The effects of herbicides on soil chemical and soil microbial properties of riparian fynbos ecosystems

By

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Thesis presented in partial fulfillment of the requirements for the degree of Master of Science in Conservation Ecology
at
Stellenbosch University

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April 2019

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Abstract

Woody invasive alien plants (IAP's) have various impacts on natural ecosystems in the Western Cape Province of South Africa. These impacts include the absorbance of large quantities of water, increased veld-fire risk, the endangerment of local indigenous flora as well as increased homogeneity of various riparian fynbos habitats. To combat the invasion of IAP's, the WfW programme has been established. Woody invasive species belonging to genera such as *Eucalyptus, Acacia*, and *Populus* are treated with specialized herbicides to eradicate the trees and to ultimately prevent further encroachment in demarcated areas.

The herbicides used have proven to be effective at controlling the targeted IAP's and is considered an agricultural or natural remedy for rehabilitating fynbos habitats. Although effective, these herbicides could have unforeseen detrimental impacts on non-target plant species as well as on the local soil environment. Many researchers have found that herbicides and other agrochemicals can affect both biotic and abiotic properties of the soil. Additionally, other researchers have shown that herbicides can in fact stimulate the soil environment and moreover induce no harm. There is an increased pressure from woody IAPs on riparian fynbos and other habitat types, which calls for increased usage of herbicides to control and effectively manage the encroaching weeds. It is, therefore, imperative that the potentially detrimental or beneficial effects of these herbicides on riparian fynbos are determined. This study therefore aimed to assess the potential impacts that conventional herbicides may have on the soil environment of riparian fynbos habitats in the Western Cape.

The herbicides tested were those registered for treating *A. mearnsii* and *E. camaldulensis* which are woody invasive plants encroaching rather frequently in riparian fynbos areas. Two different herbicide products namely, Springbok 360 SL and Garlon 480 EC, were tested in stands of *A. mearnsii*, whilst one product namely, Plenum 160 ME, was tested in *E. camaldulensis* stands. Impacts on the soil environment were determined by studying the change in various soil chemical characteristics (such as soil pH, electrical conductivity, total soil nitrogen, nitrogen mineralization, soil available phosphorus, and phosphatase activity). Additionally, soil microbial activity was studied by evaluating carbon substrate metabolism (via microtiter eco-plates). Microbial community structure was also studied, and this included evaluating the change in measures of species richness and evenness (OTU's and Shannon-index). The herbicides were applied at different concentrations [i.e. one tenth of the field rate (10 % FR), one times the field rate (100 % FR), ten times the field rate (1000 % FR), and untreated control (0 % FR)] in both *in situ* and *ex situ* experiments which ran for a duration of six weeks.

When either of the herbicides were applied, soil pH levels decreased significantly (Kruskal-Wallis ANOVA: $p < 0.05$) in both *in situ* and *ex situ* experiments. The pH generally dropped with 0.5 units or more and it appeared that the herbicides all had a slight, yet consistent acidifying effect on the soil. Additionally, soil available nitrogen increased significantly when Springbok 360 SL (glyphosate) treatment took place (Kruskal Wallis ANOVA: $p = 0.02$), where soil nitrogen levels increased from the tens to over 100 $\mu g.g^{-1}$. The acidity and nitrogen changes in the soil expressed significant changes with herbicide treatment, but other soil chemical characteristics studied showed little to no considerable impacts. An example includes the study of soil available phosphorus which revealed no changes when testing either herbicide treatment, even at the higher concentration dosages [*In situ*: Soil available P remained between 8 and 16 ppm, One Way ANOVA = $p > 0.87$; *Ex situ*: Soil available P remained between 6 and 14 ppm, One Way ANOVA = $p > 0.53$].

Similarly, soil microbial community structure seemed unaffected where both microbial carbon substrate utilization and soil microbial community structure remained stable. Soil microbial activity and community metabolic capacity in soils that were treated with herbicide showed no significant differences when compared to untreated soils. The rates of metabolism remained within a range of 1 and 4 absorbance at hour 168 of incubation. Furthermore, the microbial species richness and evenness showed variability (standard deviation values as high as 18) yet remained stable according to OTU numbers and Shannon index (One Way ANOVA: Springbok 360 SL treated, $p = 0.76$; Garlon 480 EC treated, $p = 0.19$; Plenum 160 ME treated, $p = 0.06$). An ANOSIM further presented great degrees of similarity of microbial groups before and after herbicide treatment irrespective of the concentration applied (R statistic values ranging between 0.05 and 0.15 showing for very little amounts of dissimilarity). The deduction from these results were that no detrimental or inhibitory effects occurred with treatment of either herbicide within a six-week period.

As mentioned above, the herbicides tested showed an acidifying effect on fynbos soils yet lack any other detrimental impacts on the soil environment when regarding the other various aspects studied. This study revealed that these herbicides have minor impacts on riparian fynbos soil, but the fact remains that even only to a minor extent, there are consistent and significant effects (i.e. when considering pH). There is a research gap when regarding our understanding of how herbicides can affect soils and the greater ecosystem. Herbicide operators, farmers and nature-reserve managers should be cautious when selecting chemicals to apply *in situ.* If herbicide clearing is done and the soils are rendered significantly more acidic it could hinder long term rehabilitation or planting schemes as well as inhibit the soils ability to fulfill ecosystem functions. The extent of these herbicide impacts is determined by the type of herbicide used, the concentration thereof, as well as the assemblages present

in the soil at the time of treatment. It is therefore important to further research herbicides in a variety of conditions including different application methods, different *in situ* and *ex situ* conditions, and with a broad spectrum of analyses. This is required to achieve a better understanding of how herbicides and other agrochemicals can be used in a manner that is more environmentally compatible with intricate fynbos ecosystems.

Samevatting

Houtagtige indringer uitheemse plante (IAP's) het verskeie impakte op natuurlike ekosisteme in die Wes-Kaapse Provinsie van Suid Afrika. Hierdie impakte sluit in die absorpsie van groot hoeveelhede water, verhoogde veldbrandrisiko, die bedreiging van plaaslike inheemse flora sowel as 'n toename in homogeniteit van verskeie fynbos rivieroewer habitatte. Die WfW program was gestig om hierdie indringing te beveg. Houtagtige indringer spesies wat aan genera soos *Eucalyptus*, *Acacia* and *Populus* behoort word behandel met gespesialiseerde onkruiddoders om die bome te vernietig, en om uiteindelik verdere indringing in afgebakende areas te voorkom.

Onkruiddoders wat gebruik is, is effektief om die teiken IAP's te beheer en word oorweeg as 'n landbou- of natuurlike oplossing vir die rehabilitasie van fynbos habitatte. Alhoewel die onkruiddoders effektief is, kan daar onvoorspelde nadelige impakte op nie-teiken plant spesies wees asook op die plaaslike grond omgewing. Navorsers het gevind dat onkruiddoders en ander landbouchemikalieë beide biotiese en abiotiese eienskappe van die grond kan affekteer. Daarbenewens, het ander navorsers gewys dat onkruiddoders die grond omgewing kan stimuleer en dus geen skade veroorsaak nie. Die verhoogde druk van houtagtige IAP's op rivieroewers fynbos, het tot gevolg 'n toenemende gebruik van onkruiddoders. Dit is daarom noodsaaklik dat potensiële nadelige of voordelige effekte van die onkruiddoders bestudeer word. Hierdie studie was daarom gemik om die potensiële impakte van algemene onkruiddoders op die grond omgewing van rivieroewer fynbos habitatte in die Wes-Kaap te evalueer.

Die onkruiddoders wat getoets was, is geregistreer om die houtagtige IAP's *A. mearnsii* en *E. camaldulensis* dood te maak, wat algemeen voorkom in rivieroewers fynbos areas. Twee produkte naamlik, Springbok 360 SL en Garlon 480 EC was getoets in aanplantings van *A. mearnsii*, terwyl Plenum 160 ME getoets was in aanplantings van *E. camaldulensis*. Die impak op die grond omgewing was bepaal deur die studie van die verandering in verskeie grond chemiese eienskappe (soos grond pH, elektriese geleidingsvermoë, totale grond stikstof, stikstof mineralisasie, grond beskikbare fosfor, en fosfatase aktiwitiet). Daarbenewens, grond mikrobiese aktiwiteit was bestudeer deur die evaluering van koolstof substraat metabolisme (via 'microtiter eco-plates'). Mikrobiese gemeenskap struktuur was ook bestudeer, en dit sluit in die evaluering van die verandering in spesies rykheid en eweredigheid (OTU's en Shannon-indeks). Die onkruiddoders was toegedien teen verskillende konsentrasies [d.w.s een tiende van die veld dosis (10 % FR), een keer die veld dosis (100 % FR), tien keer die veld dosis

(1000 % FR) en onbehandelde beheer (0 % FR)] in beide *in situ* en *ex situ* eksperimente wat oor ‘n tydperk van ses weke geskied het.

Wanneer enige van die bogenoemde onkruiddoders toegedien was, het die grond pH vlakke beduidend afgeneem (Kruskal-Wallis ANOVA: $p < 0.05$) in beide *in situ* en *ex situ* eksperimente. Die pH het gemiddeld met 0.5 eenhede gedaal en dit kom voor dat die onkruiddoders ‘n effense, maar konstante versurende effek op die grond gehad het. Daarbenewens het grond beskikbare stikstof beduidend toegeneem wanneer Springbok 360 SL (glifosaat) behandeling plaas gevind het (Kruskal Wallis ANOVA: $p = 0.02$) waar grond N vlakke van die tiene tot oor 100 $\mu g.g^{-1}$ gestyg het. Die waargeneemde suurheid en stikstof veranderinge in die grond het nie veel kommer gewek nie, omdat die ander grond chemiese eienskappe wat bestudeer was min tot geen beduidende impak vir enige van die onkruiddoders wat getoets was, selfs met hoër konsentrasie behandelings [*In situ*: One Way ANOVA = $p > 0.87$; *Ex situ*: One Way ANOVA = $p > 0.53$], gewys het nie.

Soortgelyk kom dit voor dat die grond mikrobiese gemeenskap struktuur ongeaffekteer gebly het waar beide mikrobiese koolstof substraat benutting en grond mikrobiese gemeenskap struktuur stabiel was. Grond mikrobiese aktiwiteit en gemeenskap metaboliese kapasiteit in grond wat behandel was met onkruiddoder het geen beduidende verskille getoon wanneer dit met onbehandelde grond vergelyk was nie. Die tempo van metabolisme het binne ‘n absorbansie van 1 en 4 by uur 168 van inkubasie gebly. Verder het die mikrobiese spesies rykheid en eweredigheid veranderlikheid gewys (standard afwyking waardes so hoog soos 18), maar het stabiel gebly volgens die OTU getalle en Shannon-indeks (One Way ANOVA: Springbok 360 SL behandeling, $p = 0.76$; Garlon 480 EC behandeling, $p = 0.19$; Plenum 160 ME behandeling, $p = 0.06$). ‘n ANOSIM het verder ‘n hoë graad van ooreenkoms tussen mikrobiese groepe voor en na onkruiddoder behandeling gewys, ongeag die konsentrasie toegedien (R statistiek waardes wissel tussen 0.05 en 0.15 toon vir baie min mate van dissimariliteit). Op grond van bogenoemde resultate was die afleiding gemaak dat geen kommerwekkende of inhiberende effekte plaasgevind het, met behandeling van enige van die onkruiddoders binne ‘n ses-week tydperk nie.

Soos genoem, het die onkruiddoders wat getoets was, gewys dat daar ‘n versurende effek op fynbos grond was, maar gebrek aan enige ander kommerwekkende impakte op die grond omgewing met verwysing na die ander verskeie aspekte wat bestudeer was. Hierdie studie het aan die lig gebring dat hierdie onkruiddoders klein impakte op oewerfynbosgrond het, maar die feit bly dat selfs in 'n mindere mate konsekwente en betekenisvolle effekte (d.w.s. by die oorweging van pH). Daar is 'n navorsingsgaping wat betref ons begrip van hoe onkruiddoders gronde en die groter ekosisteem kan beïnvloed. Onkruiddoderoperateurs, boere en

natuurreservaatbestuurders moet versigtig wees wanneer chemikalieë gekies word om *in situ* toe te pas. As onkruiddoderopruiming gedoen word en die gronde beduidend meer suur gemaak word, kan dit langtermyn rehabilitasie of plantskemas belemmer, asook om die grond se vermoë om ekosisteemfunksies te vervul, te inhibeer. Die omvang van hierdie onkruiddoder-impak word bepaal deur die tipe onkruiddoder wat gebruik word, die konsentrasie daarvan, sowel as die samestellings wat in die grond teenwoordig is tydens behandeling. Dit is daarom belangrik dat verdere navorsing die toetsing van verskillende onkruiddoders in ‘n verskeidenheid kondisies insluitend verskillende toepassingsmetodes, verskillende *in situ* en *ex situ* voorwaardes, en met 'n breë spektrum van analise. Dit is ‘n vereiste om ‘n beter begrip te kry oor hoe onkruiddoders en ander landbouchemikalieë gebruik kan word op so ‘n wyse wat meer omgewingsvreindelik is met ingewikkelde fynbos ekosisteme.

Acknowledgements:

I am very grateful for the love and support of my family and friends whom have always been there for me when needed the most. There have been many times of 'thick and thin' throughout the course of this study and I would not have been able to complete this thesis had it been for their support and encouragement.

I am also very grateful to my two supervisors, Prof. Shayne Jacobs and Prof. Karin Jacobs. They have not only been a great source of support and guidance, but also outstanding mentors whom have taught me a lot about working in the science industry. Their constructive criticism and encouragement will remain with me for the rest of my life.

I also thank the Water Research Commission of South Africa who funded my project, provided me with a bursary, and critiqued my research to further strengthen it. The National Research Foundation is also acknowledged and thanked for funding this project. I would also like to thank Mr. Earlon McCloen, Mr. Armand van Wyk, Mr. Casper Brink, Ms. Monique Henn, Mr. Thsepo Mbaune, Dr. Etienne Slabbert, and Mr. Ricardo Smart for their assistance in many of the microbial and soil chemical laboratory techniques. Furthermore, Adrian Simmers assisted greatly with fieldwork and his knowledge and field-experience is acknowledged and appreciated. I thank all the landowners of my field sites as well as the Worcester District Municipality and Department of Water Affairs for allowing me to conduct my experiments on their land and under their supervision. Gratitude also goes to Mr. Deon Malherbe and Mr. Steyn Viljoen who supervised and helped with my greenhouse experiments.

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CHAPTER 1

Introduction, Literature Review and Problem Statement

1. Introduction

Fynbos ecosystems of the Western Cape of South Africa are highly endangered by woody invasive alien plants (IAP's) (Richardson & Van Wilgen, 2004; Richardson et al., 2007). *Acacia, Eucalyptus, Salix, Populus, Hakea* and *Prosopis* species encroach fynbos habitats and, in many instances, compromise indigenous wildlife and change the dynamics of local ecosystems (Dzikiti et al., 2013; Le Maitre et al., 2000; Steenkamp & Chown, 1996; Tucker & Richardson, 1995). This is a major threat for riparian areas in particular and it is estimated that the consumptive water absorption of alien invaders in South Africa has led to a 6.7 % reduction in river flow (Le Maitre et al., 2002). Species such as *Acacia mearnsii* and *Eucalyptus camaldulensis* are commonly found encroaching in riparian fynbos areas and continue to dominate riparian fynbos habitats. The Working for Water program (WfW) is tasked with clearing invasive plant species including *A. mearnsii* and *E. camaldulensis* from nature reserves, municipal land, agricultural land, private land, as well as green urban-spaces and aim to restore natural ecosystems in the country (Marais et al., 2004; Turpie et al., 2008). The WfW is a widely-recognized restoration initiative and utilizes mechanical removal, pile-burning and chemical treatment as a means of clearing alien invasive plants from demarcated areas (Blanchard & Holmes, 2008; Roura-Pascal et al., 2009). The chemical clearing approach includes using specified herbicides to prevent growth and eventually kill targeted invasive plant populations (Blanchard & Holmes, 2008; Roura-Pascal et al., 2009; Van Wilgen et al., 2012).

The registered herbicides used by the WfW for the control of *A. mearnsii* populations include Springbok 360 SL (active ingredient: glyphosate soluble liquid formulation) and Garlon 480 EC (active ingredient: triclopyr emulsifiable concentrate formulation), whilst the herbicide Plenum 160 ME is used for *E. camaldulensis* (active ingredients: picloram and fluroxypyr micro-emulsion formulation). These herbicides are reported to be effective at managing invasive *Acacia* and *Eucalyptus* species populations, but woody IAP's are known to regenerate through regrowth or coppicing if not treated correctly or if sprayed in the incorrect season. Secondary invasion of woody IAP's may also occur from stored seedbanks long after herbicide spraying has taken place (Otto, 2014; Wolmarans & Swart, 2014). Besides the regeneration of the targeted IAP's, another concern is that the active ingredients of these herbicides have been reported to induce potential detrimental effects on soil function and local

soil chemical properties (Estok et al., 1989; George et al., 2009; Haney et al., 2000; Ragab, 1975). Additionally, these herbicides may seep into the soil and could not only affect soil chemical characteristics but could affect non-target soil organisms and have the capacity to induce detrimental legacy effects (Atlas et al., 1978; Chakravarty & Sidhu, 1987; Prado & Airoldi, 2001).

There are several reports of herbicide and other toxicants affecting soil ecology where either the soil nutrient levels and/ or the local soil microorganisms are altered to a certain extent. Toxicants such as imazapic have been reported to significantly increase the soil nitrogen levels in soils of an alluvial flood-plain (Reddy et al., 2003). Furthermore, herbicides have not only been shown to affect the nutrient levels in the soil, but soil processes too. An example includes that of Perucci et al. (2000), where the herbicides rimsulfuron and imidazolinone had adverse effects on soil chemistry, significantly decreasing phosphatase activity and microbial biomass from its pre-treated condition. Microorganisms are sensitive to changes in the environment and are expected to respond rather easily and rapidly to changes in the soil chemistry (Kirchman, 2012). This is rather concerning as herbicides could alter soil chemical characteristics which would affect the soil microorganisms that play an integral role in soil function and above-ground flora survival. Herbicides have however, in a few instances, displayed no impact on microorganisms where biomass, colony forming units (cfu) and measures of microbial diversity remain unchanged when exposed to herbicides (Haney et al., 2000; Peterson et al., 1994; Wertz et al., 2007).

From literature, it is evident that herbicides induce rather varied responses in soil depending on the active ingredient, concentration, and toxic nature of the herbicide (Busse et al., 2001; Carter, 2000). There are no set models illustrating how riparian fynbos soil chemical traits and soil micro-organisms will respond to the exposure of certain types of herbicide. Further research based upon the impact of herbicides on cleared riparian fynbos zones is important as the non-target organisms affected, and the extent to which they are affected remain largely unknown. Understanding the impacts can inform chemical control methodologies used in invaded riparian fynbos areas. A better understanding of potential herbicide effects can enlighten managers and herbicide operators of aspects to consider for a more environmentally compatible clearing operation. Effects of herbicides, pesticides, or other toxicants are usually studied in monocultural or *ex situ* laboratory conditions (Bitton & Koopman, 1992; Wardle & Parkinson, 1990) and it is, therefore, vital that research includes experiments both in on-site (*in situ*) and off-site (*ex situ*) settings and not only in a laboratory (Thompson et al., 2000).

This study aimed to evaluate the effects of herbicides used to control *A. mearnsii* and *E. camaldulensis,* on the soils of riparian fynbos habitats in the Western Cape. Aspects of soil chemical characteristics, soil microbial diversity, and soil microbial activity were studied to determine potential effects of herbicide on soil. The study is divided into two sections, one dealing with experiments *in situ*, and the other including experiments *ex situ.* The herbicide effects on soil chemical properties included assessing soil pH, electrical conductivity, soil available nitrogen, nitrogen mineralization, soil phosphorus, and acid phosphatase activity, whilst the herbicide effects on soil microorganisms included assessing bacterial community diversity and microbial carbon substrate utilization. All these characteristics were assessed before and after herbicide treatment in an effort to express any changes in the soil from before to after a specific herbicide treatment.

