non-parasitic nematode community structures were determined by identification of different trophic groups present in soil samples from each sampling area and subjected to Faunal Analysis (Ferris *et al*., 2001)

1.3. Aim and objectives.

The aim of this study was to assess whether glyphosate application had an effect on the non-parasitic nematode diversity and microbial community structures in soils where RR and CS soybeans were cultivated over the long-term, as well as in those of adjacent NV during the 2013 season. The specific objectives included:

- Determining the microbial community structure in soils that were sampled from the two soybean and NV ecosystems indicated above, using PLFA analysis;
- Characterising the nematode diversity and population densities, both plant-parasitic and non-parasitic, in soils that were sampled from the two soybean and NV ecosystems indicated above. The emphasis of this study is, however, on non-parasitic nematodes.

- Determining relationships between microbial and non-parasitic nematode community structures in the soils of the three ecosystems mentioned above.

1.4. Outline of the dissertation.

Chapter 1: Provides an introduction to the study and includes the problem statement, aim, specific objectives and a complete outline of the dissertation. The rationale for this study is also discussed in this chapter.

Chapter 2: Contains the overall literature review of the study. This includes the use of glyphosate in RR cultivars, an overview of previous studies done on the impact of glyphosate, and the importance of microbial communities and nematode diversity in soil ecosystems.

Chapter 3: Describes the experimental layout and methods used in this study. Sampling methods are described as well as PLFA analyses, nematode extraction and identification, and analyses of soil physical-chemical properties. Statistical analyses are also discussed.

Chapter 4: Contains the results obtained in terms of microbial community structure in two different soybean-based ecosystems together with that in areas where natural veld grew adjacent to the soybean crops. A general discussion of the microbial community structure in terms of glyphosate application to RR cultivars is also elaborated on.

Chapter 5: Contains the results obtained from nematode extraction and identification, including a discussion that compares the two different soybean-based ecosystems as well as that of the natural veld areas in terms of nematode diversity.

Chapter 6: The final chapter of the dissertation includes conclusions and recommendations with regard to further investigations.

References: Contains a complete list of all the references used in this dissertation.

Chapter 2: Literature Review.

2.1. The importance of soil quality and biological indicators of soil health.

Soil quality is defined as “the capacity of soil to function as a vital living system, within ecosystem and land-use boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and promote plant and animal health” (Doran and Zeiss, 2000; Sharma *et al.,* 2011). Soil is an important component of terrestrial ecosystems, including agricultural systems, due to its important role in fertility; decomposition processes; and nutrient and energy flows (Sochová *et al.,* 2006). Soil ecosystems also support a diversity of algae, arthropods, bacteria, fungi, nematodes and protozoa (Neher, 2001). Soil is a living, dynamic entity of which the functions are facilitated by various living organisms and requires proper management to sustain soil quality (Doran and Zeiss, 2000). However, worldwide, soil quality has decreased extensively due to chemical contamination, intensive agricultural management, erosion, and contaminated air and water (Sochová *et al.,* 2006). For this reason, the evaluation of soil quality has become increasingly important.

Biological indicators are appropriate for the evaluation of soil quality due to their ability to reflect the current status of vital ecological processes in soil and changes in these processes through time (Neher, 2001). A variety of soil organisms are used as indicators of soil quality since they meet the following criteria (Doran and Zeiss, 2000; Neher, 2001; Schloter *et al.,* 2003):

1. Indicators must be sensitive to environmental changes such as management practices and climate changes.
2. These indicators must be well correlated with soil and ecosystem function.
3. Indicators should also be able to reveal ecosystem processes, meaning the indicators should reflect the structure and function of ecological processes.
4. Indicators should be easy to understand and useful without needing extensive and/or specialised training of investigators.
5. Indicators should also be inexpensive and easy to use.

Based on these criteria, microorganisms and nematodes (in this case non-parasitic nematodes) are well-suited biological indicators of soil quality. Several investigations have used these organisms to illustrate links between environmental changes and quality (cause and effect) (Bending *et al.,* 2004; Bongers and Bongers, 1998; Doran and Zeiss, 2000; Sharma *et al.,* 2011).

2.2. Glyphosate.

Herbicides are used because they provide cost-effective weed control and increase the yield of economically important crops. Before the introduction of herbicide-resistant crops, broad-spectrum herbicides, such as glyphosinate and glyphosate, had limited potential because they caused injury to a variety of crops (Reddy, 2001). Crop injuries caused by these herbicides includes stunted growth, necrosis and bronzing or burning of leaves and reddening of leaf veins (Carpenter and Gianessi, 1999). Although these symptoms do not always result in yield reduction, they do in some cases delay canopy closure and increase weed competition with the crop. Subsequently, herbicide application rates were kept low in the past to reduce possible crop injury. Due to this limitation, weed control was only effective when weed infestations were low. The potential for a rotational crop to suffer damage from carry-over herbicide residues depends on soil type and environmental conditions (Carpenter and Gianessi, 1999). For these reasons some crops have been engineered to exhibit resistance to non-selective herbicides such as glyphosate. A genetically modified (GM) crop with tolerance to herbicides is a more economically viable option in agricultural industries in comparison to the high costs associated with developing new herbicides (Reddy, 2001).

Glyphosate [active ingredient (a.i): 2-(phosphonomethyl) glycine] also known as RoundUp ®, is a non-selective, broad-spectrum herbicide, commonly used for the control of annual and perennial weeds (Barriuso and Mellado, 2012; Ellis and Griffin, 2002; Kremer *et al*., 2005; Zobiole *et al.,* 2010b). Glyphosate is used in pre-plant, post-directed, spot, and pre- and post-harvest applications. The efficacy of glyphosate depends on the weed species, growth stage of the weed and environmental conditions after application. Manufacturers of glyphosate recommend that the uses of soil-applied, residual herbicides are eliminated and that glyphosate should be used only in glyphosate-tolerant crops crops (Ellis and Griffin, 2002).

The shikimate pathway is one of the most important pathways in higher plants and has been used as a target for herbicidal agents (Bonini *et al.,* 2009). Glyphosate inhibits an enzyme in the shikimate pathway, 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSPS), necessary for the biosynthesis of aromatic amino acids (phenylalanine, tyrosine, and tryptophan) in plants, some bacteria and fungi (Dill *et al.,* 2010; Hart *et al.,* 2009; Zobiole *et al*., 2010b). This leads to the inhibition of protein production and prevents the formation of secondary products (Dill *et al.,* 2010; Reddy, 2001). It also leads to the accumulation of shikimic acid (Kremer *et al*., 2005) and other hydrooxybenzoic acids in glyphosate-sensitive plants and bacteria such as *Bradyrhizobium japonicum* (Zabaloy *et al.,* 2012). In glyphosate-tolerant crops, glyphosate is systemic within the plant and only small amounts of glyphosate

binds to the EPSPS enzyme. The remaining glyphosate is readily translocated into metabolic sinks, such as seeds, nodules and roots, from where it is eventually released into the rhizophere (Reddy, 2001; Kremer *et al*., 2005; Zobiole *et al.,* 2010b). Glyphosate use in these crops may however cause negative side-effects on non-target soil biota, including microorganisms and nematodes (Zabaloy *et al.,* 2012).

Before the introduction of weed control through herbicides, traditional practices such as mechanical and cultural control methods were used (Carpenter and Gianessi, 1999). However, since its introduction, the adoption of RoundUp ® Ready (RR) soybean varieties has increased over the years, replacing tillage and cultivation practices (Carpenter and Gianessi, 1999). Herbicide-resistance is a common modification to maize (*Zea mays* (L.)), soybean (*Glycine max* (L.) Merr.), cotton (*Gossypium hirsutum* (L.)), sugar beet (*Beta vulgaris* (L.)) and canola (*Brassica napus* (L.)) (Barriuso and Mellado, 2012; Duke, 2011; Hart *et al.,* 2009). It has also become the most dominant trait in these crops since 1996 (Liphadzi *et al.,* 2005) with RR soybean being the most dominant among all transgenic crops grown commercially (Reddy, 2001).

In 1996, RR varieties of several crops were made available allowing the use of RoundUp ®, with glyphosate as an a.i., as a post-emergence herbicide without resulting in any crop injury (Carpenter and Gianessi, 1999). The first generation of RR cultivars were developed by insertion of EPSPS coding sequence derived from *Agrobacterium* spp. strain CP4. Improvements in trait selection and biotechnology lead to the development of second generation RR cultivars that promoted higher yields (Zobiole *et al.,* 2010a). Such cultivars are genetically modified to produce glyphosate-tolerant EPSPS (Bonini *et al.,* 2009; Hart *et al.,* 2009; Zobiole *et al.,* 2010a), which has a high catalytic activity in the presence of glyphosate and enables the crops to survive and remain unaffected by the herbicide (Bonini *et al.,* 2009; Reddy, 2001). RR crops represent more than 80% of the approximately 120 million ha of transgenic crops grown in the world annually (Duke and Powles, 2009). These GM crops are popular among soybean farmers as an additional weed management tool, reducing pre-emergence herbicide use and tillage (Kremer *et al*., 2005).

There are a few main reasons for the rapid adaption of RR crops:

1) Cost-savings: The use of RR crops allows farmers to use glyphosate alone to control weeds, thus reducing costs of weed management. The use of these cultivars reduces soil tillage costs substantially (Cerdeira *et al*., 2011; Duke and Powles, 2009; Ellis and Griffin, 2002).

2) Better weed management: Because glyphosate is a non-selective herbicide it can be used on a wide range of weeds with one or two appropriately-timed applications. Farmers find the profound efficacy of the RR crop / glyphosate combination very attractive (Duke and Powles, 2009).
3) Simplicity and flexibility: The RR crops / glyphosate combination is used to control virtually all weed species, eliminating the need for consultants to provide herbicide combinations, selectivity and weed spectrum. This is especially attractive to small-scale farmers and is one of the most important reasons for the adoption of RoundUp ® technology (Duke, 2011; Ellis and Griffin, 2002; Liphadzi *et al.,* 2005).
4) Most weed control programs in conventional cropping systems rely on pre-emergence herbicides applied at high rates, followed by post-emergence herbicide applications where needed (Carpenter and Gianessi, 1999; Liphadzi *et al.,* 2005). However, in RR cropping systems, weeds are allowed to grow before glyphosate application. These weeds might help increase the diversity of arthropods, soil microbes, earthworms, and nematodes. Weed residues in the soils thus not only modify soil temperature, moisture, and organic matter, but it also provides an additional substrate for microbial communities. The latter subsequently result in optimum conditions for the development of soil microbial populations (Liphadzi *et al.,* 2005).

Although there is controversy about the deleterious effects of increased glyphosate use on the environment, the benefits are said to outweigh the negatives. However, the potential benefits of RR crops depend on the crop type, geographic location, and the manner in which farmers use them (Duke, 2011). Glyphosate is reported to be the least toxic pesticide used in agriculture (Cerdeira *et al*., 2011; Duke and Powles, 2009) and therefore RR crops allow use of a more environmentally friendly herbicide at lower costs (Duke, 2011; Ellis and Griffin, 2002; Liphadzi *et al.,* 2005).

In terms of surface- and groundwater contamination, glyphosate is superior to most herbicides it has replaced. Since glyphosate has a strong sorption to soil minerals and degrades rapidly in most soils, it does not move rapidly in soil (Duke and Powles, 2009). The application of glyphosate technology and RR crops also reduce and sometimes completely eliminate soil tillage, which is one of the methods most harmful to soil fertility and conservation (Carpenter and Gianessi, 1999; Duke and Powles, 2009). There are many benefits to using RR crops in combination with glyphosate for weed management; however the misuse of this management method is risking this safe, highly effective and economical tool and may have detrimental ecological effects.

2.3. Effect of glyphosate use in RR crops on soil biological communities.

2.3.1. Soil microbial communities.

Agricultural management practices, such as herbicide use, can significantly alter soil characteristics, including the biological processes it supports (Barriuso and Mellado, 2012; Kremer and Means, 2009). Microbial communities play important roles in soil ecosystems and it is therefore important to exploit and determine the effect of broad-spectrum herbicides such as glyphosate on these organisms.

Glyphosate is effective since the compound remains intact once it has been absorbed by the plant with little degradation being reported. Glyphosate is then systemically transported to metabolically active sites throughout the plant including seeds, nodules, and roots following excretion into the rhizosphere (Kremer *et al*., 2005; Kremer and Means, 2009). This is likely to take place through diffusion along with sugars, amino acids, and other low molecular weight compounds (Kremer *et al*., 2005). The repeated use of glyphosate in RR crops may lead to changes in the soil environment that can be described in two ways: first the influx of carbon, phosphorus and nitrogen in the form of glyphosate can lead to changes in the soil environment. In the second place, the changes might be the result of the introduction of more vegetative material as a result of post-emergent treatment by glyphosate (Hart *et al*., 2009).

Short-term studies on glyphosate showed that at recommended application rates, glyphosate penetrates the upper 2 mm of the soil surface. At these rates, microbial community structure was unaffected and even at higher rates of application there was no evidence that glyphosate affected microbial community structure (Dick *et al*., 2010). Differences in soils with single glyphosate application to RR crops compared to soils of RR crops with no glyphosate application were evaluated to determine the effects on microbial communities. The soils in which no glyphosate was applied showed no shifts in microbial community structure whereas the single-application soils showed that some microorganisms were stimulated by glyphosate application (Dick *et al*., 2010). Another study also found that application of glyphosate in RR maize did not affect soil respiration and the community structure of soil bacteria (Liphadzi *et al.* 2005). This was confirmed by various studies that also suggested that glyphosate does not significantly change soil microbial communities in terms of their structure, function or activity even at application of up to three-fold the recommended rates (Hart *et al.,* 2009; Lupwayi *et al.,* 2009). Dick *et al*. (2010), however, reported that certain groups of bacteria only responded to glyphosate when the soil had never been treated with glyphosate. Conversely, soils that have been treated with

glyphosate over a longer period showed no changes in microbial community structure. These results suggest that microbial communities adapt to glyphosate application over a longer period.

On the other hand, some studies have shown that glyphosate is toxic to some bacteria and fungi and that it could adversely affect soil microbial community structure (Barriuso *et al.,* 2010; Hart *et al.,* 2009; Zobiole *et al.,* 2010a). These changes in microbial community structure may lead to other deleterious effects such as the stimulation of plant diseases and plant nutrient deficiencies (Dick *et al*., 2010). Lupwayi *et al.* (2009) studied the potential shifts in soil microbial community structure, diversity and biomass in response to the application of glyphosate and 2,4-D-amine application to RR canola. Combined use of the two herbicides resulted in shifts in the functional structure of the soil microbial community that was different from those observed when the herbicides were applied alone. These shifts in microbial communities can lead to successions that could have long-term effects on soil food webs and biological processes (Lupwayi *et al.,* 2009). However, this will depend on whether these effects are transient or long-lasting. It has been suggested that glyphosate has a benign effect on microbial community structure when applied at recommended rates, however, it resulted in a non-specific, short-term stimulation of bacteria at high concentrations (Ratcliff *et al.,* 2006).

Rhizosphere microbes might be particularly sensitive to glyphosate due to their close proximity to the roots of the RR crops (Hart *et al.,* 2009). The effect of glyphosate on the rhizobacterial communities of RR maize showed that *Actinobacteria* was affected by the herbicide and some *Actinobacteria* taxa appeared almost exclusively in untreated soils. It was also reported that in untreated soil, *Proteobacteria* and *Actinobacteria* were the most abundant rhizobacteria and that the presence of *Actinobacteria* declined in herbicide-treated soils. However, it was suggested that upon natural inactivation of the herbicide, the decrease in population levels of some bacterial species may recover (Barriuso *et al.,* 2010). Although rhizobacterial communities, occurring in soils where RR maize were grown, were affected by glyphosate over the short-term, the microbial community manifested a near recovery at the final stage of plant growth.

Glyphosate has been shown to affect various microbial species as well as root exudation and nutrient deficiencies in soils. These effects are summarised in Table 2.1.

Table 2.1: Microbial communities and other soil properties shown to be affected by glyphosate application.

Case study	Findings	Reference
Effect on arbuscular mycorrhizal fungi (AMF).	Glyphosate reduced spore viability and ability to colonise roots of ryegrass, *Lolium multiforum* Lam. Inhibition of 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSPS) may be partially responsible for reduction in spore viability. Glyphosate has an indirect effect on AMF through alterations in the flow of carbohydrates as a result of stress in the host-plant.	Druille *et al.* (2013).
Effect on symbiotic nitrogen (N_2) fixation and nickel concentration in glyphosate-tolerant soybeans.	The soybean N_2-fixing symbiont, *Bradyrhizobium japonicum*, possesses glyphosate-sensitive EPSPS and accumulates shikimic, hydroxybenzoic and proto-catechuic acids when exposed to glyphosate. Glyphosate reduced the growth of *B. japonicum* in glyphosate-amended media and had negative effects on the formation of N_2-fixing nodules on crop roots in field experiments.	Zobiole *et al.* (2010b); Powell *et al.* (2009).
Changes in root exudation and microbial communities in RoundUp ® Ready (RR) soybean cultivars.	Glyphosate was released from roots of actively-growing RR soybeans, corresponding with exudation of high concentrations of soluble carbohydrates and amino acids. This may increase the proportion of rhizosphere fungi to such an extent where they may displace nearly all bacteria.	Kremer *et al*. (2005).
Stimulation of microbial respiration.	High rate applications to RR crops shown to stimulate microbial respiration due to the ability of some microorganisms to metabolise glyphosate. Glyphosate itself is more likely to affect microbes than the RR crop. Changes could be a result of microbes responding to increased food sources, or by microbes that are out-competed by organisms that can use the additional food sources.	Ratcliff *et al.* (2006); Barriuso and Mellado, (2012); Cerdeira *et al*. (2011); Hart *et al.* (2009).

Relationship of glyphosate to nutrient deficiencies.	Potassium (K) deficiency: Negative correlations between microbial biomass K and K plant uptake which suggested that glyphosate caused microbial immobilisation of K. This may be related to stimulation of fungi because fungi can rapidly take up K. Manganese (Mn) deficiency: The possible toxic microbial effect of glyphosate is linked to Mn deficiency. Glyphosate can lead to an increase in Mn oxidizers, a decrease in Mn reducers and reduce the ratio of Mn-reducing and Mn oxidizing bacteria. Nickel (Ni) deficiency: Ni is directly related to nitrogen fixation because N_2-fixing microorganisms require Ni for hydrogen uptake. Glyphosate is a chelator of metallic cations and could affect the availability of Ni. This could explain the effect of glyphosate on N_2-fixing bacteria. Glyphosate reduced Ni concentrations.	Dick *et al*. (2010); Johal and Huber, (2009); Zobiole *et al.* (2010a); Zobiole *et al.* (2010b).

Glyphosate degradation by soil microorganisms.

Although it is suggested that glyphosate is tightly bound and inactive in soil, various studies have shown that glyphosate is available to soil microorganisms as a substrate for direct metabolism. The latter leads to increased microbial biomass and activity (Kremer and Means, 2009). Soil microbes are considered to be the only organisms that significantly degrade glyphosate (Duke, 2011). Glyphosate is immobilised once applied to soil and is rapidly degraded by soil microorganisms (Liu *et al.,* 1991). For this reason, glyphosate has a relatively short half-life, ranging from a few days to months. A wide variety of soil microbes degrade glyphosate, including actinomycetes, bacteria, and fungi (Duke, 2011). Although it is a well-documented fact that microbes degrade glyphosate, surprisingly few glyphosate-degrading bacterial strains have been isolated (Liu *et al.,* 1991; Zabaloy *et al.,* 2012.). Only a few bacterial strains have been identified that utilise glyphosate as their sole source of phosphorus for growth. Glyphosate utilisation ability is widespread in the family

Rhizobiaceae. This group of organisms use the Carbon-Phosphorus (C-P) lyase activity for glyphosate degradation (Liu *et al.,* 1991).

There are two major pathways of glyphosate degradation found in soil. The first results in the formation of sarcosine and inorganic phosphate via a C-P lyase enzyme. Examples of soil microbes that have the C-P lyase include *Pseudomonas* spp., *Rhizobium* spp., and *Streptomyces* spp. Another way the C-P bond of glyphosate can be broken, is non-enzymatically in the presence of manganese oxide. However, the latter does not represent a large share of glyphosate degradation in soil. The second pathway of glyphosate degradation is by the glyphosate oxidoreductase (GOX) pathway which results in the formation of aminomethylphosphonic acid (AMPA) and glyoxylate. AMPA is the main metabolic product found in soil. Examples of soil microbes with GOX include *Arthrobacter atrocyaneus* and *Pseudomonas* spp. (Duke, 2011). Mineralisation of glyphosate to CO_2 was studied in different agricultural soils and results showed that the mineralisation correlated with *Pseudomonas* spp. population levels in the soils (Kremer and Means, 2009). This suggests that this bacterium plays an important role in controlling the fate of glyphosate in soil.

Microorganisms and nematodes are present within the same habitat and it is thus important to investigate possible links that exist between these two soil-inhabiting microorganism groups. The soil food web is, however, extremely complex and poorly understood (Scheu *et al.,* 2005). Interactions between microorganisms and soil invertebrates may be either direct or indirect. Direct relationships include predator-prey interactions and indirect relationships are represented by competition for resources and habitat formation. There are two major compartments in the soil food web related to nematodes. The first is nematodes that prey on bacteria as primary consumers and contribute to the bacterial energy channel. The second consist of fungi, nematode fungivores and other associated predators that contribute to the fungal energy channel (Ferris *et al.,* 2001; Scheu *et al.,* 2005).

As previously discussed, various factors can influence microbial communities and because of their close proximity to nematodes in the same area, it can be assumed that nematode communities may be affected by the same factors (Briar *et al*, 2007). Organic amendments are known to have different effects on soil properties and microbial communities. These organic amendments increase the availability of nutrients, microbial biomass and the abundance to certain nematode trophic groups such as bacterivores and fungivores (Briar *et al*, 2007). With an increase in organic matter there is an increase in microbial biomass in the amended soil. Some studies have suggested that concurrently with increased microbial biomass, an increase in nematode bacterivore populations also occurs. Further evidence of

the relationship between nematodes and microorganisms were also observed in a study done by Briar *et al.* (2007), where both non-parasitic nematodes and microbial biomass decreased in a dry season but then increased, followed by an increase in nematode bacterivores. However, the increase in nematode bacterivore numbers only occurred months after the increase in microbial biomass. This may indicate that non-parasitic/beneficial nematode communities respond to that of microbes.

In contrast, Yeates *et al.* (1999) reported that an increase in microbial biomass corresponded with an increase in predacious and not bacterivorous nematodes. This could have been because the predacious nematodes were regulating other non-parasitic nematode populations. Because of this variation in various studies, it is important to link the presence and variety of nematodes that are present in soils with those of microorganisms in current and future research.

2.3.2. Nematode diversity and community structure.

Although synthetic fertilisers, pesticides and herbicides are important inputs in conventional agricultural systems, the use thereof have been shown to negatively affect diversity and abundance of nematode trophic groups. A negative correlation between non-parasitic and plant-parasitic nematodes has generally been observed in agricultural soils. This may be a result of agricultural practices such as tillage, limited application of crop rotation and use of pesticides. Therefore, the goal of sustainable agriculture should be to reduce the use of practises that reduce non-parasitic, beneficial nematode populations (Briar *et al.,* 2007).

Dewar *et al.* (2000) did a study to determine the efficacy of glyphosate against volunteer potatoes in glyphosate-tolerant sugar beet where it represents an economically important weed in such rotation systems. Results showed that the use of glyphosate in this crop reduced the number of eggs and cysts of potato cyst nematodes (PCN) (*Globodera rostochiensis* and *G. pallida*). However, these reductions were only demonstrated in soils with low to moderate infestations of the latter nematode pests. At higher PCN infestation levels the herbicide had no significant effect on these nematode populations. In addition glyphosate application also reduced the number and size of daughter potato tubers produced. Although results of this study suggested that application of glyphosate to control volunteer potatoes helps prevent the build-up of PCN, it resulted in a reduction of daughter tubers that survived to act as a weed in the follow-up rotation crop. The use of glyphosate could, however, ultimately reduce PCN populations to such an extent that there would be a reduction in the use of nematicides or fumigants currently used to control such nematode pests (Dewar *et al.,* 2000).

Conversely, other studies on the effect of glyphosate in RR crops (maize and soybean) on nematode communities showed that the total nematode density was not altered by application of RoundUp ® (Liphadzi *et al.,* 2005). However, it was suggested that the effect of herbicides on nematode densities differs, depending on the herbicide used and the cropping system. Results of the latter indicated that nematode trophic group responses to glyphosate application did not differ between early and late glyphosate applications or between sampling dates (Liphadzi *et al.,* 2005). Also found was that bacterivorous nematodes were the most prevalent after glyphosate application followed by their fungivorous counterparts. However, when compared to the non-treated control, glyphosate had no significant effect on total nematode density or relative prevalence of their trophic groups. The lack of any observed increase or decrease in total nematode or trophic group densities over the three-year study indicated that the soil ecosystem maintained a state of balance and that the soil health was not affected by glyphosate use. Liphadzi *et al.* (2005) thus concluded that because soil microorganisms and nematodes respond rapidly to environmental change, any effect of glyphosate (negative or positive) would have been observed during the duration of the study. Moreover, after the three years of continuous glyphosate application the abundance of soil microorganisms was also not altered.

On the other hand, evidence exists that the use of certain herbicides can adversely affect entomopathogenic nematodes (EPNs). These nematodes are important due to their role in the biological control of economically important insect pests. García-del-Pino and Morton (2010) proposed that herbicides applied to crops individually do not affect these nematodes. However, many herbicides are tank-mixed and application of such mixtures could increase the toxicity of herbicides to EPNs. Studies done on the effect of glyphosate and 2-methyl-4-chlorophenoxyacetic acid (MCPA®) on the survival and infectivity of the EPN species *Steinernema feltiae* and *Heterorhabditis bacteriophora* reported that the individual herbicides had no significant effect on these nematodes. However, when used in combination, both EPN species' infectivity of their insect hosts, the wax moth *Galleria mellonella* (L.), was negatively affected by the herbicides. It was further reported that *H. bacteriophora* was more sensitive to the herbicides than *S. feltiae* (García-del-Pino and Morton, 2010).

2.3.3. Effect of glyphosate on plant pathogens.

The inhibition of the EPSPS enzyme was initially considered to be the only target of glyphosate in plants (Johal and Huber, 2009). However, some characteristics of glyphosate injury to plants suggested that the herbicidal action of glyphosate was more than initially assumed (Johal and Huber, 2009). Glyphosate has been implicated in the stimulation of plant diseases, the immobilisation of micronutrients and increased excretion of substrates

from roots that may be selectively metabolised by pathogens (Dick *et al*., 2010; Johal and Huber, 2009; Kremer and Means, 2009; Zobiole *et al.,* 2010a).

Various studies have shown that the incidence of harmful microbes, such as *Fusarium* spp. and *Phytophthora* spp., on soybean was increased by application of glyphosate to glyphosate-tolerant soybean (Cerdeira *et al*., 2011; Liphadzi *et al.,* 2005). Such increased root infections by the latter pathoghens may be due to the complete inhibition of antifungal compounds known as phytoalexins which are synthesised by the plant through the shikimate pathway (Johal and Huber, 2009; Kremer *et al*., 2005; Zobiole *et al.,* 2010a). Lignification of cell walls around pathogen infection sites also depend on compounds derived from the shikimate pathway and ensure isolation of the pathogen at the infection site (Zobiole *et al.,* 2010a). Lignification may be reduced as a result of reduced photosynthesis caused by glyphosate (Zobiole *et al.,* 2010a). These changes in plant defence allow increased root colonisation by plant pathogens such as *Fusarium* spp. (Zobiole *et al.,* 2010a).

The following findings suggest that glyphosate somehow compromises the ability of plants to defend themselves against pathogens in soybeans:

- The herbicidal efficacy of glyphosate is largely due to colonisation of roots of affected plants by soil-borne pathogens (Johal and Huber, 2009).
- One of the most important pathogens in this regard is *Fusarium*, which is abundant in agricultural soils (Cerdeira *et al*., 2011; Johal and Huber, 2009).
- Some studies showed that plants grown in sterile medium do not die after glyphosate application, although their growth is stunted. After the inoculation of *Fusarium* to these sterile media, glyphosate herbicidal effect is restored and the plants die (Johal and Huber, 2009).
- *Fusarium* spp. start to colonise roots within a few days after glyphosate application (Cerdeira *et al*., 2011; Johal and Huber, 2009).

One of the suggested mechanisms whereby glyphosate cause plants to be more susceptible to disease, is that plants rely on multiple components of defence to prevent disease (Johal and Huber, 2009). Many of these components are derived from the phenylpropanoid pathway, which acquires its precursors from the shikimate acid pathway (Johal and Huber, 2009). Given this reliance on the shikimate pathway and the fact that glyphosate inhibits this pathway, it is suggested that this herbicide renders plants more susceptible to disease (Johal and Huber, 2009).

Fusarium spp. can cause serious disease such as *Fusarium* head blight, resulting in significant yield reduction in certain crops. Because glyphosate inhibits the biosynthesis of

aromatic amino acids, it has been suggested that this may result in the plant being more susceptible to plant pathogens or other stresses (Cerdeira *et al*., 2011). However, the effect of glyphosate on plant pathogens in RR soybeans is variable, sometimes reducing and other times increasing disease. In recent studies, glyphosate was reported to have both preventative and curative properties on rust disease in RR wheat and soybean (Cerdeira *et al*., 2011). Some studies have shown that glyphosate has made some crops susceptible to normally non-pathogenic isolates of *Fusarium* and that their populations increased after glyphosate application. Also, cotton growers have reported increased incidences of *Fusarium* wilt since the introduction of RR cotton with a subsequent decrease in wilt resistance after glyphosate application (Johal and Huber, 2009).