2. Literature Review

The literature review discusses five concepts appropriate for the study of alien plant invasion in riparian fynbos ecosystems and the potential impacts that chemical clearing techniques could yield (Fig. 1.1). The first concept point discusses riparian fynbos ecosystems including a brief introduction to the ecology and species of such habitats. The second point reviews the target invasive plant species of this study including *Acacia mearnsii* and *Eucalyptus camaldulensis*. In addition, the invasive attributes and impacts of the species will be discussed. The third concept point refers to the methodologies utilized for clearing the invader species from demarcated areas. The fourth and fifth concept point reviews the impacts of agrochemicals on soil chemical and soil microbial characteristics, respectively.

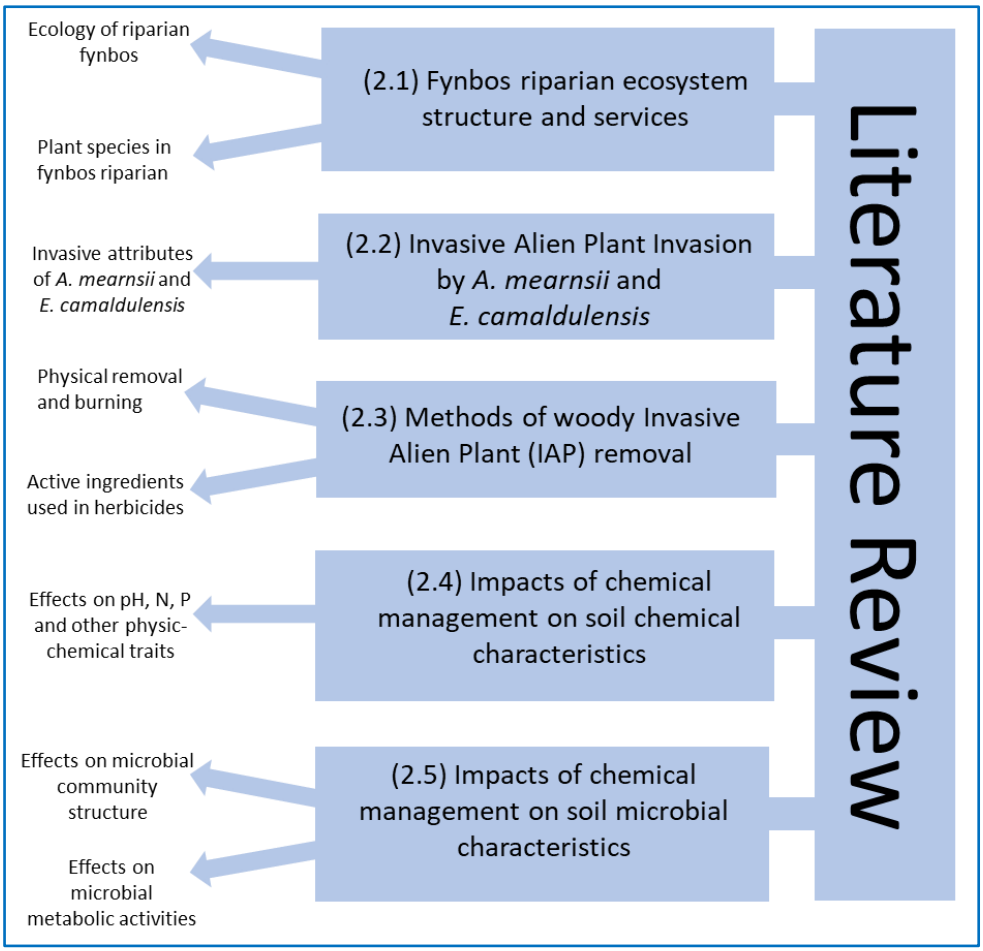

Figure. 1.1: A flow diagram illustrating the concepts discussed in the literature review below. The literature review begins with an introduction of riparian fynbos ecosystems and ends with a discussion of how herbicides used in fynbos riparian systems can induce certain impacts on soil chemical and soil microbial characteristics.

2.1. Riparian fynbos ecosystem structure and services

Riparian habitats are the interface between aquatic (riverine) and terrestrial systems (Gregory et al., 1991; Richardson et al., 2007). Riparian fynbos ecosystems, as is the case with riparian ecosystems in general, are unique in that they are transitional ecosystems and can fulfil many ecosystem services which is vital for the persistence of surrounding habitats (Catford et al., 2013; Gregory et al., 1991; Nilsson & Svedmark, 2002; Ruwanza et al., 2013b). This distinct habitat type supports many complex natural cycles including hydrological and biogeochemical cycling (McClain et al., 2003; Naiman et al., 1993; Naude, 2012), which in turn supports many riparian-associated ecosystem services (Sieben & Reinecke, 2008). For example, nutrient cycling and biogeochemical fluxes occur at greater rates in riparian systems as opposed to adjacent drier ecosystems, as a result of the riparian zone having a greater local availability of water (Stella et al., 2013). Moreover, the riparian fynbos areas of the Western Cape of

South Africa experience a Mediterranean climate with dry summers and wet winters (Stock, 2014). The species inhabiting these habitats are hence adapted to warm, dry summers with summer droughts and regular fires, whilst enduring winters that bring forth cold and wet conditions, including floods and water-logging (Pettit & Naiman, 2007; Stella et al., 2013; Stock, 2014).

Riparian fynbos ecosystems are usually dominated by woody species and herbaceous species such as *Elegia capensis, Pteridium aquilinum, Cannomois virgata*, and *Ischyrolepsis gaudichaudiana* (Dye et al., 2001), which are adapted to riparian conditions through specialized morphological and anatomical variations. The geomorphology and microclimate of these areas would be otherwise unfavourable for large woody tree species as the trees would have to deal with seasonally water-logged soil conditions and occasionally high stream power (Richardson et al., 2007). Many trees are phreatophytic in nature and have deep root system allowing water to be obtained from the zone of saturation (Meek et al., 2010). Other examples of native woody tree species thriving in these conditions within riparian fynbos zones include *Brabejum stellatifolium, Brachylaena neriifolia, Diospyros glabra*, *Kiggelaria africana*, *Metrosideros angustifolia, Olea europaea*, *Podocarpus elongatus*, and *Searsia angustifolia.* In addition, native understory plant species (graminoids, shrubs, etc.) would include *Askidiosperma chartaceum*, *Blechnum capense*, *Isolepis prolifera, Prionium serratum and Todea barbara* (Sieben & Reinecke, 2008; Tererai et al., 2015).

Native plant species that occur within riparian fynbos habitats are ecologically important in that they moderate erosion as well as base flow, which is needed to stabilize faunal species diversity and habitat diversity (Stella et al., 2013). Native plant communities are, moreover, the foundation for several different microhabitats along the course of rivers and streams and this contributes greatly to ecosystem heterogeneity. Furthermore, flood control, bank stabilization, light input and water quality are all influenced by the native flora of the riparian fynbos systems (Richardson et al., 2007). Additionally, there is great anthropogenic value as riverine landscapes are aesthetically pleasing, are used for multiple recreational purposes, and provide large amounts of freshwater. Riparian wetlands moreover contain a great intrinsic conservation value (Lant & Roberts, 1990). These aforementioned ecosystem services hold great value, yet within the local context, are threatened by large stands of invasive *Acacia* and *Eucalyptus* trees (Geldenhuys & Bezuidenhout, 2008; Richardson et al., 2000).

The soils of fynbos habitats are generally low in nutrients and plant species have consequently adopted various mechanisms to acquire nutrients and subsist (Stock et al., 2014). One such mechanism includes indigenous papilionoid legumes such as *Cyclopia, Latotomis,*

Alysicarpus, Aspalathus and *Psoralea* that form symbiotic relations with *Rhizobium, Mesorhizobium* and *Burkholderia* (Beukes et al., 2013; LeMaire et al., 2016). Both host plant and environmental factors such as soil acidity play important roles in determining the distribution of rhizobia in the soil (LeMaire et al., 2015). In addition, *Rhizobium* and *Burkholderia* species provide N and P in a soil available form that is ready for uptake by plants (Maseko & Dakora, 2013a; Maseko & Dakora, 2013b). The soil microorganisms on the other hand make use of the service of plants where plants create nodules on the roots which house and support select microorganisms (Parniske, 2000; Van de Velde et al., 2010). The microbes also consume other resources from the plant such as fixed carbon and in exchange return soluble nitrogen (Soto et al., 2009). The plant rhizosphere and associated microbial communities are hence to a great extent, interlinked and dependent on one another (Heath & Tiffin, 2009; Kiers et al., 2003).

As mentioned above, this vulnerable ecosystem type is particularly threatened by the invasion of woody invasive alien plants (IAP's) where species such as *Acacia mearnsii*, *A. saligna*, *A. longifolia*, *Eucalyptus camaldulensis, Populus canescens,* and *Salix fragilis* penetrate native habitats and outcompete native species to the detriment of ecosystem function (Kambaj Kambol, 2013; Ruwanza et al., 2013a; Tererai et al., 2015) and ecosystem services (Richardson et al., 2007). The invasive species threaten the delicate balance between native plant species populations and microbial communities (Vessey, 2003). Without these plant-microbe interactions, the system could falter, leading to secondary alien plant encroachments or an increase in the ecosystem's susceptibility to disturbance.

2.2. IAP invasion by *Acacia mearnsii* and *Eucalyptus camaldulensis*

Eucalyptus camaldulensis is a transformer species and has various mechanisms enabling it to successfully invade and establish itself within new ecosystems (Forsyth et al., 2004; Tererai et al., 2013). They are known to occur in stands with hardly any understory (native or invasive) or as stands of different ages with enough sun flecks to allow for invasive (often *Eucalyptus*) understory (Ruwanza et al., 2013b). To further compete against native plant species, *E. camaldulensis* trees utilize allelopathy, which entails the exudation of certain chemical residues into the soil environment to inhibit growth and germination of native plants. The chemicals released hinder the pathways and enzymatic activities linked to protein production and growth of the surrounding native plants and this leads to a reduction in their root and shoot growth as well as other abnormal growth patterns (Callaway and Ridenour, 2004). The *E. camaldulensis* trees essentially hamper the root and shoot growth of native plants and as a

result, outcompete the surrounding endemic plant species (Niakan & Saberi, 2009; Ruwanza et al., 2013b). Moreover, root exudation from the *E. camaldulensis* also inhibits the growth and germination of the native seeds within the local seedbank hindering germination (Niakan & Saberi, 2009).

Because of the size of *Eucalyptus* trees, the native understory can become greatly shaded. This reduces the chlorophyll content in the foliage of native plant species and this subsequently reduces photosynthetic ability of the affected plants. Consequently, this reduces the competitiveness of native plants against the growth of woody IAP's (Eker et al., 2006; Fikreyesus et al., 2011). Secondary invasion is another significant pathway which *E. camaldulensis* utilizes to persist in a new habitat as they have tough and long-lived seeds which can outlast seeds of native species in the top-soil (Tererai et al., 2013). Furthermore, the exudates and leaf-litter content of *E. camaldulensis* can disturb microbial associations with the native plant community (Blum et al., 1999). Populations of nitrogen-fixing bacteria such as *Nitrobacter* can be altered by changes in the chemical properties of the soil, in which case they are not able to fulfil certain symbiotic interactions with the above ground native plant community (Stinson et al., 2006). Allelopathic chemicals of *E. camaldulensis* may ultimately lead to the disruption of mutualistic relations that native riparian fynbos is vastly dependent on for the uptake of precious nutrients (Callaway & Ridenour, 2004).

Acacia mearnsii are also considered a transformer species and a great deal of the success in growth and persistence of this species in fynbos ecosystems stems from enhanced nitrogen fixation through microbial symbioses and producing large aboveground biomass (Yelenik et al., 2007). The microbial symbioses comprise associations between roots and nodulating microbial genera such as *Bradyrhizobium, Mesorhizobium, Microvirga, Alphaproteobacteria* and *Burkholderia* (Sprent, 2007). This tree is also known to interrupt plant-microbe and plant-resource associations for native fynbos plant species (Le Maitre et al., 2011; Richardson et al., 2000). In addition, with *A. mearnsii* newly introduced into a fynbos ecosystem, there will be an absence of pathogens and pests that would usually occur in its natural habitat (Callaway & Ridenour, 2004). Another mechanism which gives *A. mearnsii* a competitive edge over that of native vegetation includes enhanced evapotranspiration rates and nutrient acquisition which is facilitated by more extensive root systems compared to that of endemic fynbos plant species (Morris et al., 2011). In order to mine for nutrients, *Acacia* species allocate more biomass to the root system making it more extensive and able to physically get into deeper soil layers and access nutrient sources that are inaccessible to many native species (Mortimer et al., 2013; Tickner et al., 2001).

These trees are also relatively drought tolerant and this is facilitated by a greater water use efficiency. This stems from a greater safety margin against xylem cavitation and a lower water potential at certain hydraulic conductivity loss rates (Crous et al., 2012). Additionally, *A. mearnsii* grow taller and faster than the native species and are able to occupy the top of the canopy where they can gain direct access to the sunlight. This results in a bare understory which is starved of sunlight, inducing lower growth and photosynthetic rates for the native plant populations (Morris et al., 2011). *A. mearnsii* also have sclerophyllous foliage and long-lived seed-banks, which are both key traits for survival in an exotic habitat. This species is thus able to outlive and outlast the native plant species (Morris et al., 2011). Furthermore, *A. mearnsii* utilizes heteroblasty which includes high relative growth rates where biomass is conferred to bipinnate seedlings (Morris et al., 2011). These seedlings are long-lived and make use of nutrient conservation in their phyllodes as adults (Callaway & Ridenour, 2004).

2.3. Impacts of woody invasive alien plant (IAP) removal

The Working for Water program (WfW) aims to clear existing stands of IAPs and suppress the regeneration of invasive alien plants in riparian fynbos ecosystems in the Western Cape (Blanchard & Holmes, 2008), using clearing approaches and methods involving both physical and chemical means (Working for Water, 2002). These removal methodologies may bring about unintended effects on the ecosystem, especially for the plant community and associated soil environment. The felling (mechanical control) of woody IAPs could imply changes to soil nutrient stocks as the biomass of the felled trees are decomposed in the top soil layer. *Acacia* species (such as *A. saligna*) have been found to elevate soil nitrogen levels when left to decompose in Cape fynbos habitats (Witkowski, 1991). The felling of alien trees is, however, not always able to completely eradicate the targeted species in the long term. It has been observed that IAP seeds are abundantly found in the seedbank after a clearing operation and are hence able to germinate and persist for extended periods in the given ecosystem (Holmes et al., 2005). Slash and pile burning, another means of clearing woody IAPs, are aimed more at the eradication of biomass (Scott & Van Wyk, 1990). This clearing approach has also been recorded to induce effects on soil chemical properties in fynbos habitats (Korb et al., 2004; Yelenik et al., 2004).

Apart from mechanical and fire control methods, chemical control methods are also employed (Holmes et al., 2005) and are usually used in a follow-up strategy to prevent the regrowth of alien plants after a physical clearing operation. Chemical control methods include the use of specified herbicide treatments (Working for Water, 2002). Factors such as herbicide type, active ingredient, dosage of treatment, and application method are all dependent not only on

the IAP species, but also on the trees' life stage and surrounding environmental conditions (General Invasive Alien Plant Control, 2002). These herbicides could have residual effects in the soil and alter dynamics between microbes and plants, as reported in Weidenhamer & Callaway (2010). In riparian fynbos habitats, common herbicides used for controlling *A. mearnsii* populations include active ingredients such as, picloram, imazapyr, cloparylid, aminopyralid, and fluroxypyr, but more commonly glyphosate (Springbok 360 SL, Bushpig 360 SL, and Round Up herbicide), and triclopyr (Garlon 480 EC and Nuvogon 480 EC herbicide) as they work well against broadleaf invader species. Picloram and fluroxypyr micro-emulsion 'cocktails' (such as Plenum 160 ME and Gladiator 160 ME) are used for *E. camaldulensis* (Bold, 2007) (Table 1.1.), but imazapyr and triclopyr herbicide products can also be used for controlling *Eucalyptus* species. The active ingredients in the herbicide target certain enzymatic or photosynthetic processes which induces abnormal growth, the inhibition of cell division, or the disruption of certain important enzymatic pathways (Kearney & Kaufman, 1975; Peterson et al., 1994; Powles & Yu, 2010).

Table 1.1. Conventional active ingredients used by the WfW displayed along with the target plant species and the mode of action. Note that not all registered herbicides used by WfW are listed here (Master Species & Herbicide list v2.3, 2012)

Active ingredient:	Herbicide product:	Target invasive alien plant:	Mode of action for killing the target plant:
Imazapyr 200 g/L SL	Format, Eco-Imazapyr	*Callistemon rigidus, C. virminalis, Melaleuca hypericifolia, M. quinquenervia, Tamarisk aphylla, T. gallica*	Imazapyr is readily adsorbed through the foliage and roots of plants. This active ingredient controls plant growth by preventing the production of branched chain amino acids. This is done by inhibiting the enzyme AHAS (acetohydroxy acid synthase). This active ingredient is translocated via the xylem and phloem to the meristematic tissues.
Triclopyr (as butoxy ethyl ester) 480 g/L EC	Garlon, Triclon, Viroaxe	*Acacia mearnsii, A. cyclops, A. dealbata, A. melanoxylon, Solanum mauritianum, Anredera cordifolia*	Triclopyr hinders plant growth and kills the plant by limiting the nutrients available to the plant. It essentially starves the plant to death. It is known to target the enzyme peroxidase which is involved in various physiological processes.
Glyphosate (as isopropylamine salt) 360 g/L SL	Glyphosate 360, Mamba, Springbok, Ciplasite,	*Acacia mearnsii, A. saligna, A. dealbata, Ailanthus altissima, Briza maxima, Ipomoea purpurea*	The activity of the EPSP (5-enolpyruvylshikimate-3-phosphate synthase) enzyme is inhibited by this chemical and this induces the inhibition of aromatic amino acids which is necessary for protein formation. Glyphosate is adsorbed through foliage, cut-stumps or green stems, yet not through woody bark.
Fluroxypyr 80 + Picloram 80 g/L ME	Plenum, Gladiator	*Eucalyptus camaldulensis, Rubus cuneifolius, Riccinus communis, Pueraria montana, Psidium guajava*	Fluroxypyr is adsorbed through the foliage of plants and kills plants through the disruption of cell growth. This is achieved through inducing altered auxin like responses in plants. Picloram similarly mimics the plant growth hormone (auxin) which induces disorganized and uncontrollable growth of cells.
Metsulfuron methyl 600 g/kg WP	Brushoff, Climax	*Stoebe vulgaris, Verbena bonariensis, Datura ferox*	This active ingredient inhibits the growth of plants via the inhibition of cell-division in roots and shoots. Metsulfuron methyl targets the ALS (acetolactate synthase) enzyme.

2.4. Impacts of chemical control methods on soil chemical characteristics and processes in fynbos

Herbicide clearing methods aimed at controlling invasive alien plants include specialized products and dosages for an effective treatment. Herbicides are usually sprayed with care, but it can be challenging to execute a precise application and prevent the spread of these herbicides to non-target organisms or unintended areas such as fresh-water bodies or croplands. When using herbicides in alien clearing-operations, the chemicals sprayed could travel to non-target localities through spray-drift, spillages, or even run-off (DeLorenzo et al., 2001). These occurrences have lesser known impacts on the soil environment, especially for soil processes (Rhoades et al., 2002). It is possible that agro-chemicals used can persist in the environment and consequently induce legacy effects (Pratt et al., 1997). Several studies have concluded that the active ingredients of these herbicides have potential detrimental effects on soil functioning and soil processes (Araújo et al., 2003; George et al., 2009). The active ingredients or adjuvants contained in an herbicide could settle in the soil-matrix for a certain amount of time after being applied (e.g. the active ingredient triclopyr has a residual life span of approximately 39 days in the soil) (Johnson et al., 1995).

Triclopyr based herbicides have shown detrimental impacts on species of ectomycorrhizal fungi where radial growth is significantly reduced by exposure to high concentrations of triclopyr (Chakravarty & Sidhu, 1987; Estok et al., 1989). In addition, triclopyr treatments also impact the growth and survival of bryophyte and lichen microbial species in forest soils where treatments led to a decrease in species richness. These effects lasted for as long as two years and clearly showed a long-term effect on soil microorganisms (Newmaster et al., 1999). Picloram is also an active ingredient that has been noted to have harmful effects not only on agricultural crops and land, but on soil microorganisms as well. Heightened treatment levels of picloram (as high as 200 $\mu g.g^{-1}$) proved to temporarily depress substrate induced respiration (SIR) levels (Wardle & Parkinson, 1990). Another study assessed potential effects of fluroxypyr, an active ingredient thought to be degraded in soil rather rapidly. This herbicide stimulated growth of *Chlamydomonas reinhardtii* at low levels of exposure yet inhibited the growth of the same green micro-algae at higher levels of treatment (Zhang et al., 2011).

Certain herbicides containing paraquat, primeextra, glyphosate and atrazine have also revealed a potential to impact soil related processes such as dehydrogenase activity (Mijangos et al., 2006). Additionally, herbicides containing atrazine, paraquat and glyphosate have shown to decrease organic matter in the top soil layer (Sebiomo et al., 2011). Furthermore, chemical residues from the herbicide could impart negative impacts on biochemical properties

of the soil to an extent that important processes are hindered. Soil processes such as symbiotic mycorrhizal interactions and nutrient cycling are all directly linked to the health of the soil (Van Der Heijden et al., 2008). Rhizosphere interactions including plant and microbial communities have also expressed sensitivity to herbicide exposure, which could imply that xenobiotic substances or herbicides (and the adjuvants involved) have the capacity to disturb or hinder mutualisms and symbiotic relations in the soil environment.

Herbicide treatment has furthermore been reported to adversely affect microbial activity (phosphatase activity) when applied at an elevated concentration (ten times the recommended field rate) (Perucci et al., 2000). A potential negative impact of the usage of herbicides containing these active ingredients includes its ability to alter the acidity of the soil (Wolmarans & Swart, 2014). The toxic nature of the herbicide could be strong and enduring enough to increase the acidity of the soil, decrease soil fertility or even weaken native plant species metabolism, making them more susceptible to pathogen attack (Wolmarans & Swart, 2014). In addition to modifying nutrient availability for plants, this would directly impact soil functioning and might be unfavourable for the persistence of local microorganisms and native plant populations alike. Factors such as soil pH and soil available nutrients are all critical for keeping within tolerance ranges specific for supporting life of various soil micro- (Moroenyane et al., 2016) and macro-fauna (e.g. annelids, insects, protozoans and other nematodes) (Tate III, 1995).

Herbicides have been reported to stimulate different aspects of the soil as opposed to an inhibitory effect as mentioned above. Reddy et al. (2003) reported effects on soil nutrients where the given herbicide treatment (including paraquat and imazapic) significantly increased the available nitrogen levels in the soil. Barreiro & Pratt (1994) similarly showed that the addition of a toxicant (diquat) to a microcosm resulted in elevated nitrogen and phosphorus levels. Glyphosate and derivative herbicides have also been recorded to elevate carbon and nitrogen mineralization rates in the top-soil (Haney et al., 2000). Further, other studies have reported that the xenobiotic nature of herbicides can alternatively be stimulatory and be utilized as a carbon, nitrogen or phosphorus source by local microbes (Gaertner et al., 2011; Magadlela et al., 2015).

2.5. Impacts of chemical control methods on soil microbial organisms

Microbial community structure is of great importance for fynbos ecosystems. The soil microbial communities are critical for controlling carbon, nitrogen, sulphur, and phosphorus cycles in the soil, and partake in various immobilization, decomposition, and mineralization processes (Allison & Martiny, 2008; Perucci et al., 2000). Soil microorganisms are therefore vital for maintaining certain nutrient levels and other physicochemical properties of the soil, as they drive the processing of various biotic and abiotic compounds through biogeochemical cycling (Naude, 2012; Stock et al., 2014; Widenfalk, et al., 2008). At the end of these cycles, certain end-products are released into the free environment which is essential for the growth and persistence of native plant communities (Höflich et al., 1995; Stafford et al., 2005). These end-products are also vital for maintaining soil quality, especially in fynbos habitats where soils are known for being nutrient poor.

Soil microorganisms are involved in both direct and indirect means of providing nutrients in the soil and rhizosphere (Jeffries et al., 2003). Their direct influences stems from mutualistic relationships with the plant's roots where microbes provide plant available elements such as nitrogen, phosphorus, and potassium, which are rather limited in fynbos soils. Stafford et al. (2005) showed that rhizosphere soils which host intensive plant-microbial associations, can be increasingly enriched in nutrients and more fertile when compared to non-rhizosphere soils. Indeed, it has been recorded that mycorrhizal fungi and nitrogen-fixing bacteria can contribute to up to 90% of plant phosphorus uptake and up to 20% of nitrogen fixation (Van Der Heijden et al., 2008). Moreover, productivity in above ground plant communities is also driven by indirect mechanisms such as plant-disease suppression through the production of antifungal metabolites as seen in *Pseudomonas* species (Dowling & O'Gara, 1994; O'Sullivan & O'Gara, 1992).

Soil microorganisms are additionally noted to be strongly selected by plant species where the plant releases specific exudates in an effort to cultivate a select group of microorganisms (Hartmann et al., 2009). Thorpe & Callaway (2006) discussed this in detail, distinguishing between two separate feedback systems. Plant and microbial interactions can in many instances include a positive feedback system where plant species accumulate microbes that are beneficial for fixing nitrogen and other nutrient compounds in the soil. This could result in a plant community shift towards a monoculture of IAP's as the diversity of microbial communities are diminished, because the chosen bacterial and fungal species become dominant over the more sensitive microbial species (Reinhart & Callaway, 2006). In addition, negative feedback systems have been recorded, where microbial species diversity is

promoted as plants accumulate pathogenic microbes in the rhizosphere. These feedback systems are dependent on factors such as the degree of invasion, litter-fall, phenology, tissue chemical composition of the plant, and nutrient uptake (Thorpe & Callaway, 2006).

General plant rhizosphere organisms such as *Alcaligenes, Arthrobacter, Burkholderia, Enterobacter, Pseudomonas, Rhizobium*, and *Serratia;* and non-symbiotic N-fixing bacteria including *Azospirilium, Azotobacter, Acetobacter diazotrophicus,* and *Cyanobacteria,* are all important for the above-mentioned functions in the soil environment (Buée et al., 2009; Slabbert et al., 2014). The prescence of invasive alien plants such as *Acacia* can be strongly correlated with specific bacterial phyla such as *Bradyrhizobium* which fix nitrogen for uptake by the host invader plant (Slabbert et al., 2014). Furthermore, certain disturbances can however, induce strong impacts on microorganisms as seen in actinomycete populations in disturbed soils being significantly different when compared to undisturbed soils (Zabinski & Gannon 1997). Microorganisms can withstand disturbances with a high metabolic versatility and tolerance for changes in certain environmental conditions, but the resilience of microbial communities can succumb to transformations in carbon, nitrogen, sulphur, phosphorus cycles and the associated processes (Allison & Martiny, 2008). The addition of xenobiotic compounds (such as herbicides) applied to the soil has been reported to act as a disturbance and can induce microbial community shifts or changes in the microbial communities' ability to perform in crucial soil processes (Chowdhury et al., 2008; Wardle & Parkinson, 1990).

In addition, herbicide and other agrochemicals including atrazine, and glyphosate have been revealed to affect microbial parameters such as substrate induced respiration (SIR), alkylhydroxybenzenes (AHB), fluorescein diacetate hydrolysis (FDA), and dehydrogenase activity (DHA) (Cole, 1976; Wardle & Parkinson, 1990; Zabaloy et al., 2008). Anderson & Domsch (1990) and Saratchandra et al. (1993) also recorded effects of xenobiotic compounds including metabolic quotients (qCO 2 and qD) and super phosphate fertilizer on microbial reduction-activities as well as mineralization and immobilization rates. Picloram application has also shown to have a toxic effect on soil micro-flora where increased dosage treatments led to increased lag phase periods, consequently resulting in population death of micro-flora communities (Prado & Airoldi, 2001). Moreover, herbicides such as phenylurea have been shown to decrease the microbial diversity and colony forming units (cfu's) of soil microbial communities (El Fantroussi et al., 1999). In addition, fungal growth inhibition due to treatment with herbicides has been described where the herbicide suppressed spore germination and the rate of hyphal extension of the mycelia (Wilkinson & Lucas, 1969). The regulatory ability of microorganisms such as *Rhizobium*, which partake in these important metabolic activities is vital for the survival of many native plant species (Barton & Northup, 2011; Van Der Heijden

et al., 2008; Wolmarans & Swart, 2014).

One should also consider that microbial communities have a high metabolic versatility and could show resilience and persistence in the face of disturbance or extreme environmental conditions (Allison & Martiny, 2008; Barton & Northup, 2011). As mentioned above, microorganisms are also strongly determined by specific plant relations. Because of these strong bonds and metabolic capabilities, certain soil microorganisms can, to an extent, remain unaffected when exposed to xenobiotic compounds (Grayston et al., 1998). Fynbos soils are highly heterogenous, which could imply a rather diverse distribution of bacteria and fungi in the soil environment (Moroenyane et al., 2016). This diverse microbial community found within fynbos soils could, therefore, show variable responses when exposed to specific herbicides. As an example, a picloram based herbicide could have no recorded effects on microbial growth, yet when applying triclopyr to the same soil, bacterial growth is stimulated (Peterson et al., 1994). In addition, a study illustrated a glyphosate herbicide having no significant effect on microbial genera such as *Alcaligenes* and *Pseudomonas* yet showed for an inhibitory effect on *Chlamydomonas reinhardtii* (Schulz et al., 1985).

On the contrary, there have been reports of herbicides having no effect on soil microbial composition (Wertz et al., 2007). Glyphosate for example was shown to have no significant effect on bulk rhizosphere soil (Weidenhamer & Callaway, 2010). This study revealed no microbial community shifts, even at three times the field rate (FR). Another study based on the effect of a metsulfuron methyl herbicide also presented no effect on microbial communities in the soil (Zabaloy et al., 2008). Haney et al. (2000) similarly reported no effect of a glyphosate herbicide on microbial biomass. Many prior research studies illustrate the harmlessness of herbicides to soil microorganisms (Schulz et al., 1985; Weaver et al., 2007). Further research on the matter is essential as we have no set model for how microorganisms are to respond to certain herbicide treatments in specific conditions. Evaluating the potential impacts that herbicides may have on the soil environment and the ecosystem in its entirety will enhance our understanding of how herbicides fit into the dynamic of successful IAP removal and the rehabilitation that takes place thereafter. It is of great significance that any detrimental impacts induced by herbicide be brought to light so that future management of IAPs can have minimal negative effects, be more cost effective, and more environmentally compatible (Gaertner et al., 2011; Thompson et al., 2000).

3. Problem Statement

To combat the encroachment of woody IAPs the WfW uses several specialized herbicides (Turpie et al., 2008). *A. mearnsii* and *E. camaldulensis* are of the most threatening woody IAPs in riparian fynbos habitats and the herbicides used to chemically control them could have unseen detrimental impacts on the ecosystem. While woody IAPs are being removed, these secondary effects could create space for other IAP species (such as graminoids or forbs), encouraging further fragmentation of native plant communities (Otto, 2014). Herbicides and the respective adjuvants and active ingredients involved, could thus shift the ecosystem to a state that is more unnatural and disturbed (Ehrenfeld, 2003).

The success of the chemical-control methods is therefore questionable as the toxic nature of the herbicide could lead to impacts on soil microorganisms, soil chemical properties, as well as the local plant community. We do not fully understand the extent to which chemical residues of herbicides can alter certain important characteristics of the soil in riparian fynbos zones. The toxicological profiles of the different herbicides used by WfW differ from one another and could behave differently in the soil once it has seeped into the top-soil layer (Sebiomo et al., 2011; Zabaloy et al., 2008). We need to make an effort to better understand the effects on the soil environment as it forms a great part of the ecosystem and acts as a core component for terrestrial and semi-aquatic ecosystems (Groves, 1983; Kruger et al., 2012; Yelenik et al., 2004).

Moreover, herbicides can find their way to non-target organisms as well as non-target localities (such as riverine habitats) through spray drift, run-off, and spillages during treatment operations. Most studies focusing on the impact of herbicides are based *ex situ* in a laboratory setting where conditions are controlled and homogenous. Hence there is a research gap for evaluating herbicides in a more natural or simulated environment where the effects measured are more representative of the responses that occur on-site. It is important that research studies put effort into evaluating agrochemicals both *in situ* and *ex situ* as this will improve our efficacy for the chemical-control methodologies used as well as expose future research gaps. Assessing the potential drawbacks or disadvantages to the methodologies used will provide prospects for future development and enhance the positive impacts that IAP management can generate.

4. Objectives and Key Questions

4.1. Main aim:

The aim of this project is to determine potential impacts of herbicides used to control *Acacia mearnsii* and *Eucalyptus camaldulensis,* on the soils of riparian fynbos ecosystems. These potential impacts are to be determined by assessing soil chemical and soil microbial properties.

4. 2. Specific aims*:*

- Evaluate the potential effect of different concentrations and types of herbicides on soil chemical characteristics, *ex situ* and *in situ*. The soil chemical properties studied include soil pH, electrical conductivity, total soil nitrogen, nitrogen mineralization, soil available PO_4^{3-}, and phosphatase activity.

- Evaluate the potential impacts of different concentrations and types of herbicides on soil microbial community structure, *in situ* and *ex situ*. This aim deals with evaluating aspects of microbial communities' diversity and richness.

- Assess the potential impacts of different concentrations and types of herbicides on soil microbial activity and function *ex situ*. The microbial communities' metabolic potential is used as a metric to evaluate the effects of herbicides on microbial function.

4.3. Key questions:

- How would nutrients and chemical properties of riparian fynbos soil change over time when treated with certain concentrations and types of herbicides?

- Does certain types and concentrations of herbicide treatments affect soil microbial structure in riparian fynbos ecosystems?

- How does metabolic potential in microbial communities respond to certain types and concentrations of herbicides?

- What implications for future chemical management of invasive alien plants can be inferred, especially for that of the management of *Eucalyptus camaldulensis* and *Acacia mearnsii*?

5. Thesis structure

5.1. Chapter 1

This chapter includes an introduction to the study covering a summary, a brief description of each of the chapters, the aims and objectives, research questions, as well as a literature review. The literature review is focused upon aspects relevant to the clearing of invasive alien plants (IAPs) in riparian fynbos ecosystems. The literature review includes the following themes: Riparian Fynbos ecosystem structure and services; IAP invasion of *Acacia mearnsii* and *Eucalyptus camaldulensis*; Impacts of woody invasive alien plant (IAP) removal; Impacts of chemical control on soil-chemical characteristics and processes in fynbos; and the effects of chemical control on soil-microbial communities.

5.2. Chapter 2

The *in situ* section of the study is covered in this chapter. The experiments took place at infield trial sites that were characterized by riparian fynbos habitats. The methods and experimental design of the *in situ* experiments are discussed along with a description of the field sites. The results obtained from these on-site experiments are expressed and includes results of the effects of herbicide on both soil chemical and soil microbial community structure.

5.3. Chapter 3

This chapter deals with the *ex situ* section of the study. The experiments were conducted off-site in a nursery and in a temperature and light controlled room so that certain conditions could to an extent be controlled and simulated. The methods of the *ex situ* experiments are discussed as well as the results obtained. The effects of herbicide on aspects including soil chemical properties, soil microbial community structure, and soil microbial community function are covered in this chapter.

5.4. Chapter 4

This chapter deals with the conclusions and implications of the findings of the study. Aspects such as the main findings are discussed, and the most important implications of the results

are highlighted. In addition, recommendations for future research is discussed as well as what the results infer for herbicide-management operations.

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CHAPTER 2

The potential effects of herbicide on Riparian Fynbos soil *in situ*

1. Introduction

The effects of herbicides on the soils of fynbos and other habitat types are unknown and, therefore, of great concern. Herbicides, depending on their active ingredients and inert chemicals, have the potential to be broken down by soil microbial communities and essentially have no long-term or permanent effects on the soil environment. This can be said for most herbicides, but research has shown that the active ingredients and other additive chemicals can persist in soil longer than expected (Curran, 2016). Some compounds have displayed toxic effects on certain microbial groups and hence can indirectly influence other fauna and flora at higher trophic levels. An example includes DeLorenzo et al. (2001), whom reported atrazine herbicide and organophosaphate/ chlorine insecticide to impact microorganisms which are responsible for decomposition, nutrient cycling, and act as a food source for genera in higher trophic levels. With the said impacts occurring, the food web can be disturbed when facing herbicide exposure and organisms in higher trophic levels such as fungivores, bacterivores, and other predatory nematodes can be affected (Zhao et al., 2013).

Studies evaluating the effects of herbicides on the environment including soils have been conducted mostly in monocultural agricultural or laboratory microcosms where conditions are controlled to a considerable extent (Pratt et al., 1997). Few studies have focused on the effects of herbicides in a more natural environment (DeLorenzo et al., 2001; Pratt et al., 1997). It is important that more attention be paid to determining potential impacts of herbicide application in field conditions (Pratt et al., 1997). *In situ* studies can show how herbicide treatment could affect natural ecosystem components. Many studies on herbicide impact are conducted *ex situ* which could bias results by not fully taking into consideration the environmental heterogeneity as well as the environmental factors exerted on the soil and its inhabitant organisms *in situ* (Mekwatanakarn & Sivastihamparm, 1987).

In situ studies hold value in the assessment of herbicide toxicology/ ecotoxicology. It signifies effects in an actual natural environment, and soils treated historically for years with herbicides can act as long-term evaluation sites (Cole, 1976). A few *in situ* studies have been used to evaluate the effect of herbicides on microbial communities and on various soil chemical characteristics (Reddy et al., 2003; Sebiomo et al., 2011). Additionally, other on-site studies have not only looked at the long-term effects of herbicides on microbial populations, but also effects on aspects such as soil seed banks (Cardina et al., 1991; Roberts & Neilson, 1981).

Studies evaluating the microbial community's response to herbicide application *in situ* are crucial as changes in community structure and microbial activity could significantly alter the soil environment and cascade into the entire ecosystem (Ratcliff et al., 2006; Seghers et al., 2003). In some cases, a secondary aspect such as soil available nitrogen can be linked to the effects of herbicides observed on a primary aspect such as rhizobium biomass (Rhoades et al., 2002).

An *in situ* study mimicking the application of herbicides in a riparian fynbos habitat (as done by the WfW or other private landowners and reserve managers) can account for the heterogenous field conditions and 'natural state' when *A. mearnsii* and *E. camaldulensis* trees are being treated with conventional herbicides. In addition, a study found that captan and glyphosate exposure on-site induced multi-directional shifts including changes in soil nutrient levels and significant shifts in soil microbial community composition (Widenfalk et al., 2008). The potential effects of agrochemicals such as pesticides are usually assessed in laboratory conditions focusing on individual bacterial strains or only on functional responses such as microbial biomass or overall respiration levels. These *ex situ* experiments are valuable but could fail to detect subtle interactive changes between microbial communities and soil chemical aspects when responding to herbicide exposure. The *in situ* detection of these interactive and potential functionally important responses are important for understanding how agrochemicals such as herbicides can affect dynamic ecosystems.