Some scientists thus reported that the extensive use of glyphosate has increased the severity of diseases that was once effectively managed (Johal and Huber, 2009). The presence of glyphosate in association with RR crops also reduced Mn uptake and physiological efficiency. This has resulted in the gradual recognition of increased disease severity (Johal and Huber, 2009). Listed below are some diseases increased by glyphosate use in RR crops:

- *Corynespora* root rot of soybean: The prevalence of this fungal disease has increased in RR soybean since glyphosate application, suggesting that the glyphosate increased the severity of the disease. This fungal root rot is more severe when glyphosate is applied to soybeans under weedy conditions, with the weeds serving to translocate and release more glyphosate into the rhizosphere environment. This increase in glyphosate results in a reduction in Mn-reducing and an increase in Mn-oxidising organisms. These changes lead to changes in the soil biology, limiting Mn availability for plant uptake and host-plant defence reactions against disease (Johal and Huber, 2009).
- Take-all disease of cereal crops (caused by the fungus, *Gaeumannomyces graminis* var. *tritici* (Ggt)): an increased incidence of take-all disease in cereal crops is another example of the interaction of micronutrients affected by glyphosate application. It was also recorded that this disease is increased after the application of glyphosate to RR soybeans. Low levels of residual glyphosate in soil also reduce root uptake and translocation of iron (Fe), copper (Cu) and Mn. Increased take-all disease, following glyphosate applications may also be the result of reduced resistance from induced Mn deficiency, inhibited root growth from glyphosate accumulation in root tips or modified virulence of the pathogen (Johal and Huber, 2009).
- Diseases caused by the endophytic bacterium *Xylella fastidiosa:* this bacterium colonises xylem tissues and restricts nutrient translocation when plants are stressed.

These diseases are also known as “emerging” or “re-emerging” diseases as glyphosate weed management programs for their respective crops have intensified. These diseases are characterised by a loss of vigour, slow decline, micronutrient deficiency, and reduced productivity of infected crops it infects (Johal and Huber, 2009).

2.4. The role of soil microorganisms in soil quality.

The rhizosphere is a habitat that consists of diverse microbial communities responsible for a variety of important processes that can affect plant growth and viability (Barriuso *et al.,* 2010). Soil microorganisms perform the following important functions (Altieri, 1999):

- The decomposition of organic matter, transformation of soil organic matter, mineralisation and nutrient cycling;
- Converting atmospheric nitrogen into organic forms and reconverting nitrogen to gaseous nitrogen;
- Suppressing soil-borne pathogens through antagonisms;
- Synthesising enzymes, vitamins, and hormones that regulate populations and processes;
- Altering soil structure;
- Directly interacting with each other and plants through mutualism, commensalism, competition and pathogenesis.

It is well-known that some agricultural practices result in the reduction of biodiversity of soil-inhabiting organisms and that it can alter soil microbial community structures (Altieri, 1999; Barriuso and Mellado, 2012). This is caused by the loss of a stratified soil microhabitat and other agricultural practices such as pesticide use and others. The recycling of nutrients and proper balance between organic matter, soil organisms and plant diversity are important components of an ecologically balanced soil environment (Altieri, 1999). Thus, the reduction in soil biodiversity may influence soil health adversely.

Microbial communities respond rapidly to environmental disturbances of soil due to their short generation time, their intimate relation with their environment and their composition and activity (Sharma *et al*., 2011). Microbial communities largely determine biogeochemical cycles, the turnover processes of organic matter and the fertility and quality of soils (Neher, 2001; Sharma *et al*., 2011; Zelles, 1999). Therefore, microbial community structure is commonly used as an indicator of soil health and possible contamination. Based on the theory that there is functional redundancy in high quality soil, it has been suggested that good soil quality is indicated by a medium to high microbial diversity. Functional redundancy

(illustrated in Figure 2.1) means that the soil ecosystem will recover from disturbances that change the microbial communities. The higher the functional redundancy of microbial communities, the quicker the ecosystem can return to its initial condition after a disturbance. Also, microbial communities may be resilient to changes due to the fast growth rate, physiological flexibility and rapid mutation of most microbial species (Sharma *et al*., 2011).

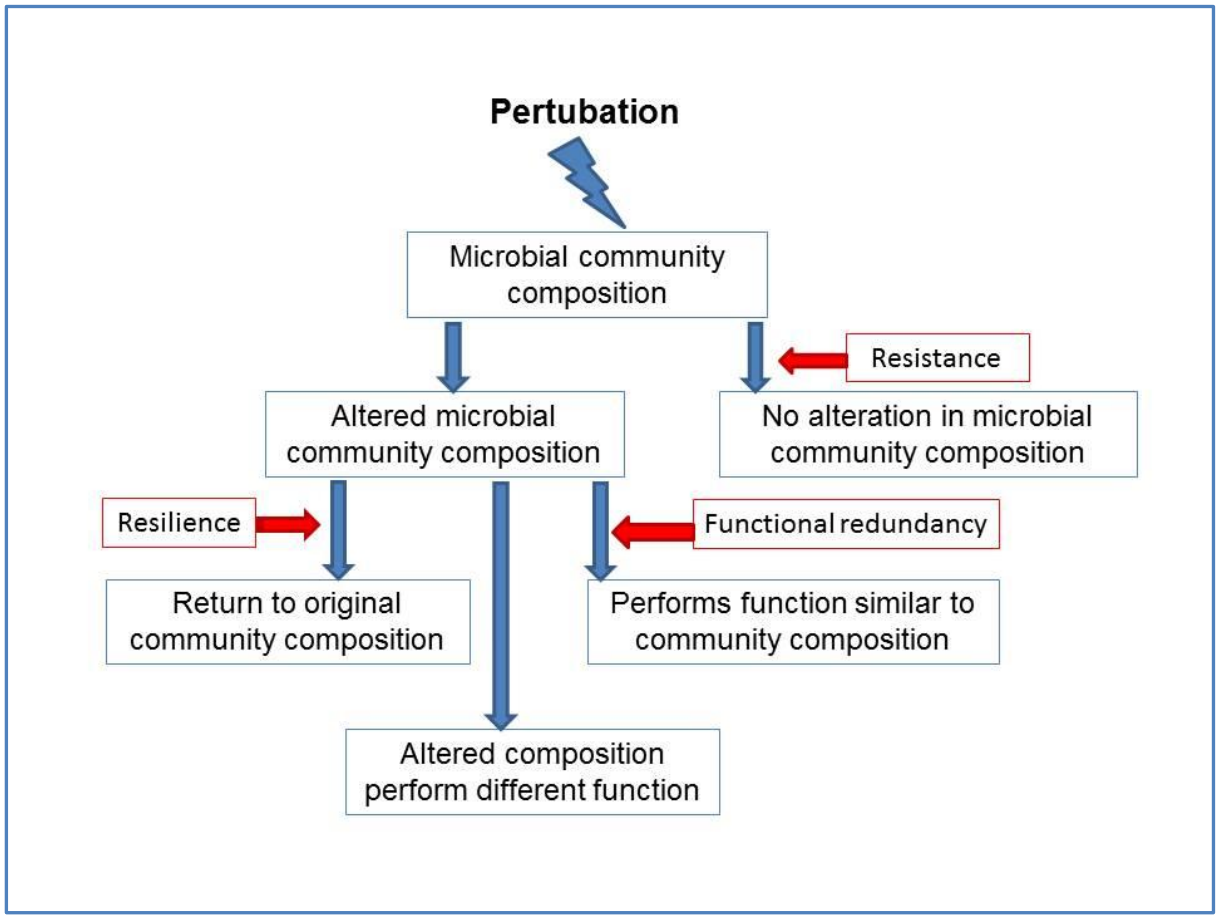

Figure 2.1: Illustration of the impact of environmental disturbances on changes in soil microbial community structure and function (Sharma *et al*., 2011).

The microbial properties of soil are used as indicators of soil quality due to the important relationships between soil and plant quality, microbial diversity and ecosystem sustainability (Anderson, 2003; Hill *et al.,* 2000). There are also complex interactions between individual microbial species relying on the presence and function of other species (Hill *et al.,* 2000). The relationship between microorganisms and their abiotic environment forms part of a self-regulatory process which determines soil fertility (Anderson, 2003). Thus, it is important to reduce agricultural practices that may lead to a reduction in microbial diversity and encourage sustainable agricultural practices that enhance this diversity.

2.4.1. Methods to characterise soil microbial communities.

Traditional soil health indicators such as soil physical-chemical analyses, do not take soil microorganisms into account. An understanding of microbial properties such as microbial activity, biomass and diversity can not only increase knowledge of factors contributing to soil health, but it can also be useful in devising practical measures of soil quality (Hill *et al.,* 2000). For these reasons, a wide range of methods to determine soil microbial community structure have been developed (Drenovsky *et al.,* 2004). These methods include phospholipid fatty acid (PLFA) analyses, community-level physiological profiling (CLPP) and polymerase chain reaction (PCR) amplification, as well as other molecular profiling methods (Hill *et al.,* 2000).

Soil microbial properties have been commonly studied at process level, where biomass, enzyme activities and respiration rates are examined. However process-level measurements represent limited information about qualitative community-level changes since any given microbial process may be carried out by diverse microbial taxa. Process-level measurements are also limited in its ability to describe a particular microbial ecosystem. In contrast community-level interactions are complex, with individual microbial species relying on the function, interaction and presence of many other species (Hill *et al.,* 2000). Therefore, community-level interactions at microbial level may serve as important and sensitive indicators of both short and long-term changes in soil health.

Culture-dependent methods of community analysis.

There are many culture-dependent methods to analyse microbial communities. In the past, the analysis of soil microbial communities relied on culture dependent techniques using dilution plating and various culture media designed to maximise the recovery of different microbial species. However, it has been estimated that less than 0.1% of microorganisms found in agricultural soils are cultivable using culture media formulations (Hill *et al.,* 2000; Sharma *et al*., 2011).

One of the more widely used culture-dependent methods for analysing microbial communities in soil is CLPP. Bacterial species are identified based on their utilisation of different carbon sources to assess their physiological profiles and to reflect their functional diversity (Sharma *et al*., 2011). These profiles have been facilitated by the use of a commercial taxonomic system known as the BIOLOG® system (Hill *et al.,* 2000; Sharma *et al*., 2011). This system is based on the utilisation of a set of 95 different carbon sources. Utilisation of each substrate is detected by the reduction of a tetrazolium dye, which results

in a colour change quantified by spectrophotometry (Hill *et al.,* 2000; Sharma *et al*., 2011). The pattern of substrates that are oxidised can be compared among different soil samples from a series of times or locations as an indication of differences in physiological functions of microbial communities (Hill *et al.,* 2000). However, there are some considerations in the use of this method namely: (1) The density of the initial inoculum must be standardised because it affects the rate at which colour develops in the wells; this may lead to negatives if the wells are read too soon; (2) It should be considered that an analysis of functional diversity is based on the assumption that colour development in each well is exclusively a function of the portion of organisms present in the sample which are able to utilise a particular substrate; (3) the substrates present in commercially available BIOLOG® plates are not necessarily ecologically relevant and most likely do not reflect the diversity of substrates found in the environment (Sharma *et al*., 2011).

Culture-independent methods of community analysis.

Due to the limitations of culture-based methods, culture-independent methods are being focussed on for community-level analysis of microbial populations. Composition of such communities can be determined by a number of methods which include the extraction, quantification, and identification of molecules from soil that is inherent to certain microbial groups, or advanced fluorescence microscopy techniques (Hill *et al.,* 2000). The methods discussed here will include PLFA, nucleic acid techniques and fluorescent *in situ* hybridisation (FISH).

The genetic diversity of soil microorganisms is used as an indicator that provides the basis of all actual and potential functions of soil (Kent and Triplett, 2002; Sharma *et al*., 2011). Of all the cell component molecules, nucleic acids have been the most useful in providing an understanding of microbial community structures (Hill *et al.,* 2000; Schloter *et al.,* 2003). The determination of the sequence of 16S ribosomal RNA (rRNA) genes in prokaryotes and 5S or 18S rRNA genes in eukaryotes are the most commonly used methods to study the taxonomic diversity of soil microorganisms on a genetic level (Kent and Triplett, 2002; Sharma *et al*., 2011) and is also the most useful of the nucleic acid techniques.

There are several benefits to using these small subunit rDNA molecules, namely:(1) such molecules are universally found in all three forms of life such as *Bacteria, Archaea*, and *Eucarya;* (2) these molecules are composed both of highly conserved regions and also of regions with sequence variation; (3) also the phylogenetic information held in the rDNA molecule is further enhanced by its relatively large size; and finally (4) rDNA can be easily amplified using PCR and rapidly sequenced (Hill *et al.,* 2000; Schloter *et al.,* 2003).

However, there are also many limitations to the usefulness of these nucleic acid techniques, *viz.* the storage of samples prior to processing can bias results; comparisons of activity among organisms or soil samples may be confounded by several factors; this method has been applied largely to investigations of prokaryotes and ribosomes of eukaryotes and have not been well studied (Hill *et al.,* 2000; Kent and Triplett, 2002).

Fluorescent *in situ* hybridisation (FISH) allows the direct identification and quantification of specific and/or general taxonomic groups of microorganisms within their natural microhabitat (Hill *et al.,* 2000; Kent and Triplett, 2002). Microorganisms can be detected using FISH across all phylogenetic levels and it can be used to visualise soil microorganisms that have not yet been cultured. It is also useful in studying the ecological distribution of microorganisms throughout diverse habitats (Hill *et al.,* 2000). This method uses oligonucleotide hybridisation probes corresponding to regions of the 16S rRNA gene for the determination of *in situ* abundance (Kent and Triplett, 2002). The FISH method is mostly used for studying individuals within a population, but can also be used for studying population dynamics, tracking microorganisms released into the environment, epidemiology, and microbial ecology of economically important plant pathogens in agricultural soils (Hill *et al.,* 2000; Kent and Triplett, 2002).

Another culture-independent method, PLFA analysis, is used to determine microbial community structure and to determine the effect of soil disturbances such as cropping practices, pollution, fumigation, and changes in soil quality (Frostegård *et al.,* 1996; Hill *et al.,* 2000). This type of analysis overcomes the limitations of culture-dependant techniques (Sharma *et al*., 2011; Xue *et al.,* 2005). Phospholipids are membrane components essential to all living cells and make up a relatively constant proportion of the biomass of organisms. These lipids and their associated fatty acids are useful as biomarkers due to their structural diversity and high biological specificity (Sharma *et al*., 2011; Zelles, 1999). In microorganisms, phospholipids are found only in cell membranes (Sharma *et al*., 2011; Hill *et al.,* 2000). This is important because cell membranes are rapidly degraded and the component PLFAs are rapidly metabolised following cell death (Hill *et al.,* 2000). For this reason phospholipids can serve as important indicators of viable microbial biomass.

The total amount of PLFAs indicates viable microbial biomass in various environmental samples (Zelles, 1999; Xue *et al.,* 2005). Schloter *et al.* (2003) defined microbial biomass as organisms living in the soil that are generally smaller in size than 10 µm. Such organisms are generally dominated by fungi and bacteria, however, most microbial biomass estimates do not exclude protozoa. Microbial biomass is ultimately important because it is one of the

fractions of soil organic matter that is biologically meaningful and responds to agricultural practices or pollution (Schloter *et al.,* 2003).

Phospholipids are easily extracted from microbial cells in soils and unique fatty acids are indicative of specific groups of organisms as indicated in Table 2.2 (Drenovsky *et al.,* 2004; Hill *et al.,* 2000; Sharma *et al*., 2011). The presence and abundance of signature fatty acids in soils reveals the presence and abundance of particular organisms or groups of organisms in which those signatures can be found (Drenovsky *et al.,* 2004; Frostegård *et al.,* 1996; Hill *et al.,* 2000; Zelles, 1999). These lipid profiles give insight into the microbial community structure of soils due to the relative abundance of certain PLFAs, which varies between specific groups of organisms (Table 2.2).

Table 2.2: Major phospholipid fatty acid analyses groups representing specific microbial groups (Drenovsky *et al.*, 2004; Frostegård *et al*., 1996; Hill *et al.,* 2000; McKinley *et al.*, 2005; Ratcliff *et a*l., 2006; Zelles, 1999).

Fatty acid	**Marker**
Mid-chain branched saturated fatty acids (MBsats) 10me16:0 10me18:0	Actinomycetes
Terminally branched saturated fatty acids (TBsats) i14:0; i15:0; a15:0; i16:0; i17:0; a17:0.	Gram-positive bacteria
Monounsaturated fatty acids (Monos) 16:1ω9c; 16:1ω7c; 16:1ω 7t; 16:1 ω 5c; cy17:0; 18:1ω7c; 18:1ω7t; 18:1ω5c; cy19:0.	Gram-negative bacteria
Polyunsaturated fatty acids (Polys) 18:2ω6,9 and 18:1ω9c	Fungi
Normal saturated fatty acids (Nsats)	Found in all microorganisms

Straight-chain fatty acids are widely distributed among organisms, whereas long straight-chain fatty acids are characteristic of eukaryotes and higher plants. Bacteria for example, contain straight-chain fatty acids that are also common in other organisms. However, fatty acids that are unique to bacteria include β-OH, cyclopropane and branched-chain fatty

acids. Most of the latter fatty acids present in bacteria are linked to phospholipids and glycolipids. Branched-chain fatty acids on the other hand are commonly found in Gram-positive bacteria and sulphate-reducing Gram-negative bacteria. Cyclopropyl fatty acids are common in some Gram-negative bacteria and some anaerobic Gram-positive bacteria. Actinomycetes are identified by methyl branching on the tenth carbon atom in the molecule while linoleic acid has been used as an indicator fatty acid for fungi (Zelles, 1999).

Certain PLFA markers, such as certain fatty acids, can also be used as environmental stress indicators. The physiological or nutritional status of microbial communities can be determined by knowledge of specific lipid biosynthetic pathways (Kaur *et al.,* 2005; Navarrete *et al*., 2000). When gram-negative bacteria are experiencing nutritional stress, it can be observed in an increased ratio of sat / unsat fatty acids and also an increased *trans*- to *cis* ratio (Kieft *et al.*, 1994). The ratio of sat / unsat fatty acids is generally higher in environments where organic carbon and nutrients are limiting factors (Fierer *et al*., 2003).

It is known that bacteria alter their membrane fatty acid components in response to environmental stresses, for example the conversion of monoenoic fatty acids to cyclopropyl fatty acid products (Kieft *et al.,* 1994). The isomerisation of *cis* unsaturated fatty acids (16:1ω7c and 18:1ω7c) to its complementary *trans* unsaturated fatty acids (16:1ω7t and 18:1ω7t) is a mechanism used by various bacterial species to adapt their membranes to organic solvents (Heipieper *et al.,* 1996; Kaur *et al.,* 2005). Due to the steric differences between *cis*- and *trans* unsaturated fatty acids, the conversion of *cis* to *trans* reduces the membrane fluidity and acts against the increase in fluidity caused by organic compounds (Heipieper *et al.,* 1996; Kaur *et al.,* 2005). Various studies have shown that this conversion can be used as an indicator of environmental stress, especially an indicator to measure toxicity of organic compounds (Heipieper *et al*., 1996; Navarrete *et al.,* 2000). For example, a correlation has been found among the hydrophobicity of organic compounds, the toxic potential of the compounds and changes in the *trans* / *cis* ratios of unsaturated fatty acids (Heipieper *et al*., 1996).

Most studies done to determine the effects of agricultural management practices on microbial communities used PLFA analyses (Drenovsky *et al.,* 2004; Sharma *et al.,* 2011). However, as with any other method, there are some limitations to this method. First, appropriate signature molecules are not known for all organisms in a soil sample and in some cases, a specific fatty acid present in a soil sample cannot be linked with a specific microorganism or group of microorganisms. Secondly, any variation in these signature fatty acids may give rise to false community structures created by artifacts in the methods (Hill *et al.,* 2000).

2.5. The role of nematodes in agricultural soil.

Nematodes (non-parasitic and plant-parasitic) are the most abundant organisms in soil and they play an important role in the soil food web (Briar *et al.,* 2007; Ferris *et al.,* 2012; Rasmann *et al.,* 2012; Sochová *et al.,* 2006). A healthy soil food web should consist of a variety of nematode genera, of which non-parasitic nematodes will be predominant, with different life strategies and feeding behaviours. Such nematode genera generally range from fast-growing and fast-breeding bacterivores that are represented at the bottom of the food web, to slow-growing, long generation and low fecundity predators that are typically at the top of the food web. Nematode fauna can be influenced by several factors, including agricultural practices such as soil cultivation, crop rotation, soil fumigation, nematicide application, the use of herbicides and other biotic and abiotic factors (Ferris *et al.,* 2012).

These organisms are important to the soil biotic community and assessing nematode communities may provide unique insights into soil biological processes (Briar *et al.,* 2007). Nematode communities are usually large and because of various biological characteristics they respond rapidly to environmental changes. Thus, these organisms are frequently used as indicators of soil health especially in terms of short-term responses to environmental changes. However, there have been little research on the long-term effects of agricultural management practices and other environmental changes on non-parasitic nematodes that occur in soil (Briar *et al.,* 2007; Cheng *et al.,* 2008; García-del-Pino and Morton, 2010; Sánchez-Moreno *et al*., 2006; Sánchez-Moreno *et al.,* 2009).

Some of the characteristics that make non-parasitic nematodes successful as biological indicators of soil quality include:

- Their abundance in almost all environments including soil;
- Morphology, which reflects their different types of feeding behaviour;
- High diversity of life strategies and feeding habits (food specificity) of these organisms;
- Life cycles that range from only a few days to several months for different species over a broad range;
- The relative ease with which nematodes can be isolated from different substrates such as soil, water, and the plant parts they infect;
- Their permeable cuticles allowing response to a range of pollutants;
- The ability to survive extremes (also known as cryptobiosis / anhydrobiosis);
- The presence of heat shock proteins which aid in their survival in high temperatures;
- Their close interaction with other soil organisms

(Bongers and Bongers, 1998; Neher, 2001; Porazinska *et al.,* 1999; Schloter *et al.,* 2003; Sochová *et al.,* 2006).

In the past, ecological research was dominated by three main topics: interactions of nematodes with other organisms, nematode population dynamics, and nematodes as model systems (Ferris *et al.,* 2012). This research was concerned with the study of only a few nematode species in relation to functional groups of organisms such as arbuscular mycorrhizal fungi, rhizobia, plant pathogens, viruses and other specifically defined groups of nematodes. The focus was on plant-parasitic nematodes as they are economically more important in terms of disease management (Ferris *et al.,* 2012). However, there was a shift in focus in recent years as entire nematode faunae are used to study conditions of the food web status and function in managed and natural ecological systems.

The activities of nematodes in soil substrates also affect primary crop production, decomposition of organic material, energy flows and nutrient cycling (Neher, 2001; Sochová *et al.,* 2006). Non-parasitic nematodes in particular play important roles in several aspects of soil quality. These include the following:

- Nutrient mineralisation: this is the result of two processes following the consumption of prey, including bacteria, fungi, nematodes and other fauna, by soil-inhabiting nematodes (Rasmann *et al.,* 2012). The ingested carbon is used by nematodes for respiration and assimilation whereas ingested nutrients are used only for assimilation (Ferris *et al.,* 2012). The carbon:nutrient ratio of nematodes is larger than that of their microbial prey, resulting in more nutrients being ingested by nematodes than required and the excesses are excreted in a mineral form (Ferris *et al.,* 2012; Sochová *et al.,* 2006). Non-parasitic nematodes are responsible for up to 25 % of nitrogen mineralisation in soil. Some studies revealed that bacterial-feeders are involved in nitrogen mineralisation and fungal-feeders are involved in phosphorus mineralisation. Predatory nematodes are indirectly involved in nutrient cycling following the pattern of population densities of their prey (Ferris *et al.,* 2012). Bacterivorous and predator nematodes contribute approximately 8 % and 19 %, respectively, to nitrogen mineralisation in agricultural soils (Briar *et al.,* 2007; Neher, 2001). Omnivorous nematodes contribute to nitrogen mineralisation directly by the release of nitrogen from prey and indirectly through accelerated turnover and predation by microbial grazers (Ferris *et al.,* 2012; Sochová *et al.,* 2006).
- Redistribution of other soil microorganisms involved in nutrient cycling: although nematodes are relatively immobile in soil, moving only centimetres per day, they can be easily moved across ecosystems either by water, farming implements or

phoretically on insects. Nematodes themselves can also redistribute and inoculate bacterial and fungal spores as well as pathogenic viruses into new soil patches (Ferris *et al.,* 2012; Sochová *et al.,* 2006). For example, some bacterivorous nematodes are vectors for four strains of beneficial rhizobacteria (Ferris *et al.,* 2012).

Nematode community patterns are sensitive to environmental changes and may indicate any fluctuations in the soil environment caused by agricultural management practices (Porazinska *et al.,* 1999; Rasmann *et al.,* 2012; Sánchez-Moreno *et al.,* 2006). Environmental disturbances in agriculture can be divided into two groups, *viz*. chemical and physical. Chemical disturbances include nutrient enrichment and chemical pollutants such as pesticides, while physical disturbances include practices associated with the cultivation of crops (Neher, 2001).

The most sensitive traits of nematodes as biological indicators are their community structure and diversity (Sochová *et al.,* 2006). Studying nematodes on community level are ecologically relevant as they integrate interactions of all soil factors, including management and pollutants. Nematode community analyses can offer insights into abundance of individuals or species, biomass of species, species composition, feeding strategies, presence and abundance of key species (Schloter *et al.,* 2003). In agricultural fields, nematode abundance and diversity are used to evaluate soil process rates, soil functions and the effects of environmental changes on soil fauna (Sánchez-Moreno *et al.,* 2006).

Nematodes are a very diverse group of organisms and there is extensive knowledge available in terms of their taxonomy and feeding habits (Neher, 2001; Yeates *et al.,* 1999). Their species composition can reflect substrate texture, climate, organic inputs and environmental disturbances (Yeates, 2003). However, extensive taxonomic knowledge is not needed to study nematode community structure, due to their allocation into functional groups (Table 2.3) (Sochová *et al.,* 2006). A functional group can be defined as groups of species that have similar effects on ecosystem processes (Bongers and Bongers, 1998).

Each functional group demonstrates varying sensitivity to environmental changes. Research suggests that management practices are the greatest drivers of changes in nematode community structure. Other factors may however, also contribute to these changes, including substrate structure and climate (Sochová *et al.,* 2006).

Table 2.3: Examples of nematode genera belonging to different nematode functional groups (Bongers and Bongers, 1998; Neher, 2001; Rasmann *et al*., 2012; Sochová *et al.,* 2006; Yeates *et al.,* 1993).

Functional group	**Selected examples of nematode genera belonging to functional groups**
Plant feeders or plant-parasitic nematodes	*Helicotylenchus* spp., *Heterodera* spp., *Meloidogyne* spp., *Pratylenchus* spp.
Bacterial feeders or bacterivores	*Cephalobus* spp., *Caenorhabditis* spp., *Plectus* spp., *Rhabditis* spp.
Fungal feeders or fungivores	*Aphelenchus* spp., *Aphelenchoides* spp., *Diphtherophora* spp., *Leptonchus* spp.,
Substrate ingesters	*Daptonema* spp., *Eumonhystera* spp., *Neodiplogaster* spp.
Animal predators	*Diplogaster* spp., *Labronema* spp., *Laimaphelenchus* spp., *Mononchus* spp., *Nygolaimus* spp., *Seinura* spp.,
Omnivores	Nematodes belonging to the order Dorylaimida

Various species of nematodes are known to be deleterious to agriculture and human health. However, most nematodes have beneficial roles in ecosystems and are not pests or parasites. For example, bacterial feeding nematodes regulate microbial activity which in turn regulates rates of decomposition and nutrient mineralisation (Neher, 2001). Thus, changes in the abundance of bacterial and fungal feeding nematodes indicate changes in the decomposition route and energy flow (Bongers and Bongers, 1998; Ferris *et al.,* 2012). Some laboratory and field studies have suggested that bacterial and fungal feeding nematodes play important roles in the turnover of microbial biomass in soil and availability of nutrients to plants. Bacterial feeding nematodes in particular enhance nutrient release and approximately 40 % of nutrient mineralisation is due to nematodes feeding on microbial populations (Yeates, 2003).

Several studies reported that bacterial feeders increase in number as a result of increasing microbial activity due to agricultural practices such as fertilisation. In contrast, a decrease in

bacterivorous and an increase in fungivorous nematodes may be caused by heavy-metal-induced stress. The most sensitive functional groups regarding pollutants are the carnivorous and omnivorous nematodes. These functional groups have a regulating function in the soil ecosystem (Bongers and Bongers, 1998). The carnivores regulate other soil biota by feeding on the most dominant prey, while omnivores assist several other functional groups where needed. These functional groups are also the first to disappear as a result of environmental change and this may lead to an unstable environment in terms of soil-borne pathogens (Bongers and Bongers, 1998).