This chapter reports two separate *in situ* experiments. The first experiment solely evaluated soil chemical characteristics at four separate riparian fynbos localities. The second experiment however, included a greater array of analyses for both soil chemical and soil microbial aspects with a focus on two separate riparian fynbos localities. The two experiments were aimed at answering how soil chemical properties and microbial community structure of riparian fynbos could change over time when treated with certain dosages of conventional herbicides. Additionally, these experiments were conducted to determine whether there are any significant links between the response of microbial communities and the response of soil chemical characteristics when exposed to herbicide treatment. Further, this chapter discusses the methods and experimental design of the *in situ* experiments including a description of the chosen infield trial-sites. The results obtained from these *in situ* experiments are discussed and reviewed in a discussion.

2. Materials and Methods

2.1. Experimental design

The experimental design consisted of five basic steps (Fig. 2.1). Step 1 included the selection of a site within a riparian fynbos habitat. Step 2 involved the specified herbicide application to *E. camaldulensis* and *A. mearnsii* trees within the chosen trial site. Step 3 included the collection of soil samples from the site of treatment at set assessment stages. Step 4 was the storage of the soil samples into appropriately sealed and labelled bags and placed into refrigeration. Step 5 included conducting select microbial and soil-chemical analyses at the separate assessment stages (soil collected before and after treatment).

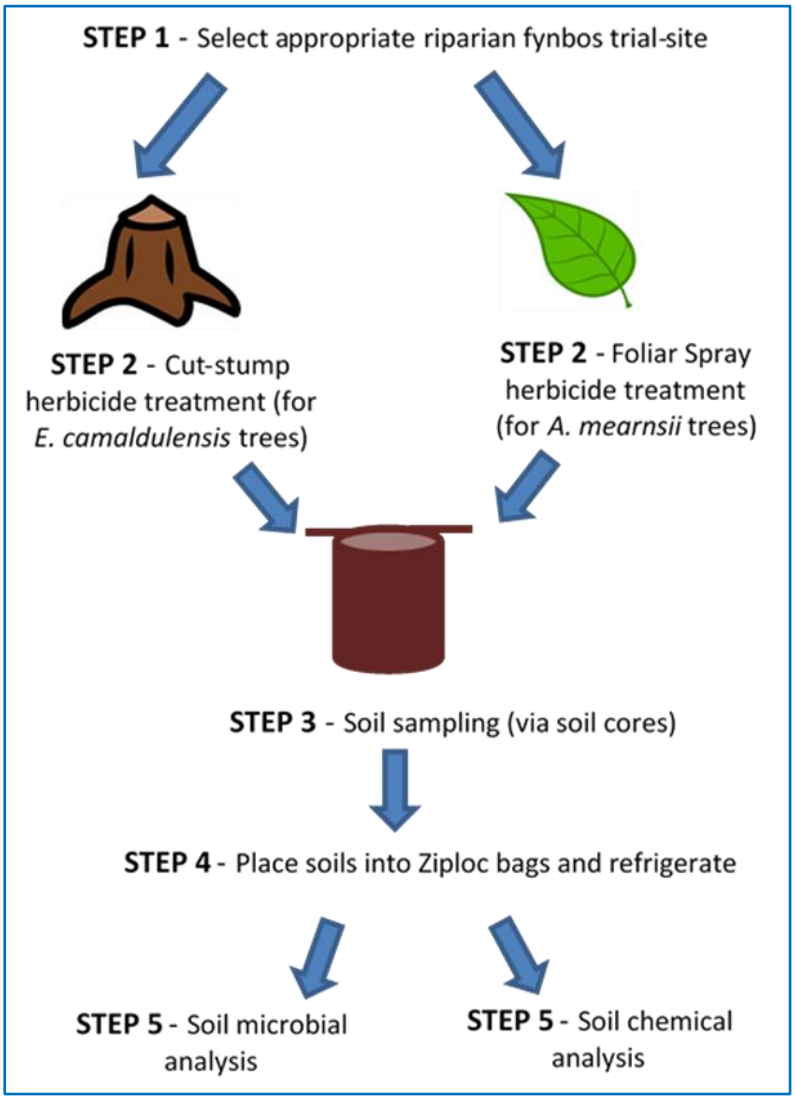


Figure. 2.1. A flow diagram displaying the experimental procedure of this study. It begins with selecting a suitable site and leads onto the respective herbicide treatments of *A. mearnsii* and *E. camaldulensis* trees. Thereafter, soils are collected and preserved in Ziploc bags and placed into refrigeration. The process ends with select microbial and soil-chemical analyses.

2.1.1. Experimental conditions

2.1.1.1 Experiment 1

Experiment 1 took place during the summer months of 2015/ 2016. The sites chosen were within riparian fynbos habitats along the Breede River and Wit River catchments in the Western Cape. A total of four *in situ* sites were chosen, two of which were heavily invaded by *Acacia mearnsii* and the other two sites invaded by *Eucalyptus camaldulensis* (see Table 2.1). Additionally, the sites had to be within close vicinity of a river (to be considered 'riparian') but had to be far enough from the river-banks so that herbicide spillage into water-bodies could be prevented.

The chosen individual trees were treated with a full recommended dosage of herbicide (100 % of the registered field rate). Another selection of trees were untreated controls with no herbicide application (0 % of the registered field rate). Three herbicides were tested, each with a different active ingredient. *A. mearnsii* trees were treated with separate applications of Garlon 480 EC (triclopyr) and a Springbok 360 SL (glyphosate), whilst *E. camaldulensis* trees were treated with Plenum 160 ME (an emulsion of fluroxypyr and picloram). The herbicides were applied with a 16-liter knapsack sprayer with a solid-conical nozzle. A pressure of 200 kPa was maintained and together with the cone-nozzle, the herbicide was sprayed in a fine mist to increase absorption by the tree tissues. Herbicide application took place in warm, cloudless and windless conditions to prevent spray-drift and to enhance the penetration of the herbicide in the targeted plant and surrounding soil.

A. mearnsii trees were treated with herbicide in a foliar spray where sapling trees not higher than 1.5 meters were sprayed on all the foliage to the point of run-off. *E. camaldulensis* plants that were chosen were mature trees of approximately 10 cm diameter and the herbicide treatment for these specimens was a cut stump application. The trees were cut down to ankle height exposing the cambium and were similarly sprayed to the point of run-off. All treatments were replicated five times (n = 5). The herbicide field rates that were applied are displayed in Table 2.1.

Table 2.1. The separate herbicide treatments are shown along with the respective active ingredients for *in situ*, experiment 1. The locality and targeted invasive tree species are included. Each of the herbicides were applied at 2 different rates including 100 % of the recommended field rate and an untreated control (0% of the field rate).

Locality	**Target IAP species:**	**Treatment field rate (FR):**	**Active ingredient:**
Alfalfa A (Breede River)	*Acacia mearnsii* seedlings	Garlon 0.75 l/ ha: • 0 % FR • 100 % FR	Triclopyr (as butoxy ethyl ester) 480 g/L EC (emulsifiable concentrate)
		Springbok 4.5 l/ ha: • 0 % FR • 100 % FR	Glyphosate (as isopropylamine salt) 360 g/L SL (soluble concentrate)
Alfalfa B (Breede River)	*Eucalyptus camaldulensis* adults	Plenum 9 l/ ha: • 0 % FR • 100 % FR	Fluroxypyr + Picloram 80 g/L ME (micro-emulsion)
Bainskloof (Wit River)	*Acacia mearnsii* seedlings	Garlon 0.75 l/ ha: • 0 % FR • 100 % FR	Triclopyr (as butoxy ethyl ester) 480 g/L EC (emulsifiable concentrate)
		Springbok 4.5 l/ ha: • 0 % FR • 100 % FR	Glyphosate (as isopropylamine salt) 360 g/L SL (soluble concentrate)
Worcester Airfield (Breede River)	*Eucalyptus camaldulensis* adults	Plenum 9 l/ ha: • 0 % FR • 100 % FR	Fluroxypyr + Picloram 80 g/L ME (micro-emulsion)

2.1.1.2. Experiment 2

Experiment 2 was conducted *in situ* in autumn/ spring of 2016 at the Alfalfa A and Alfalfa B sites. Alfalfa A was an area of riparian fynbos vegetation dominated by *A. mearnsii* trees and Alfalfa B was heavily invaded by *E. camaldulensis* trees. Similar to experiment 1, the sites had to be within the vicinity of a river or stream. The herbicides used, remained the same as tested in experiment 1 where *A. mearnsii* was treated with two separate products namely, Garlon 480 EC (triclopyr) and Springbok 360 SL (glyphosate), whilst *E. camaldulensis* was treated with Plenum 160 ME (picloram and fluroxypyr). Each of these herbicides were applied at three different rates including 0 % field rate (untreated control), 10 % of the registered field rate and 100 % of the registered field rate (Crouzet et al., 2010). The 10 % field rate simulated under application and was tested to see what happens to the various soil aspects when

herbicides are under applied infield or mixed incorrectly with low ratios. The herbicides were applied to the targeted trees in the same manner as described in experiment 1. Similarly, all treatments were replicated five times over. The field rates used per herbicide product are displayed in Table 2.2. below.

Table 2.2. The separate treatments are shown with the respective active ingredient for *in situ*, experiment 2. The locality as well as the targeted invasive tree species are additionally shown. Each of the herbicides were applied at 3 different rates including, 0 % of the field rate (untreated control), 10% of the registered field rate, and 100 % of the registered field rate.

Locality	Target IAP species:	Treatment FR (field rate):	Active ingredient:
Alfalfa A (Breede River)	*Acacia mearnsii* seedlings	Garlon 0.75 l/ ha: • 0 % FR • 10 % FR • 100 % FR	Triclopyr (as butoxy ethyl ester) 480 g/L EC (emulsifiable concentrate)
		Springbok 4.5 l/ ha: • 0 % FR • 10 % FR • 100 % FR	Glyphosate (as isopropylamine salt) 360 g/L SL (soluble concentrate)
Alfalfa B (Breede River)	*Eucalyptus camaldulensis* adults	Plenum 9 l/ ha: • 0 % FR • 10 % FR • 100 % FR	Fluroxypyr + Picloram 80 g/L ME (micro-emulsion)

2.1.2. Trial site localities

2.1.2.1. Bainskloof

The Bainskloof trial site is found within the Wit River catchment area, which is one of the major tributaries of the Breede River in the Western Cape. The Wit River is a perennial river found within the mountain transitional zone at the foot of Bainskloof Pass. The GPS co-ordinates of the chosen site are: 33°32’37.93” S; 19°10’28.19” E. It is privately owned and managed, and efforts are ongoing to clear woody invasive alien plant (IAP) biomass from the site. The primary invasive alien plant species dominating the site is *Acacia mearnsii*, and seedlings and adult trees are found in dense thickets within the wet-bank and dry-bank zones. The soil pH levels range between 5.8 and 6.3, and the lithology consists of sandstone. In addition, the channel features of the river are comprised of a moderately steep gradient with a mixture of boulders and bedrock. Furthermore, prominent disturbances for this stretch of the Wit River include fires and floods.

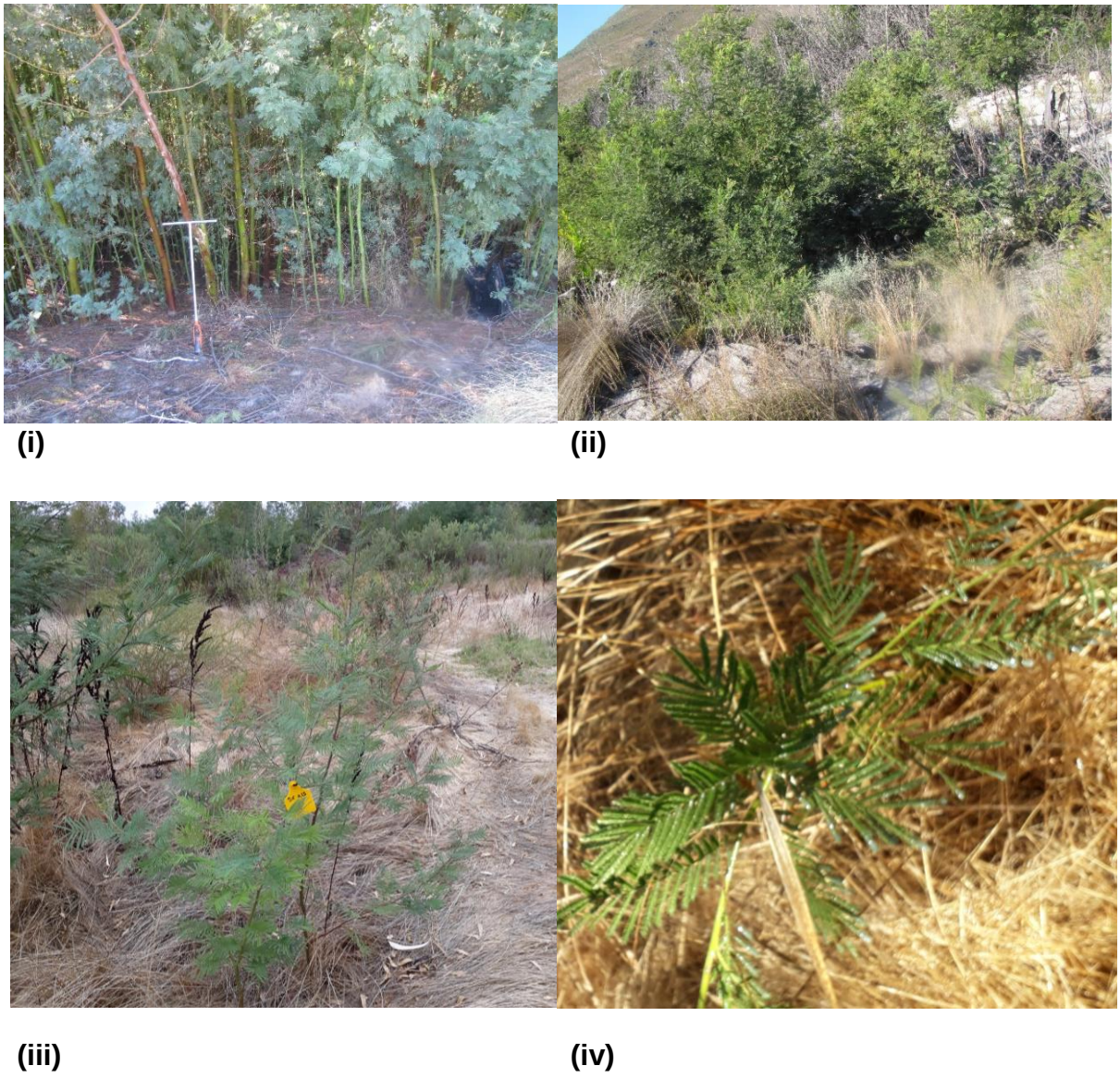


Figure. 2.2. (i) *A. mearnsii* thickets at the Bainskloof site found within the wet-bank zone forming a wall of trees on the banks of the river. (ii) The *A. mearnsii* thickets are also found in scattered stands in the more dry-bank/ upland terrestrial zones. (iii) Picture of well-established *A. mearnsii* seedlings in the upland terrestrial zones (iv) Picture of *A. mearnsii* foliage just after being sprayed to the point of run-off.

2.1.2.2. Alfalfa (A) and (B)

These trial sites are found along the dry bank zones of the Breede River east of Worcester and Rawsonville. The two sites are found along the same stretch of river opposite the Alfalfa Dairy. Site 'Alfalfa A' contains a heavily *A. mearnsii* invaded habitat, whilst 'Alfalfa B' found further downstream, is largely dominated by stands of mature *E. camaldulensis* trees. Alfalfa A is found at the following GPS co-ordinates: 33°46'5.12" S; 19°32'6.107" E. Alfalfa B can be

found at 33°46'1.528" S; 19°32'23.243" E. The channel features of the river include moderately steep to steep channels with a combination of bedrock, boulders and sandstone. The soil is sandy and rather acidic with an average soil pH of 6. The primary disturbances for the Alfalfa A and Alfalfa B site appears to differ despite being in close proximity to one another. Alfalfa A contains various herbaceous plant species in-between stands of *A. mearnsii* making herbivory and grazing a prominent disturbance. Alfalfa A also seems to be inundated easily in periods of extended high rainfall. Alfalfa B however, is more woody and densely populated site and the thick *E. camaldulensis* stands have little to no understory hosting a top-soil layer that is covered with a thick leaf-litter layer. The most recent disturbance for this site has been fire. Furthermore, the land is privately owned, and efforts have been made to manage these two alien species through mechanical felling and the removal of biomass through chipping and the sale of chopped wood.

(i)

(ii)

(iii)

Figure. 2.3. (i) *A. mearnsii* densley populated at Alfalfa A with a mixture of adult and sapling trees. (ii) Alfalfa B contains dense thickets of large *E. camaldulensis* trees. (iii) Both sites are found along the Breede River east of the town of Worcester.

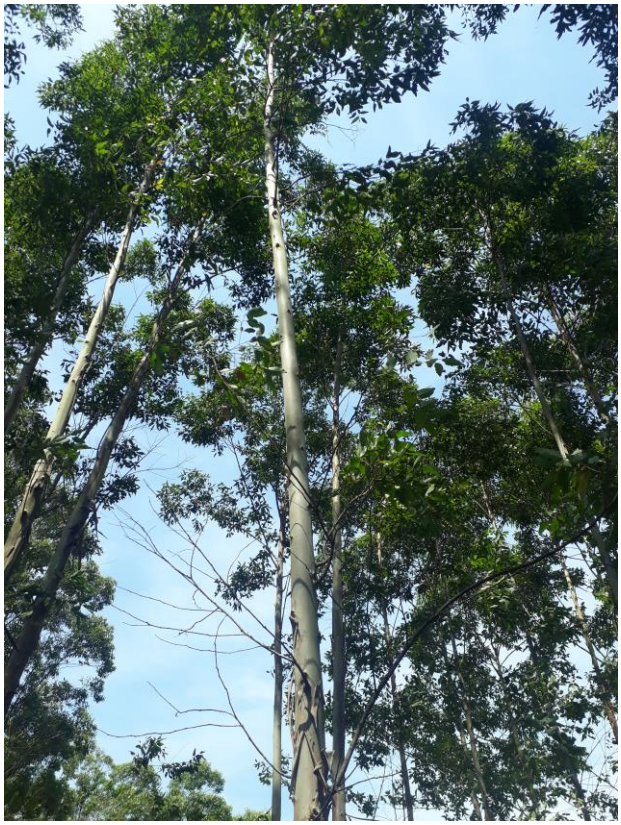
2.1.2.3. Rainbow

This trial site is found a few kilometers from the Worcester Airfield on Rainbow Worcester Chicken Farm along the Breede River in the Western Cape. The site can be found at the following GPS co-ordinates: 33°39'57.83" S; 19°24'28.15". *Eucalyptus camaldulensis* and *Acacia saligna* are the primary invasive plant species here. The trial sites chosen, were however, kept within the *E. camaldulensis* thickets so that samples could be collected from an '*E. camaldulensis* dominated' habitat. The soil pH is fairly acidic at a pH of 6.1 and the soil derives from a sandstone lithology. The slope of the river banks are relatively gentle which results in inundation across the wetland during high rainfall in winter months. Furthermore, the river contains a mixture of sandstone boulders and bedrock. This site is owned by the Worcester municipality and efforts to clear the woody IAP's through mechanical means were made shortly after the completion of this study by the WfW.

(i)

(ii)

(iii)



(iv)

Figure. 2.4. (i) *E. camaldulensis* thickets found along the banks of Breede River on Rainbow Chicken Farm near the Worcester airfield. (ii) Trees at the Rainbow trial-site are exposed to high degrees of water logging and flooding in winter. (iii) Picture of exposed cambium of *E. camaldulensis* just before herbicide treatment. (iv) *E. camaldulensis* trees at Rainbow ranged between heights of 2 and 5 metres tall.

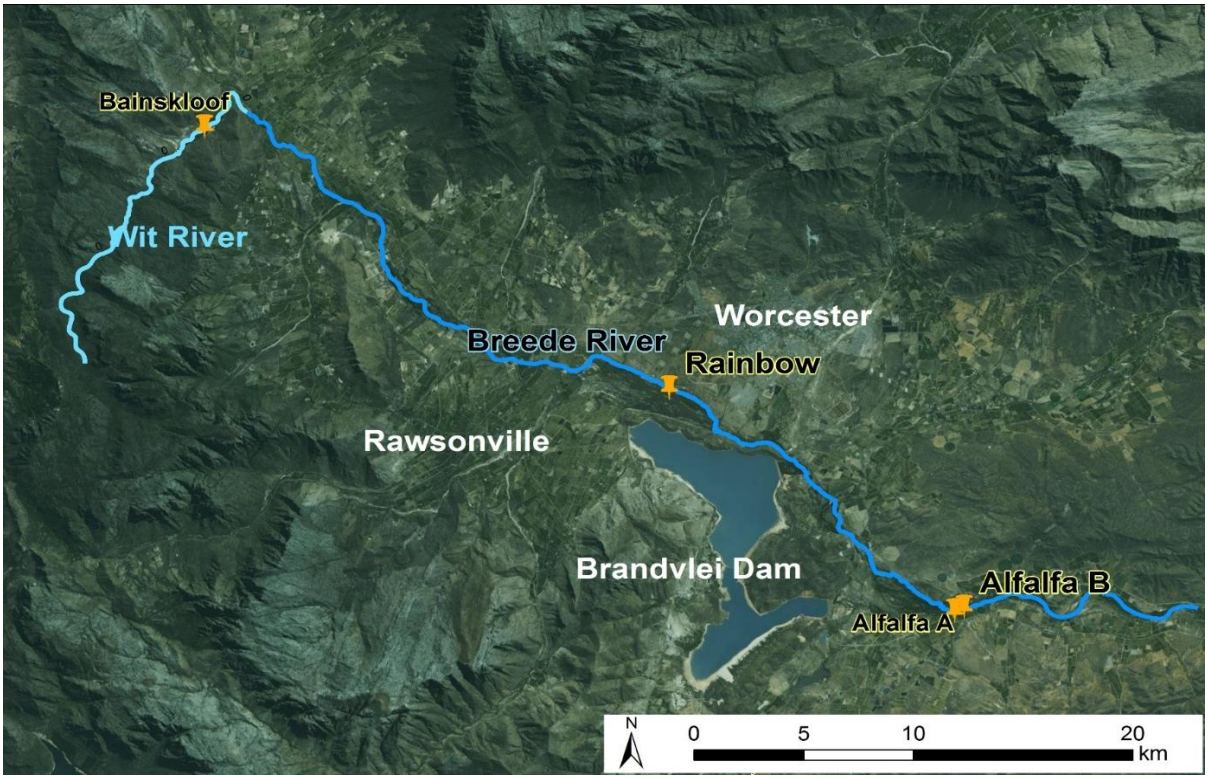


Figure. 2.5. A map displaying the four different trial sites with reference to Worcester and Rawsonville in the Western Cape, South Africa. Additionally, the major river catchments, namely the Wit River and the Breede River are shown. Map drawn via ArcMap GIS software.

2.1.3. Sampling and storage methods

Soil samples were collected using a steel cylinder within 5 cm of the tree stems that were treated so that the soils collected represented a soil sample that was exposed to the given herbicide treatment. The steel cylinder was driven into the top soil layer and standardized to a depth of 4 cm (Sebiomo et al, 2011; Crouzet et al, 2010); this is where most microbial activity takes place. Once the soils were collected, they were placed into labelled Ziploc-bags and stored in a 4°C refrigerator within two hours of collection to preserve the soils properties. All soil chemical analysis was done within 48 hours of collection as certain properties of the soil such as nitrogen mineralization or phosphatase activity are sensitive to environmental conditions and could change over-time when placed into refrigerated conditions. The sampling stages were as follows: (i) Experiment 1 - soil collected before herbicide treatment and again one month after treatment; (ii) Experiment 2 - soil collected before herbicide treatment, one week after treatment, and again six weeks after treatment.

2.2. Soil chemical analysis

2.2.1. pH

A HI-8424 pH meter was used to measure the pH of soil samples. The soil samples were sieved (2 mm) and thereafter weighed into 10 g samples. The 10 g sample was then mixed into solution with distilled water in a 1:5 ratio in a 50 ml plastic vial (Robertson et al., 1999). The soil mixture was then thoroughly stirred for 30 minutes to mix, after which the solution was left to settle for a minimum of 30 minutes. Once soil particles were completely settled at the bottom of the vial, the recordings were taken. The HI-8424 pH meter was calibrated to a range of pH 4-7 (Buurman et al., 1996). After calibration, the pH of each sample was recorded to one decimal place.

2.2.2. Electrical conductivity

Electrical conductivity was recorded with a HI-8733 conductivity meter. Each sample was sieved (2 mm) and weighed to 10 g (Robertson et al., 1999). Distilled water was added to each soil sample in a plastic vial to a ratio of 1:5. The solution was then shaken to allow for thorough mixing of the soil with the distilled water, after which the solution was left for at least 30 minutes to give the soil a chance to settle on the bottom of the vial. The calibration was set to 12.88 microSiemens (mS) and a temperature of 20°C. After calibration, recordings were then taken to one decimal place.

2.2.3. Total soil available nitrogen

Total soil available nitrogen was determined via the summation of soil available nitrate, NO_3^-, and soil available ammonium, NH_4^+, to units measured in µg $N.g^{-1}$.

(i) *Nitrate*:

Soil available nitrate, NO_3^- was determined spectrophotometrically. Sieved soil samples (2 mm) of 10 g was weighed and thoroughly mixed with 25 ml of 0.5 M K_2SO_4 in sterile 50 ml plastic vials (Keeney, 1982). The solution was subsequently shaken for an hour and thereafter filtered through Whatman no.40 filter paper. Two reagents were mixed into the filtrate before final measurements were taken. The first of the reagents was a 5 % salicylic acid solution, whilst the second was a solution of 4 M sodium hydroxide. 0.5 ml of each sample's filtrate was mixed with 1 ml of the salicylic acid and left for a total of 30 minutes. Thereafter, 10 ml of the sodium hydroxide reagent was added to the mixture and left for an hour before reading the absorbance at 410 nm using a Thermo Genesys 20 spectrophotometer and converted to units

of NO_3^- µg.g^{-1}.

(ii) Ammonium:

Soil available ammonium, NH_4^+ was determined spectrophotometrically. Soil samples were sieved (2 mm) and weighed to 10 g each. Thereafter, soils were thoroughly mixed with K_2SO_4 solution and filtered through Whatman no.40 filter paper as mentioned above in the nitrate protocol. The filtrate extracted was mixed with two reagents to induce colour development. The reagents included 'reagent N1' and 'reagent N2'. N1 consisted of sodium salicylate, sodium citrate, sodium tartate, sodium nitroprusside and distilled water. The N2 reagent consisted of sodium hydroxide, sodium hypochlorite and distilled water. A volume of 0.1 ml of each samples filtrate was mixed with 5 ml of reagent N1 and left for 15 minutes. After 15 minutes, 5 ml of reagent N2 was mixed into solution and thereafter left for an hour for the mixture to stabilize and to allow for colour development. Each of the samples were subsequently measured with a spectrophotometer at an absorbance of 655 nm and converted to units of NH_4^+ µg.g^{-1} (Buurman et al., 1996).

2.2.4. Nitrogen mineralization

Nitrogen mineralization was determined by measuring the daily rate at which ammonium was mineralized. As mentioned in the above ammonium protocol, soils were sieved to 2 mm and weighed to 10 g samples. Each sample was mixed with 25 ml of distilled water in a sterile 50 ml plastic vial. The samples were thereafter placed into incubation at a constant temperature of 27 ˚C for seven days (Schomberg et al., 2009). After the seven days, the sample solutions were extracted with 0.5 M K_2SO_4 and filtered through Whatman no.40 filter paper (Keeney, 1982). The filtrate extracted was mixed with reagents N1 and N2. Having measured the absorbance on the first and seventh day, one could obtain the change in available ammonium overtime and hence the rate at which ammonium was being mineralized daily N-µg.g^{-1}.day^{-1} (Schomberg et al., 2009).

2.2.5. Soil available phosphorus

To evaluate soil available inorganic phosphorus, PO_4^{3-}, the Bray no.2 method was used (Tabatabai, 1982). Soil samples were sieved (2 mm) and weighed to 6.7 g each. Each soil sample was mixed with 50 ml Bray no.2 solution, which includes a solution of HCl^- and NH_4F, in sterile plastic vials. Each sample solution was mixed for 40-50 seconds with a high-speed shaker. The solution was filtered through Whatman no.40 filter-paper. Thereafter, 20 ml of

the filtrate was mixed with 10 ml of Boric acid, which is a mixture consisting of boric acid and ammonium molybdate. ANSA (amino-naphol sulphonic acid) reducing agent was then added secondarily at a volume of 10 ml. The ANSA reducing agent consists of sodium sulphite (anhydrous) and potassium metabisulphite. The complete solution mixture was then adjusted to a total volume of 50 ml with distilled water. Thereafter, the complete mixture was left for 20 minutes for colour development to take place. After the 20 minutes elapsed, the absorbance was determined at 660 nm with a Thermo Genesys 20 spectrophotometer and PO_4^{3-} was determined in ppm.

2.2.6. Acid phosphatase activity

The soils from the trial-sites of this study were proven to be fairly acidic. The 'acid-phosphatase method' was hence used instead of using the 'alkaline-phosphatase activity method'. The extraction procedure included weighing 1 g of 2 mm sieved soil and mixing it with 0.2 ml toluene in a sterile 50 ml plastic vial. After mixing, 4 ml of MUB (modified universal buffer) was secondarily added. The MUB reagent is a solution consisting of 1 N NaOH, citric acid, boric acid, maleic acid, tris (hydroxymethyl) aminomethane and distilled water. The MUB was modified to a pH of 6.5 to continue with the acid-phosphatase method. After this reagent was added, 1 ml of 0.025 M p-nitrophenyl phosphatase solution was added, and the content shaken for a few seconds to allow mixing. The 0.025 M p-nitrophenyl reagent was produced by dissolving disodium p-nitrophenyl phosphate tetrahydrate in MUB at the pH level of 6.5. After mixing all the above, the vials were sealed and incubated for an hour at 37°C. Thereafter, the vials were uncapped and 1 ml of 0.5 M $CaCl_2$ and 4 ml of 0.5 M NaOH was added (Tabatabai & Bremner, 1969). This was shaken again to allow for mixing, after which the soil suspension was filtered through no.12 filter paper. A Thermo Genesys 20 spectrophotometer was used to read the absorbance values of the samples at 402 nm and readings were converted to units of µg-p-NP.g^{-1}.

2.3. Soil microbial analysis

2.3.1. DNA extraction

A Zymo Soil Microbe DNA MiniPrep™ kit was used for the extraction and isolation of DNA from a specified soil sample. For each sample, 0.25 g of fresh soil was used, and the extraction was done according to the manufacturer's instructions including the following:

The method included weighing 0.25 g of a fresh soil sample and adding it to a ZR Bashing-Bead Lysis Tube. Thereafter, 750 µl lysis solution was added to the tube, which was secured

in a bead beater and processed at maximum speed. After five minutes, the tubes were centrifuged for one minute at 10,000 g. The supernatant was transferred to a Zymo-Spin IV Spin Filter (at a volume of 400 µl per sample) and centrifuged at 7000 g for one minute. The filtrate from the above-mentioned Spin Filter had 1200 µl of Soil DNA Binding Buffer added to it. Thereafter, 800 µl of the mixture was transferred into a Zymo-Spin IIC Column and centrifuged for one minute at 10000 g. The flow through of the mixture was discarded and the previous step was repeated.

Two hundred microliters DNA Pre-Wash Buffer was then added to the Zymo-Spin IIC Column and centrifuged once again for one minute at 10000 g. Once the pre-wash step was complete, 500 µl Soil DNA Wash Buffer was added to the Spin IIC Column and centrifuged for one minute at 10000 g. The Zymo-Spin IIC Column then had 100 µl of DNA Elution Buffer added directly to the column matrix and centrifuged for 30 seconds at 10000 g. This would result in eluted DNA, which was transferred to a prepared Zymo-Spin IV-HRC Spin Filter and centrifuged for one minute at 8000 g. The filtered DNA (solution) after this final step was now suitable for PCR and other further DNA analyses (such as ARISA-PCR).

2.3.2. Gel electrophoresis

After DNA extraction, gel electrophoresis was conducted to check for the presence of DNA. A 1 %-agarose gel was used, stained with 0.05 µg/ ml EtBr (ethidium bromide). The EtBr binds to the DNA and allows for the DNA to be visualized under UV light.

2.3.3. Automated Ribosomal Intergenic Spacer Analysis

The Automated Ribosomal Intergenic Spacer Analysis (ARISA) method is based on the Polymerase Chain Reaction. Extracted DNA was amplified using a fluorescently labelled (6-carboxy-fluorescein) forward primer (ITSF-FAM-(5'-GTCGTAACAAGGTAGCCGTA-3')) and the reverse primer (ITSReub-(5'-GCCAAGGCATCCACC-3')). A 10 µl reaction mixture was prepared as follows: 4.1 µl ddH2O, 5 µl KapaTaq ready-mix (Kapa Biosystems, South Africa), 0.2 µl of each of the forward and reverse primers and 0.5 µl genomic DNA. The PCR conditions were as follows: 95°C for 5 min (1 cycle), followed by 38 cycles of 95°C for 45 seconds, 56°C for 50 seconds and 72°C for 70 seconds and a final cycle at 72°C for 7 minutes. The presence of DNA was confirmed on an 1% agarose gel stained with Ethidium Bromide (EtBr) under UV light.

An automated Genetic Analyzer ABI 3010XI (Central Analytical Facility (CAF) at Stellenbosch University) was used for capillary analysis using the Liz 1200 size standard. The raw data

was expressed as electropherograms using different fluorescent intensities and fragment lengths and analyzed using Gene Mapper® Version 5 Software (Applied Biosystems, United States). After performing size calling according to the size marker, the genotypes table was exported to Microsoft Excel (2016) for further analysis. Furthermore, peak height was preferred over peak size.

2.4. Statistical analysis

All statistical analysis was done through excel (2016), Statistica (version. 12) and 'R' statistic. The distribution of the data was tested through a Shapiro-Wilks test. This test gave the normality of the data-set based around significance values ($p < 0.05$ is significant and hence non-parametric, whilst $p > 0.05$ is parametric). If the data revealed a non-parametric distribution, then a Kruskal-Wallis Analysis of Variance (ANOVA) was conducted. An ANOVA was done to test for any significant differences between samples before and after treatment (pre, post 1, and post 2 assessment stages). Significant difference between soils treated at different field rates (0 % FR, 10 % FR, or 100 % FR) were also determined. Furthermore, data proven to be parametric had a One-Way ANOVA conducted. Additionally, an Analysis of similarity (ANOSIM) was used to evaluate the degree of similarity between microbial communities before and after herbicide treatment as well as compare untreated control sample communities to the treated sample communities (which were treated with 10 % or 100 % FR).

3. Results

3.1. *In situ* experiment 1

In situ experiment 1 was conducted in summer at the four riparian fynbos trial sites namely Alfalfa A and Bainskloof which was invaded with *A. mearnsii*, as well as Alfalfa B and Rainbow which was invaded with *E. camaldulensis*. The *A. mearnsii* trial sites recieved two separate herbicide treatments including glyphosate and triclopyr, whilst the *E. camaldulensis* trial sites recieved one herbicide treatment including a fluroxypyr and picloram emulsion. The results of *in situ* experiment 1 include an assessment of soil available nitrogen and soil available phosphorus before (pre) and after (post) the specified herbicide applications.

3.1.1. Soil chemical

3.1.1.1. Total soil available nitrogen

Total soil available N was measured in units of N-µg.g^{-1} which is the summation of soil available NO_3^- and NH_4^+. This was done to assess the impacts on available nitrogen stocks in the soil as a whole. Nitrogen is an important component of the soil to consider for this study as it directly affects the fertility of the soil.

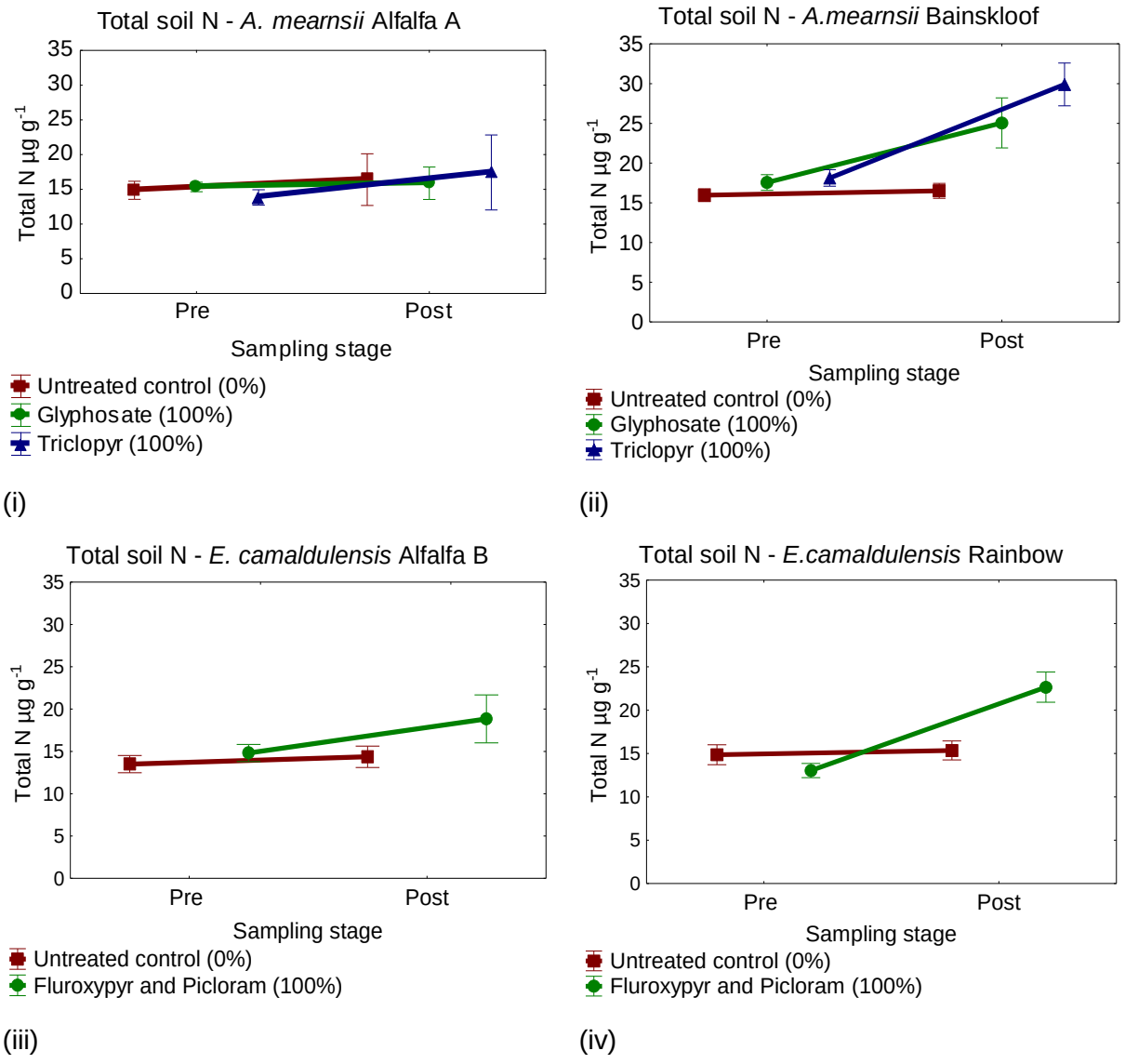


Figure. 2.6. The total soil available nitrogen expressed in units of N-µg g^{-1} for *A. mearnsii* sites namely (i) Alfalfa A and (ii) Bainskloof, as well as for *E. camaldulensis* sites namely (iii) Alfalfa B and (iv) Rainbow. *A. mearnsii* site treatments included an untreated control (0 % of the field rate), glyphosate (100 % of the field rate), as well as triclopyr (100 % of the field rate). *E. camaldulensis* site treatments included an untreated control (0 % of the field rate) and fluroxypyr and picloram emulsion (100 % of the field rate). The graph displays mean and standard error of total soil available N before herbicide treatment (pre) and a month after treatment (post).