2.5.1. Methods to investigate nematode diversity and community structure.

Nematodes are globally recognised as good soil quality indicators. Therefore, in the 20th century, nematode community indices were developed and have been used to study the impact of various management practices on soil quality (Ferris *et al.,* 2012). The interpretation of nematode community structure through faunal analysis not only provides the opportunity to evaluate the condition of the soil environment in terms of effects of pollution and other environmental stresses, but also acts as an indicator of the soil food web function (Bongers, 1999; Ferris and Matute, 2003). Comprehensive analyses are required to understand the soil health condition by using nematode community analyses and include identification of different nematode feeding groups, fungivore to bacterivores ratio as well as indices for richness, diversity, dominance and maturity of soil food webs (Ferris *et al.,* 2012).

To investigate nematode diversity and community structure within the soil ecosystem it is usually useful to evaluate the soil food web. The structure and function of the soil food web can be altered by environmental changes and can be assessed using structural or functional analysis. Structural analysis is done through the determination of the presence and abundance of individual taxa present in the soil or as an alternative, to determine the presence and abundance of non-parasitic nematode indicator guilds. On the other hand, functional analysis includes rates of various soil processes such as soil respiration, organic matter decomposition, and biologically-mediated mineralisation (Ferris *et al*., 2001). The latter authors developed a framework for weighted nematode faunal analysis to use as an indicator of the condition of the soil food web. To explain this framework, nematode coloniser-persister (cp)-values and various indices used in faunal analyses will be discussed first.

Coloniser-persister (cp) values.

The life strategies of nematodes are generally categorised with cp values (Bongers and Ferris, 1999), which is defined as the assignment of nematode taxa to a 1-5 linear scale which corresponds to r-K strategies (Ferris *et al*., 2001; Neher, 2001). Colonisers and opportunists (cp-1) are r-strategists (colonisers of new resources). Persisters (cp-5) are K-strategists and are dominant in undisturbed habitats (Bongers and Ferris, 1999; Neher, 2001; Sochová *et al*, 2006). Colonisers produce many small eggs and exploit nutrient-rich environments at high rates. On the other hand, persisters hardly react at transient conditions of high food availability (Bongers, 1999; Bongers and Bongers, 1998).

Since nematode species within a community have different sensitivities to toxins and stressors, the categorisation of nematodes in groups based on their cp-value has been a useful tool to predict sensitivity of certain nematode taxa to environmental stress factors (Ferris *et al.,* 2012).The cp-value system is based on the recognition that nematode taxa of monophyletic families are adapted to certain environmental conditions and nutrient status of the soil in similar ways (Ferris *et al.,* 2001) and is summarised in Table 2.4.

The cp-values also generally correspond to the sensitivity of taxa to environmental disturbance. Taxa with low cp-values are relatively tolerant to disturbances, whereas taxa with high cp-values are more sensitive to the same disturbances. Nematode taxa with cp-value of 1 are considered as enrichment opportunists and their population densities increase rapidly in response to added nutrients to the soil (Bongers and Ferris, 1999; Bongers and Bongers, 1998; Ferris *et al*., 2001; Neher, 2001). Therefore, the latter non-parasitic nematodes may not reflect long-term changes in soil ecological condition. Non-parasitic nematodes in the cp-2 scale are considered to be general opportunists, while those belonging to cp-3 to cp-5 are categorised as persisters (Bongers and Ferris, 1999).

Non-parasitic nematodes with cp values between 2 and 5 are considered to be more stable indicating the absence of environmental stress (Bongers and Bongers, 1998) and, therefore, may provide long-term information on environmental conditions. Those with a cp-value of 5 are considered to have lower fecundity and appear later in succession (Neher, 2001). Based on the cp-values, various indices can be calculated to evaluate soil nematode communities (Bongers, 1999), including the maturity index of nematode fauna (Ferris *et al*., 2001) as discussed below.

Table 2.4: Description of coloniser-persister (cp) scale classification of nematodes (Bongers, 1999; Bongers and Bongers, 1998; Ferris *et al*., 2001; Neher, 2001).

cp-Value	Description of nematodes	Example
1	Short generation time; produce many small eggs, resulting in high population growth rates in nutrient-rich environments. This group is relatively tolerant to pollution-induced stress and have high metabolic activity. These nematodes generally characterised as enrichment opportunists and are only active with high microbial activity. Dauerlarvae are form in conditions where microbial activity is low.	Rhabditid diplogastrid and panagrolaimid bacterial feeders.
2	Short generation times with high fecundity. They do not form dauerlarvae, but may become cryptobiotic in undesirable/non-optimal conditions. These nematodes occur in nutrient-rich and nutrient-poor environments. This nematode group has a high tolerance for pollution and other environmental disturbances and include mainly bacterivores and fungivores.	Smaller tylenchids predominantly feeding on epidermal cells; fungal feeding aphelenchoids and anguinids; and the bacterial feeding cephalobids, plectids and monhysterids.
3	Longer generation times and are more sensitive to environmental disturbances, including fungivores, bacterivores and carnivores.	Bacterial feeding Araeolaimida and Chromadorida; larger tylenchid nematodes that feed on deeper root cells; the diphtherophorids; and the carnivorous tripylids.
4	Long generation times and a high sensitivity to pollution and disturbances. Characterised by having a permeable cuticle and includes smaller omnivores.	Larger carnivores; bacterial feeding Alaimidae and Bathyodontidae; smaller dorylaimid nematodes; and plant-feeding trichodorids.
5	Very long generation times with low fecundity, low metabolic activity and motility. Have permeable cuticles and are very sensitive to pollution and other environmental disturbances. Predominantly omnivores and carnivores.	Larger dorylaimids including omnivores, predators and plant feeders.

Maturity indices.

Maturity indices offer both quantitative and biological-ecological aspects of the individual nematode species in a community. These indices provide the quantitative means to characterise ecosystems and also provide the means to compare various ecosystems (Porazinska *et al.,* 1999). However, nematode faunae can be misinterpreted if these indices are not used properly (Ferris *et al.,* 2012). Maturity indices are based on the principle that different taxa have contrasting sensitivities to environmental stress or disruption of the successional sequence because of their life-history characteristics. The history of disturbance can be reflected by the successional status of soil communities since succession can be interrupted at various stages by common agricultural practices, including pesticide application (Ferris *et al.,* 2012; Neher, 2001)

The Maturity Index (MI) and the Plant-Parasitic Index (PPI) are indices used in nematode faunal analyses to assess the response of nematode assemblages to environmental disturbances. These indices were initially designed for the comparison of conditions in adjacent plots; however, they have also been applied among a range of plots, soil types and regions (Yeates, 2003). Both the PPI and the MI are calculated as $\Sigma((v_i*f_i)/n)$, where v_i is the cp-value assigned to a family, f_i is the frequency of the family in the sample and n is the total number of individuals present in a sample (Bongers, 1999).

The MI is represented by the weighted, mean cp-value for all individual nematodes in a representative soil sample and is based on the relative abundance of non-parasitic nematode taxa (Bongers, 1999; Bongers and Ferris, 1999; Bongers and Bongers, 1998; Ferris *et al.,* 2012; Neher, 2001). The PPI index is referred to as a sensitive index of ecosystem development (Bongers and Bongers, 1998; Neher, 2001; Sochová *et al.,* 2006). Maturity indices values are also linked to the life strategies of nematodes. Low values of maturity indices are generally associated with either rare K-strategists or predominant r-selected nematodes (Porazinska *et al.,* 1999).

The MI generally ranges from 1 (nutrient-enriched, polluted and disturbed environments) to 5 (indicating undisturbed ecosystems) and is used as an indicator for the persistence of colonisers or for the life strategies of nematodes (Bongers and Ferris, 1999; Bongers and Bongers, 1998; Neher, 2001 Schloter *et al.,* 2003; Sochová *et al.,* 2006). Disturbances are generally indicated by a low valued index. The MI index has also been used as an indicator of soil recovery after an environmental disturbance, variations in agricultural management practices, or measuring pollution-induced stress (Bongers and Bongers, 1998; Schloter *et al.,* 2003; Sochová *et al.*, 2006). The MI has been used in various studies, including: soil

health and sustainability, functional aspects, biodiversity, ecosystem management, effects of agricultural practices and ecotoxicology (Bongers, 1999).

The plant-feeding taxa were originally excluded from the calculation of the MI due to the inverse relationship of MI and PPI under certain conditions. However, some studies included plant-feeding taxa in MI calculations (Bongers and Bongers, 1998). The plant-parasitic nematodes were also excluded from the MI calculations because their occurrence and abundance is mainly determined by their host status (Bongers and Ferris, 1999). The ratio of PPI to MI has been used for comparison of various tillage regimes and also for indicating the nutrient status of soil samples (Bongers and Bongers, 1998). Since nematode communities change in response to these practices, maturity indices vary with various land management practices (Neher, 2001). Various factors may influence the MI and PPI values. Under N-fertilisation the MI decreases due to higher microbial activity and the PPI increases due to higher carrying capacity of plants on which plant-parasitic nematodes feed (Bongers, 1999; Bongers and Bongers, 1998; Bongers and Ferris, 1999). On the other hand, pollution may also cause a decrease in the MI value since pollution causes a community shift favouring opportunists and lowers the presence of taxa with higher cp-values (Bongers and Ferris, 1999).

Ratio of fungivorous to bacterivorous nematodes.

Decomposition processes in soil are often distributed in one of two pathways, namely the bacterial-based or in the fungal-based channel. The ratio between the abundance of bacterivorous and fungivorous nematodes provides an index of the relative contribution of the above mentioned pathways to decomposition processes (Ferris and Matute, 2003; Ferris *et al*., 2001; Porazinska *et al.*, 1999; Yeates, 2003).

According to Yeates (2003), this ratio is the nematode channel ratio (NCR) and its expressed as NCR = B/(B+F), where B is the relative contributions of bacterivores and F is the relative contribution of fungivores to total non-parasitic nematode abundance. This ratio can have a value of between 0 and 1, where closer to 1 indicates bacterivore-dominated and 0 indicates fungivore-dominated. Some studies showed that the soil microbial communities in managed soils are dominated by bacterial-based pathways. On the other hand, soils that are completely unmanaged are dominated by fungal-based pathways (Ferris *et al*., 2004).

The Channel Index (CI) is an alternative way to indicate the relative flow of substrates along bacterial and fungal decomposition pathways. This index provides the opportunity of tracking

succession between fungivore and bacterivore nematodes as organic resources are supplied and depleted in agricultural systems (Ferris *et al*., 2004; Ferris and Matute, 2003).

The ratio of bacterivore to fungivore nematodes can also reflect some aspects of the surrounding soil environment. Soils dominated by fungivores generally reflect more favourable moisture content for fungi and a slow rate of organic matter turn-over. On the other hand, soils dominated by bacterivores indicate relatively high rates of organic matter turn-over (Porazinska *et al.,* 1999).

Nematode faunal analysis model.

The most recent nematode faunal analysis includes the integration of non-parasitic nematode feeding groups and cp-values into a matrix classification of functional guilds. Functional guilds are defined as a grouping of taxa of similar biology within the food web (Ferris *et al*., 2001).

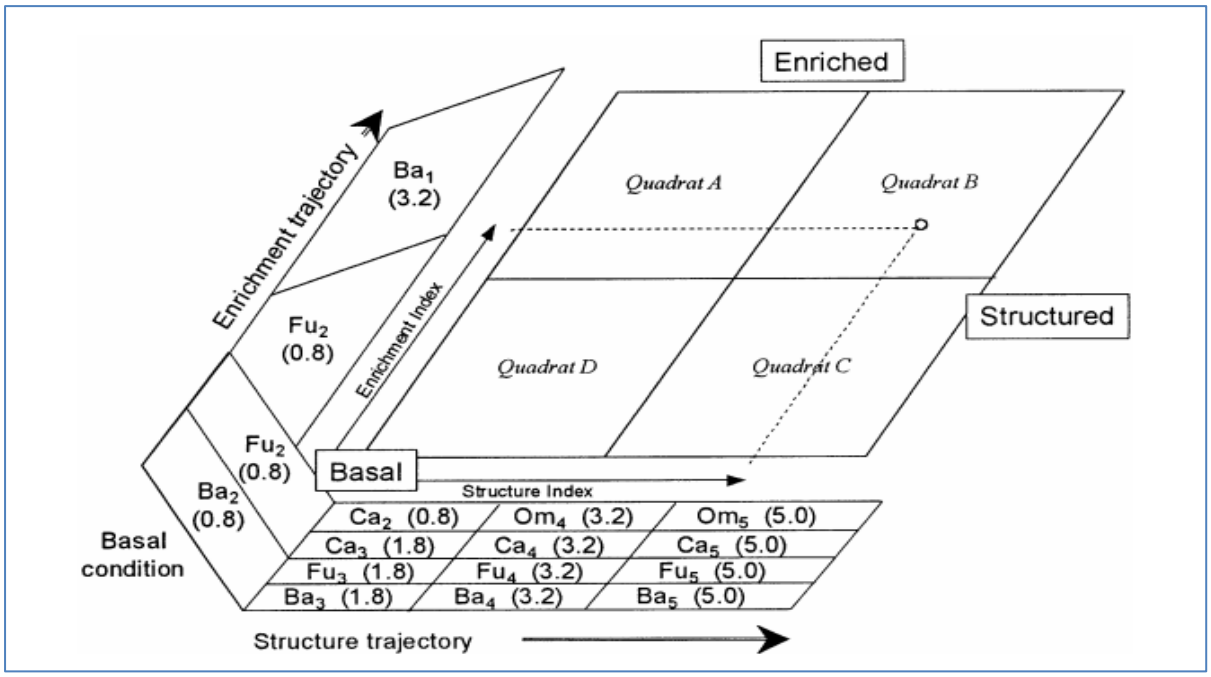

Figure 2.2: Functional guilds of non-parasitic soil nematodes characterised by feeding habit, coloniser–persister (cp) scale and food web structure, where Ba = bacterivores; Fu = fungivores, Om = omnivores; Ca = carnivores and numbers after each acronym indicates the cp-value for that functional group (Ferris *et al*., 2001).

Graphical illustration of nematode faunal analysis provides a fundamental summary of the structure of the soil food web and may be an indication of environmental conditions (Ferris *et al.*, 2001). Figure 2.2 is a graphical representation of functional guilds of nematodes based

on trophic groups and life history characteristics. These functional guilds are expressed along a cp-scale. The faunal profile (Figure 2.2) can be divided into four quadrats. Quadrat A indicates an enriched but unstructured soil nematode community, whereas Quadrat B indicates an enriched and structured community. Quadrat C is indicative of a resource-limited and structured community and the final quadrat (Quadrat D) indicates a resource-depleted community with minimal structure (Ferris *et al*., 2001). Some of the different characteristics of these four quadrats are summarised in Table 2.5. This graphical illustration allows the response of enrichment opportunists (cp-1), general opportunists (cp-2) and persisters (cp-3 to 5) to be distinguished and provides an overview of the state of the surrounding environment (Bongers and Ferris, 1999).

Table 2.5: Characteristics of four different quadrats representing the non-parasitic food web structures and functions (Ferris *et al*., 2004).

Quadrat	Characteristics
A (High Enrichment Index (EI) and low Structure Index (SI)	Disturbed environment, nitrogen (N)-enriched with a low carbon:nitrogen (C:N) ratio. Generally bacterial dominated.
B (High EI and high SI)	Maturing environment, N-enriched with a low C:N ratio. Generally bacterial dominated.
C (Low EI and high SI)	Environment undisturbed and not enriched by organic input. C:N ratio is high with fungal dominated decomposition pathways.
D (Low EI and low SI)	Resources are depleted and the environment may be under stress due to disturbances. Moderate C:N ratio and both bacterial and fungal dominated decomposition pathways.

In Figure 2.2, three qualitative soil food web conditions are demonstrated that are associated with non-parasitic nematode indicator guilds. These are the basal-, structured- and enriched-soil food web classes. The basal class is where a soil food web has been weakened due to environmental stresses including limitation of nutrients, and contamination (Ferris and Matute, 2003; Ferris *et al*., 2001). Non-parasitic nematode groups present under these

conditions are generally cp-2 and are adapted to stress conditions. According to Ferris *et al*. (2001), the indicator guilds in this soil food web class are the bacterivores (Ba_2) and fungivores (Fu_2) (Table 2.6). The Ba_2 and the Fu_2 are guilds also present in other soil food web classes and are defined as general opportunists.

Table 2.6: Soil food web classes based on coloniser-persister (cp)-values with examples in each class (Ferris *et al*., 2001; Ferris and Matute, 2003).

Soil food web class	Functional guild with Cp-values	Example of nematode group
Basal	Ba_2*	Cephalobidae
	Fu_2**	Aphelenchidae, Aphelenchoididae and Anguinidae
Structured	Ba_3	Prismatolaimidae
	Fu_3	Diphtherophoridae
	Ca_3***	Tripylidae
	Ca_2	Aphelenchidae
	Ca_4	Mononchdae
	Om_4****	Dorylaimidae
	Fu_4	Leptonchidae
	Ca_5	Discolaimidae
	Om_5	Thornenematidae and Qudsianematidae
Enriched	Ba_1	Rhabditidae, Panagrolaimidae, and Diplogasteridae
	Fu_2	Aphelenchidae, Aphelenchoididae and Anguinidae

*Ba – Bacterivores (Ferris *et al*., 2004);
**Fu – Fungivores (Ferris *et al.,* 2004);
***Ca – Carnivores / Predators (Ferris *et al.,* 2004);
****Om – Omnivores (Ferris *et al*., 2004).

Structured food webs are where nutrients are more abundant and where recovery from disturbances is occurring. Food webs in this class contain more trophic links than that of a basal food web and nematodes representing cp groups 3 to 5 are generally present (Ferris

and Matute, 2003; Ferris *et al*., 2001). This class is represented by the indicator guilds Ba_3, Fu_3, Ca_3 and Ca_2. At a higher structure level within the nematode community (more links in the food web and the occurrence of multi-trophic interactions) nematode indicator guilds Ca_4, Om_4 and Fu_4 are also included in this soil food web class. The highest level of community structure (achieved by environmental stability and homeostasis) is represented by Ca_5 and Om_5 nematode guilds. The third and final class of the soil food web are the enriched food webs (Ferris and Matute, 2003; Ferris *et al*., 2001) and represent conditions where nutrients become more available due to organism mortality, organic matter turn-over or there are favourable shifts in environmental conditions (Ferris and Matute, 2003). Indicator guilds here are represented by Ba_1, however Fu_2 indicator guild may also increase here when complex organic material becomes available in the soil. In enriched food webs there is an increase in microbial activity and bacterivore nematodes, that are enrichment opportunists (Ferris and Matute, 2003; Ferris *et al*., 2001).

Nematode faunal analysis also includes the recognition of enrichment- and structure-trajectories (Figure 2.2). A structure trajectory measures the abundance of trophic linkages in the soil food web and the effects of exploitation and competition on opportunist populations. The enrichment trajectory reflects resource characteristics of the soil food web and the increase of primary consumers of added organic material (Ferris and Matute, 2003). In Figure 2.2 the structured trajectory is calculated as the degree of trophic connectance (l) in the food web related to species richness (s) i.e. $l = \alpha s^2$ where α is 0.8 to keep the maximum value at 5.0 (Ferris *et al*., 2004; Ferris and Matute, 2003). This relationship between connectance and species richness can be used to estimate the respective degrees of connectance if only cp-2 were present, cp-2 and cp-3 were present, cp-2 to 4 were present or only cp-5 were present. According to these calculations the following weights were applied to the cp-classes 2 to 5: 0.8 (cp-2); 1.8 (cp-3); 3.2 (cp-4) and 5.0 (cp-5) (Ferris *et al*., 2004). In the enrichment trajectory the cp-2 indicator guilds received the same value of 0.8, whereas cp-1 nematodes of the enrichment trajectory received four times that value (3.2) (Ferris *et al*., 2004).

Based on the weighted abundance of nematode guilds in structured (s), enriched (e), and basal (b) food webs, two indices can be calculated. These indices represent the SI and the EI (Figure 2.2) (Ferris and Matute, 2003). The EI is the distance along the enrichment trajectory and is a measure of the resources available to the soil food web. The EI is calculated as 100(e/(e+b)). The distance along the structure trajectory, also known as the structure index (SI), is calculated as 100(s/(s=b)) (Ferris *et al*., 2004).

The use of faunal analyses, representing community, diversity and ecosystem function indices of non-parasitic nematodes to categorise soil quality in various terrestrial ecosystems, are becoming more popular. Therefore, this approach was used in the curent study to categorise soil quality of agro-ecosystems where RR and CS soybean were planted and compared to that of natural veld ecosystems. Ultimately, the faunal nematode and microbial PLFA data were linked to exploit the role and contribution of these microorganisms in soils where glyphosate-treated soybean crops were grown.

Chapter 3: Materials and Methods.

3.1. Experimental design and site description.

Soils managed in three different ways were compared to determine the effect of glyphosate (RoundUp ®) on soil microbial communities and nematode diversity. These included two agro-ecosystems, RoundUp ® Ready (RR) soybean and conventional soybean (CS) fields as well as surrounding natural veld (NV). The RR fields sampled during this study have been treated with glyphosate for no less than 5 years in all localities. The CS fields have not been been treated with glyphosate and no RR crops have ever been planted in these soils for at least four years prior to this study. Natural veld refers to uncultivated grassland where no agricultural activity took place for at least 10 years.

Soybean cultivars that were growing at the 23 sites during sampling were either PAN1664, PAN1583 and/or PAN6164 in terms of RR, while Egret, Mukwa, MC555, SNK500 and/or Superboon were the conventional cultivars used by farmers. Only the farmers in Bothaville (RR1 and CS1) applied nematicides to the soybean crops during the 2013 growing season. Crop history at these sampling sites generally included the planting of maize before soybean as well as sunflower during the summer. In Brits, however, wheat was planted during the winter seasons. Soil preparation generally included chisel plough, disc, minimum tillage, rip and tilling.

Rhizosphere soil samples from fields of each of the three ecosystems were collected at six different localities (Figure 3.1) within the soybean production areas of South Africa (Table 3.1). The term rhizosphere describes the soil in close proximity to the roots (Barriuso and Mellado, 2012) that were collected during this study. Rhizosphere soil samples were ultimately collected from 23 sites, including six RR fields, seven CS fields and ten NV sites.

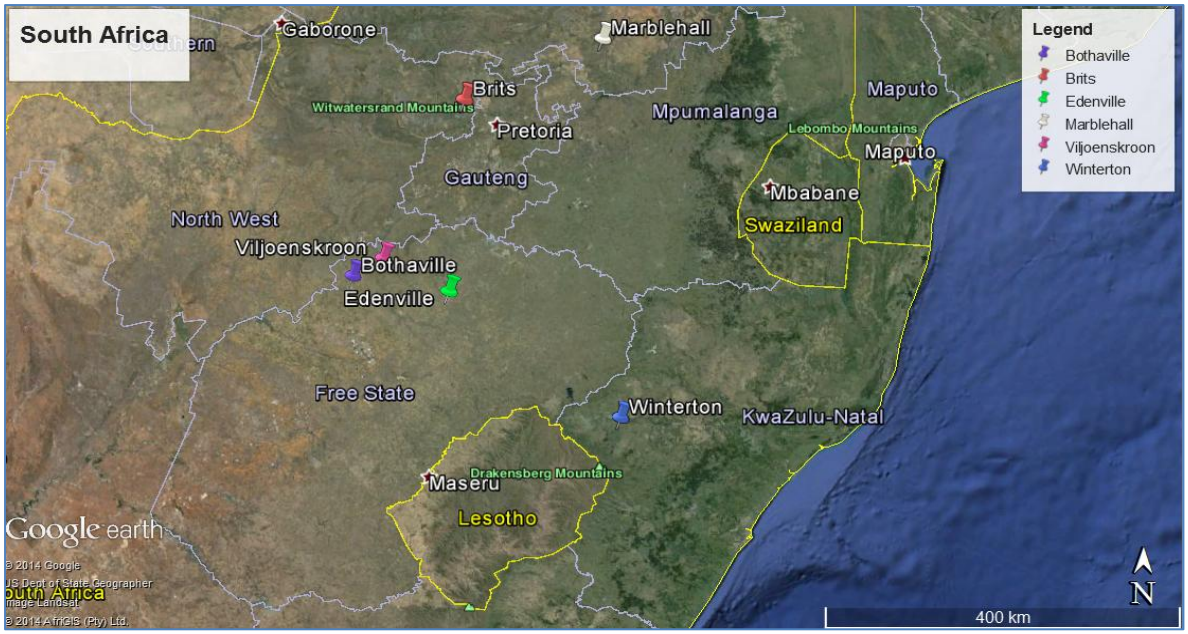

Figure 3.1: Illustration of the six localities where samples were obtained in the soybean production areas of South Africa. Respective provinces are described in Table 3.1 (Google Earth Pro, Google Inc).

From each of the three ecosystems at the various localities two different rhizosphere soil samples were obtained from the 80 samples collected per site. One was to determine microbial community structure using phospholipid fatty acid (PLFA) analyses, and the second to determine nematode diversity through extraction, identification and quantification. Samples were collected from soybean crops that were in the reproductive stage (R6 to R7) (Liebenberg, 2012).

Table 3.1: Description of localities and sites.

Locality	Date of sampling	Site/Ecosystem
Bothaville (Free State province)	20/02/2013	Conventional soybean
	15/02/2013	RoundUp ® Ready soybean
	20/02/2013	Natural veld
Brits (North-West province)	25/03/2013	Conventional soybean
	25/03/2013	Natural veld
Edenville (Free State province)	12/03/2013	RoundUp ® Ready soybean
	12/03/2013	Conventional soybean
	12/03/2013	Natural veld (1)
	1203/2013	Natural veld (2)
Marble Hall (Limpopo province)	19/03/2013	Conventional soybean
	19/03/2013	RoundUp ® Ready soybean
	20/03/2013	RoundUp ® Ready soybean
	20/03/2013	Conventional soybean (1)
	20/03/2013	Conventional soybean (2)
	19/03/2013	Natural veld (1)
	20/03/2013	Natural veld (2)
	20/03/2013	Natural veld (3)
Viljoenskroon (Free State Province)	11/02/2013	RoundUp ® Ready soybean
	11/02/2013	Natural veld
Winterton (Kwazulu-Natal Province)	02/04/2013	RoundUp ® Ready soybean
	02/04/2013	Conventional soybean
	02/04/2013	Natural veld (1)
	02/04/2013	Natural veld (2)

3.2. Microbial community structure analysis.

3.2.1. Sampling procedure for microbial community structure analysis.

Four composite samples from soils of RR and CS soybean fields as well as the surrounding natural veld were prepared directly from 80 rhizosphere samples taken randomly across each of the 23 sites to obtain representative data. The samples were obtained during the months of February to April 2013, from R6 to R7 soybean during the soybean production season. The top layer of organic matter was removed with a small garden spade and approximately 50 g soil was obtained from the upper 10 to 15 cm of rhizosphere soil.

Soil samples were placed in plastic bags, on ice and transported to the laboratory at the North-West University, Potchefstroom. The four composite soil samples from each site were stored at -80 °C for 24 hours. After 24 hours, the samples were freeze-dried, mixed thoroughly, homogenised and passed through a 2 mm mesh sieve (Drenovsky *et al.,* 2004) before being analysed.

3.2.2. Phospholipid fatty acid analysis.

Microbial biomass was determined by the total extractable PLFAs and the microbial community structure was determined by groups of signature lipid biomarkers (McKinley *et al*., 2005). This method was used to detect differences in the microbial community structure (Zelles, 1999) in soils from fields where RR and CS soybean were planted and those of the surrounding natural veld.

Glassware used in PLFA analysis was washed with phosphate-free soap and rinsed with tap water, distilled water and finally nano-pure water. The glassware was then baked in a muffle furnace for 4 hours at 450 °C. For extraction of lipids, 5 g soil was weighed off from the freeze-dried samples. Lipids were extracted with a single-phase chloroform-methanol-phosphate buffer system, in a ratio of 1:2:0.8 (v:v:v) (5 ml:10 ml:4 ml), using a modified Bligh and Dyer (1959) method as cited by McKinley *et al.* (2005). Nano-pure water (5 ml) and chloroform (5 ml) were then added to each sample and centrifuged to separate the two phases. The chloroform phase (containing the lipids) was then extracted, reduced by evaporation and stored at -20 °C until lipid fractionation.

Silicic acid columns were used to fractionate the total lipids (dissolved in chloroform) into neutral lipids, glycolipids and polar lipids using chloroform (5 ml), acetone (5 ml), and methanol (5 ml), respectively. The polar lipids (including phospholipids) were isolated in

methanol and transesterified into fatty acid methyl esters (FAMEs) using alkaline methanolysis (McKinley *et al.,* 2005).

The FAMEs were dissolved in hexane (100 µl) and analysed by capillary gas chromatography with flame ionisation detection on an Agilent 7890A gas chromatograph, using a 60 m SPB-1 column. The injector and detector temperature was maintained at 270 °C and 290 °C, respectively. Hydrogen was the carrier gas used and sample injection (1 µl of each sample was injected) was splitless. Gas flow was at a constant pressure of 300 kPa. Column temperature was programmed to start at 60 °C for 2 minutes, ramped at a rate of 10°C / minute to 150 °C and then increased at 3 °C / minute to 320 °C. Methyl nonadecanonate (19:0) was used as the internal standard in this procedure. Definitive peak identification was made for representative samples by gas chromatography / mass spectrometry using an Agilent 6890 series ll gas chromatograph interphased with an Agilent 5973 mass selective detector under the same column and temperature programme described previously. Mass spectra were determined by electron impact at 70 eV (McKinley *et al.,* 2005).