Total soil available N levels revealed varying responses to herbicide exposure where *A. mearnsii* site, Alfalfa A and *E. camaldulensis* site, Alfalfa B, showed no considerable changes. The *A. mearnsii* site, Bainskloof and *E. camaldulensis* site, Rainbow, however, showed marked increases in nitrogen levels from before and after herbicide treatment (Fig. 2.6). A Kruskal-Wallis ANOVA further revealed that the triclopyr treatment lead to a significant increase in total soil available nitrogen from before to one month after application at the Bainskloof site ($H = 6.82$; $p = 0.01$). A similar trend was detected at the Rainbow site where the fluroxypyr and picloram emulsion application lead to a significant increase from before to after treatment (Kruskal-Wallis ANOVA: $H = 6.82$; $p = 0.01$).

Table 2.3. Kruskal-Wallis ANOVA showing differences in total soil available nitrogen between pre and post treatment with a given herbicide dose. Significance at $p<0.05$ ($n = 10$).

Alfalfa A			**Bainskloof**			**Alfalfa B**			**Rainbow**		
Treatment	**H**	**p**	**Treatment**	**H**	**p**	**Treatment**	**H**	**p**	**Treatment**	**H**	**p**
Untreated control (0 %)	0,01	0,92	Untreated control (0 %)	0,27	0,6	Untreated control (0 %)	0,53	0,46	Untreated control (0 %)	0,27	0,6
Glyphosate (100 %)	0,01	0,92	Glyphosate (100 %)	3,94	0,05	Fluroxypyr / Picloram (100%)	0,89	0,35	Fluroxypyr/ Picloram (100%)	6,82	0,01
Triclopyr (100 %)	0,01	0,92	Triclopyr (100 %)	6,82	0,01						

3.1.1.2. Soil available phosphorus

Soil available P is a limited resource in fynbos soils and native plant species are hugely dependent on phosphorus for growth. Soil available phosphorus (PO_4^{3-}) was measured in ppm over two separate assessment stages including pre (before), and post (one month after herbicide treatment). Soil available phosphorus was generally unaffected by either of the herbicides and seemed to remain stable from before to after herbicide application (Fig. 2.7).

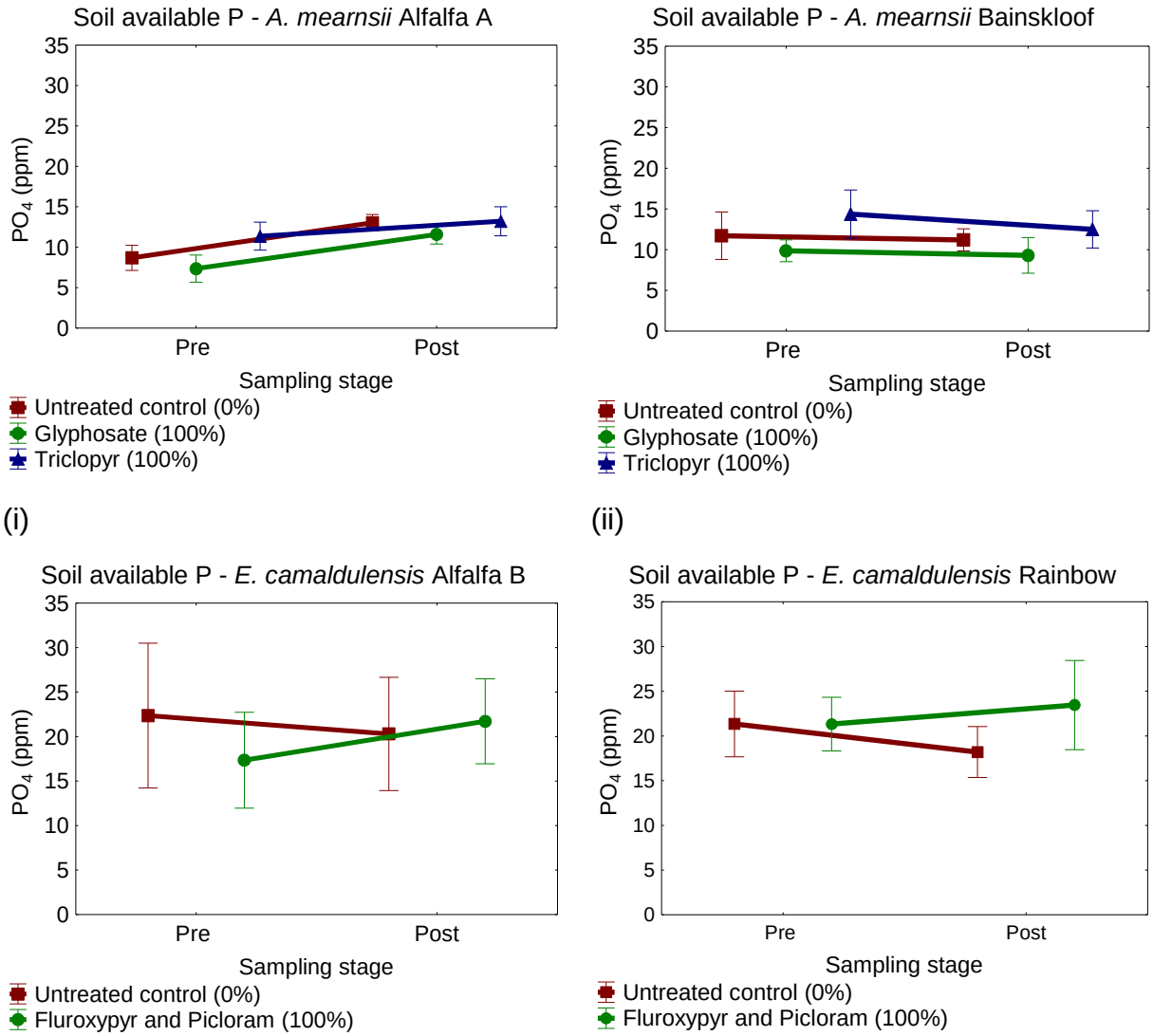


Figure. 2.7. The total soil available phosphorus expressed in units of ppm for *A. mearnsii* sites namely (i) Alfalfa A and (ii) Bainskloof, as well as for *E. camaldulensis* sites namely (iii) Alfalfa B and (iv) Rainbow. *A. mearnsii* site treatments included an untreated control (0 % of the field rate), glyphosate (100 % of the field rate), as well as triclopyr (100 % of the field rate). *E. camaldulensis* site treatments included an untreated control (0 % of the field rate) and fluroxypyr and picloram emulsion (100 % of the field rate). The graph displays mean and standard error of soil available phosphorus before herbicide treatment (pre) and a month after treatment (post).

The *A. mearnsii* soils at Alfalfa A and Bainskloof showed phosphorus levels ranging between 5 and 15 ppm with no significant effect between before and after glyphosate or triclopyr treatments at 100 % field rates (Kruskal-Wallis ANOVA: $p < 0.05$). The *E. camaldulensis* soils at Alfalfa B and Rainbow similarly showed no significant difference in phosphorus levels from before to after treatment with the fluroxypyr and picloram herbicide application (Kruskal-Wallis ANOVA: $p < 0,05$) (Table 2.4).

Table 2.4. Kruskal-Wallis ANOVA showing differences in soil available phosphorus between pre and post treatment with a given herbicide dose. Significance at $p<0.05$ (n = 10).

Alfalfa A			**Bainskloof**			**Alfalfa B**			**Rainbow**		
Treatment	**H**	**p**	**Treatment**	**H**	**p**	**Treatment**	**H**	**p**	**Treatment**	**H**	**p**
Untreated control (0 %)	3,94	0,05	Untreated control (0 %)	0,01	0,92	Untreated control (0 %)	0,01	0,92	Untreated control (0 %)	0,27	0,6
Glyphosate (100 %)	3,15	0,08	Glyphosate (100 %)	2,47	0,12	Fluroxypyr/ Picloram (100%)	0,27	0,6	Fluroxypyr/ Picloram (100%)	0,1	0,75
Triclopyr (100 %)	0,89	0,35	Triclopyr (100 %)	0,01	0,92						

3.2. *In situ* experiment 2

In situ experiment 2 was conducted in autumn and spring at the two riparian fynbos trial sites namely Alfalfa A which was invaded with *A. mearnsii*, as well as Alfalfa B which was invaded with *E. camaldulensis*. The *A. mearnsii* trial site, Alfalfa A, recieved two separate herbicide treatments including glyphosate and triclopyr, whilst the *E. camaldulensis* trial site, Alfalfa B, comprising a fluroxypyr and picloram emulsion. The results of *in situ* experiment 2 include an assessment of various soil chemical aspects including pH, electrical conductivity, total soil available nitrogen, nitrogen mineralisation, soil available phosphorus, and acid phosphatase activity. In addition, this study includes microbial analysis using ARISA to assess soil bacterial species richness and diversity. All measurements are shown from before herbicide teratment (pre), one week after treatment (post 1), and six weeks after treatment (post 2).

3.2.1. Soil chemical properties

3.2.1.1. pH

The pH of soil is a good indicator of soil health. Fynbos soils are known to be fairly acidic and other soil characteristics such as nitrogen and phosphorus concentrations and soil cations are directly associated with the pH of the soil. Soil pH was assessed in units rounded off to the nearest one decimal place. Measurements were taken before treatment, one week after treatment and six weeks after treatment. Studying the soil pH levels revealed that the three herbicides each induced an increase in the soil's acidity. The pH levels decreased consistently from before to after all glyphosate and fluroxypyr and picloram treatments (Fig. 2.8 (i) and (iii) respectively). The 10 % and 100 % triclopyr treatments also induced a significant decrease in soil pH and the untreated control decreased, but not as much as the treated soils (Fig. 2.8 (ii)).

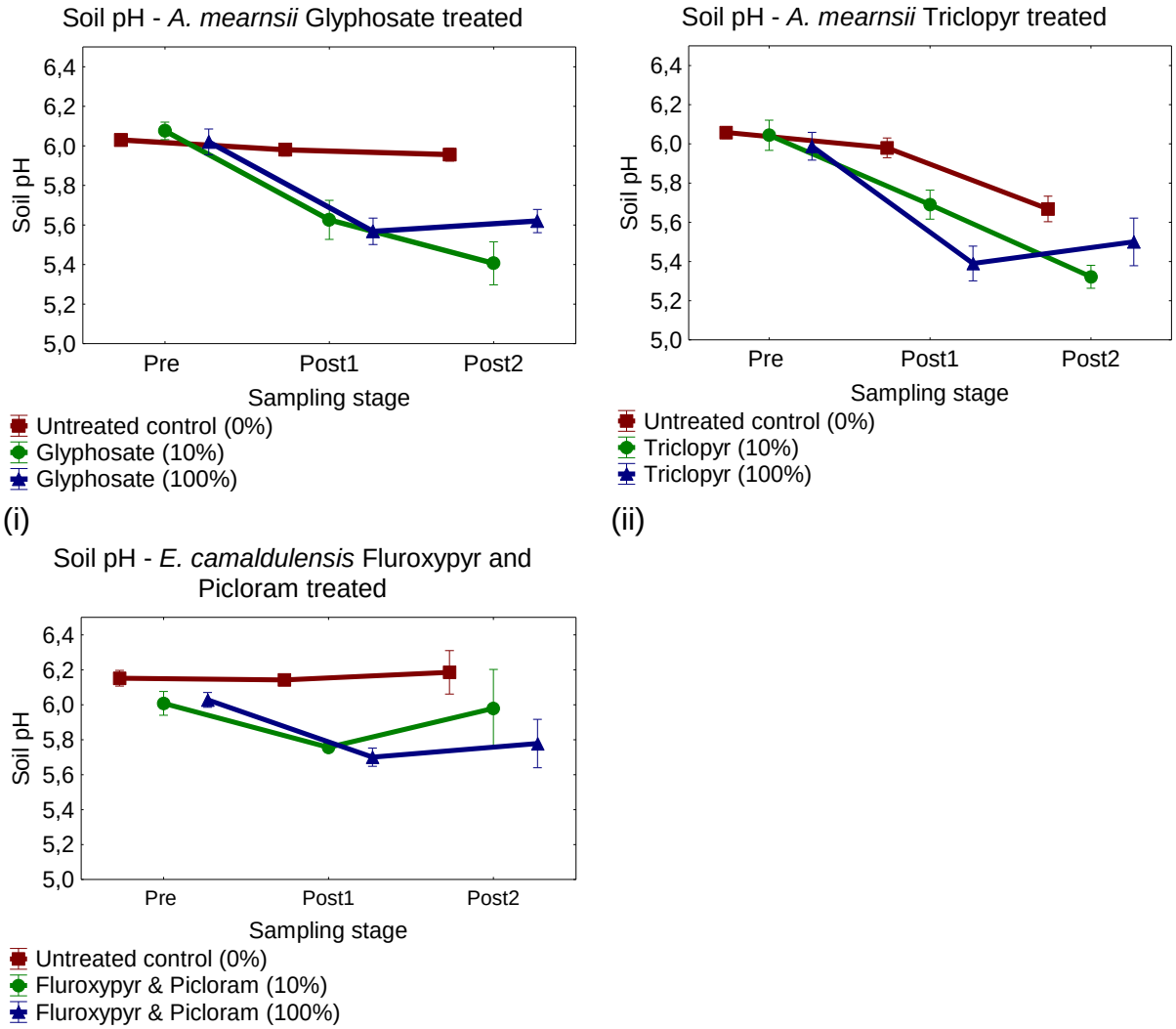


Figure. 2.8. The pH levels expressed to the nearest decimal for the *A. mearnsii in situ* site, Alfalfa A, as well as for the *E. camaldulensis in situ* site, Alfalfa B. The graph shows pH levels for the different treatments including (i) *A. mearnsii* glyphosate treatments of untreated control, 10 % field rate, and 100 % field rate; (ii) *A. mearnsii* triclopyr teratments of untreated control, 10 % field rate, and 100% field rate; (iii) *E. camaldulensis* treatments of untreated control, 10 % field rate, and 100 % field rate. Mean soil pH along with standard error is shown before herbicide treatment (pre), one week after treatment (post 1), and six weeks after treatment (post 2).

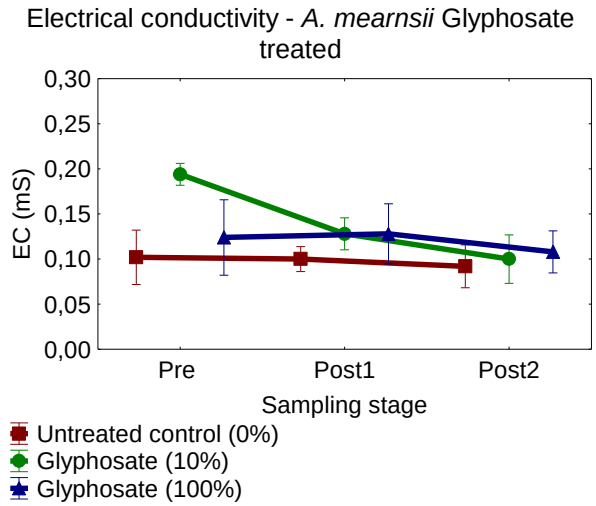
Soil pH in *A. mearnsii* soils treated with glyphosate showed for a significant decrease from before treatment to six weeks after when treated with 10 % and 100% field rates (Kruskal-Wallis ANOVA: H = 10.22; p = 0.01 and H = 9.47; p = 0.01, respectively). Triclopyr field rates of 10 % and 100 % also showed a significant acidifying effect on the soil decreasing the pH from 6 before treatment to as low as 5.4 six weeks after treatment (Kruskal-Wallis ANOVA: H = 12.02; p = 0.00 and H = 8.9; p = 0.01, respectively). The fluroxypyr and picloram treatment however revealed no signicifcant effect on soil pH at the *E. camaldulensis* Alfalfa B site, but pH did decrease with 10 % and 100 % field rates from 6 to as low as 5.6 (Fig. 2.8).

Table 2.5. Kruskal-Wallis ANOVA showing differences in soil pH between pre, post 1 and post 2 when treated with a specific herbicide dose. Significance at $p<0.05$ (n = 15).

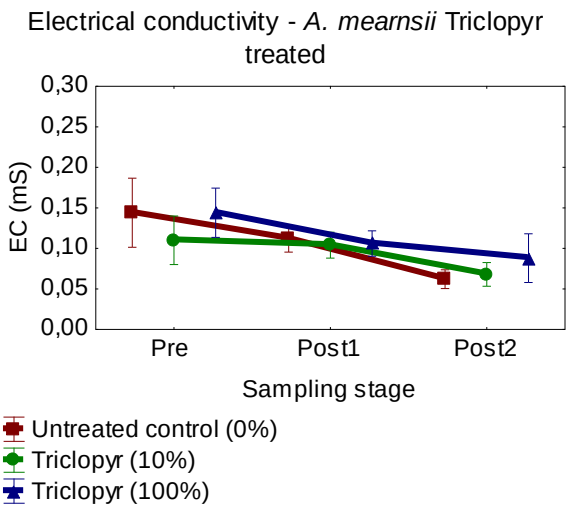
Alfalfa A, *A. mearnsii*						**Alfalfa B, *E. camaldulensis***		
Treatment	**H**	**p**	**Treatment**	**H**	**p**	**Treatment**	**H**	**p**
Untreated control (0 %)	3,53	0,17	Untreated control (0 %)	9,50	0,01	Untreated control (0 %)	0,29	0,87
Glyphosate (10 %)	10,22	0,01	Triclopyr (10 %)	12,02	0,00	Fluroxypyr/ Picloram (10%)	4,56	0,1
Glyphosate (100 %)	9,47	0,01	Triclopyr (100 %)	8,90	0,01	Fluroxypyr/ Picloram (100%)	4,84	0,09

3.2.1.2. Electrical conductivity

Soil electrical conductivity is a measurement of the soil's ability to transmit electrical currents. It serves as an indirect indicator of soil salinity in the soil. Electrical conductivity was measured in microSiemens (mS) and was rounded to the nearest decimal. Electrical conductivity for the soils treated appeared to have little to no response when faced with herbicide treatment and additionally showed no inhibitory effect from herbicide application.



(i)



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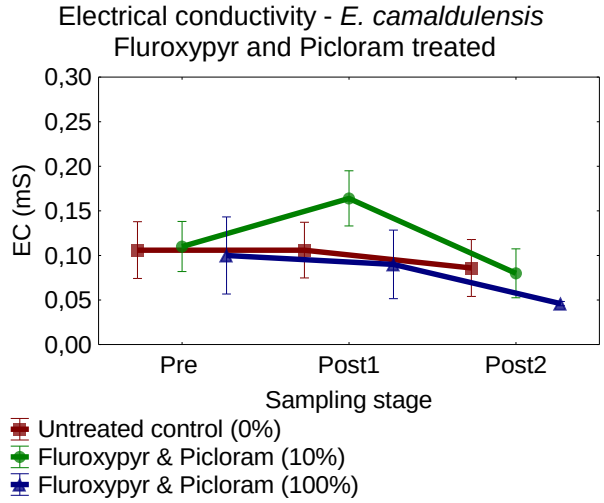


(iii)

Figure. 2.9. The electrical conductivity expressed in mS for the *A. mearnsii in situ* site, Alfalfa A, as well as for the *E. camaldulensis in situ* site, Alfalfa B. The graph shows electrical conductivity for the different treatments including (i) *A. mearnsii* glyphosate treatments of untreated control, 10 % field rate, and 100 % field rate; (ii) *A. mearnsii* triclopyr treatments of untreated control, 10 % field rate, and 100% field rate; (iii) *E. camaldulensis* treatments of untreated control, 10 % field rate, and 100 % field rate. Mean and standard error of Electrical conductivity is shown before herbicide treatment (pre), one week after treatment (post 1), and six weeks after treatment (post 2).