A:BωC represents the format of fatty acid nomenclature. Fatty acids are designated in terms of the total number of carbon atoms ("A") and the number of double bonds ("B") followed by the position of the first double bond from the aliphatic (methyl) end ("C") (Frostegård *et al.,* 1996). The prefixes "c" and "t" indicate *cis* and *trans* geometry, respectively. The prefixes "a" and "i" refers to the anteiso- and iso-branching, respectively, whereas "br" refers to an unknown branch position. "OH" refers to the hydroxyl group and "Me" refers to the methyl group. The cyclopropyl fatty acids are represented by "cy". For example, 10Me indicates a methyl group on the 10^{th} carbon atom from the carboxyl end (Xue *et al.,* 2005; Zelles, 1999). The various PLFAs present in the samples, were used to calculate specific ratios and representative groups of microorganisms, which are described in section 3.4.

3.3. Nematode population and diversity analysis.

3.3.1. Sampling procedure for nematode analyses.

To determine the effect of glyphosate on nematode populations and diversity in in RR, CS and NV soils, rhizosphere soil samples were taken from each of these ecosystems. A total of 80 samples were collected from each site concurrently with those used for PLFA analyses (see 3.2.1). These 80 samples per site were combined in the laboratory to obtain 20 soil composite samples. The soil samples were stored in a walk-in fridge set at a temperature of between 6 °C and 8 °C until nematode extractions were done.

After preparation of composite samples, nematodes were extracted using Cobb's decanting and sieving method (Mahesh *et al.,* 2012) followed by the sugar centrifugal-flotation method (Jenkins, 1964). Parastic and non- parasitic nematodes were then counted by using a dissection microscope. A compound microscope (1 000x magnification) was used to classify nematodes into various functional feeding groups based on specific morphological characteristics. Ultimately, genus-level identifications were done for the majority of the nematodes extracted.

3.3.2. Nematode extraction.

In this study plant-parasitic as well as non-parasitic / beneficial nematodes were extracted from soil samples using a combination of two extraction methods. The latter were Cobb's decanting and sieving method as described by Mahesh *et al.* (2012); followed by the sugar centrifugal-flotation method (Jenkins, 1964).

3.3.2.1. Decanting and sieving method.

The principle of this method is based on the density and size of the nematodes in the soil samples and permits the recovery of active, sluggish and some dead nematodes (Mahesh *et al.,* 2012). During this method, approximately 20% of nematodes originally present in the soil samples are lost.

After soaking the soil samples in water, soil particles with a diameter of more than 1 mm were removed by passing the samples through a 710 µm-mesh sieve that was nested on a 5 L-bucket. The residue on the sieve was washed with tap water for about 2 minutes and then discarded. The bucket was filled up to 5 L with tap water and the soil sample was thoroughly mixed and allowed to settle for about 30 seconds. The mixture was then decanted through a 25 µm-mesh sieve, leaving behind the sediment that had settled to the bottom of the bucket. The procedure was repeated once more. The nematodes and fine soil particles retained on the sieves were washed into 40 ml centrifuge tubes and centrifuged for 5 minutes at 1800 rpm. After centrifugation the supernatant was decanted and discarded. Nematodes were subsequently present at the bottom of the centrifuge tubes and these samples were then immediately subjected to the sugar centrifugal-flotation method (Jenkins, 1964).

3.3.2.2. Sugar centrifugal-flotation method.

The principle of this method is based on the specific gravity of nematodes. Terrestrial nematodes have a specific gravity of about 1.08 and marine nematodes about 1.13. After centrifugation in water, only organic materials with a specific gravity lower than 1.0 will remain in suspension and can be discarded. When centrifuged in a solution with a higher

specific gravity than the nematodes' density (i.e. sucrose solution with a specific gravity of 1.15) the nematodes will remain in suspension and be separated from soil particles with a specific gravity larger than 1.15.

A sucrose solution with a specific gravity of 1.15 was added to the centrifuge tubes, containing the nematodes. The sucrose solution was prepared by adding 624 g sugar to 1000 ml tap water (Jenkins, 1964). This solution and the sediment containing the nematodes at the bottom of the centrifuge tubes were then thoroughly stirred with a spatula and centrifuged for 1 minute at 1800 rpm. After centrifugation, the supernatant (with nematodes in suspension) was decanted on a 25 µm-mesh sieve and gently rinsed with tap water in order to remove the sucrose as quickly as possible. Suspension in the sucrose solution for too long (longer than 2 minutes) may distort and kill the nematodes due to the osmotic pressure (Jenkins, 1964). This will compllicate counting and identification of nematodes. The nematodes were finally washed into a sample bottle for examination and counting.

3.3.3. Nematode counting and identification.

After extraction, nematodes were counted using a stereomicroscope at 110 x magnification and assigned into functional groups. These functional groups included bacterivores, predators, fungivores and plant-parasitic nematodes based on specific morphological features. Nematodes were killed and fixed with an 4% formaldehyde solution (Bridge and Starr, 2007). To enable identification of nematodes to genus level, temporary slides were prepared by fishing several nematodes from the various functional groups during the counting process and placing it in a drop of tap water on a microscope slide. The microscope slide was then covered with a cover slip and sealed with clear fingernail polish (Ferris and Matute, 2003). Parasitic and non-parasitic nematode individuals were finally identified to genus level as described by Heyns (1971) using a light microscope (1000x magnification). Nematode identification was based on morphological characteristics, which included basal bulb position and structure; lip region (head) structure and position; tail shape; amphid presence or absence; spinneret absence or presence; stoma (pharynx) shape and size; vulva postion (females); and bursa presence or absence (male) (Heyns, 1971). Some of these characteristics are illustrated in Figure 3.2.

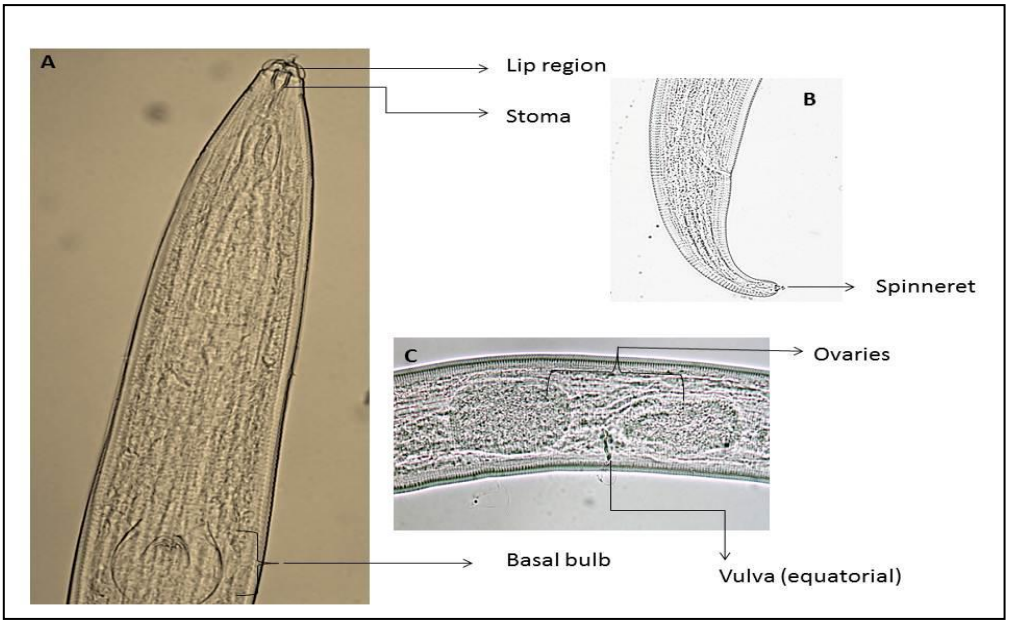

Figure 3.2: Photographs of *Plectus* spp. female A: Anterior part including basal bulb, lip region and stoma; B: Tail with spinneret; C: Vulva position with two opposing ovaries (1000 x magnification) (Photographs by Chantelle Jansen).

3.4. Soil physical-chemical properties.

Physical and chemical analyses of the soil samples were conducted by an independent laboratory, Eco-Analytica (North-West University, Potchefstroom) according to standard procedures (Table 3.2).

Extractable and exchangeable micro-elements were determined using the 0.02M $(NH_4)_2$ EDTA.H_2O method (Black, 1965b). The P-Bray 1 extraction method was used to determine the P concentration in the soil samples. Exchangeable cation concentration was determined using the 1 M NH_4-Acetate extraction method at a pH of 7 (Black, 1965b).

The particle size distribution of the soil was determined using a hydrometer-method with a soil hydrometer (Black, 1965a).

Table 3.2: Soil physical-chemical properties analysed for this study.

Physical – Chemical properties		
Nutrient status	Exchangeable cations	Particle size
Ca (mg/kg)	Ca (cmol(+)/kg))	>2 mm (%)
Mg (mg/kg)	Mg (cmol(+)/kg))	Sand (% < 2mm)
K (mg/kg)	K (cmol(+)/kg))	Silt (% < 2mm)
Na (mg/kg)	Na (cmol(+)/kg))	Clay (% < 2mm)
P (mg/kg)	CEC (cmol(+)/kg))	
pH (H_2O)	S-Value (cmol(+)/kg))	
EC (mS/m)	Base saturation (%)	
Organic % C	NO_3 (mg/l)	

The exchangeable ion status was then used to determine the percentage base saturation, which expresses the content of exchangeable bases as a percentage of the cation exchange capacity (CEC) with the equation:

$$\text{Base saturation (\%)} = (X_b / \text{CEC}) * 100$$

Where X_b is the sum of the exchangeable bases (Ca, Mg, K, and Na) and CEC (measured at pH of 7.0, using a 1 M Na-acetate method) is the cation exchange capacity. NO_3 concentration was determined using the 1:2 water extraction method (Sonneveld and Van Den Ende, 1971). The pH (H_2O/KCl) was determined with a 1:2.5 extraction method. Electrical conductivity (EC) was determined by saturated extraction.

3.5. Statistical analyses.

3.5.1. Phospholipid fatty acid data analyses.

To analyse the results obtained during PLFA analysis, various ratios and microbial biomass was calculated. The ratios included the fungal to bacterial biomass ratio (F / B ratio); The ratios of saturated to unsaturated fatty acids (sat / unsat); gram + to total PLFAs (gram + / total); iso – to anteiso branched PLFAs (iso / anteiso) and finally trans to cis-monoenoic

unsaturated fatty acids (*trans*- to *cis*). The PLFA markers used for these calculations are indicated in Table 3.3.

Table 3.3: Phospholipid fatty acid markers for biomass and various ratios calculated in this study.

Ratios and microbial biomass calculated	PLFA markers used
Bacterial biomass[1]	Sum of 15:0, i15:0, a15:0, i16:0, 16:1ω9, 16:1ω7t, 17:0, i17:0, a17:0, cy17:0, 18:1ω7 and cyc19:0
Fungal biomass[1]	18:2ω6
F / B ratio	Fungal biomass / Bacterial biomass
Gram + / total PLFAs ratio[2]	i15:0 + a15:0 + i16:0 + i17:0 + a17:0 / total biomass
Iso / anteiso PLFAs ratio[2]	i15:0 +i17:0 / a15:0 + a17:0
Sat / unsat PLFAs ratio[3]	14:0 + 15:0 + 16:0 + 17:0 + 18:0 + 19:0 + 20:0 + 21:0 + 21:0 + 22:0 + 23:0 + 24:0 / 14:1ω5c + 16:1ω9c + 16:1ω7c + 16:1ω7t + 16:1ω5c + 18:1ω9c + 18:1ω7c + 18:1ω7t + 18:1ω5c + 20:2ω6 + 20:1ω9c
Trans / *cis* PLFAs ratio[2]	16:1ω7t / 16:1ω7c

[1]Frostegård *et al.* (1996); Ratcliff *et al.* (2006).

[2] McKinley *et al*. (2005).

[3]Bach *et al*. (2010).

3.5.2. Nematode data analyses.

Nematode data was log (x+1) transformed to limit natural variation (Liphadzi *et al.,* 2005). Coloniser-persister (cp) values were assigned to all nematode taxa according to Bongers and Bongers (1998) (see Table 2.4 section 2.5.2 in Chapter 2). These cp-values were then used to calculate several nematode indices including the maturity index (MI), the channel index (CI), the enrichment index (EI) and the structure index (SI).

The MI was calculated as the weighted mean of the cp-values of non-parasitic nematodes (Bongers and Bongers,1998):

$$MI = \Sigma_{i=1} v(i) \times f(i)$$

where v(i) is the cp-value of the i'th taxon in the sample and f(i) is the frequency of the i'th taxon. The CI was calculated as (Ferris *et al*., 2004; Ferris and Matute, 2003):

$$100(k_eFu_2/(k_eBa_1+k_eFu_2))$$

where k_e is the enrichment weightings of the bacterivores with cp-1 value (Ba_1) and the fungivores with a cp-2 value (Fu_2). According to Ferris *et al*. (2004) the k_e for Ba_1 and Fu_2 is 3.2 and 0.8 respectively. The EI and SI were calculated as:

$$EI = 100(e/(e+b))$$

where e is calculated as $\Sigma k_e n_e$ (k_e is the structure indicator weights (according to Ferris *et al*., 2004) and n_e are the abundance of nematodes in those guilds) (Ferris and Matute, 2003).

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

where s is calculated as $\Sigma k_s n_s$ (k_s is the structure indicator weights (according to Ferris *et al*., 2004) and n_s are the abundance of nematodes in those guilds) (Ferris and Matute, 2003).

A faunal analysis was performed using the SI and EI indices in Microsoft Excel 2010. This was based on the feeding strategies of the nematodes found as well as the enrichment weightings (Ferris and Matute, 2003).

Data obtained was subjected to parametric and non-parametric statistical analysis using Statistica 10.0 (Statsoft Inc., Tulsa, Oklahoma, USA, 2012). The data was tested for normality using Shapiro-Wilk's test. Normally distributed (parametric) data was then subjected to one-way breakdown ANOVA using Tukey's Honest Significant Difference (HSD) test. The latter was to test for statistically significant differences between the sample sites ($p<0.05$). Non-parametric data was subjected to the Kruskal-Wallis ANOVA and the Median test was applied to determine statistically significant differences between the sample sites.

The relationship between microbial community and nematode community structures was determined using multivariate statistical analyses by using the statistical programme CANOCO (Canoco for Windows 4.56, Biometris – Plant Research International, Wageningen, The Netherlands, 2009).

Chapter 4: Microbial community structures in rhizosphere soil samples - Results and discussion.

Three ecosystems were investigated to determine the microbial community structure. Each of these ecosystems at the various localities was assigned a specific code. CS refers to conventional soybean fields, RR refers to RoundUp ® Ready soybean fields and NV refers to natural veld. In Table 4.1 below the different localities with their respective codes for each site are listed.

Table 4.1: Various localities where rhizosphere soil was sampled during this study with their respective site codes for the RoundUp ® Ready (RR) and conventional soybean (CS) fields as well as adjacent natural veld (NV) treatments sampled.

Locality	Site code
Bothaville	RR1; CS1; NV1
Brits	CS2; NV2
Edenville	RR2; CS3; NV3; NV4
Marblehall	RR3; RR4; CS4; CS5; CS6; NV5; NV6; NV7
Viljoenskroon	RR6; NV8
Winterton	RR5; CS7; NV9; NV10

4.1. Soil physical-chemical properties.

The particle size distribution of each soil sample is provided in Table 4.2. The samples collected consisted primarily of sandy soils. The RR soils ranged from 61.4 % (RR6) to 94.5 % (RR1) sand; CS soil ranged from 44.8 % (CS7) to 94.6 % (CS1) and the NV had a sand content of between 45.9 % (NV10) and 94.7 % (NV1). Bothaville soil samples (RR1, CS1 and NV1) had the highest sand content, whereas Winterton (RR5; CS7; NV9; NV10) soil samples had the lowest sand content (Table 4.2).

Table 4.2: Particle size distribution of the various sites in soils of RoundUp ® Ready (RR) and conventional soybean (CS) fields and adjacent natural veld (NV).

Site	Sand	Silt	Clay
		(% < 2mm)	
RR1	94.5	0.7	4.7
RR2	85.9	3.4	10.7
RR3	86.1	1.4	12.6
RR4	69.2	9.3	21.5
RR5	61.4	23.3	15.3
RR6	94.1	1.2	4.7
CS1	94.6	0.7	4.7
CS2	81.4	7.6	11.0
CS3	70.7	9.3	20.0
CS4	91.0	3.7	5.2
CS5	88.7	1.3	10.0
CS6	78.6	8.7	12.7
CS7	44.8	38.4	16.7
NV1	94.7	0.7	4.6
NV2	79.9	5.5	14.6
NV3	59.3	19.1	21.6
NV4	86.6	3.2	10.1
NV5	88.7	3.7	7.6
NV6	84.9	6.7	8.4
NV7	91.2	3.7	5.1
NV8	84.4	5.9	9.7
NV9	81.2	10.4	8.5
NV10	45.9	31.4	22.8

In Table 4.3 the physical and chemical properties of the various soil samples are provided. Only a few selected properties will be discussed. The percentage organic carbon (C) in the RR fields ranged from 0.20 % (RR1) to 1.62 % (RR5); for the CS fields 0.22 % (CS1) to 2.77 % (CS7); and finally for the NV % organic C ranged from 0.21 % (NV1) to 3.89 % (NV3). Overall the NV soil samples generally showed a higher organic C compared to the agricultural fields (RR and CS). It should also be noted that samples collected from Winterton (RR5,CS7, NV9 and NV10) contained the highest organic C with the exception of one NV in Edenville (NV3). The rest of the localities had similar organic C.

Table 4.3: Chemical properties of the various sites sampled from soils of RoundUp ® Ready (RR) and conventional soybean (CS) fields and adjacent natural veld (NV).

Nutrient status

Site	Ca	Mg	K	Na	P	pH(H_2O)	EC[1](mS/m)	Organic %C
			(mg/kg)					
RR1	445.5	78.0	194.0	0.5	168.9	6.77	30	0.21
RR2	396.0	67.0	268.0	3.0	92.8	6.15	19	0.20
RR3	455.0	104.0	192.0	24.5	164.4	6.07	123	0.40
RR4	967.5	237.5	345.5	35.5	151.1	6.64	46	0.84
RR5	751.0	120.0	85.5	0.5	335.3	5.67	18	1.62
RR6	381.0	95.5	329.5	5.1	295.2	6.82	130	0.20
CS1	373.0	96.0	203.0	0.5	203.1	6.43	36	0.22
CS2	1698.5	345.5	290.5	58.5	399.4	7.49	47	0.76
CS3	511.5	212.5	292.0	9.5	117.9	4.97	31	0.49
CS4	540.5	148.5	146.0	23.0	117.9	6.74	52	0.54
CS5	545.5	170.5	179.5	30.0	60.5	6.62	48	0.55
CS6	1000.5	401.5	389.5	34.5	66.8	7.05	53	0.58
CS7	1826.5	138.5	550.5	5.5	531.6	5.92	41	2.77
NV1	381.0	106.5	204.5	0.5	204.0	6.48	30	0.21
NV2	1434.0	304.0	496.5	2.0	499.5	7.11	36	1.51
NV3	1486.0	400.5	419.0	60.5	39.5	5.92	25	3.89
NV4	222.0	51.5	271.0	1.5	86.0	5.64	36	0.34
NV5	826.0	212.0	291.0	176.0	335.8	5.93	390	2.58
NV6	665.5	227.5	389.5	20.0	401.9	6.52	119	1.76
NV7	1012.0	244.0	508.5	14.5	155.7	6.83	81	1.80
NV8	432.5	194.5	396.5	9.5	227.3	6.14	54	1.14
NV9	1782.0	400.5	380.5	20.5	84.6	6.85	36	2.49
NV10	887.0	208.0	295.0	4.5	358.4	5.65	14	2.59

Exchangeble cations

	Ca	Mg	K	Na	CEC[2]	S-value	Base saturation (%)	pH(H_2O)	NO_3 (1:2Extract) mg/l
			(cmol(+)/kg)						
RR1	2.22	0.64	0.50	0.00	6.51	3.36	51.66	6.77	13.18
RR2	1.98	0.55	0.69	0.01	6.28	3.23	51.41	6.15	4.67
RR3	2.27	0.86	0.49	0.11	7.11	3.73	52.37	6.07	173.69
RR4	4.83	3.30	1.00	0.15	9.32	9.45	101.33	7.05	42.01
RR5	3.75	0.99	0.22	0.00	11.02	4.96	44.99	5.67	19.66
RR6	1.90	0.79	0.84	0.02	6.57	3.55	54.13	6.82	68.78
CS1	1.86	0.79	0.52	0.00	5.70	3.17	55.64	6.43	16.33
CS2	8.48	2.84	0.74	0.25	12.02	12.32	102.50	7.49	20.78
CS3	2.55	1.75	0.75	0.04	10.64	5.09	47.84	4.97	11.59
CS4	2.70	1.22	0.37	0.10	7.16	4.39	61.39	6.74	64.67
CS5	2.72	1.40	0.46	0.13	8.00	4.72	58.95	6.62	45.64
CS6	4.99	1.95	0.89	0.15	9.74	7.82	80.32	6.64	37.56
CS7	9.11	1.14	1.41	0.02	23.91	11.69	48.88	5.92	53.01
NV1	1.90	0.88	0.52	0.00	5.86	3.30	56.38	6.48	14.49
NV2	7.16	2.50	1.27	0.01	10.86	10.94	100.72	7.11	2.57
NV3	7.42	3.30	1.07	0.26	24.47	12.05	49.24	5.92	3.59
NV4	1.11	0.42	0.69	0.01	7.79	2.23	28.66	5.64	24.31

NV5	4.12	1.74	0.75	0.77	14.99	7.38	49.22	5.93	3.76
NV6	3.32	1.87	1.00	0.09	10.56	6.28	59.48	6.52	24.66
NV7	5.05	2.01	1.30	0.06	10.68	8.43	78.90	6.83	42.24
NV8	2.16	1.60	1.02	0.04	8.80	4.82	54.74	6.14	51.76
NV9	8.89	3.30	0.98	0.09	14.68	13.25	90.29	6.85	1.11
NV10	4.43	1.71	0.76	0.02	15.85	6.91	43.62	5.65	0.59

[1]EC = Electrical conductivity

[2]CEC = Cation exchange capacity

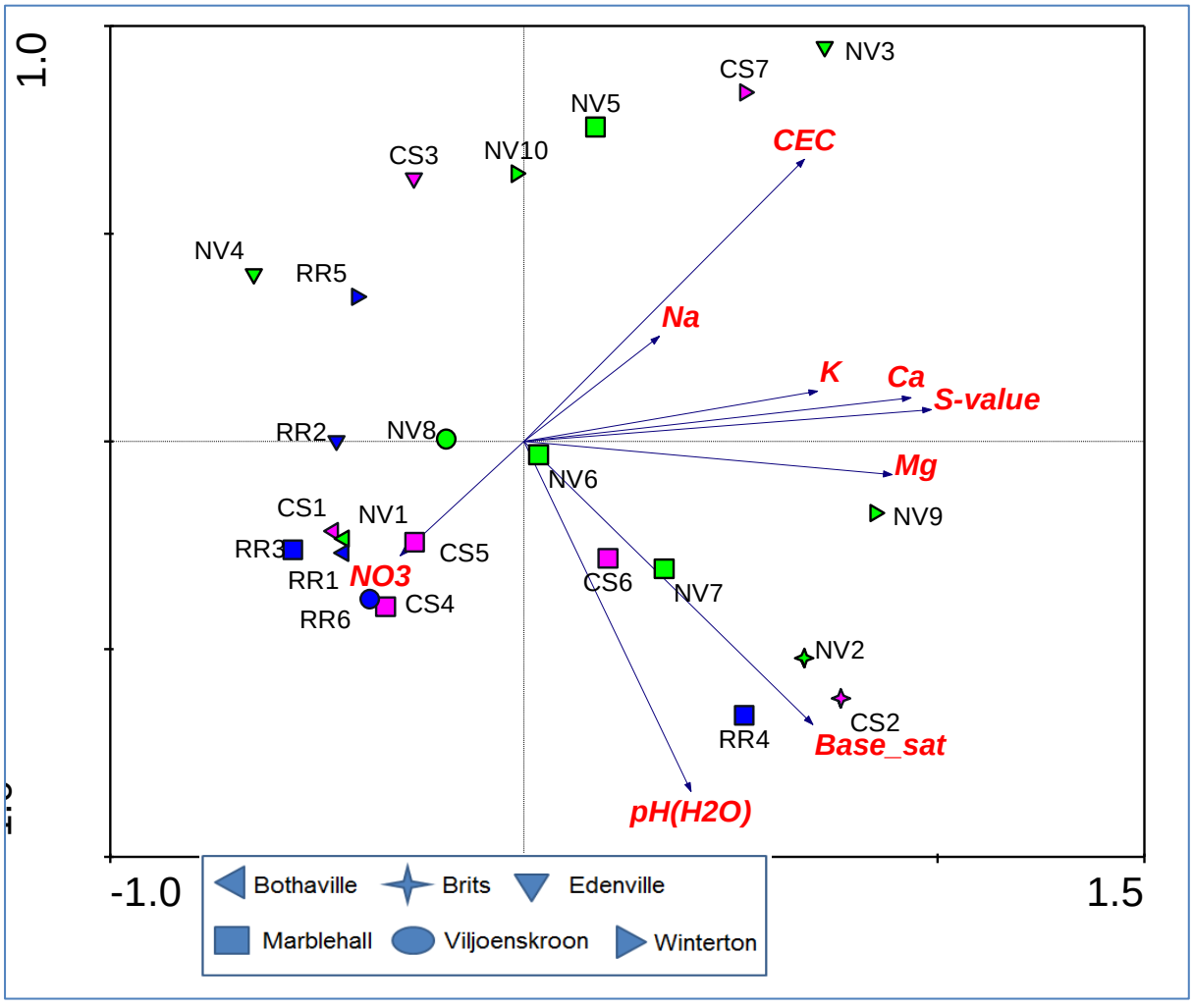

Figure 4.1: Principal Component Analysis (PCA) ordination diagram illustrating the relationship between the exchangeable cations and the different sites. Key to symbols: Green – natural veld (NV); Blue – RoundUp ® Ready (RR) soybeans; and Pink – conventional soybeans (CS); Na - Sodium; K - Potassium; Ca - Calcium; Mg - Magnesium; Base_sat – Base-saturation; NO_3 - Nitrate.

The relationship between the different sites and the exchangeable cations are illustrated based on a principal component analysis (PCA) (Figure 4.1). The different symbols in the

ordination diagram represent the different localities. While some of the localities clustered together, there were no trends distinguishing samples from RR, CS or NV sites based on the physical and chemical soil properties. The three fields in Bothaville clustered together and had similar physical and chemical soil properties (Tables 4.2 and 4.3). The same applied to samples from Brits and Viljoenskroon.

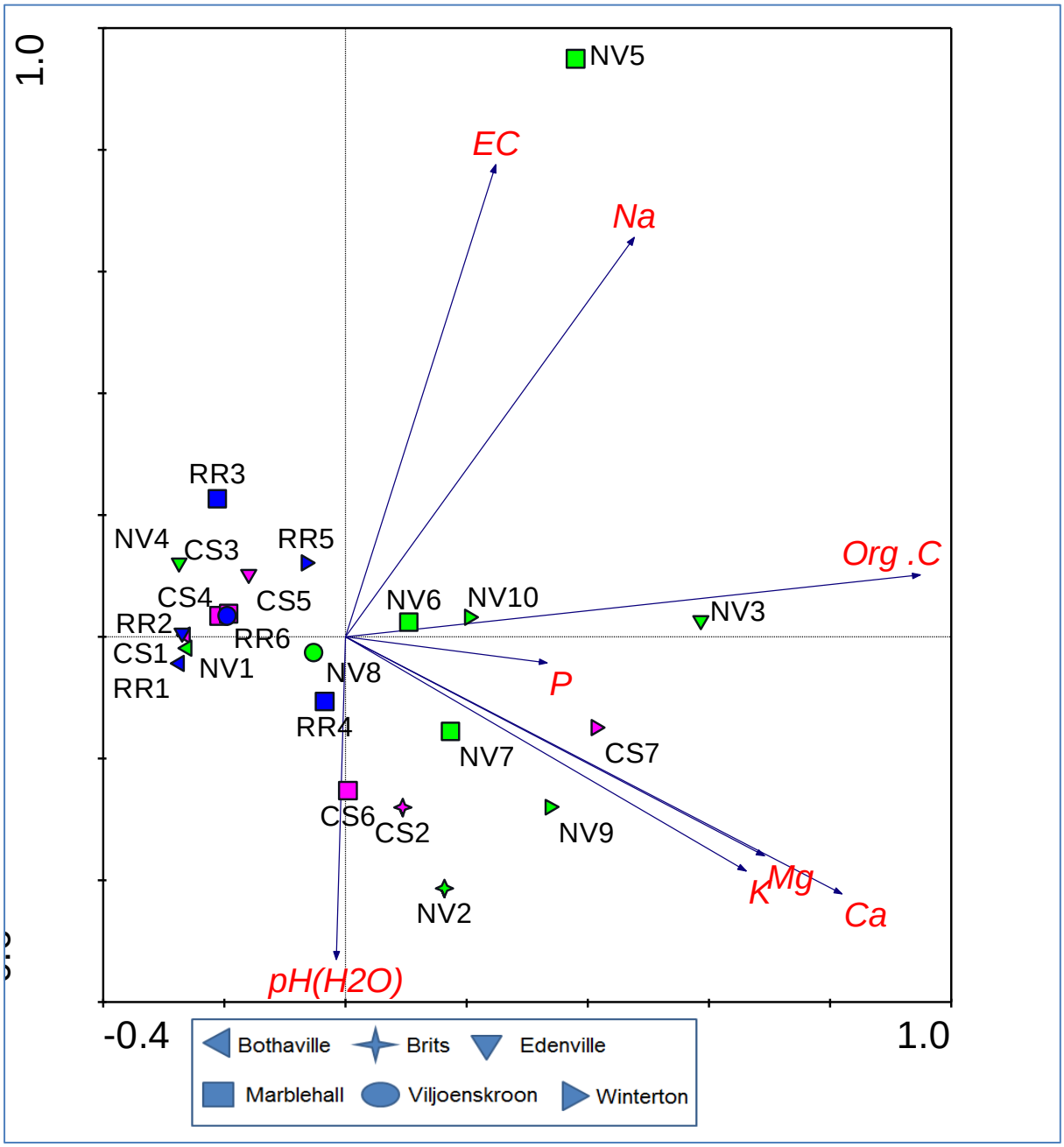

Figure 4.2: Principal Component Analysis (PCA) ordination diagram illustrating the relationship between the nutrient status and the different sites where soils samples were obtained during this study. Key to symbols: Green – natural veld (NV); Blue – RoundUp ® Ready (RR) soybeans; and Pink – conventional soybeans (CS); EC – electronic conductivity; K - Potassium; P - phosphorus ; Org C – Organic carbon; Mg - Magnesium; Ca - Calcium; Na – Sodium.

In Figure 4.2, relationships between the different samples and the nutrient status of the soils are illustrated based on a PCA. The RR and CS samples clustered together, illustrating that the nutrient status of both showed similarities. The analysis indicated that the nutrients status of the NV fields differed from each other and from that of the agricultural fields (RR and CS). Samples from the same localities clustered together, indicating similar nutrient status properties of soils in each locality (Table 4.3).

4.2. Estimated viable microbial biomass and fungal to bacterial biomass ratio.

Estimated viable microbial biomass.

The estimated viable biomass is shown in Figures 4.3 and 4.4. For the RR soybean fields the biomass ranged between 5224.38 (RR1) and 28718.51 (RR5) pmol.g^{-1} (Figure 4.3). In the CS fields, between 6874.89 (CS1) and 38.754.40 (CS7) pmol.g^{-1} (Figure 4.3) and the NV samples, from 6306.57 (NV1) to 64327.26 (NV3) pmol.g^{-1}. Tukey's honest significant difference (HSD) test showed some significant differences between localities ($p \leq 0.05$).

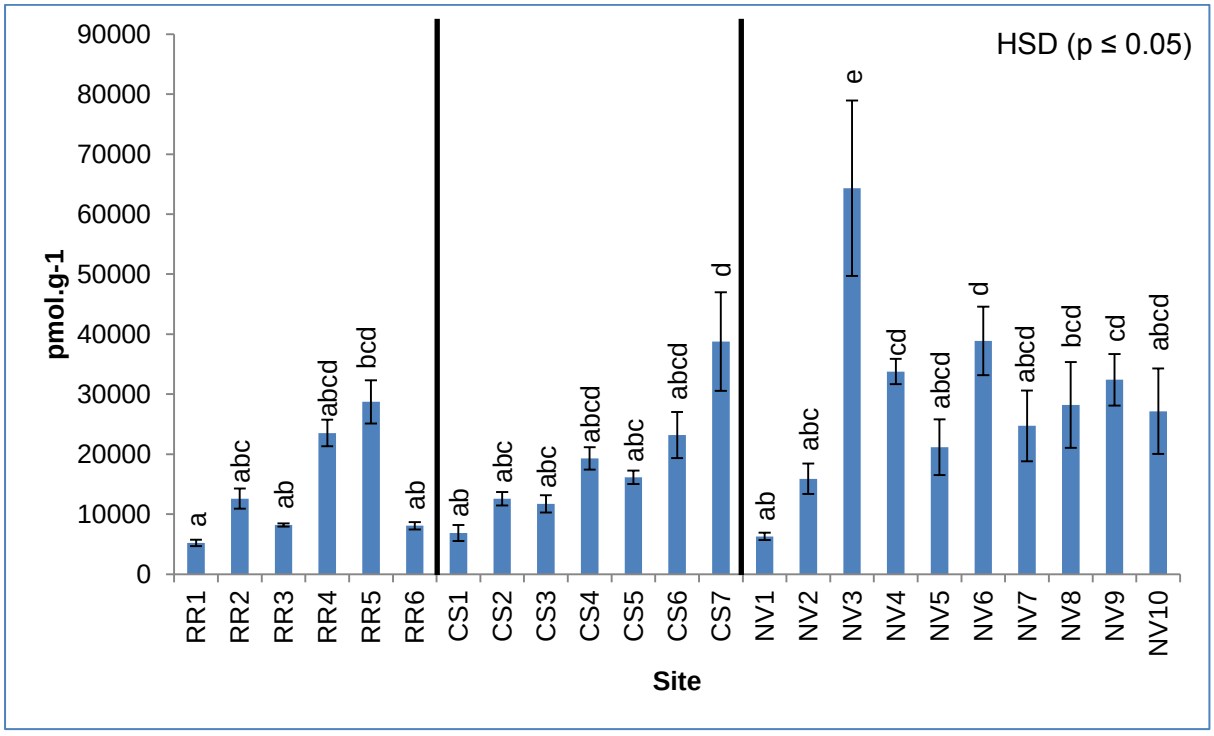

Figure 4.3: Estimated viable microbial biomass for each site at the different localities. Statistically significant differences are indicated by alphabetic letters using Tukey's HSD test at $p \leq 0.05$.The same letters indicate no significant differences. NV = Natural veld; RR = RoundUp ® Ready soybean; CS = Conventional soybeans. Values for each site are indicated in Table 1 in Appendix A.

Soil physical and chemical properties differed among the localities (see Table 4.3). This may explain some of the differences found between sites in various localities. The localities with the highest microbial biomass in the RR and CS fields (Winterton) also showed the highest

organic C, known to increase microbial biomass. The NV3 site (Figure 4.3) had the highest microbial biomass which corresponded with the highest organic C of 3.89 % (Table 4.3). The same trend was visible in the localities with low microbial biomass where sites in Bothaville had corresponding low organic C. Similar results were found in other studies (Altieri, 1999; Briar *et al.,* 2007; Fierer *et al*., 2003; Lupwayi *et al.,* 2007).

The average microbial biomass for each of the three ecosystems is illustrated in Figure 4.4. The NV showed the highest microbial biomass (29286.56 pmol.g^{-1}), followed by the CS (18368.73 pmol.g^{-1}) and RR ecosystems (14393.20 pmol.g^{-1}). Although differences were observed in terms of viable microbial biomass between the three ecosystems, these were not statistically significant ($p \leq 0.05$).

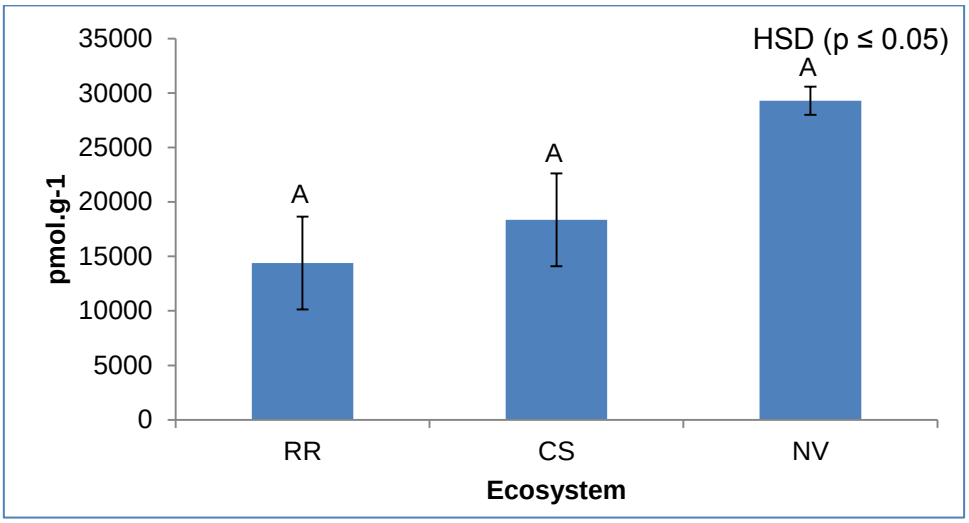

Figure 4.4: Average estimated viable microbial biomass for each ecosystem. NV = Natural veld; RR = RoundUp ® Ready soybean; CS = Conventional soybeans. Statistically significant differences are indicated by alphabetic letters ($p \leq 0.05$). The same letters indicate no significant differences. Values are shown in Table 2 in Appendix A.

In this investigation, no differences in microbial biomass could be observed in the soils of RR fields compared to that of CS fields (Figure 4.4). There were also no significant differences evident in microbial community structure between soils from NV and those from agricultural soils (RR and CS).

Related investigations have shown that soils subjected to glyphosate may not show altered levels of microbial biomass, but will experience changes in function of microbial communities (Sharma *et al.,* 2011). For this reason, further investigation is required to investigate the microbial community function and activity associated with RR crops and glyphosate application.

It has also been suggested that glyphosate application can lead to microbial stimulation and increased microbial biomass in soils (Lupwayi *et al.,* 2007). Due to the availability of glyphosate to rhizosphere microorganisms as a substrate for direct metabolism, microbial biomass and activity might be increased after increased glyphosate application (Kremer and Means, 2009). The weeds killed by glyphosate may also add organic C to the soil, which can increase microbial biomass due to the decomposition of such weeds (Lupwayi *et al.,* 2009). However, in the present study the microbial biomass in the RR ecosystem showed no significant differences from CS and NV ecosystems indicating that glyphosate application did not cause an increase in microbial biomass, since the NV and CS ecosystem showed higher microbial biomass than the RR ecosystem (Figure 4.4).

Fungal to bacterial biomass ratios.

The PLFA markers used to determine fungal and bacterial biomass are shown in Chapter 3, Table 3.3. The fungal to bacterial (F/B) biomass ratio for the various samples, is shown in Figures 4.5 and 4.6. This was determined by dividing the mole % of the fungal fatty acid marker by the sum of the mole % of the bacterial fatty acid markers.

For a majority of the sites in the different localities (Figure 4.5), the F/B ratio was below 0.01. However, there were some localities and sites with higher values. There were no significant ($p \leq 0.05$) differences in F/B ratio between the different sites excluding (RR1, RR3, RR6, CS1 and NV1). These low F/B ratios indicated that bacteria dominated the soils. It has been suggested that the application of glyphosate leads to an increase in bacterial biomass due to the addition of substrates that favour bacteria (Ratcliff *et al.,* 2006). Although all F/B ratios were low, some of the RR fields (RR1, RR3, RR6) differed significantly ($p \leq 0.05$) from the CS and NV fields. However, it should be noted that CS1 and NV1 also significantly differed from the rest of the sites indicating that glyphosate was not responsible for these differences as no glyphosate was applied to CS1 and NV1.

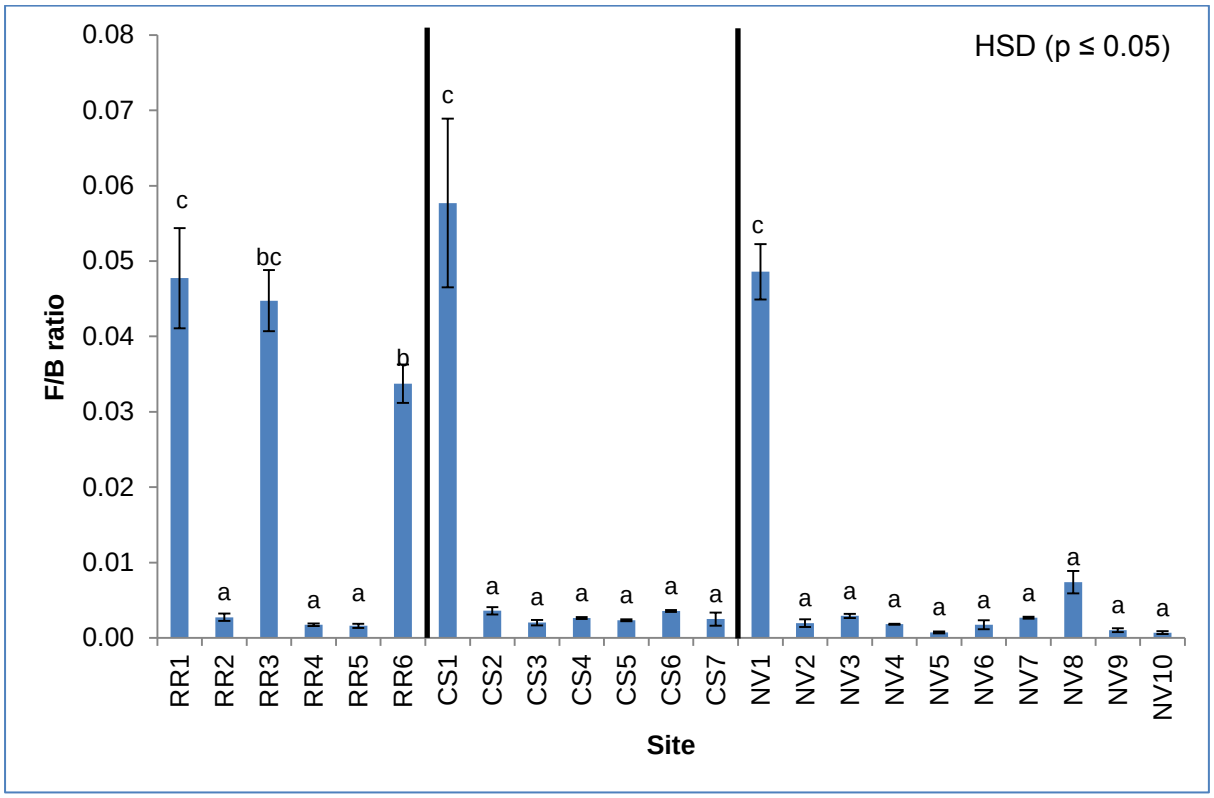

Figure 4.5: Fungal to bacterial (F/B) biomass ratio of the different sites at various localities. Statistically significant differences are indicated by alphabetic letters (p ≤ 0.05).The same letters indicate no significant differences. NV = Natural veld; RR = RoundUp ® Ready soybean; CS = Conventional soybeans. Values for each site are indicated in Table 1 in Appendix A.

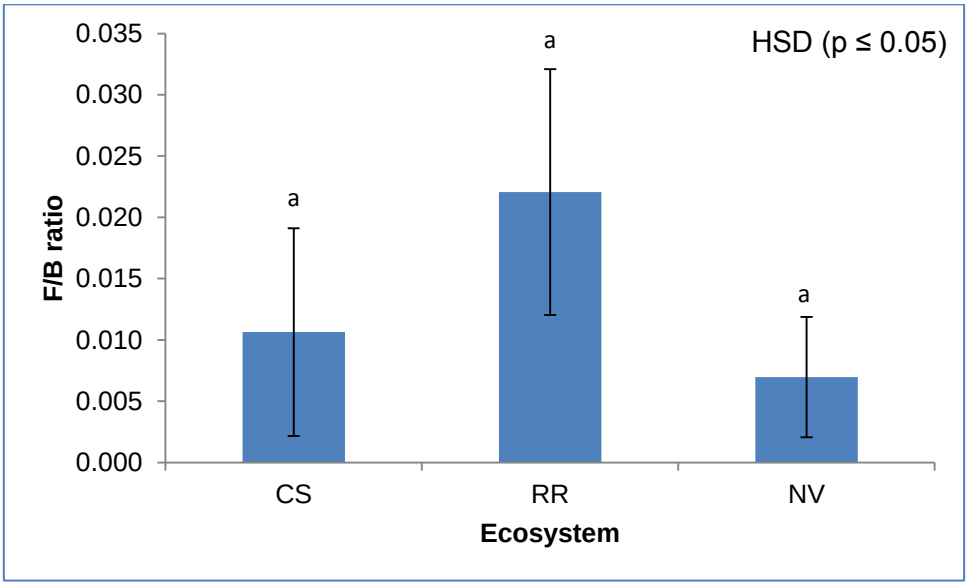

Figure 4.6: Average fungal to bacterial (F/B) biomass ratio of the different ecosystems. Statistically significant differences are indicated by alphabetic letters (p ≤ 0.05).The same letters indicate no significant differences. NV = Natural veld; RR = RoundUp ® Ready soybean; CS = Conventional soybeans. Values are shown in Table 2 in Appendix A.

Bacteria-dominated soils are common in agricultural systems that rely on application of fertilisers or manure (Ratcliff *et al.,* 2006), which supports results found in this study. In agricultural soils bacteria are generally more efficient colonisers of organic resources, especially where tillage results in newly available organic matter (Strickland and Rousk, 2010).

Although it was found that the F/B ratios in this study were not affected by glyphosate application, several other factors such as nutrient availability may affect the F/B ratios in soils (Strickland and Rousk, 2010). Soil pH is another factor that may influence the F/B ratio in both natural and agricultural soils. Soils with a low pH have been found to be fungal dominated as fungi are more acid tolerant than bacteria (Strickland and Rousk, 2010). However, in this study the soils with the higher pH values also had the highest F/B ratio (RR1, RR3, RR6, CS1, and NV1).

Another factor to consider when investigating the F/B ratios, is soil moisture. Fungal and bacterial biomass are both positively correlated with soil moisture in cultivated soils, however bacteria are less affected by dry and wet conditions (Strickland and Rousk, 2010). It must be taken into account that during the 2013 growing season, when samples for this study was collected, relatively dry conditions occurred which might explain the dominance of bacteria. During July 2012 to April 2013, below-normal precipitation to drought conditions occurred in South Africa. This was found especially in the North West province as well as the Free State province with rainfall between 50mm and 150mm. Compared to the same time in the following year (2014) where these regoins obtained an excess of 100 mm precipitation with between 150 mm to 200 mm rainfall (Agricultural Research Council, 2013; Agricultural Research Councill, 2014).

4.3. Microbial community structure.

The microbial community structure for each site was based on the mole % fraction of the various PLFA groups and is illustrated in Figures 4.7 and 4.8. When focussing on each individual site for the different localities in Figure 4.7 it is clear that there were some differences between localities regarding the microbial community structure. Bothaville (represented by RR1, NV1 and CS1) significantly differed from the other localities in terms of monounsaturated fatty acids and polyunsaturated fatty acids. It was also observed that this locality had the highest percentage polyunsaturated fatty acids, representative of fungi, among all the localities.

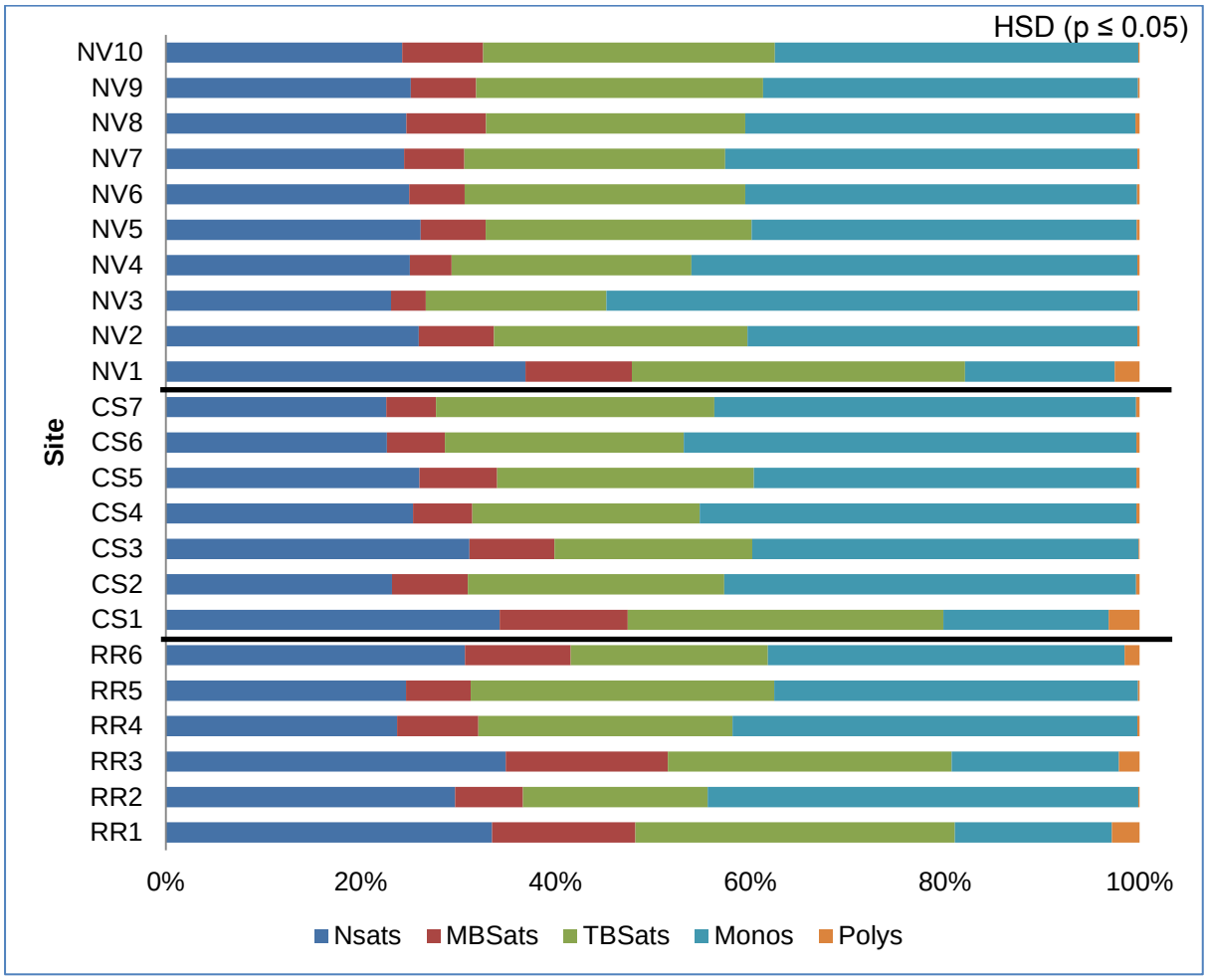

Figure 4.7: Microbial community structure determined by the mole % fraction of the major phospholipid fatty acid groups of each site in the various localities. NV = Natural veld; RR = RoundUp ® Ready soybean; CS = Conventional soybeans. Nsats = Normal saturated fatty acids; MBsats = Mid-branched saturated fatty acids; TBsats = Terminally-branched fatty acids; Monos = Monounsaturated fatty acids; Polys = Polyunsaturated fatty acids. Values and significant differences are shown in Table 4 in Appendix A.

When investigating the average of each PLFA group to determine the microbial community structure in the RR, CS and NV ecosystems (Figure 4.8) the following could be observed. Monounsaturated fatty acids, representative of gram-negative bacteria (Zelles, 1999) dominated in all three soil types making up as much as 38% of the microbial community structure. Microbial communities can be characterised based on growth strategies of the specific microorganisms present (Dorodnikov *et al.,* 2009). The r-strategists grow rapidly on easily available substrates, whereas K-strategists use the resources slowly but more efficiently and are able to metabolise more complex carbon substrates (Dorodnikov *et al.,* 2009; Waldrop *et al.,* 2000). Because these two groups of microorganisms are both abundant in soil, changes in the growth rate of the whole microbial community can reflect

whether the community shifts to r or K strategists (Dorodnikov *et al.,* 2009). Gram-negative bacteria are considered to be r-strategists and are the first colonisers in an environment. It has been found that in agricultural soils microorganisms are generally r-strategists, because they are characteristic of disturbed environments (Waldrop *et al.,* 2000; Zornoza *et al.,* 2009). Gram-positive bacteria are considered K-strategists and tolerate stress better than gram-negative bacteria. In this study, results correspond with previously published work, showing that the agricultural soils (CS and RR) were dominated by r-strategists (gram-negative bacteria). However, there is no evidence that the application of glyphosate influenced the r-strategists ecology as the soils of CS soybean cultivars and NV sites were also dominated by r-strategists.

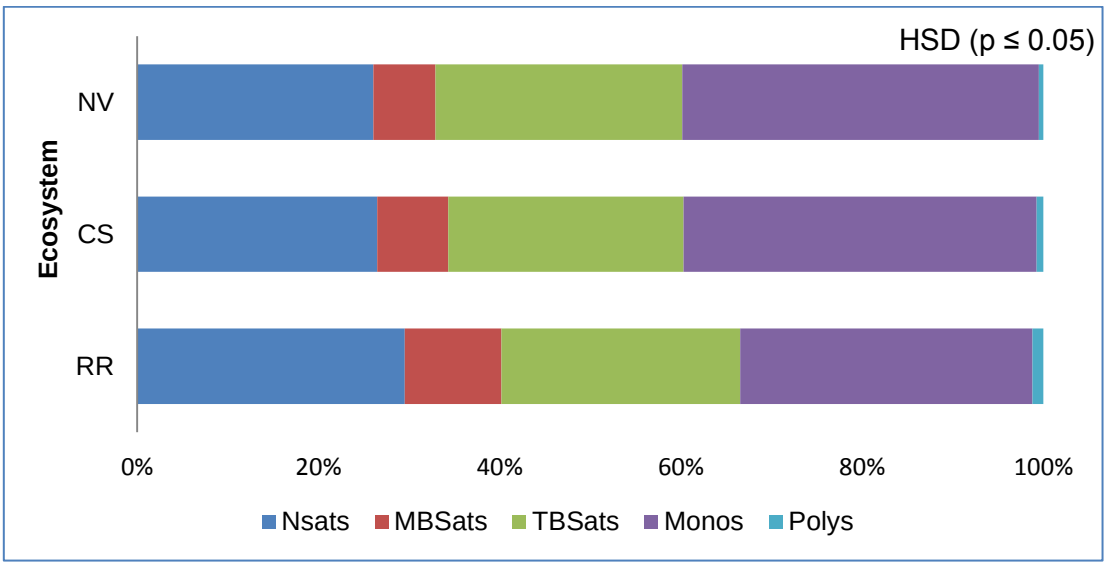

Figure 4.8: Microbial community structure determined by the average mole % fraction of the major phospholipid fatty acid groups of each ecosystem. NV = Natural veld; RR = RoundUp ® Ready soybean; CS = Conventional soybeans. Nsats = Normal saturated fatty acids; MBsats = Mid-chain branched saturated fatty acids; TBsats = Terminally-branched fatty acids; Monos = Monounsaturated fatty acids; Polys = Polyunsaturated fatty acids. Values and significant differences are shown in Table 3 in Appendix A.

In Figure 4.8 normal saturated fatty acids (found in all organisms) represented 28.9%, 25.9% and 25.4% of the RR, CS and NV ecosystems, respectively. Terminally branched saturated fatty acids (representative of gram-positive bacteria) represented 25.7%, 25.4% and 26.5% of the RR, CS and NV ecosystems, respectively. Mid-chain branched saturated fatty acids (representative of Actinomycetes) (Frostegård *et al.,* 1996) represented 10.4%, 7.6% and 6.6% of the RR, CS and NV ecosystems, respectively. In all three soils, polyunsaturated fatty acids which are representative of fungi, were the lowest of all fatty acid groups ranging from 0.46 % (NV) to 1.16 % (RR). Soil texture and pore size have been shown to affect

microbial community structure. Fungi are found to be dominant in macroaggregates (soils with small to large pores) due to the fact that their hyphae cannot penetrate the small pores of microaggregates (Bach *et al.,* 2010; Dorodnikov *et al.,* 2009). Bacteria are found more commonly in microaggregates because of enhanced survival due to protection from predators and desiccation. Some studies have suggested that microbial biomass is greater in macroaggregates in comparison to that of microaggregates (Dorodnikov *et al.,* 2009). In this study soils were dominated by bacteria and physical-chemical properties of the soils (Table 4.2) showed that all of the soils investigated were primarily sandy soils. Literature suggested that soils with larger pores should contain higher fungal populations; however this was not the case except in one locality, Bothaville (Figure 4.7).

Xue *et al.* (2005), studying the microbial community structure associated with two genetically engineered crops namely maize and potatoes, found that there were changes in the microbial community structure. There was an increase in bacteria and a decrease in fungi in the soils associated with these crops. In the present study, some differences between RR, CS and NV were found. Although no statistically significant differences were found, Figure 4.8 illustrates that the community structures of CS and NV were similar and showed some differences to RR fields. This could indicate that although differences were small, the RR plants might be the factor that resulted in differences between CS and RR fields rather than the application of glyphosate to these plants. However, further investigation is needed to determine whether the RR plants had a direct or indirect effect on the microbial community structure.

On average there were no statistically significant differences ($p \leq 0.05$) observed between the PLFA groups for all three ecosystems (Figure 4.8). These results indicated that glyphosate did not drastically affect or permanently alter microbial community structure in soil over the long-term. This could be ascribed to microbial resilience where microbial communities tend to return to normal after an environmental disturbance (Sharma *et al.,* 2011), in this case the glyphosate application. In the current study this could be the case, because all the fields have been treated with glyphosate for no less than 5 years. Over this time frame, microbial communities have been resilient and have recovered from the disturbance of glyphosate application.