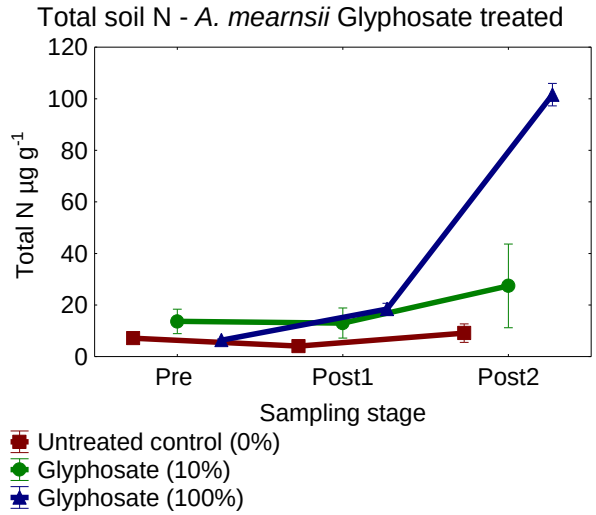
Electrical conductivity was unaffected by herbicide treatment, but a significant difference was, however, detected in *A. mearnsii* soil from before to six weeks after treatment with 10 % field rate of glyphosate (Kruskal-Wallis ANOVA: H = 7.83; p = 0.02). Electrical conductivity did, however, remain stable across all other herbicide treatments including the triclopyr and fluroxypyr and picloram herbicide with a range of 0.05 and 0.20 mS (Fig. 2.9). Furthermore, no significant effects could be detected from the triclopyr and fluroxypyr and picloram treatments (Table 2.6).

Table 2.6. Kruskal-Wallis ANOVA showing differences in soil electrical conductivity between pre, post 1 and post 2 when treated with a specific herbicide dose. Significance at $p<0.05$ ($n = 15$).

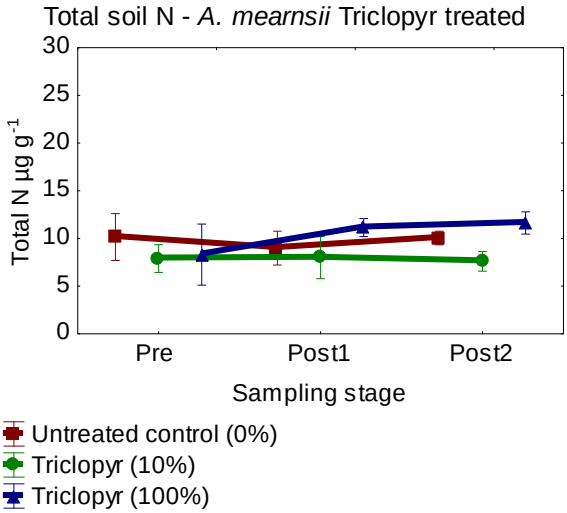
Alfalfa A, *A. mearnsii*						**Alfalfa B, *E. camaldulensis***		
Treatment	**H**	**p**	**Treatment**	**H**	**p**	**Treatment**	**H**	**p**
Untreated control (0 %)	0,11	0,95	Untreated control (0 %)	5,19	0,07	Untreated control (0 %)	0,25	0,88
Glyphosate (10 %)	7,83	0,02	Triclopyr (10 %)	1,95	0,38	Fluroxypyr/ Picloram (10%)	4,04	0,13
Glyphosate (100 %)	0,55	0,76	Triclopyr (100 %)	1,2	0,55	Fluroxypyr/ Picloram (100%)	1,14	0,57

3.2.1.3. Total soil available nitrogen

Total soil available nitrogen was hugely affected by glyphosate treatment *in situ*. The 100 % field rate treatment lead to a significant increase in total soil available nitrogen from before treatment to six weeks thereafter (Kruskal-Wallis ANOVA: $H = 12.50$; $p = 0.00$). Soil nitrogen levels ranged between 5 and 25 µg $N.g^{-1}$, but increased to as high as 100 µg $N.g^{-1}$ six weeks after the 100 % glyphosate treatment was applied (Fig. 2.10 (i))



(i)



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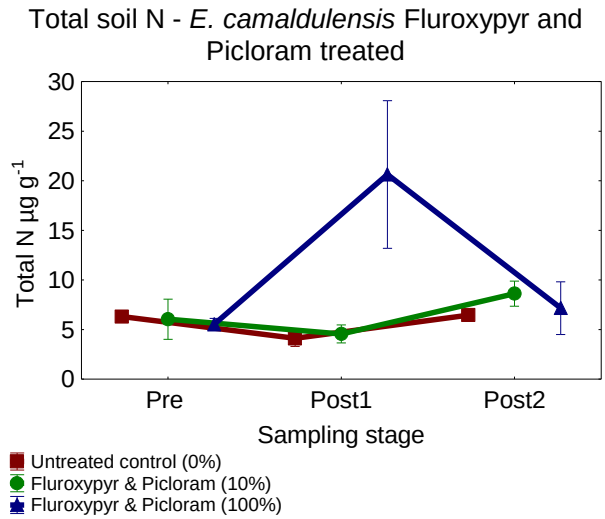


(iii)

Figure. 2.10. The total soil available nitrogen is expressed in µg $N.g^{-1}$ for the *A. mearnsii in situ* site, Alfalfa A, as well as for the *E. camaldulensis in situ* site, Alfalfa B. The graph shows total soil available N for the different treatments including (i) *A. mearnsii* glyphosate treatments of untreated control, 10 % field rate, and 100 % field rate; (ii) *A. mearnsii* triclopyr teratments of untreated control, 10 % field rate, and 100% field rate; (iii) *E. camaldulensis* treatments of untreated control, 10 % field rate, and 100 % field rate. Mean and standard error of total soil available N is shown before herbicide treatment (pre), one week after treatment (post 1), and six weeks after treatment (post 2).

Triclopyr treatments showed for no significant effects on total soil available nitrogen (Table 2.7) remaining between a range of 5 to 15 µg $N.g^{-1}$ (Fig. 2.10). Further, a Kruskal-Wallis ANOVA revealed no significant differences in soil nitrogen levels when faced with the fluroxypyr and picloram emulsion treatment. There was, however, a marked increase in soil nitrogen at the 100 % field rate treatment from before to one week after treatment increasing from 5 to 20 µg $N.g^{-1}$, but again dropped to precondition levels by week six (Fig. 2.10 (iii)).

Table 2.7. Kruskal-Wallis ANOVA showing differences in total soil available nitrogen between pre, post 1 and post 2 when treated with a specific herbicide dose. Significance at $p<0.05$ (n = 15).

Alfalfa A, *A. mearnsii*						**Alfalfa B, *E. camaldulensis***		
Treatment	**H**	**p**	**Treatment**	**H**	**p**	**Treatment**	**H**	**p**
Untreated control (0 %)	1,82	0,40	Untreated control (0 %)	1,94	0,38	Untreated control (0 %)	5,66	0,06
Glyphosate (10 %)	0,42	0,81	Triclopyr (10 %)	0,18	0,91	Fluroxypyr/ Picloram (10%)	3,92	0,14
Glyphosate (100 %)	12,5	0,00	Triclopyr (100 %)	1,58	0,45	Fluroxypyr/ Picloram (100%)	5,46	0,07

3.2.1.4. Nitrogen mineralization

Nitrogen mineralization rate was measured in units of µg g^{-1} day^{-1}. It essentially expresses the rate at which organic nitrogen was being converted to inorganic forms of nitrogen. These forms of nitrogen are readily available for uptake by plants, making this mineralization process fundamental for plant growth. Nitrogen mineralization was assessed to see how rates are influenced and accordingly how end products may be impacted. The nitrogen mineralization rates displayed large amounts of variability, particularly in *A. mearnsii* soils that recieved 10 % and 100 % field rate treatments of glyphosate and triclopyr (Fig. 2.11 (i) and (ii) respectively).

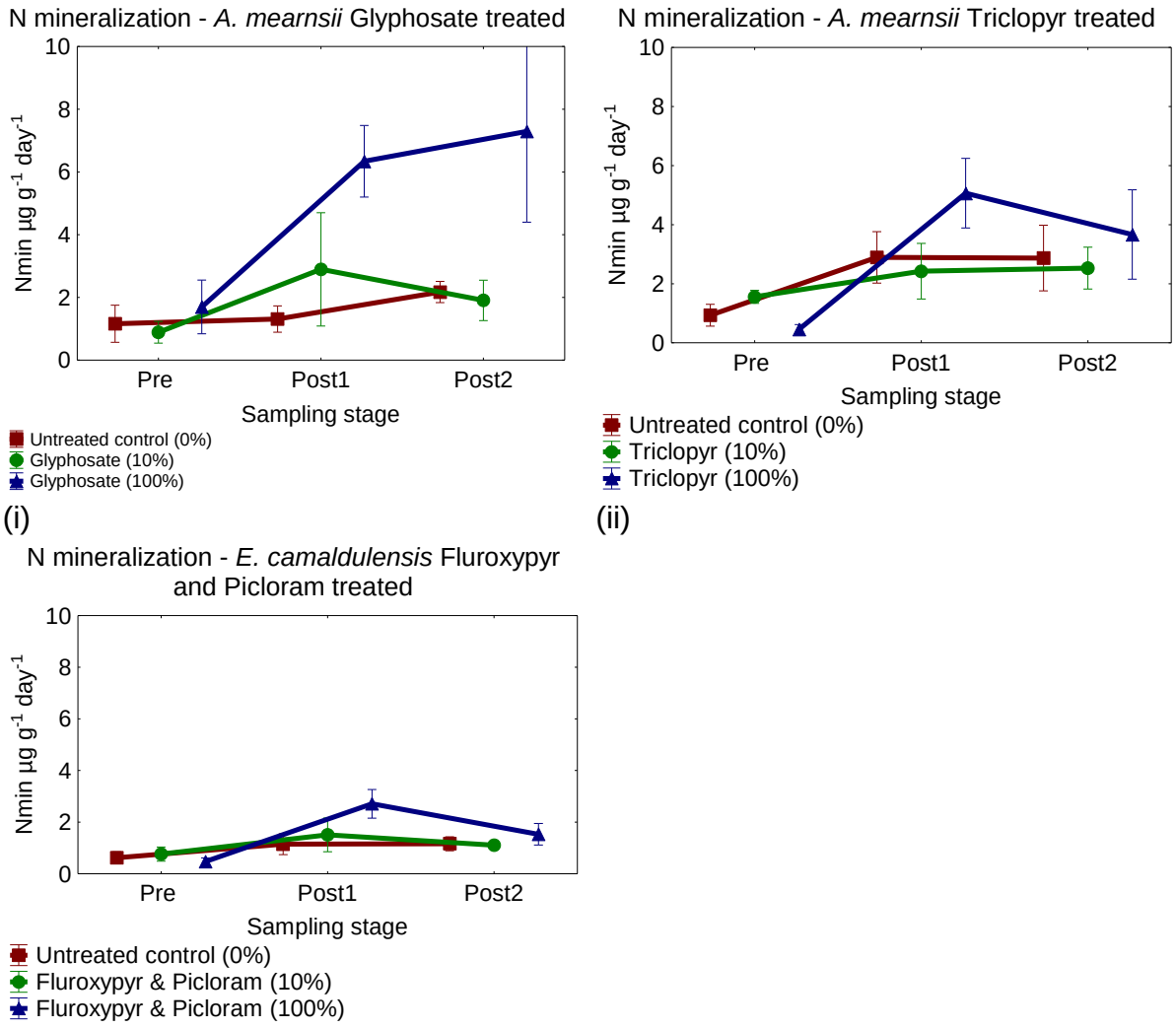


Figure. 2.11. The nitrogen mineralization rates are expressed in µg g^{-1} day^{-1} for the *A. mearnsii in situ* site, Alfalfa A, as well as for the *E. camaldulensis in situ* site, Alfalfa B. The graph shows nitrogen mineralization for the different treatments including (i) *A. mearnsii* glyphosate treatments of untreated control, 10 % field rate, and 100 % field rate; (ii) *A. mearnsii* triclopyr teratments of untreated control, 10 % field rate, and 100% field rate; (iii) *E. camaldulensis* treatments of untreated control, 10 % field rate, and 100 % field rate. Mean and standard error of nitrogen mineralization is shown before herbicide treatment (pre), one week after treatment (post 1), and six weeks after treatment (post 2).

Mineralization rates in *A. mearnsii* soils treated with glyphosate treatment showed large amounts of variability and no significant effects between the 10 % or 100 % field rate treatments (Kruskal-Wallis ANOVA: significance at $p < 0.05$). Despite no significant effects revealed, there was a mean increase in nitrogen mineralization from 2 to 7 µg g^{-1} day^{-1} from before treatment to six weeks thereafter (post 2). Triclopyr, however, significantly increased mineralization rates from before treatment to one week after treatment when exposed to the 100 % field rate dose (Krusal-Wallis ANOVA: $H = 9.98$; $p = 0.01$). In addition, the 100 % treatment with the fluroxypyr and picloram emulsion of *E. camaldulensis* soils at Alfalfa B also

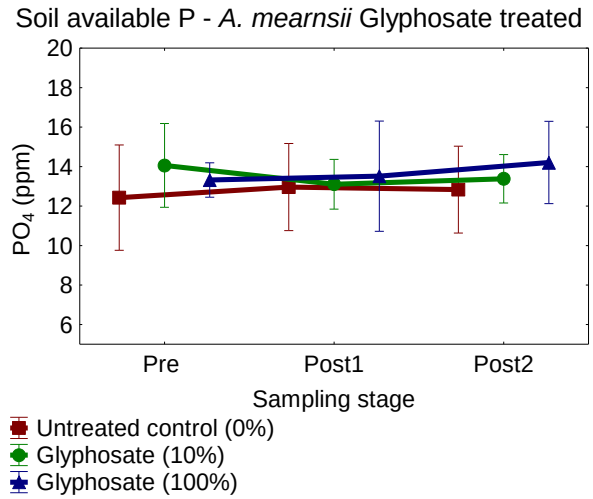
revealed a significant increase in nitrogen mineralization rates from before to one week after treatment (post 1) (Kruskal-Wallis ANOVA: H = 8.24; p = 0.02).

Table 2.8. Kruskal-Wallis ANOVA showing differences in nitrogen mineralisation between pre, post 1 and post 2 when treated with a specific herbicide dose. Significance at p<0.05 (n = 15).

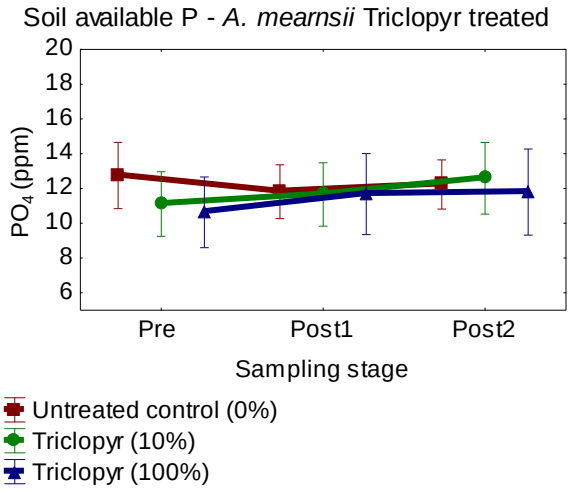
Alfalfa A, *A. mearnsii*						**Alfalfa B, *E. camaldulensis***		
Treatment	**H**	**p**	**Treatment**	**H**	**p**	**Treatment**	**H**	**p**
Untreated control (0 %)	2,42	0,3	Untreated control (0 %)	4,38	0,11	Untreated control (0 %)	2,62	0,27
Glyphosate (10 %)	1,82	0,47	Triclopyr (10 %)	0,26	0,88	Fluroxypyr/ Picloram (10%)	1,58	0,45
Glyphosate (100 %)	3,38	0,18	Triclopyr (100 %)	9,98	0,01	Fluroxypyr/ Picloram (100%)	8,24	0,02

3.2.1.5. Soil available phosphorus

Soil available phosphorus remained unaffected by either of the three different herbicide products tested with no significant effects resulting from the 10 % and 100 % field rate treatments (One-Way ANOVA: p < 0.05). Soil available phosphorus ranged between 8 and 16 ppm in *A. mearnsii* soils at the Alfalfa A trials site, while ranging between 9 and 16 ppm in *E. camaldulensis* soils at the Alfalfa B trial site. The stability of soil available phosphorus was clearly observed where post 1 and post 2 phosphorus levels maintained similar ranges when compared to phosphorus levels before herbicide treatment (Fig. 2.12).



(i)



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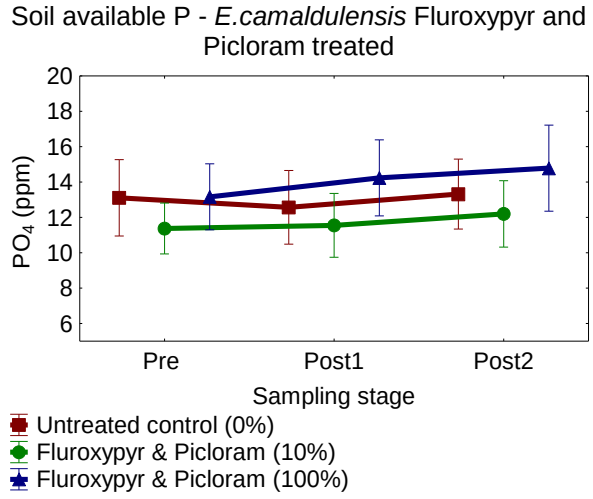

(iii)

Figure. 2.12. The soil available phosphorus is expressed in ppm for the *A. mearnsii in situ* site, Alfalfa A, as well as for the *E. camaldulensis in situ* site, Alfalfa B. The graph shows soil available P for the different treatments including (i) *A. mearnsii* glyphosate treatments of untreated control, 10 % field rate, and 100 % field rate; (ii) *A. mearnsii* triclopyr teratments of untreated control, 10 % field rate, and 100% field rate; (iii) *E. camaldulensis* treatments of untreated control, 10 % field rate, and 100 % field rate. Mean soil available P and standard error is shown before herbicide treatment (pre), one week after treatment (post 1), and six weeks after treatment (post 2).

Table 2.9. One-Way ANOVA showing differences in soil available phosphorus between pre, post 1 and post 2 when treated with a specific herbicide dose. Significance at p<0.05 (n = 15).

Alfalfa A, *A. mearnsii*						**Alfalfa B, *E. camaldulensis***		
Treatment	**F**	**p**	**Treatment**	**F**	**p**	**Treatment**	**F**	**p**
Untreated control (0 %)	0,01	0,99	Untreated control (0 %)	0,08	0,92	Untreated control (0 %)	0,03	0,97
Glyphosate (10 %)	0,10	0,91	Triclopyr (10 %)	0,15	0,86	Fluroxypyr/ Picloram (10 %)	0,06	0,94
Glyphosate (100 %)	0,05	0,95	Triclopyr (100 %)	0,08	0,92	Fluroxypyr/ Picloram (100 %)	0,15	0,87

3.2.1.6. Acid phosphatase activity

The acid phosphatase activity was measured in units of µg-p-NP.g^{-1} dry soil. This hydrolysis process induces the production of phosphate in a form that is available for plant uptake. Herbicide treatment appeared to have no detrimental effects on the acid phosphatase activity rates as the activity levels remained constant regardless of rate of application or assessment stage. The activity rates remained relatively stable although displaying fair amounts of variablity (Fig. 2.13).