The results of both short- and long- term studies support present results that indicated that glyphosate application to RR soybeans did not alter the microbial community structure in these ecosystems (Lane *et al.,* 2012; Liphadzi *et al.,* 2005; Ratcliff *et al*., 2006).

4.4. Phospholipid fatty acid ratios used as stress indicators.

Various ratios calculated from fatty acid data, can be applied as indicators of stress and are shown in Figures 4.9 to 4.12. These ratios were calculated as described in chapter 3. Stress indicators, including the iso to anteiso PLFA ratio, ratio of trans to cis-monoenoic fatty acids (*trans*- to *cis*) and the ratio of saturated to monounsaturated PLFAs (sat / unsat ratio) can be used to evaluate nutrient stress (Fierer *et al*., 2003; McKinley *et a.l,* 2005). These ratios have also been linked to physical or chemical disturbances or communities undergoing changes in composition (McKinley *et al.,* 2005).

The *trans* / *cis* ratio can be used as an indicator of starvation and other environmental stresses such as metal contamination (Heipieper *et al.,* 1996) high temperatures, organic compound toxicity, osmotic stress and low pH (Kaur *et al.,* 2005). Healthy, non-stressed microbial communities usually have a *trans* / *cis* ratio of less than 0.05, whereas nutritional stress is indicated by values higher than 0.12 (Kaur *et al.,* 2005; Navarrete *et al.,* 2000). In this study, all the sites in the various localities had *trans* / *cis* ratios of between 0.2 and 1 (Figure 4.9) which indicated high levels of stress as these values are significantly higher than that of the proposed 0.05 value of healthy non-stressed microbial communities. This also rules out that glyphosate was the only cause of stress in microbial communities, since all three soils (RR, CS and NV) had *trans* / *cis* ratios of higher than 0.01.

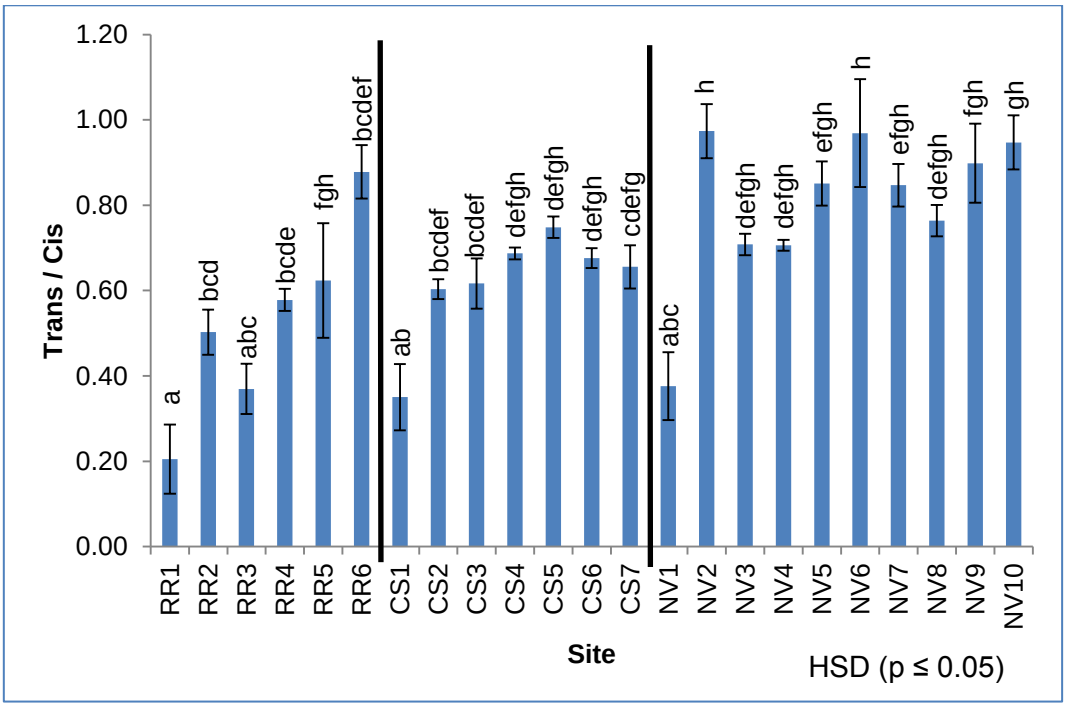

Figure 4.9: The *trans* to *cis* phospholipid fatty acid ratio for each of the 23 sites. Significant differences are indicated by alphabetical letters. The same letters indicate no significant difference. Standard error values are given in Table 1 in Appendix A. NV = Natural veld; RR = RoundUp ® Ready soybean; CS = Conventional soybeans.

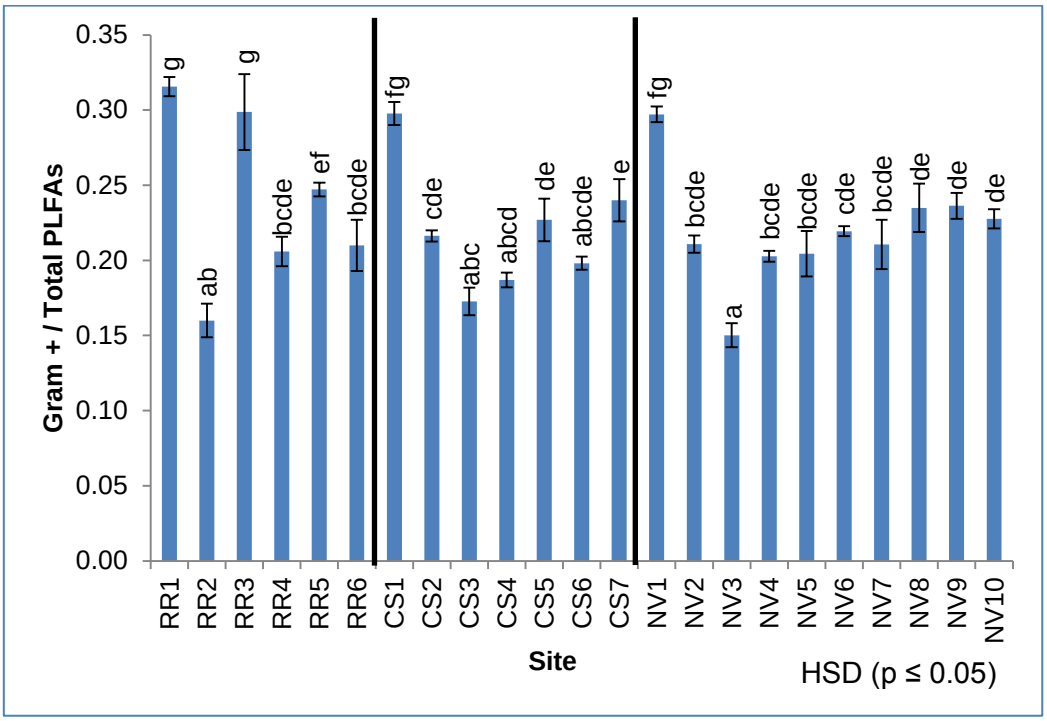

Figure 4.10: The gram-positive to total phospholipid fatty acid ratio for each of the 23 sites. Significant differences are indicated by alphabetical letters. The same letters indicate no significant difference. Standard error values are given in Table 1 in Appendix A. NV = Natural veld; RR = RoundUp ® Ready soybean; CS = Conventional soybeans.

The ratio of gram-positive to total PLFAs is indicated in Figure 4.10. Various stress conditions such as pesticide application, tillage and heavy metal contamination may result in an increase in gram-negative PLFAs. This can be attributed to the presence of cyclo-fatty acids in the cell envelopes of gram-negative bacteria, which gives them the ability to adapt to stress better than gram-positive bacteria (Kaur *et al.,* 2005). A decrease in gram-positive PLFAs will follow. There were some significant differences in these ratios between the different localities which indicated that the various localities were under different amounts of stress. No trends corresponding to glyphosate application was evident.

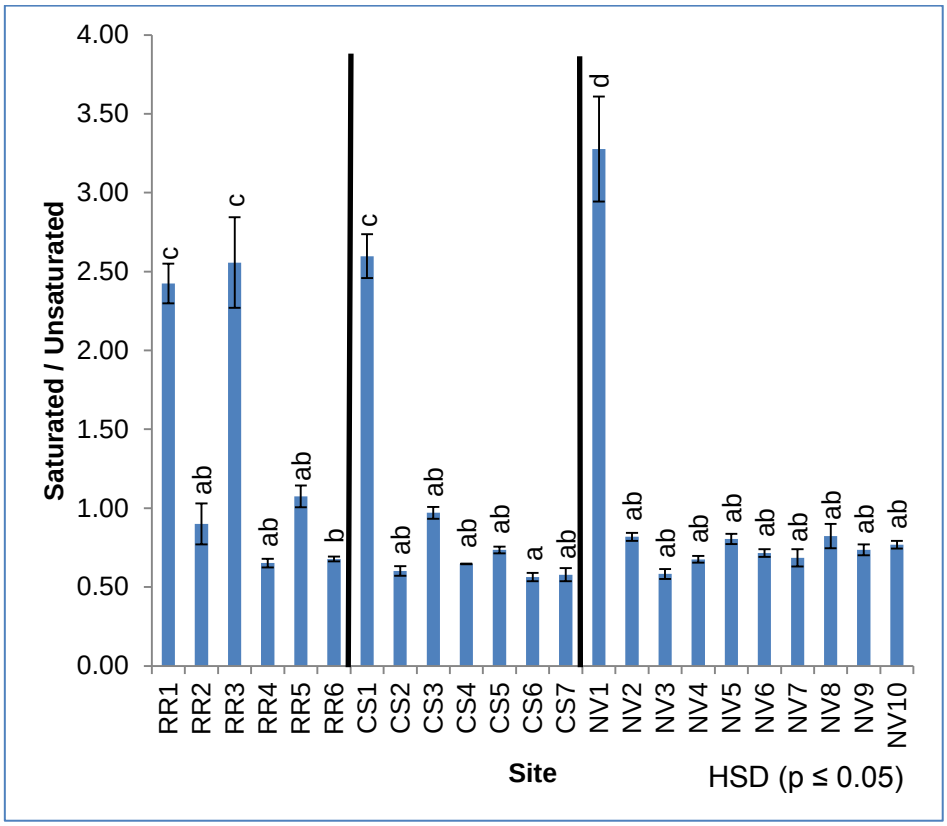

Figure 4.11: The saturated to unsaturated phospholipid fatty acid ratio for each of the 23 sites. Significant differences are indicated by alphabetical letters. The same letters indicate no significant difference. Standard error values are given in Table 1 in Appendix A. NV = Natural veld; RR = RoundUp ® Ready soybean; CS = Conventional soybeans.

The sat / unsat ratio can be also be used as an indicator of physiological or nutritional stress in microbial communities (Figure 4.11). This stress ratio reflect the degree to which microbial communities are affected by physiological stress (Moore-Kucera and Dick, 2008). Three samples from cultivated fields (RR1, RR3, and CS1) differed significantly from NV. However no trends corresponding to glyphosate application was evident.

To produce saturated fatty acids, a large amount of energy is required. In a study by Moore-Kucera and Dick (2008), it was suggested that to obtain this large amount of energy there must be a large turnover of microbial biomass. This indicates that with an increase in the saturated PLFAs (increase in sat/unsat ratio) there will be a decrease in total microbial biomass (Moore-Kucera and Dick, 2008). This supports the results illustrated in Figure 4.11. The samples with the highest sat/unsat ratio also had the lowest viable microbial biomass (Figure 4.3).

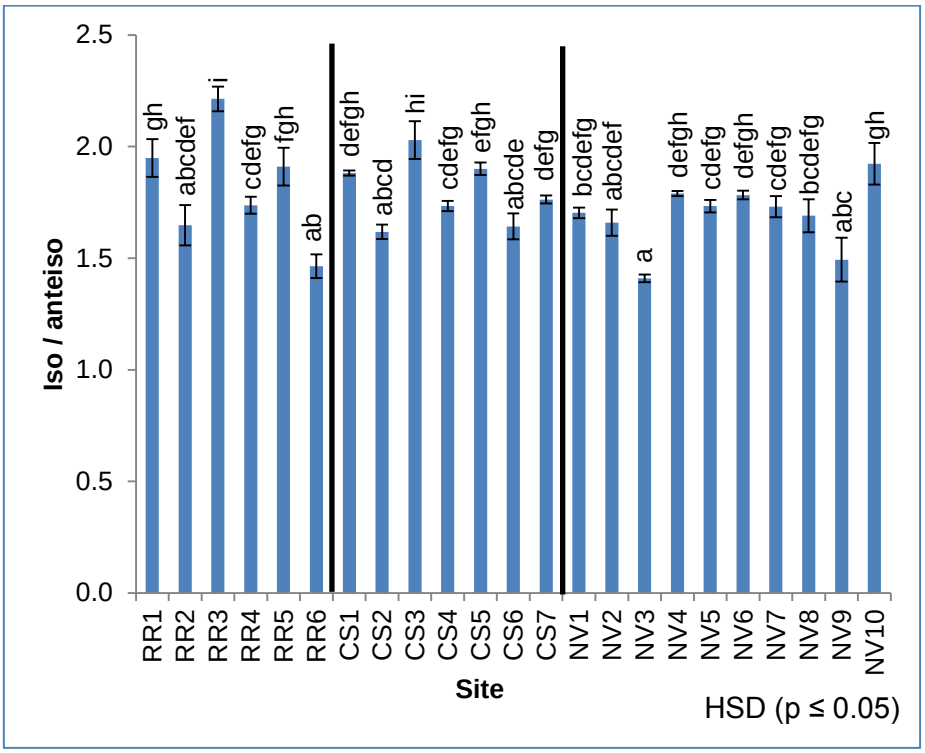

Figure 4.12: The iso to anteiso phospholipid fatty acid ratio for each of the 23 sites. Significant differences are indicated by alphabetical letters. The same letters indicate no significant difference. Standard error values are given in Table 1 in Appendix A. NV = Natural veld; RR = RoundUp ® Ready soybean; CS = Conventional soybeans.

Literature suggests that the ratio of sat / unsat and iso / anteiso (Figure 4.11 and Figure 4.12, respectively) will be higher in cultivated fields in comparison to natural veld (McKinley *et al.,* 2005). However, Figure 4.12 indicated that there were no significant differences in the iso / anteiso PLFA ratios between cultivated (RR and CS) fields and natural veld (NV). In McKinley *et al.* (2005), results indicated that cultivated soils significantly differed from natural veld soils in terms of three stress ratios (iso / anteiso; sat / unsat; and gram + / total PLFA ratios). In this study, (Figures 4.10, 4.11 and 4.12) however, there were no significant differences between the cultivated fields and the natural veld. This could indicate that glyphosate may not have induced any physiological or nutritional stress on the microbial communities and that the stress may have been induced by other environmental factors.

In conclusion, based on the viable microbial biomass; F/B ratio; the mole % fraction of the major PLFA groups and the various stress ratios it may be concluded that the long-term application of glyphosate to RR soybeans did not alter the microbial community structure when compared to conventional soybeans and natural veld.

Chapter 5: Nematode assemblages and corresponding microbial community structures - Results and discussion.

Since this study is focussed on nematode assembladges that occur in terrestrial ecosystems, the following terms will be used throughout: i) plant-parasitic nematodes and ii) non-parasitic and / or beneficial nematodes. In this case non-parasitic and / or beneficial nematodes refer to those that are present in soil and do not attack / infect plant hosts. Each of the three ecosystems investigated at the various localities has been assigned a specific code. CS refers to conventional soybean fields, RR to RoundUp ® Ready soybean fields and NV to natural veld. In Table 5.1 below the different localities with their respective codes for each site are listed. All physical and chemical properties for the soil samples discussed in this chapter are included in Chapter 4, Tables 4.2 and 4.3 of which only selected characteristics will be emphasised, namely % organic carbon (C), clay and sand.

Table 5.1: The various localities sampled during this study with the respective site codes for the RoundUp ® Ready (RR) and conventional soybean (CS) fields as well as adjacent natural veld (NV) sites sampled.

Locality	Site code
Bothaville	CS1; RR1; NV1
Brits	CS2; NV2
Edenville	CS3; RR2; NV3; NV4
Marblehall	CS4; CS5; CS6; RR3; RR4; NV5; NV6; NV7
Viljoenskroon	RR6;NV8
Winterton	CS7; RR5; NV9; NV10

5.1. Nematode community structures.

Nematode families and genera identified from soil samples obtained from the 23 sites where RR and CS were planted as well as from NV are listed in Table 5.2. Individuals from 32 nematode genera, representing 17 families were identified. Non-parasitic nematode genera that were present in soil samples that are listed as bacterivores (Ferris *et al*., 2001) were *Acrobeles, Acrobeloides, Alaimus, Butlerius, Cephalobus, Cruznema, Deontolaimus, Diploscapter, Eucephalobus, Mesorhabditis, Monhystera, Panagrolaimus, Plectus, Prismatolaimus, Rhabditis, Teratocephalus,*

Turbatrix, Wilsonema and Zeldia. Fungivorous nematode genera listed represented *Aphelenchus, Aphelenchoides, Paraphylenchus* and *Tylenchus*, while *Achromadora*, *Discolaimus, Dorylaimus, Labronema, Mesodorylaimus* and *Paraxonchium* represented omnivorous genera. The genus *Tripyla* is representive of predatory nematodes, while *Prionchulus* and *Mononchus* represent carnivorous nematodes (Bongers and Bongers, 1998). According to this data, nematode individuals from 22 of the 33 genera were present in soils from RR fields, while individuals from 26 of the genera were present in the soils from CS and NV fields. The genera *Alaimus, Teratocephalus, Tripyla, Turbatrix* and *Wilsonema* were only found in soil samples from NV, *Diploscapter* only in soils from RR fields and *Discolaimus, Cruznema, Mesodorylaimus* and *Paraxonchium* only in soils from CS fields. *Panagrolaimus* were only found in soils from both CS and NV fields and *Achromadora* only in soils from both CS and RR fields.

Table 5.2: Non-parasitic nematode families and genera present in soil samples from RoundUp ® Ready (RR) and conventional soybean (CS) fields as well as from adjacent natural veld (NV) ecosystems with their respective coloniser-persister (cp) values as listed by Bongers and Bongers (1998).

Families	Genera	cp -values	RR	CS	NV
Achromodoridae	*Achromadora* (Fu[2]+Om)	3	X	X	
Alaimidae	*Alaimus* (Ba[1])	4			X
Aphelenchidae	*Aphelenchus* (Fu)	2	X	X	X
Aphelenchoididae	*Aphelenchoides* (Fu)	2	X	X	X
Cephalobidae	*Cephalobus* (Ba)	2	X	X	X
	Eucephalobus (Ba)	2	X	X	X
	Acrobeles (Ba)	2	X	X	X
	Acrobeloides (Ba)	2	X	X	X
	Zeldia (Ba)	2	X	X	X
Diplogasteridae	*Butlerius* (Ba + P[3])	1	X	X	X
Dorylaimidae	*Labronema* (P+Om[4])	4	X	X	X
	Dorylaimus (Om)	4	X	X	X
	Mesodorylaimus (Om)	4		X	
	Discolaimus (Om)	4		X	
	Paraxonchium (P+Om)	4		X	
Leptolaimidae	*Deontolaimus*	2	X	X	X
Monhysteridae	*Prismatolaimus* (Ba)	2	X	X	X
	Monhystera (Ba)	2	X	X	X
Mononchidae	*Prionchulus* (Ca[5])	4	X	X	X
	Mylonchulus (Ca)	4	X	X	X

Panagrolaimidae	*Panagrolaimus* (Ba)	1		X	X
	Turbatrix (Ba)	1			X
Paraphylenchidae	*Paraphylenchus* (Fu)	2	X	X	X
Plectidae	*Plectus* (Ba)	2	X	X	X
	Wilsonema (B)	2			X
Rhabditidae	*Cruznema* (Ba)	1		X	
	Rhabditis (Ba)	1	X	X	X
	Mesorhabditis (Ba)	1	X	X	X
	Diploscapter (Ba)	1	X		
Teratocephalidae	*Teratocephalus* (Ba)	3			X
Tripylidae	*Tripyla* (P)	3			X
Tylenchidae	*Tylenchus* (Fu)	2	X	X	X

[1]Ba=bacterivore; [2]Fu=fungivore; [3]P=predator; [4]Om=omnivore; [5]Ca=carnivore; X indicate the presence of the respective nematode genera in one, two or all three ecosystems sampled.

The mean number of nematodes (parasitic and non-parasitic) identified from soil samples from each site is illustrated in Figure 5.1. Significant ($p \leq 0.05$) differences were evident among the 23 sites (Figure 5.1 and Table 5.3) with regard to nematode population levels in soil samples from RR, CS and NV ecosystems.

For the RR fields the mean number of nematodes ranged from 1129 for RR6 to 10525 individuals per 200 cm^3 soil for RR5. In the CS fields the mean number of nematodes per 200 g soil ranged from 1481 for CS4 to 11892 for CS7 and for NV from 1061 for NV5 to 4299 for NV4.

According to Table 5.3, significant ($p<0.05$) differences were evident in terms of nematode population levels (plant-parasitic and non-parasitic) in soil samples for each of the nematode trophic groups (plant-parasitic, bacterivores, fungivores, omnivores and carnivores) within the different sites that included RR, CS and NV. Such differences will, however, not be discussed in detail since various factors, excluding the use of glyphosate, could have been responsible for these tendencies.

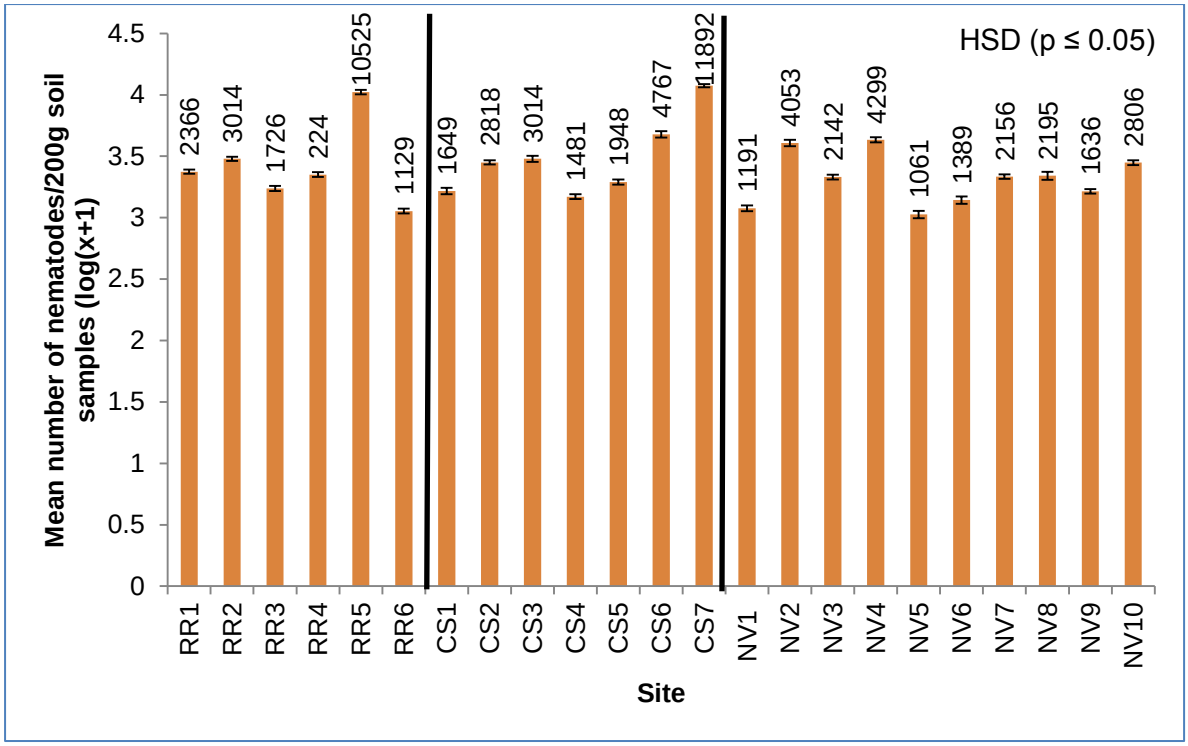

Figure 5.1: Mean number of nematodes (parasitic and non-parasitic) that were present in soil samples from each site at the various localities where NV = Natural veld; RR = RoundUp ® Ready soybean; CS = Conventional soybeans. The graph illustrates mean log(x+1) transformed values, while actual mean values are indicated in parenthesis. Statistically significant differences are illustrated using Tukey's HSD test at $p \leq 0.05$ and indicated in Table 5.3.

Plant-parasitic nematode numbers per 200 g soil ranged from 106 (RR4) to 838 (RR2) for RR sites; from 168 (CS2) to 2526 (CS7) for CS sites; and from 104 (NV5) to 1288 (NV10) for NV sites (Table 5.3). Bacterivore population levels ranged from 492 (RR6) to 8348 (RR5) for RR fields; from 602 (CS1) to 8352 (CS7) for CS fields; and from 465 (NV9) to 3335 (NV2) for NV sites (Table 5.3). The latter trophic group also represented the predominant nematodes identified during this study.

Fungivores followed in terms of non-parasitic nematode dominance with population levels ranging from 107 (RR6) to 1632 (RR5) for RR sites; from 48 (CS5) to 1100 (CS3) for CS sites; and from 63 (NV6) to 1394 (NV4) from NV sites (Table 5.3). Omnivore numbers ranged from 38 (RR1) to 80 (RR4) for RR sites; from 4 (CS4 and CS5) to 159 (CS1) for CS sites; and from 4 (NV5) to 111 (NV2) from NV sites (Table 5.3). Finally the least prominent non-parasitic nematode groups, carnivores ranged from 0 (RR1, RR2, RR3, and RR6) to 92 (RR4) for RR sites; from 0 (CS1, CS5, CS6, CS7 and CS8) to 23 (CS3) for CS sites; and from 0 (NV1, NV3, NV4, NV5, and NV6) to 60 (NV7) for the NV sites (Table 5.3).

Table 5.3: Actual mean and total number of nematodes in soil samples representing various functional groups for each of the three ecosystems sampled, namely RoundUp ® Ready (RR) and conventional soybean (CS) as well as natural veld (NV).

Sites	Plant-parasitic nematodes	Bacterivores	Fungivores	Omnivores	Carnivores	Total number of nematodes (plant-parasitic + non-parasitic)
RR1	485 (fghij)	1583 (fg)	260 (cdef)	38 (bcdef)	0 (a)	2366 (ghi)
RR2	838 (ijk)	1116 (cde)	1019 (ghi)	41 (bcde)	0 (a)	3014 (i)
RR3	219 (a)	1289 (def)	167 bcd)	51 (cdefg)	0 (a)	1726 (def)
RR4	106 (ab)	1772 (fg)	194 (cde)	80 (defgh)	92 (d)	2244 (gh)
RR5	458 (defghi)	8348 (j)	1632 (i)	78 (defgh)	9 (abc)	10525 (k)
RR6	461 (fghij)	492 (a)	107 (bc)	69 (efgh)	0 (a)	1129 (ab)
CS1	184 (abcd)	602 (a)	704 (fghi)	159 (h)	0 (a)	1649 (de)
CS2	168 (abcd)	2477 (h)	117 (bcd)	42 (bcdef)	14 (abc)	2818 (i)
CS3	400 (efghi)	1470 (efg)	1100 (hi)	44 (bcdefg)	0 (a)	3014 (i)
CS4	415 (efghi)	892 (c)	170 (bcd)	4 (a)	0 (a)	1481 (cd)
CS5	734 (hijk)	1162 (cde)	48 (a)	4 (a)	0 (a)	1948 (efg)
CS6	644 (ghijk)	3729 (i)	347 (defgh)	47 (bcdef)	0 (a)	4767 (j)
CS7	2526 (l)	8352 (j)	844 (fghi)	110 (gh)	60 (d)	11892 (k)
NV1	433 (efghi)	593 (a)	141 (bcd)	24 (abcd)	0 (a)	1191 (abc)
NV2	434 (fghij)	3335 (hi)	156 (bcd)	111 (fgh)	17 (c)	4053 (j)
NV3	457 (fghij)	1121 (cde)	494 (efghi)	47 (cdefgh)	23 (bc)	2142 (fg)
NV4	223 (cdef)	2593 (h)	1394 (i)	84 (cdefgh)	5 (abc)	4299 (j)
NV5	104 (abc)	881 (bc)	72 (ab)	4 (a)	0 (a)	1061 (a)
NV6	328 (defgh)	983 (cd)	63 (a)	15 (ab)	0 (a)	1389 (bcd)
NV7	221 (bcde)	1783 (g)	128 (bcd)	24 (abc)	0 (a)	2156 (fg)
NV8	1103 (jk)	655 (ab)	351 (defg)	86 (defgh)	0 (a)	2195 (fg)
NV9	303 (defg)	465 (a)	822 (fghi)	41 (bcdef)	5 (abc)	1636 (de)
NV10	1288 (kl)	902 (c)	531 (efghi)	83 (efgh)	2 (ab)	2806 (hi)

*Significant difference indicated by alphabetic letters ($p \leq 0.5$).

**NV = Natural veld; RR = RoundUp ® Ready soybean; CS = Conventional soybeans.

When data for the mean number of nematodes (plant-parasitic and non-parasitic) identified from soil samples obtained from the three respective ecosystems were pooled (Figure 5.2), no significant differences were evident among the three ecosystems. Soil samples from the CS fields had the highest number of nematodes (3938), followed by those for RR fields and NV fields with 3501 and 2293 nematodes per 200 g soil, respectively. In terms of this study, the latter pooled data thus suggest that glyphosate application did not have an adverse impact on nematode population levels in soils where RR soybeans were grown.

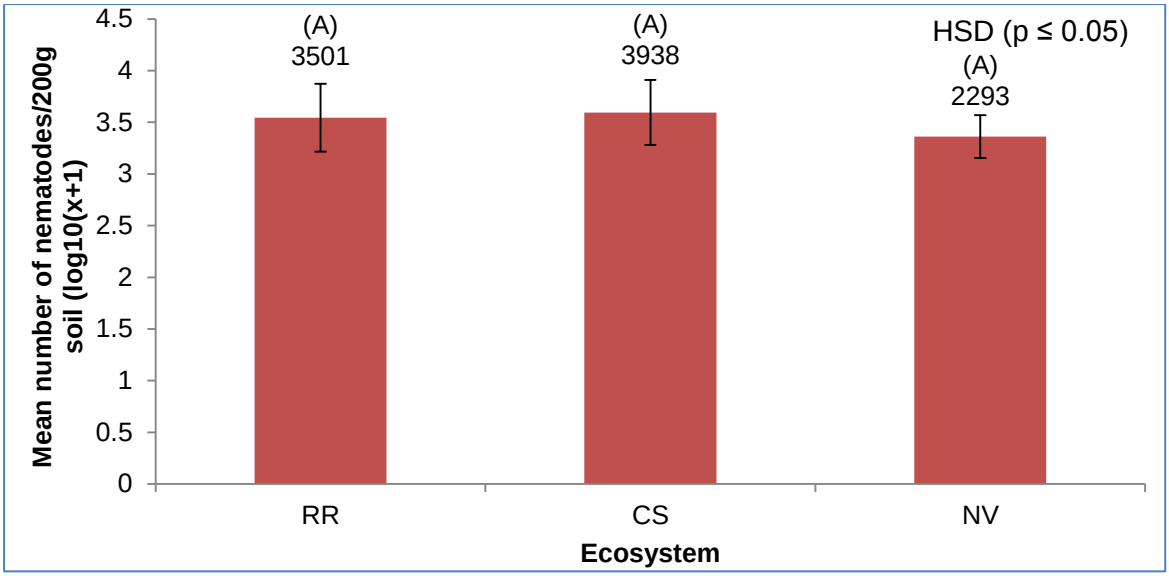

Figure 5.2: Average for mean numbers of nematodes (parasitic and non-parasitic) present in the three ecosystems. NV = Natural veld; RR = RoundUp ® Ready soybean; CS = Conventional soybean. The graph illustrates mean log transformed values, while actual mean values are indicated in parenthesis. Statistically significant differences are indicated by alphabetic letters using Tukey's HSD test at p ≤ 0.05.The same letters indicate no significant differences.

Ultimately, nematode data listed in Table 5.3 were pooled for the different trophic groups that were identified in soils samples from the three ecosystems (RR, CS and NV) and their community structures displayed in Fig. 5.3.

No significant differences were evident among the three ecosystems for each of the respective nematode trophic groups. Bacterivore nematode population levels were, however, the highest, followed by plant-parasitic nematodes, fungivores, omnivores and carnivores.

In terms of the plant-parasitic nematodes extracted from soils samples, the spiral nematodes (belonging to the genera *Helicotylencus* and *Rotylenchus*) generally dominated in all three ecosystems followed by *Pratylenchus, Tylenchorhynchus, Rotylenchulus* and *Meloidogyne.* Data for the latter genera are, however, not shown since the focus of this study is on non-parasitic nematodes.

Illustration of the average nematode community structure (including plant-parasitic and non-parasitic nematodes) for each of the three ecosystems showed similarities for CS, RR and NV fields. Both the latter were dominated by bacterivorous nematodes, followed by plant-parasitic – and fungivorous nematodes (Fig. 5.4). Omnivores and carnivores were the least dominant functional groups in both RR and CS fields as also been illustrated in Fig. 5.3.

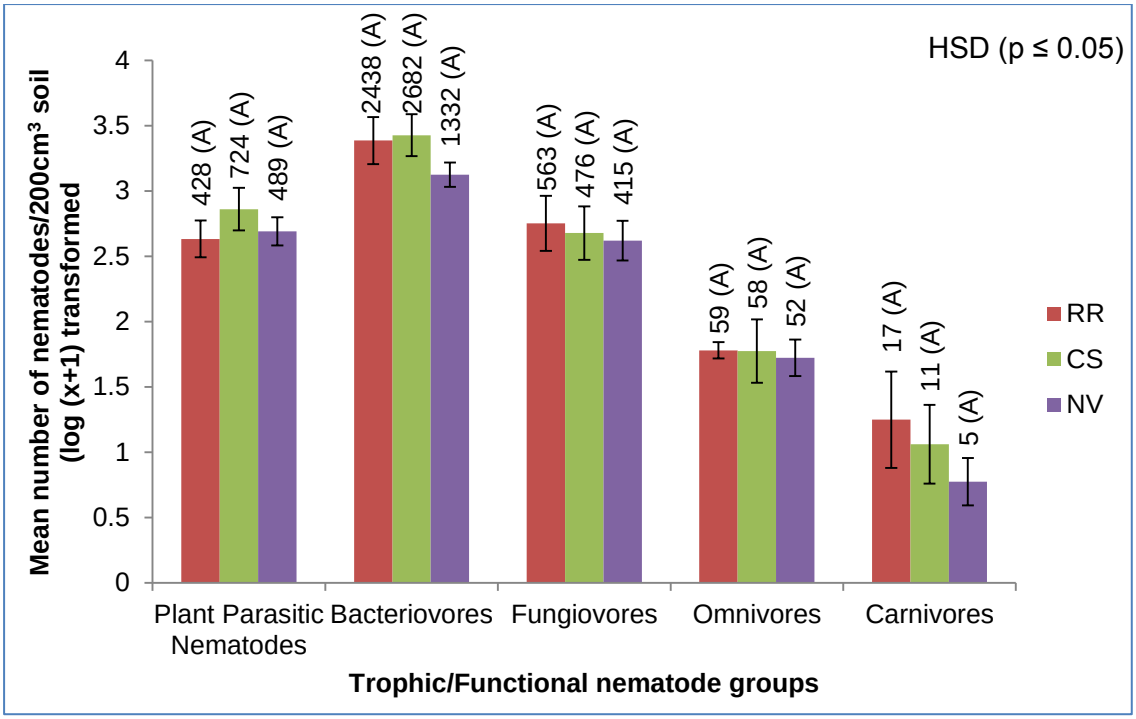

Figure 5.3: Average of mean nematode population levels for different trophic groups for the three ecosystems where NV = Natural veld; RR = RoundUp ® Ready soybean; CS = Conventional soybean. The graph illustrates log transformed values with actual means indicated in parenthesis. Statistically significant differences are indicated by alphabetic letters using Tukey's HSD test at $p \leq 0.05$.The same letters indicate no significant differences.

Bacterivores contributed to 68 % and 69 % of the community structure of both RR and CS fields, respectively, and 58 % of the nematode community structure in soils from NV. Soils from the latter sites had the highest percentage of omnivores (2.7 %), followed by RR and CS which had 1.7 % and 1.5 %, respectively. Soils from RR fields had the highest percentage of carnivores (0.6 %), followed by CS and NV (0.3 % and 0.2 %, respectively). Plant-parasitic nematode contributed 21 % of the total nematode community structure in NV, followed by CS with 18 % and RR with 12 %.

Nematode data obtained for the different trophic groups further compliment earlier results and suggest that glyphosate application did not have an adverse impact on nematode population levels (both parasitic and non-parasitic) in soils where RR soybeans were grown. Ultimately, soils from most of the sites sampled were dominated by bacterivores (Table 5.3; Figures. 5.3 and 5.4) followed by fungivores, plant-parasitic nematodes, omnivores and carnivores.

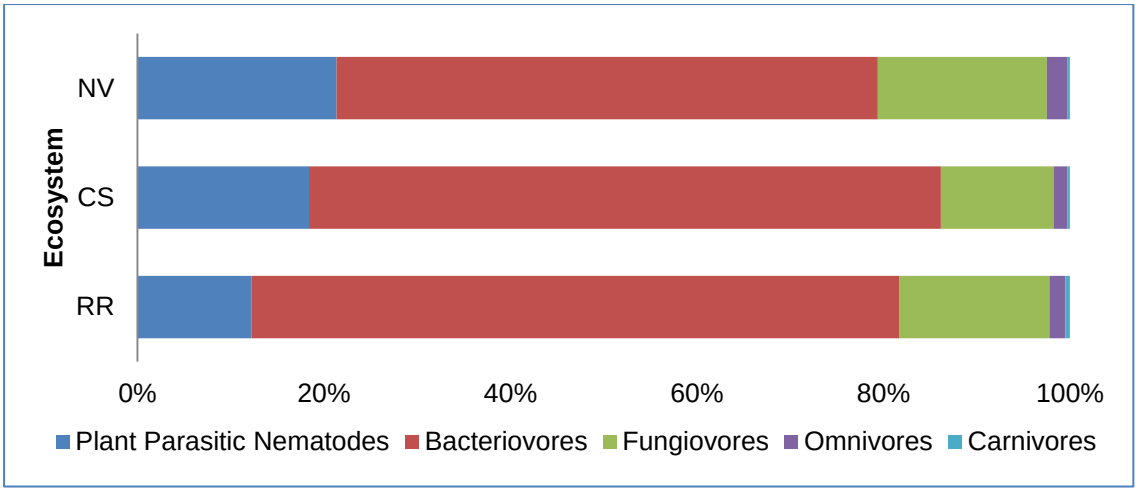

Figure 5.4: Nematode community structure for each ecosystem, including parasitic and non-parasitic nematodes, based on the percentage fractions of each functional group where NV = Natural veld; RR = RoundUp ® Ready soybean; CS = Conventional soybeans. Mean values used are illustrated in Table 5 in Appendix A.

Collective results from traditional nematode data analyses referred to thus far in terms of this study indicated that bacterivore nematodes from the family Cephalobidae was the most abundant of the bacterivore trophic group in soil samples from RR and CS as well as NV sites obtained during this study and is in agreement with results from Yeates (2003). These nematodes are able to survive in conditions where food is less abundant and respond to environmental factors more rapidly than those belonging to the Rhabditidae family (Bongers, 1999.; Porazinska *et al.,* 1999). Within this family various genera responds to pollution in different ways. For example *Acrobeloides* is more tolerant to environmental stress than *Acrobeles* (Bongers, 1999). The least abundant non-parasitic nematode trophic groups in soils from the sites obtained during this study, namely omnivores and carnivores are also in agreement with results by Ferris *et al*. (2001) and other authors (Bongers and Bongers, 1998; Bongers, 1999) due to their sensitivity to disturbance. In terms of plant-parasitic nematode assemblages in soils from NV sites sampled in this study, their high population numbers can be explained by the host-status of the natural vegetation present in the NV ecosystem. It is well-known that various natural plants are hosts to some plant-parasitic nematodes (Van der Putten and Van der Stoel, 1998).

It is ultimately suggested from results obtained that glyphosate did not have an adverse effect on nematode community structures in soils where RR cultivars were planted. This phenomenon is in agreement with results of a 15 year study (1989 – 2003) study by Cheng *et al.* (2008) who examined the effects of various pesticides, including the herbicides pendimethalin and a broadleaf herbicide (containing MCPA, mecoprop and Dicamba) on nematode communities in turfgrass.

Results from the latter study showed that such pesticides had no detrimental effects on the soil nematode community structure or on microbial biomass. In addition, a study by Liphadzi *et al.* (2005) reported that the total number of non-parasitic nematodes as well as the individual trophic groups they represented, identified from glyphosate-treated soils did not significantly differ from those present in untreated soils. Bacterivores dominated in all soils followed by fungivores and suggested that the effects of herbicide application depend on the type of herbicide applied (Liphadzi *et al.,* 2005). In contrast to the latter phenomena, a seven-year study by Yeates *et al.* (1999) in which the long-term effects of three agricultural practices, *viz*. cultivation, herbicide application and mulching, on parasitic and non-parasitic nematode communities were studied, showed that soils treated with terbumeton / terbythylazine herbicides generally had the highest total nematode numbers with lower diversity as well as fewer bacterivore numbers than untreated soils. Furthermore, the application of other herbicides (atrazine and primisulfuron) had no detrimental effect on nematode populations (parasitic and non-parasitic) according to the latter study.

5.2. Faunal analyses of food web structure.

Faunal analyses is an alternative and additional statistical means that relate the soil nematode communities to soil food-web health (Ferris *et al*., 2012) and were thus also calculated for nematode data obtained in this study. Non-parasitic nematode data, *viz.* population levels and diversity, only are included in faunal analyses since the latter gives an indication of the quality of soils in terms of the beneficial nematode assemblages present (Ferris *et al*., 2012).

In Figure 5.5 the calculated Enrichment Index (EI) and Structure Index (SI) of soil samples from each of the RR and CS fields as well as from natural veld sites (Table 5.4) obtained from the various localities are illustrated based on the cp-values assigned to non-parasitic nematode families identified. This figure illustrates the faunal profile based on the abundance of nematode guilds, which is defined by Ferris *et al.* (2001) as the grouping of nematode species that share similar biological characteristics / attributes and also respond to environmental conditions in a similar way(s).

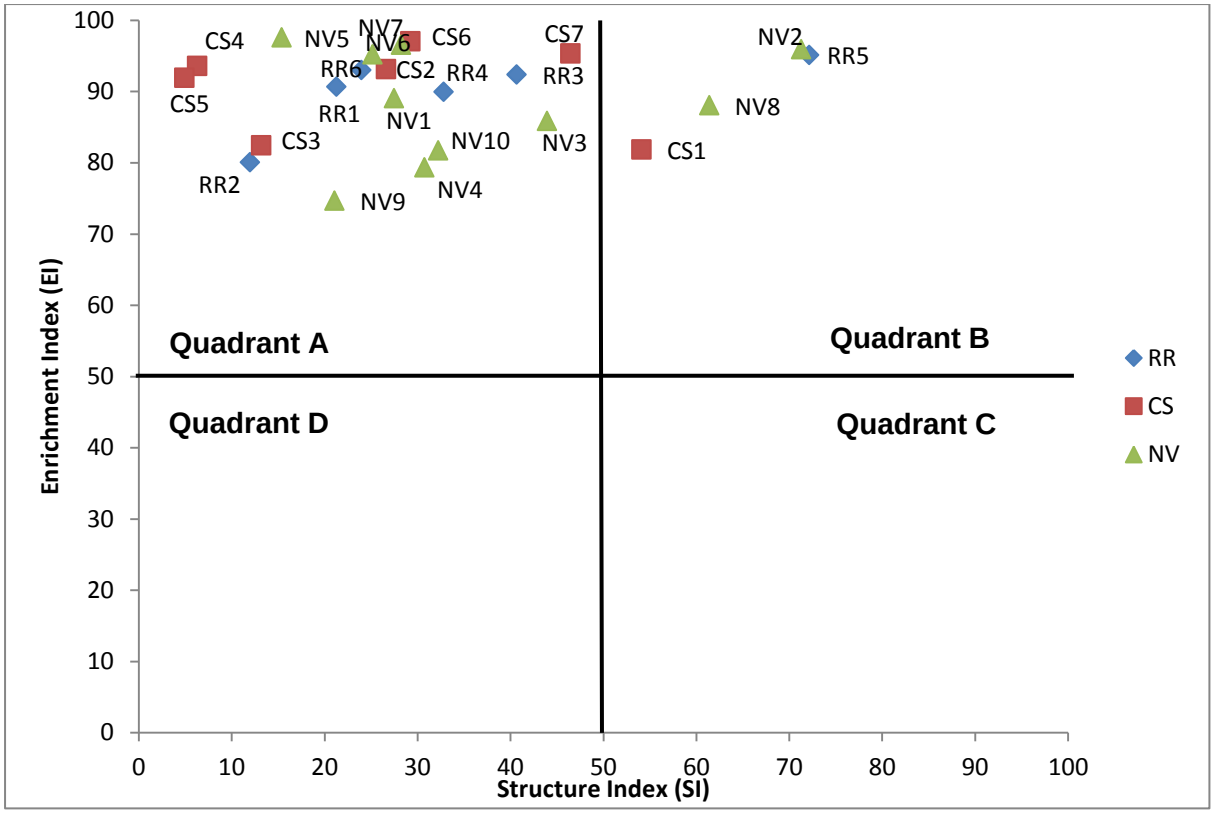

Figure 5.5: The Structure Index (SI) and Enrichment Index (EI) of each site according to coloniser-persister (cp) values assigned to non-parasitic nematodes that were identified from soil samples obtained from natural veld (NV) as well as RoundUp ® Ready (RR) and conventional soybean (CS) ecosystems at 23 localities within the soybean production area of South Africa during the 2013 growing season.

According to the EI and SI indices (Table 5.4), 83% of the localities sampled were grouped in Quadrant A (Fig. 5.5), indicating that soils from such fields had enriched but unstructured soil nematode communities. The latter scenario further implicates that enriched soil food webs and high rates of bacterial-mediated decomposition pathways were present in soils from the majority of the samples obtained at the specific sampling interval during the sixth growth stage (R6) of the soybean crops. The latter fields generally had high EI (ranging from 74.7% for NV9 to 97.6 % for NV5) and low SI (ranging from 4.9% for CS5 to 40.7 % for RR3) (Table 5.4). High EI are generally the result of high population levels of bacterivores belonging to the Ba_1 (Ferris *et al*., 2001). In this study, nematodes that represent Ba_1 guilds in the majority of soil samples with high EI included individuals from the genera *Butlerius, Cruznema, Diploscapter, Mesorhabditis, Panagrolaimus, Rhabditis* and *Turbatrix* (Table 5.2). The latter non-parasitic nematodes generally occur in soils where recovery from environmental stress is occurring. Conversely, in agricultural soils the SI is usually low, indicating low numbers and diversity of predator nematodes mainly, and is often

correlated with management practices such as application of pesticides (Sánchez-Moreno *et al.,* 2009).

Table 5.4: Maturity (MI), Enrichment (EI), Structure (SI) and Channel index (CI) values for sites sampled from RoundUp ® Ready (RR) and conventional soybean (CS) as well as natural veld (NV) in terms of the presence of non-parasitic nematodes.

Sites	MI[1] including cp-1	MI[2] excluding cp-1	EI[3]	SI[4]	CI[5]
RR1	1.0	0.3	90.7	21.3	4.8
RR2	1.1	0.7	80.1	12.0	21.3
RR3	1.2	0.6	92.4	40.7	3.5
RR4	1.3	0.5	89.9	32.8	3.3
RR5	0.9	0.4	95.1	72.2	5.1
RR6	1.2	0.5	93.0	24.0	5.2
CS1	1.7	1.4	81.9	54.1	18.0
CS2	1.2	0.5	93.2	26.6	1.4
CS3	1.3	0.8	82.4	13.2	17.8
CS4	0.9	0.3	93.6	6.3	4.9
CS5	0.8	0.3	91.9	4.9	1.3
CS6	1.0	0.2	97.1	29.2	2.3
CS7	1.0	0.3	95.3	46.4	2.7
NV1	0.8	0.3	89.0	27.5	6.8
NV2	1.2	0.6	95.9	71.3	0.6
NV3	1.2	0.8	85.9	43.9	12.5
NV4	1.5	1.1	79.3	30.7	17.0
NV5	1.0	0.2	97.6	15.4	2.0
NV6	0.9	0.3	95.2	25.2	1.8
NV7	1.0	0.2	96.5	28.2	1.9
NV8	0.9	0.7	88.0	61.4	13.6
NV9	1.4	1.2	74.7	21.1	33.0
NV10	0.9	0.7	81.7	32.2	16.9

[1]Maturity index including nematodes with a cp-value of 1 - MI = Σi=1 v(i) x f(i)
[2]Maturity index excluding nematodes with a cp value of 1 - MI = Σi=1 v(i) x f(i)
[3]Enrichment index - EI = 100(e/(e+b))
[4]Structure index - SI = 100(s/(s+b))
[5]Channel index - 100(keFu2/(keBa1+keFu2))

For the soils of sites mapped in quadrant A (Fig. 5.5), MI ranged from 0.8 (NV1) to 1.7 (CS1) and CI from a low 1.3 (CS5) to a high 33 (NV9). In general, MI ranges from 1 to 4, with the latter

highest value indicating undisturbed conditions (Bongers and Bongers, 1998; Bongers, 1999; Bongers and Ferris, 1999; Mulder *et al.,* 2005; Neher, 2001). No soil samples from the 23 sites sampled had MI higher than 1.7 (CS1), indicating that all sites are generally representative of disturbed environments and either a dominance of r-selected nematodes (nematode genera with high reproduction rates) or low populations of rare K-strategists (nematodes with low reproduction rates) (Bongers and Ferris, 1999; Porazinska *et al.,* 1999). Further, the general low MI values for soils from all of the 23 sites sampled during this study relates to general high population levels of bacterivore colonisers with low cp-values (Bongers and Ferris, 1999).

Pooled MI data (Table 5.5) ultimately suggest that soil in these fields were dominated by r-strategists and were under stressed environmental conditions. It can thus be concluded that glyphosate applied in RR fields was not responsible for this stress as soil from the latter did not differ significantly ($p<0.05$) from those obtained from CS and / or NV fields sampled during this study. The latter is further supported by the presence of similar functional groups of nematodes, food web analyses and nematode community indices from soils obtained in RR, SC and NV fields. Ultimately, it is suggested that glyphosate application did not seem to have long-term detrimental effects on the diversity and community structure of beneficial nematodes in soils obtained from RR fields during this study.

For soils from four of the sites sampled, however, high EI and SI indices were calculated, viz. those from natural veld sites NV2 (95.9%; 71.3%) and NV8 (88%; 61.4%) as well as those from RR5 (95.1%; 72.1%) and CS1 (81.9%; 54 %) (Table 5.4). These soils thus proved to contain enriched but structured non-parasitic nematode communities, representing more structured food webs at the time of sampling. Soils with high SI are generally due to the presence of high numbers of Fu3 and Fu4 nematode guilds, which is representative of uncultivated fields that occur in natural ecosystems (Cheng *et al.,* 2008). In this study only fungivores, representing the Fu3 guild were identified as belonging to the *Achromadora* genus and were present in soils from RR5 and CS1 fields that were mapped in Quadrant B (Table 5.2). However, the greatest contribution to high SI values of soils from NV2 and NV8 that were also mapped in the latter quadrant was due to the presence of omnivore and carnivore nematodes with cp-values of 1, 3 and 4. The latter trophic nematode groups included individuals from the genera *Dorylaimus* and *Labronema* as omnivores; and *Mylonchulus* and *Prionchulus* as carnivores. In this study, high CI for soils from CS1 and NV8 is thus indicative of lower organic matter decomposition mediated by fungi while low CI for RR5 and NV2 relates to domination by bacteria in such ecosystems. The latter trend is agreement by those reported by Ferris *et al*. (2012) and Ferris and Matute (2003) since it contained healthy soil food webs by sustaining nematodes from different functional groups (ranging from colonisers to persisters). However, soils from these four fields are still not superior in terms of their healthy food-web status since they have moderate SI indices. These four fields are more stable in terms of non-

parasitic / beneficial nematode assemblages than those mapped quadrant A. However, mapping of these fields in Quadrant B still indicate that such soils exhibited high rates of primary production (resulting from high numbers of bacterivores) and high numbers of general bacterivore nematode opportunists (Ferris *et al.,* 2001). The latter was represented by the genera *Butlerius, Cruznema, Diploscapter, Mesorhabditis, Panagrolaimus, Rhabditis* and *Turbatrix* (cp - 1) as well as *Acrobeles, Acrobeloides, Aphelenchus, Aphelenchoides, Cephalobus, Deontolaimus, Eucephalobus, Monhystera, Paraphylenchus, Plectus, Prismatolaimus, Tylenchus* and *Wilsonema* (cp - 2) (Table 5.2) in this study.

In terms of optimal beneficial nematode community structures, soils that map in quadrant B are superior to those that map in the other three quadrants, in terms of soil quality (related to beneficial nematodes) and should be aimed to achieve by farmers for sustainable crop production. Such soils, representing structured food webs are generally present in sites where natural vegetation grow and no / minimal disturbances were imposed (Ferris *et al.,* 2001). None of the soils sampled during this study mapped either in quadrants C (indicative of resource-limited but structured beneficial nematode community) or D (indicative of a resource-depleted beneficial nematode community with minimal structure) (Ferris *et al.,* 2001).

Table 5.5: Maturity (MI), Enrichment (EI), Structure (SI) and Channel index (CI) values pooled for soil samples obtained from RoundUp ® Ready (RR) and conventional soybean (CS) as well as natural veld (NV) ecosystems sampled during this study in terms of the presence of non-parasitic nematodes.

Ecosystems	MI (including cp1)	EI	SI	CI
RR	1.1 (a)	90.2 (a)	33.8 (a)	7.2 (a)
CS	1.1 (a)	90.8 (a)	25.8 (a)	6.9 (a)
NV	1.1 (a)	88.4 (a)	35.7 (a)	10.6 (a)

When nematode data for MI, EI, SI and CI were pooled, it again emphasised that glyphosate application in soils where RR field crops were planted did not affect nematode community structures (both plant-parasitic and non-parasitic) adversely. The latter statement is based on no significant differences (p<0.05) being evident among the three ecosystems with regard to any of these calculated nematode community indices.Ultimately, most (83%) of the soils sampled from RR, CS and NV ecosystems in this study represent unhealthy and unstructured food webs apart from their enriched status in terms of nematodes. Non-significant differences among indices (EI, CI, MI, SI), between the pooled non-parasitic nematode data for the three ecosystems (Table 5.5), used to qualify soil food webs in this study complements the latter findings and furthermore substantiate the conclusion that glyphosate applications in fields where RR soybeans were grown

did not affect nematode soil-food webs adversely. MI, which is a useful tool to measure effects of disturbances, differences between agricultural systems and pollution-induced stress using nematode assemblages (Bongers and Bongers, 1998; Bongers, 1999; Bongers and Ferris, 1999; Neher, 2001; Porazinska *et al.,* 1999) obtained for soils in this study further proved that factors other than glyphosate most probably could be responsible for significant differences in nematode assemblages among sites from the various areas.

In terms of associations between nematodes and soil physical characteristics, some relationships were evident. For example, soils from RR5 and CS7 that contained the highest number of nematodes (Figure 5.1) also exhibited high clay contents of 15.3 and 16.7, respectively, and the lowest sand contents of 61.4 and 44.8, respectively, compared to those for the other sites. Also, soil organic (C) content for soils from RR5 and CS7 had relatively high mean values of , with 1.62 and 2.77, respectively (Table 4.3; Chapter 4). Interestingly, these two sites also showed relative high viable microbial biomass of 28718.51 pmol.g^{-1} for RR5 and 38754.40.pmol.g^{-1} for CS7 (see Chapter 4 Figure 4.3 and Table 1 in Appendix A). These finding were interesting and warrants further investigation.

In conclusion, based on traditional and alternative (faunal analysis) data analyses conducted to describe and interpret the presence and role of nematode assemblages (parasitic and non-parasitic) in soil samples, it is suggested that the application of glyphosate to RR soybean fields did not adversely alter nematode community structures in such soils. Other abiotic and biotic factors, which are not known at this stage and have not been investigated in this study, most probably impacted on biota (including nematodes) in soils of these sites and caused the significant differences in nematode communities within and among the 23 sites sampled. Also, more sampling intervals should be included in future studies to limit the seasonal effect in terms of variation among nematode populations as has been done in a fifteen-year study by Cheng *et al.* (2008).

5.3. Integration of nematode and microbial community data.

To determine whether relationships between microbial (see Chapter 4) and nematode (parasitic and non-parasitic) community structures (discussed earlier in this chapter) existed, integration of such data sets were done by subjecting it to Redundancy Analyses (RDA). Figure 5.6 illustrates relationships between the microbial- and nematode community structures based on phospholipid fatty acid (PLFA) analyses and nematode functional groups identified from soil samples from RR, CS and NV ecosystems.

The eigenvalues for the first two ordination axes were 0.098 and 0.071, respectively. These two axes accounted for 71 % of the total observed variance for the species and environmental

variables (Fig. 6.1). The species-environment relation was determined by the cumulative percentage variance for the four axes which was 50.6, 87.4, 98.5, and 100 %, respectively.

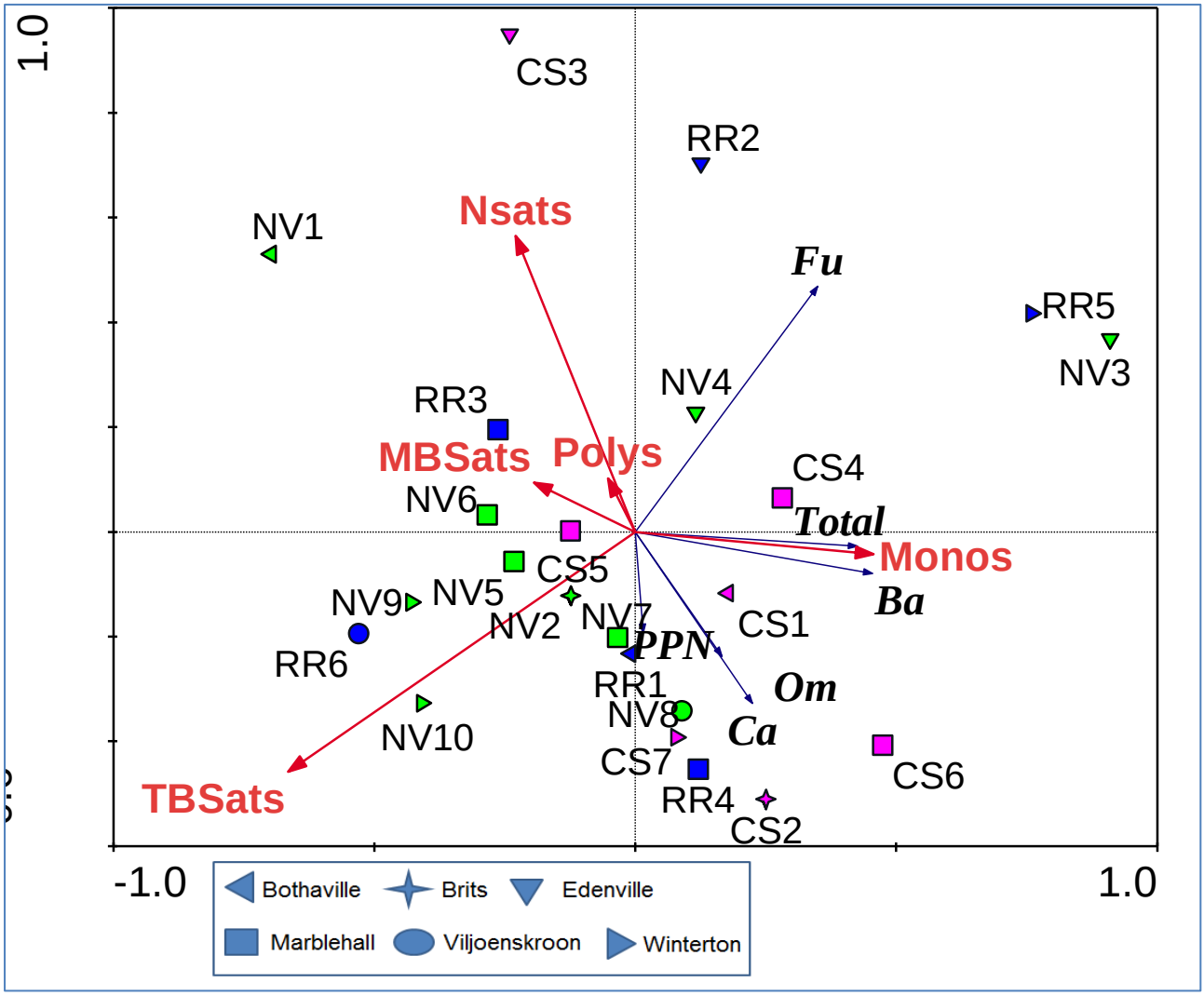

Figure 5.6: Redundancy Analyses (RDA) ordination diagram illustrating the relationship between the major phospholipid fatty acid groups and the nematode functional groups (including parasitic and non-parasitic nematodes) that were identified in each site. PPN = Plant-parasitic nematodes; Om = Omnivores; Ca = Carnivores; Ba = Bacterivores; Fu = Fungivores; Nsats = normal saturated fatty acids (all organisms); MBsats = mid-branched saturated fatty acids (Actinomycetes); TBsats = terminally-branched fatty acids (gram-positive bacteria); Polys = polyunsaturated fatty acids (fungi); Monos = monounsaturated fatty acids (gram-negative bacteria); RR = RoundUp ® Ready soybeans; NV = Natural veld ; CS = Conventional soybeans.

A positive relationship existed between some nematode functional groups and their respective microbial prey (Fig. 6.1), for example, bacterivore nematodes had a strong positive relationship with monounsaturated fatty acids that is indicative of gram-negative bacteria (Zelles, 1999). Similar but weaker relationships also existed between carnivores, omnivores, plant-parasitic nematodes and monounsaturated fatty acids. This fatty acid identified gram-negative bacteria as the dominant bacterial group in soils from all three ecosystems (see Chapter 4 Figure 4.7 and 4.8). A positive relationship also existed between fungivores and fungal, microbial biomass (polyunsaturated fatty acids (Polys)).

Relationships that were evident between non-parasitic nematodes and their microorganism prey in soil habitats of RR and CS as well as NV sites agreed with reports by Neher (2001) who emphasised that these organisms play major roles in the functioning of soils ecosystems. These two groups of soil microbiota are frequently used as indicators of soil quality. The inability to correlate nematode and microbial data in soil sampled from RR fields to which glyphosate were applied and those without application of the herbicide (CS and NV), support reports from other studies to date that herbicide application generally does not alter soil community structures of microbes (Cheng *et al.,* 2008; Dick *et al*., 2010; Lane *et al.,* 2012; Liphadzi *et al.,* 2005) and nematode assemblages (Cheng *et al.,* 2008; Liphadzi *et al*., 2005; Yeates *et al*., 1999).

In previous studies the population size of some functional non-parasitic nematode groups has been linked to microbial biomass that were also present in the same ecosystem at the time of sampling. For example, an increase in microbial biomass and activity resulted in an increase in numbers of nematode bacterivores, carnivores, and omnivores (Bongers, 1999; Yeates *et al.,* 1999). The latter trend was observed in this study since population levels of these two groups of soil microbiota were both high in soils from RR5 and CS7. Furthermore, the organic C content of soils from the latter two sites was also the highest of all the sites sampled during this study.

In Figure 5.6 it was also illustrated that the nematode functional groups bacterivores, plant-parasitic nematodes, carnivores and omnivores clustered together in the same quadrant, while fungivores mapped in a different quadrant. In terms of microorganisms the monounsaturated fatty acids (gram-negative bacteria) clustered in the same quadrant as the former nematode functional groups. Gram-negative bacteria was the most dominant microbial groups in all soils types as a result from this study, which is in agreement with reports that an increase in microbial activity has been linked to an increase in abundance of the non-parasitic nematodes (bacterivores, carnivores and omnivores) (Bongers, 1999; Yeates *et al.,* 1999).

Chapter 6: Conclusions.

6.1. Conclusions.

The aim of this study, viz. to determine and compare microbial and non-parasitic / beneficial nematode community structures in soil samples from RoundUp ® Ready (RR) and conventional soybean (CS) fields as well as adjacent natural veld (NV) areas in soybean production areas of South Africa during the reproductive stage of soybean plants has been met. Ultimately, results from this study that the long-term (no less than 5 years) application of glyphosate to RR crops did not result in changes to the nematode- and microbial community structures in the soils where such soybean crops were planted. Microbial community structures in soil samples obtained from the three ecosystems were determined using phospholipid fatty acid (PLFA) analysis. On the other hand, nematode community structure and diversity were established by extraction of nematodes from soil samples, followed by counting and identifying it to genus level. Ultimately, nematode community indices and faunal analysis were used to determine the foodweb structures of soils from the three ecosystems.

Results obtained from PLFA analyses indicated that no significant ($p<0.05$) differences were evident for microbial biomass, fungal to bacterial (F / B) ratios, stress ratios and major PLFA groups from soil samples from RoundUp ® Ready (RR) and conventional soybean (CS) fields as well as and natural veld (NV) ecosystems. The F / B ratio and the major PLFA groups indicated that the soils from all three ecosystems (RR, CS and NV) were dominated by bacteria. Bacteria-dominated soils are common in agricultural systems, which were also indicated by results of this study. Monounsaturated fatty acids were the dominating PLFA group in soil samples obtained from the three ecosystems sampled during this study and are indicative of gram-negative bacteria, which are considered to be r-strategists. The latter organisms are considered to be the first colonisers in an environment. It has been reported that in agricultural soils microorganisms present are generally r-strategists, referred to as fast colonisers with high growth rates that is characteristic to disturbed soil environments.

Similar to microbial community structure results, no significant ($p<0.05$) differences existed in terms of nematode (parasitic and non-parasitic) community structures and population levels among soils collected from the various sites in. In terms of non-parasitic nematodes, bacterivores dominated in soils from the 23 sites with individuals of the Cephalobidae and Rhabditidae families generally being the most abundant. Nematodes belonging to these families are considered to be r-strategists, which is also supported by the r-strategist bacterial microbe domination as a result of PLFA results.

Fungivores followed in dominance, while omnivores and carnivores were the least dominant non-parasitic nematodes identified from soil samples. The latter two trophic nematode groups are

considered as K-strategists (persisters) and are the most sensitive to environmental disturbances and stress and the presence of these nematodes also increases the structure of soil food webs, supporting results from this study that the soil food webs in 83% of the sites sampled were enrhiched but not structured. The maturity index as well as the stress ratios calculated for the three ecosystems in this study were relatively low, which further support the soil food-web analysis indicating disturbed environments.

Plant-parasitic nematode assemblages (identified from soils of all three ecosystems) were dominated by the spiral nematodes (belonging to the genera *Helicotylencus* and *Rotylenchus*) followed by *Pratylenchus, Tylenchorhynchus, Rotylenchulus* and *Meloidogyne.* High plant-parasitic numbers can be explained by the presence of natural vegetation hosts in NV sites. This scenario may, however, be challenging in terms of damage that may be inflicted by these pests when such sites are in future considered for the cultivation of crops.

Important to bear in mind is that some of the physical and chemical properties of the soil showed trends of association with the microbial and nematode results found. Soils that contained high microbial biomass and the highest nematode numbers also had the highest clay and organic C content. The latter is in agreement with reports that the population size of some functional nematode groups has been linked to the microbial biomass in soil samples. Research on this topic to date proved that soil texture and the organic C content influence microbial biomass and fungal or bacterial dominance, of which the trends were also found in this study.

Based on all microbial and nematode parameters determined, no significant differences existed between the cultivated (RR and CS) and non-cultivated (NV) fields, which suggests that glyphosate application was not responsible for adversely influencing nematode community structures in RR fields. This may indicate that environmental conditions that prevailed during the time that this study was conducted in, were not favourable for microbiota in NV soils. This conclusion is drawn from literature that shows that globally, soils from natural vegetation usually contain higher population levels of non-parasitic nematodes compared to soil from cultivated fields. During the time of sampling in this study, below-normal precipitation to drought conditions occurred, which may explain why the microbial mass and nematode community structures in soils from NV ecosystems did not meet expectations.

6.2. Recommendations for future studies.

Limited cultivation of conventional soybean complicated this study and will definitely have the same effect on future studies similar to this one. Only a few producers these days grow conventional soybean and other conventional crops. Growing RoundUp® Ready crops such as soybean, maize and cotton dominate, since it is more cost-effective and easier to manage. After careful consideration of all the results obtained in this study, a few recommendations are made for future studies.

i) Since the physical and chemical properties determined in this study had such an effect on the results found, the focus of future studies should rather be shifted from the effects of herbicides to the effects of various physical and chemical properties of the soils where RR crops are grown. Various physical and chemical properties of the soils might affect the mechanisms of the herbicide itself. Greenhouse trails should be executed where these properties can be controlled, intensified or reduced to determine the effect of physical and chemical properties on herbicide use and the surrounding soils.

ii) Because it has been suggested that root exudates of RR crops may be affected by glyphosate application (Kremer *et al*., 2005), studies determining the structure of these exudates should be conducted. This should be followed by the extraction of these exudates and the determination of the direct effect of these exudates on soil microbial communities and beneficial nematodes. Microbial activity could be determined using community-level physiological profiles (CLPP) and substrate utilisation using BIOLOG® systems (Hill *et al.,* 2000; Sharma *et al*., 2011). DNA analyses may also be valuable in both nematode and microbial community analyses.

iii) If glyphosate has direct effects on plant disease (Johal and Huber 2009), the direct effect on specific plant-parasitic nematodes can be determined to indicate whether this herbicide makes the environment more favourable to beneficial nematodes. Studies should also be conducted specifically on beneficial nematode guilds that are more sensitive to agricultural practices such as the omnivorous and carnivorous nematodes. It has been shown that glyphosate adversely affects other beneficial organisms such as arbuscular mycorrhizal fungi (AMF) (Druille *et al*., 2013) and symbiotic organisms such as *Bradyrhizobium japonicum* (Powel *et al.,* 2009).

iv) Ultimately, should field studies similar to this one should be executed it should include more sampling intervals during the growing season of soybean crops. The reason for this is that beneficial nematode community structures may change over time (from planting to harvesting of the crop) as a result of biotic and abiotic factors that influence such organisms. In a study by Cheng *et al.* (2008), sampling occurred over three seasons to limit seasonal variation among nematode populations.

The abovementioned recommendations may not only lead to more insight into the effect and impact of glyphosate on agricultural systems as a whole, but can also provide soybean producers with more information on the benefits of healthy soils that contain beneficial organisms. However, since various short-term studies and the present long-term study have suggested that glyphosate do not alter microbial and nematode community structures, it may be more beneficial to shift the focus of future studies from the effects of herbicides on soil organisms to other environmental impacts, such as the physical and chemical environment, on beneficial and harmful microorganisms found in agricultural soils. This could lead to more information to create more sustainable agricultural management and food security for future generations.

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Appendix A.

Table 1: Estimated viable microbial biomass and phospholipid fatty acid ratios of the sites sampled from RoundUp ® Ready (RR) and conventional soybean (CS) fields as well as from adjacent natural veld (NV).

Site	pmol	F / B ratio[1]	Sat / Unsat[2]	Gram +[4] / Total	Iso / a-iso[5]	*Trans / cis*[3]
	5224.38	0.05	2.42	0.32	1.95	0.21
RR1	± 532.27 (a)	± 0.007 (c)	± 0.125 (c)	± 0.006 (g)	± 0.085 (gh)	± 0.081 (a)
	12600.69	0.003	0.90	0.16	1.65	0.50
RR2	± 1684.45 (abc)	± 0.001 (a)	± 0.129 (ab)	± 0.011 (ab)	± 0.090 (abcdef)	± 0.053 (bcd)
	8202.32	0.04	2.56	0.30	2.21	0.37
RR3	± 267.61 (ab)	± 0.004 (bc)	± 0.287 (c)	± 0.025 (g)	± 0.055 (i)	± 0.059 (abc)
	23533.62	0.002	0.65	0.21	1.74	0.58
RR4	± 2195.91 (abcd)	± 0.0001 (a)	± 0.028 (ab)	± 0.010 (bcde)	± 0.038 (cdefg)	± 0.026 (bcde)
	28718.51	0.002	0.68	0.25	1.91	0.88
RR5	± 3587.52 (bcd)	± 0.0002 (a)	± 0.015 (ab)	± 0.004 (ef)	± 0.085 (fgh)	± 0.063 (fgh)
	8079.69	0.0337	1.07	0.21	1.46	0.62
RR6	± 615.52 (ab)	± 0.003 (b)	± 0.069 (b)	± 0.017 (bcde)	± 0.053 (ab)	± 0.134 (bcdef)
	6874.89	0.06	2.60	0.30	1.88	0.35
CS1	±1354.13 (ab)	± 0.011 (c)	± 0.139 (c)	± 0.008 (fg)	± 0.011 (defgh)	± 0.078 (ab)
	12583.29	0.004	0.60	0.22	1.62	0.60
CS2	±1125.66 (abc)	± 0.001 (a)	± 0.031 (ab)	± 0.004 (cde)	± 0.032 (abcd)	± 0.023 (bcdef)
	11722.38	0.002	0.97	0.17	2.03	0.62
CS3	± 1433.93 (abc)	± 0.0004 (a)	± 0.037 (ab)	± 0.009 (abc)	± 0.085 (hi)	± 0.059 (bcdef)
	19294.77	0.003	0.65	0.19	1.73	0.69
CS4	± 1879.65 (abcd)	± 0.0001 (a)	± 0.001 (ab)	± 0.005 (abcd)	± 0.022 (cdefg)	± 0.014 (defgh)
	16152.92	0.002	0.74	0.23	1.90	0.75
CS5	± 1118.02 (abc)	± 0.0001 (a)	± 0.022 (ab)	± 0.014 (de)	± 0.028 (efgh)	± 0.025 (defgh)
	23198.43	0.004	0.56	0.20	1.64	0.68
CS6	± 3860.37 (abcd)	± 0.0001 (a)	± 0.026 (a)	± 0.004 (abcde)	± 0.058 (abcde)	± 0.023 (defgh)
	38754.4	0.05	0.58	0.24	1.76	0.66
CS7	± 8212.90 (d)	± 0.001 (a)	± 0.041 (ab)	± 0.014 (e)	± 0.0182 (defg)	± 0.051 (cdefg)
	6306.57	0.04	3.28	0.30	1.70	0.38
NV1	± 635.05 (ab)	± 0.004 (c)	± 0.333 (d)	± 0.005 (fg)	± 0.024 (bcdefg)	± 0.079 (abc)
	15908.01	0.002	0.82	0.21	1.66	0.97
NV2	± 2544.70 (abc)	± 0.001 (a)	± 0.025 (ab)	± 0.006 (bcde)	± 0.059 (abcdef)	± 0.064 (h)
	64327.26	0.003	0.58	0.15	1.41	0.71
NV3	± 14630.21 (e)	± 0.0002 (a)	± 0.032 (ab)	± 0.008 (a)	± 0.017 (a)	± 0.025 (defgh)

	33771.13	0.002	0.68	0.20	1.79	0.71
NV4	± 2094.69 (cd)	± 0.0001 (a)	± 0.021 (ab)	± 0.002 (bcde)	± 0.012 (defgh)	± 0.013 (defgh)
	21186.66	0.0007	0.81	0.20	1.73	0.85
NV5	± 4641.39 (abcd)	± 0.0001 (a)	± 0.032 (ab)	± 0.015 (bcde)	± 0.028 (cdefg)	± 0.052 (efgh)
	38875.99	0.002	0.72	0.22	1.78	0.97
NV6	± 5684.91 (d)	± 0.0006 (a)	± 0.024 (ab)	± 0.003 (cde)	± 0.021 (defgh)	± 0.126 (h)
	24734.45	0.003	0.69	0.21	1.73	0.85
NV7	± 5894.91 (abcd)	± 0.0001 (a)	± 0.055 (ab)	± 0.0163 (bcde)	± 0.048 (cdefg)	± 0.049 (efgh)
	28195.11	0.007	0.82	0.24	1.69	0.76
NV8	± 7139.21 (bcd)	± 0.0015 (a)	± 0.078 (ab)	± 0.016 (de)	± 0.074 (bcdefg)	± 0.037 (defgh)
	32407.21	0.001	0.74	0.24	1.49	0.90
NV9	± 4300.99 (cd)	± 0.0003 (a)	± 0.032 (ab)	± 0.009 (de)	± 0.098 (abc)	± 0.093 (fgh)
	27153.22	0.001	0.77	0.23	1.92	0.95
NV10	± 7132.65 (abcd)	± 0.0002 (a)	± 0.024 (ab)	± 0.006 (de)	± 0.093 (gh)	± 0.064 (gh)

* All values ± standard error represent results obtained for each site

** Statistically significant differences are indicated by alphabetic letters ($p \leq 0.05$).The same letters indicate no significant differences.

[1] F/B ratio determined using: Fungi: 18:2ω6; Bacteria: Sum of i15:0, a15:0, 15:0, i16:0, 16:1ω9, 16:1ωt, i17:0, a17:0, cy17:0, 17:0, 18:1ω7 and cy19:0 (Frostegård *et al.,* 1996; Ratcliff *et al.,* 2006).

[2] Straight chain saturated: Monounsaturated PLFAs (PLFA markers used are shown in Table 3.3) (Bach *et al*., 2010)

[3] 16:1ω7t: 16:1ω7c (McKinley *et al.,* 2005).

[4] Gram-positive PLFA indicators (i15:0, a15:0, i16:0, 10me16:0 and 17:0) to total PLFAs (McKinley *et al.,* 2005).

[5] Iso: Anteiso branched PLFAs (i15:0 + i17:0 / a15:0 + a17:0) (Frostegård *et al.,* 1996; McKinley *et al.,* 2005).

Table 2: Estimated viable microbial biomass and PLFA ratios the three ecosystems - RoundUp ® Ready (RR) and conventional soybean (CS) as well as natural veld (NV).

Ecosystem	**RR**	**CS**	**NV**
Viable microbial biomass pmol/g^{-1} soil dry weight	14393.20 ± 4262.53 (a)	18368.73 ± 4263.61 (a)	29286.56 ± 5150.57 (a)
PLFA ratios			
Fungal / Bacterial [1]	0.0221 ± 0.01 (a)	0.0106 ± 0.01 (a)	0.0070 ± 0.01 (a)
Saturated / Unsaturated [2]	1.3809 ± 0.39 (a)	0.9559 ± 0.30 (a)	0.9888 ± 0.27 (a)
trans / cis [3]	0.5262 ± 0.10 (a)	0.6197 ± 0.05 (ab)	0.8040 ± 0.06 (b)
Gram (+) / Total PLFAs [4]	0.2396 ± 0.03 (a)	0.2198 ± 0.01 (a)	0.2194 ± 0.01 (a)
Iso / Anteiso [5]	1.8201 ± 0.12 (a)	1.7954 ± 0.06 (a)	1.6916 ±0.05 (a)

* All values ± standard error represent results obtained from averages for each ecosystem

** Statistically significant differences are indicated by alphabetic letters ($p \leq 0.05$).The same letters indicate no significant differences.

[1] F/B ratio determined using: Fungi: 18:2ω6, 9; Bacteria: Sum of i15:0, a15:0, 15:0, i16:0, 16:1ω9, 16:1ωt, i17:0, a17:0, cy17:0, 17:0, 18:1ω7 and cy19:0 (Frostegård *et al.,* 1996; Ratcliff *et al.,* 2006).

[2] Straight chain saturated: Monounsaturated PLFAs (Bach *et al*., 2010)

[3] 16:1ω7t: 16:1ω7c (McKinley *et al*., 2005).

[4] Gram-positive PLFA indicators (i15:0, a15:0, i16:0, 10me16:0 and 17:0) to total PLFAs (McKinley *et al.,* 2005).

[5] Iso: Anteiso branched PLFAs (i15:0 + i17:0 / a15:0 + a17:0) (Frostegård *et al.,* 1996; McKinley *et al.,* 2005).

Table 3: Phospholipid fatty acid (PLFA) composition of the three ecosystems - RoundUp ® Ready (RR) and conventional soybean (CS) as well as natural veld (NV).

Average	**RR**	**CS**	**NV**
Normal saturated	28.92 (a)	25.93 (a)	25.41 (a)
Mid-chain branched saturated	10.44 (b)	7.66 (ab)	6.65 (a)
Terminally branched saturated	25.77 (a)	25.42(a)	26.53 (a)
Monounsaturated	31.60 (a)	38.16 (a)	38.35 (a)
Polyunsaturated	1.16 (a)	0.70 (a)	0.46 (a)

Table 4: Phospholipid fatty acid (PLFA) composition for sites sampled in RoundUp ® Ready (RR) and conventional soybean (CS) as well as natural veld (NV) during this study.

Site	Normal saturated	Mid-chain branched saturated	Terminally branched saturated	Monounsaturated	Polyunsaturated
RR1	32.51 ± 0.91 (bc)	14.28 ± 2.09 (ef)	31.85 ± 1.53 (hi)	15.65 ± 0.62 (a)	2.76 ± 0.36 (cd)
RR2	30.00 ± 2.87 (b)	7.02 ± 0.39 (abc)	19.16 ± 1.67 (ab)	44.60 ± 1.49 (b)	0.15 ± 0.05 (a)
RR3	33.81 ± 0.17 (bc)	16.11 ± 3.49 (f)	28.22 ± 1.53 (efgh)	16.62 ± 2.02 (a)	2.08 ± 0.17 (bc)
RR4	23.23 ± 0.35 (a)	8.17 ±0.35 (abcd)	25.53 ± 0.99 (cdefg)	40.69 ± 1.14 (b)	0.23 ± 0.01 (a)
RR5	23.67 ± 0.52 (a)	6.38 ± 0.42 (abc)	29.89 ± 0.69 (ghi)	35.85 ± 0.86 (bc)	0.18 ± 0.01 (a)
RR6	30.34 ± 0.86 (b)	10.71 ± 2.19 (bcde)	19.99 ± 1.37 (abcd)	36.17 ± 0.95 (b)	1.53 ± 0.21 (b)
CS1	33.52 ± 0.81 (bc)	12.86 ± 0.94 (def)	31.73 ± 1.41 (hi)	16.59 ± 0.55 (a)	3.12 ± 0.52 (d)
CS2	22.68 ± 0.60 (a)	7.62 ±0.37 (abcd)	25.71 ± 0.29 (efg)	41.31 ± 0.93 (bc)	0.37 ± 0.02 (a)
CS3	30.81 ± 1.00 (b)	8.63 ±0.58(abcd)	20.07 ± 0.95 (abc)	39.23 ± 2.73 (bc)	0.10 ± 0.15 (a)
CS4	25.00 ± 0.23 (a)	5.98 ± 0.23 (abc)	23.01 ± 0.56 (abcde)	44.17 ± 0.39 (bc)	0.33 ± 0.01 (a)
CS5	25.29 ± 0.51 (a)	7.75 ±0.68(abcd)	25.61 ± 0.63 (efg)	38.19 ± 0.91 (b)	0.31 ± 0.01 (a)
CS6	22.32 ± 0.42 (a)	5.85 ± 0.20 (abc)	24.14 ± 0.74 (bcdef)	45.70 ± 0.33 (bc)	0.33 ± 0.01 (a)
CS7	21.90 ± 0.45 (a)	4.9 6 ± 1.09 (ab)	27.68 ± 1.53 (fghi)	41.96 ± 2.61745 (bc)	0.35 ± 0.02 (a)
NV1	36.14 ± 0.94 (c)	10.68 ± 0.66 (cde)	33.44 ± 0.36 (i)	15.04 ± 0.99 (a)	2.52 ± 0.18 (cd)
NV2	25.60 ± 0.42 (a)	7.60 ±0.41 (abcd)	25.66 ± 0.99 (defg)	39.47 ± 1.11 (bc)	0.23 ± 0.02 (a)
NV3	22.50 ± 0.79 (a)	3.46 ± 0.48 (a)	18.05 ± 1.019 (a)	53.05 ± 1.42 (b)	0.19 ± 0.01 (a)
NV4	24.42 ± 0.35 (a)	4.20 ± 0.09 (a)	24.01 ± 0.47 (cdef)	44.69 ± 0.51 (c)	0.22 ± 0.01 (a)
NV5	25.71 ± 0.18 (a)	6.58 ± 0.15 (abc)	26.85 ± 1.59 (efg)	38.87 ± 1.71 (b)	0.29 ± 0.02 (a)
NV6	24.00 ± 0.30 (a)	5.48 ± 0.29 (abc)	27.70 ± 0.22 (efgh)	38.65 ± 0.55 (d)	0.26 ± 0.03 (a)
NV7	23.95 ± 0.30 (a)	6.06 ± 0.51 (abc)	26.26 ± 2.11 (efg)	41.46 ± 2.98 (b)	0.22 ± 0.02 (a)
NV8	24.13 ± 0.68 (a)	8.00 ±1.54 (abcd)	26.02 ± 0.88 (efg)	39.15 ± 2.74 (bc)	0.43 ± 0.08 (a)
NV9	24.36 ± 0.51 (a)	6.48 ± 0.72 (abc)	28.55 ± 0.99 (fghi)	37.30 ± 0.94 (bc)	0.16 ± 0.04 (a)
NV10	23.31 ± 0.63 (a)	7.96 ±1.05 (abcd)	28.79 ± 1.14 (fghi)	35.83 ± 2.03 (b)	0.13 ± 0.03 (a)

* All values ± standard error represent results obtained from averages for each ecosystem

** Statistically significant differences are indicated by alphabetic letters ($p \leq 0.05$).The same letters indicate no significant differences.

Table 5: Percentage fraction for each functional group in the sites sampled in RoundUp ® Ready (RR) and conventional soybean (CS) as well as natural veld (NV) during this study.

Site	Plant-Parasitic Nematodes	Bacterivores	Fungivores	Omnivores	Carnivores
RR1	20.51	66.93	10.97	1.59	0.00
RR2	27.80	37.05	33.81	1.34	0.00
RR3	12.68	74.71	9.65	2.96	0.00
RR4	4.74	79.01	8.63	3.55	4.08
RR5	4.35	79.32	15.51	0.74	0.09
RR6	40.85	43.61	9.43	6.11	0.00
CS1	11.15	36.50	42.69	9.65	0.00
CS2	5.95	87.93	4.15	1.49	0.48
CS3	13.28	48.78	36.49	1.44	0.00
CS4	28.02	60.23	11.48	0.27	0.00
CS5	37.67	59.66	2.46	0.21	0.00
CS6	13.51	78.25	7.27	0.98	0.00
CS7	21.24	70.23	7.10	0.92	0.50
NV1	36.34	49.79	11.85	2.02	0.00
NV2	10.71	82.30	3.85	2.74	0.41
NV3	21.34	52.37	23.07	2.17	1.05
NV4	5.18	60.34	32.43	1.95	0.10
NV5	9.78	83.05	6.79	0.38	0.00
NV6	23.64	70.75	4.54	1.08	0.00
NV7	10.25	82.70	5.94	1.11	0.00
NV8	50.26	29.84	16.00	3.90	0.00
NV9	18.51	28.45	50.29	2.48	0.28
NV10	45.92	32.15	18.94	2.94	0.05