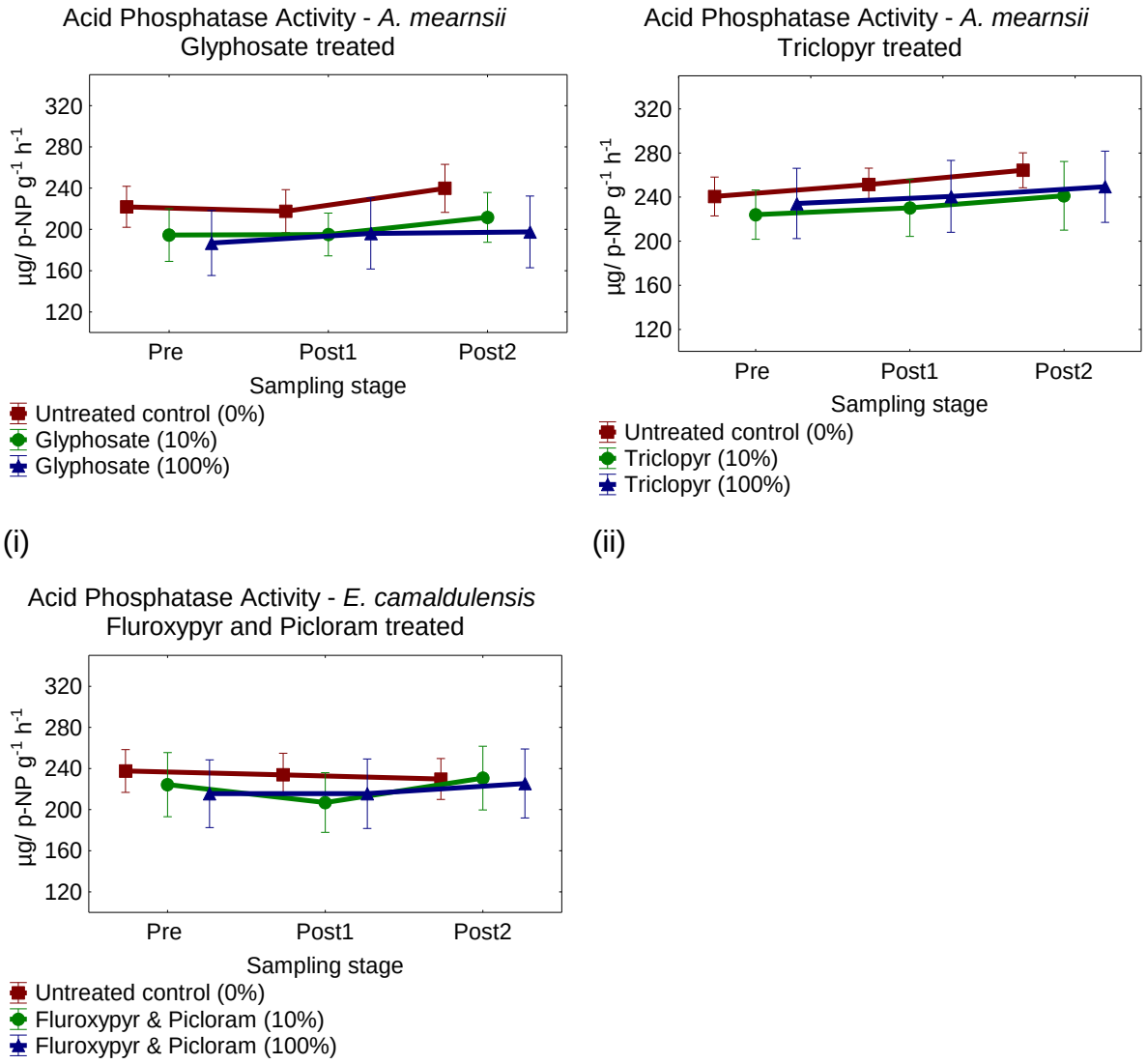

Figure. 2.13. The acid phosphatase activity rates are expressed in µg *p*-NP g^{-1} dry soil for the *A. mearnsii in situ* site, Alfalfa A, as well as for the *E. camaldulensis in situ* site, Alfalfa B. The graph shows acid phosphatase activity for the different treatments including (i) *A. mearnsii* glyphosate treatments of untreated control, 10 % field rate, and 100 % field rate; (ii) *A. mearnsii* triclopyr teratments of untreated control, 10 % field rate, and 100% field rate; (iii) *E. camaldulensis* treatments of untreated control, 10 % field rate, and 100 % field rate. Mean acid phosphatase activity and standard error is shown before herbicide treatment (pre), one week after treatment (post 1), and six weeks after treatment (post 2).

Glyphosate treated *A. mearnsii* soils maintained phosphatase activity levels between 160-260 µg *p*-NP g^{-1} (Fig. 2.13 (i)) and a One-Way ANOVA showed no significant difference between before and after glyphosate application (Table 2.10). The triclopyr treated *A. mearnsii* soil also showed no significant difference between before and after application for all concentration treatments and ranged between 190 and 320 µg *p*-NP g^{-1} across the six week assessment period (Table 2.10). The fluroxypyr and picloram emulsion similarly showed an acid phosphatase activity rate ranging between 180 and 280 µg *p*-NP g^{-1} in *E. camaldulensis* soils (Fig. 2.13 (iii)). A One-Way ANOVA further revealed no significant difference among the triclopyr and fluroxypyr and picloram treatments (Table 2.10).

Table 2.10. One Way ANOVA showing differences in acid phosphatase activity between pre, post 1 and post 2 when treated with a specific herbicide dose. Significance at $p<0.05$ (n = 15).

Alfalfa A, *A. mearnsii*						**Alfalfa B, *E. camaldulensis***		
Treatment	**H**	**p**	**Treatment**	**H**	**p**	**Treatment**	**H**	**p**
Untreated control (0 %)	1,22	0,54	Untreated control (0 %)	1,46	0,48	Untreated control (0 %)	0,00	1,00
Glyphosate (10 %)	0,72	0,70	Triclopyr (10 %)	0,26	0,88	Fluroxypyr/ Picloram (10%)	0,86	0,65
Glyphosate (100 %)	0,26	0,88	Triclopyr (100 %)	0,72	0,70	Fluroxypyr/ Picloram (100%)	0,26	0,88

3.2.2. Soil microbiology

3.2.2.1. Shannon diversity index (H')

The mean Shannon diversity index values are shown for the bacterial communities present in the soils treated with herbicides *in situ*. The diversity index is shown for the bacterial community structure before herbicide treatment (pre), a week after treatment (post 1), and six weeks after treatment (post 2). The diversity index is a quantitative measure used to show the diversity in sample sets, whilst showing the distribution of these species. The number of OTUs (operational taxonomic units) are expressed alongside the Shannon diversity values. The OTUs classify closely related individuals, grouped by DNA sequence length similarity. The Shannon diversity values and number of OTU are displayed below with the respective means as well as relative standard error of the mean (Table 2.11).

Table 2.11. The Shannon diversity values and number of OTUs for bacterial communities in soils treated with specified herbicides are shown with the mean and relative standard error for the different assessment stages including before treatment (pre), one week after (post 1), and six weeks after (post 2). Results are displayed for the different treatments including (i) *A. mearnsii* glyphosate treatments of untreated control, 10 % field rate, and 100 % field rate; (ii) *A. mearnsii* triclopyr teratments of untreated control, 10 % field rate, and 100% field rate; (iii) *E. camaldulensis* fluroxypyr and picloram treatments of untreated control, 10 % field rate, and 100 % field rate.

(i)	***A. mearnsii* Glyphosate treated**	
Pre	**Shannons-diversity index:**	**Number of OTUs:**
0 %	1.94 ± 0.03	42.33 ± 1.70
10 %	1.96 ± 0.01	39.67 ± 3.30
100 %	2.00 ± 0.06	50.67 ± 8.99
Post 1	**Shannons-diversity index:**	**Number of OTUs:**
0 %	1.97 ± 0.06	40.00 ± 6.53
10 %	2.03 ± 0.06	36.33 ± 3.68
100 %	1.94 ± 0.01	51.33 ± 3.09
Post 2	**Shannons-diversity index:**	**Number of OTUs:**
0 %	2.01 ± 0.11	56.33 ± 3.86
10 %	1.95 ± 0.01	49.67 ± 4.92
100 %	1.93 ± 0.01	49.33 ± 1.70
(ii)	***A. mearnsii* Triclopyr treated**	
Pre	**Shannon-diversity index:**	**Number of OTUs:**
0 %	2.43 ± 0.01	57.00 ± 2.94
10 %	2.49 ± 0.04	49.00 ± 4.24
100 %	2.65 ± 0.13	49.33 ± 8.01
Post 1	**Shannon-diversity index:**	**Number of OTUs:**
0 %	2.46 ± 0.03	55.33 ± 4.50
10 %	2.50 ± 0.13	51.33 ± 14.43
100 %	2.70 ± 0.16	22.67 ± 5.19
Post 2	**Shannon-diversity index:**	**Number of OTUs:**
0 %	2.56 ± 0.05	42.67 ± 5.73
10 %	2.61 ± 0.07	42.67 ± 10.21
100 %	2.54 ± 0.01	48.33 ± 9.39
(iii)	***E. camaldulensis* Fluroxypyr and picloram treated**	
Pre	**Shannon-diversity index:**	**Number of OTUs:**
0 %	1.72 ± 0.01	47.00 ± 7.26
10 %	1.72 ± 0.00	53.67 ± 0.94
100 %	2.00 ± 0.03	35.67 ± 15.58
Post 1	**Shannon-diversity index:**	**Number of OTUs:**
0 %	1.78 ± 0.10	46.67 ± 2.36
10 %	1.81 ± 0.10	33.33 ± 2.36
100 %	1.89 ± 0.11	28.33 ± 12.04
Post 2	**Shannon-diversity index:**	**Number of OTUs:**
0 %	1.74 ± 0.01	49.67 ± 3.68
10 %	1.80 ± 0.04	36.33 ± 7.54
100 %	1.73 ± 0.02	53.33 ± 5.31

Treatment with glyphosate had little effect on the diversity of the bacterial community. However, there was a decrease in the number of OTUs from 49.33 to 22.67 a week after treatment at 100% concentration, which was followed by a recovery to the initial numbers, six weeks after the herbicide application (post 2). Additionally, a One-Way ANOVA revealed that the number of OTUs present in the bacterial community at the 100 % field rate treatment of

glyphosate a week after treatment, were significantly lower than the bacterial communities present in the soils of the 0 % and 10 % concentration treatments (One-Way ANOVA: F = 7.46; p = 0.03). This suggests that the glyphosate treatment at a 100 % field rate could influence the number of OTUs present in the soil when applied, but the effects do not persist longer than a week and eventually return to pre-application levels by week six.

The bacterial communities in soils treated with triclopyr expressed no change with the treatment at 10 % or 100 % field rate treatments (Table 2.11 (ii)). The Shannon-diversity scores remained consistent from before (pre) to a week after (post 1), to six weeks after treatment (post 2), irrespective of the concentration of the triclopyr herbicide applied. A One-Way ANOVA did however indicate a significant difference in the 10 % concentration treated samples (One-Way ANOVA: F = 5.58; p = 0.04). This suggests that there may have been an effect of the triclopyr based herbicide on the OTUs in the soil one week after treatment, but the number of OTU's increased again six weeks after treatment.

The treatment of fluroxypyr and picloram, similar to the glyphosate and triclopyr treatments, showed very little impact on the soil bacterial diversity as minor variation was observed and the Shannon-diversity scores remained consistent regardless of the concentration of herbicide applied (Table 2.11 (iii)). The Shannon diversity scores ranged between 1.7 and 2.0 and did not show any considerable effects when treated with the herbicide. The treatment of fluroxypyr and picloram additionally showed no significant impact on the number of OTUs as there were considerable variation within samples. A One-way ANOVA showed no significant differences between bacterial community's diversity exposed to 0 %, 10 % and 100 % field rates of fluroxypyr and picloram treatment at the different assessment stages (pre, post 1 and post 2) (Table 2.12). This suggests that the fluroxypyr and picloram emulsion could have no harmful effect on the diversity of soil bacterial communities over a short term period of time (a six-week period).

Table 2.12. One-Way ANOVA of Shannon diversity showing for significant difference between Shannon diversity for the *in situ A. mearnsii* soils treated with separate treatments of glyphosate and triclopyr at 0 %, 10 % and 100 % field rates, as well as for the *in situ E. camaldulensis* soils treated with fluroxypyr and picloram at 0 %, 10 % and 100 % field rates. Significance is shown at each of the separate assessment stages namely, pre (before), post 1 (one week after treatment), and post 2 (six weeks after treatment). [Significance at $p < 0.05$; df = 12]

Assessment stage	**Glyphosate *A. mearnsii***		**Triclopyr *A. mearnsii***		**Fluroxypyr and Picloram *E. camaldulensis***	
	F	**p**	**F**	**p**	**F**	**p**
Pre	1.35	0.33	2.09	0.21	1.68	0.26
Post 1	7.46	0.03	5.58	0.04	3.45	0.10
Post 2	0.29	0.76	2.23	0.19	4.87	0.06

3.2.2.2. Analysis of Similarity

Non-metric dimensional scaling plots (NMDS plots) is a gradient analysis which plots samples according to a dissimilarity matrix. The NMDS plots were generated through a Whittaker-dissimilarity index, which produced a dissimilarity matrix. The points on the figure below (Fig. 2.14) displays the similarity between the microbes from before to after being treated with a given herbicide treatment. The more similar microbial communities are ordinated closer to one another. The similarity of samples was essentially compared to one another in an effort to display any grouping in the microbial community before and after a given herbicide treatment. An analysis of similarity (ANOSIM) was done to show the R statistic, which values range between -1 and 1 to express similarity between or within microbial communities. A value of 1 shows complete dissimilarity between sites, whilst a value of -1 displays large amounts of variation within sample sets. In addition, values that are closer to 0 express similarity between the microbial communities at the given assessment stage.

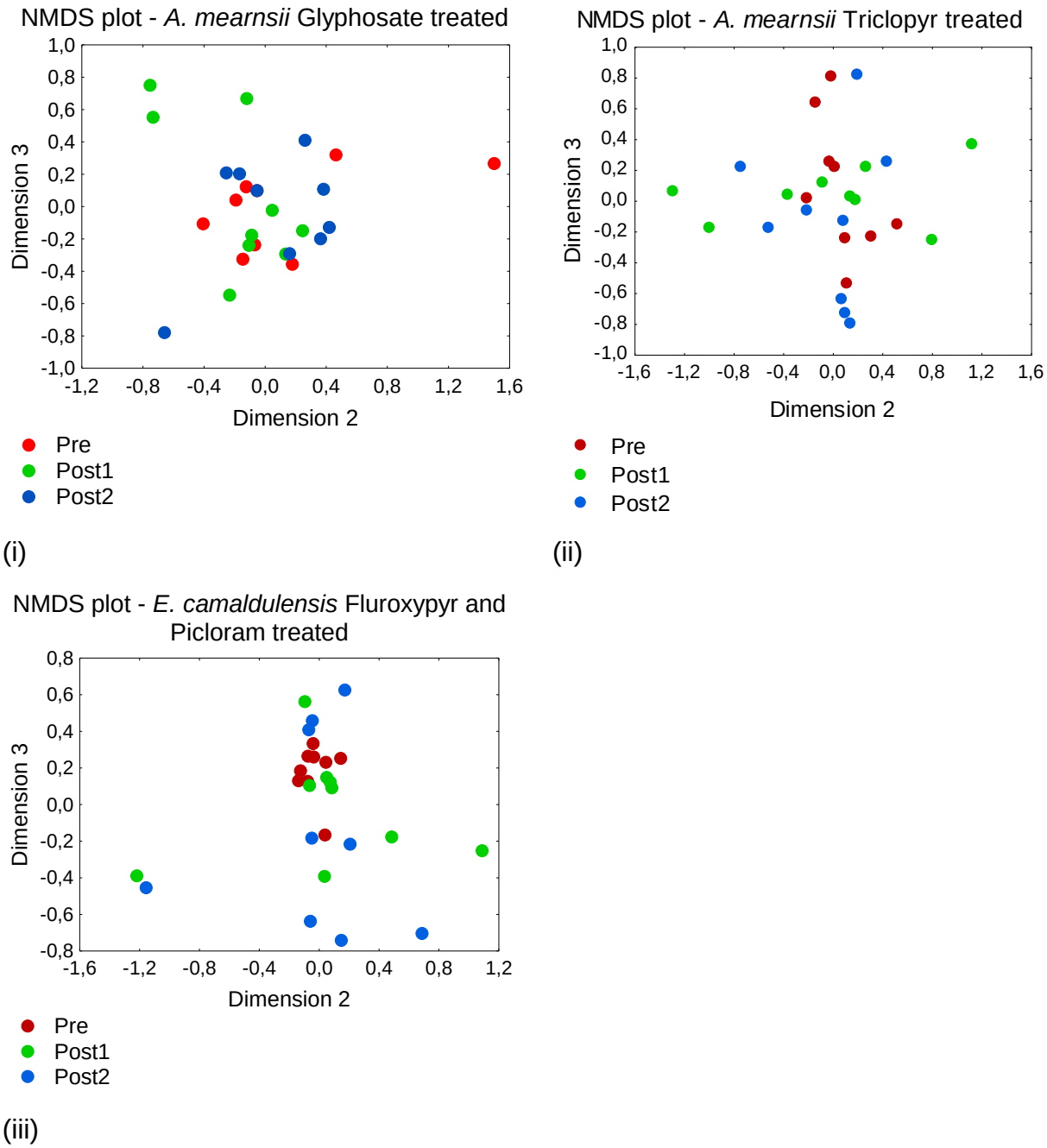

Figure. 2.14. Non-metric dimensional scaling (NMDS) plots for the microbial communities present in the soils of the following sites: (i) *A. mearnsii* site, Alfalfa A, treated with glyphosate; (ii) *A. mearnsii* site, Alfalfa A, treated with triclopyr; (iii) *E. camaldulensis* site, Alfalfa B, treated with fluroxypyr and picloram emulsion. The microbial communities are compared from before treatment (pre) to one week after treatment (post 1) to six weeks after treatment (post 2).

The bacterial communities found within soils treated with glyphosate display no convincing clusters when regarding the community structure at the pre, post 1, and post 2 sampling stages. An ANOSIM revealed that the bacterial community structure was rather similar to one another when comparing the community in *A. mearnsii* soil from before 10 % and 100 % glyphosate treatment (pre) to after treatment (post 1 and post 2) (ANOSIM: R = 0.11; p = 0.01).

The bacterial community of the *A. meanrsii* treated soils were rather randomly distributed and no considerable effeacts could be observed (Fig. 2.14 (i)).

Triclopyr treatments similarly had no effect on the bacterial community structure within six weeks of the application. Similar to the glyphosate herbicide, the bacterial communities displayed significant similarity when comparing the community structure from before treatment to after treatments with triclopyr (ANOSIM: R = 0.19 and p = 0.00). Furthermore, no effect of the triclopyr based herbicide on soil microbial community structure could be detected as there are no clusters formed between separate assessment stages (pre, post 1, post 2) (Fig. 2.14 (ii)).

The soils treated with fluroxypyr and picloram displayed the same trend as seen in the glyphosate and triclopyr treated soils, where the microbial community structure remained unchanged with herbicide treatment (Fig. 2.14 (iii)). An ANOSIM further showed a great similarity amongst bacterial communities in the soil when comparing pre and post application samples to one another. Additionally, no significant difference between these groups were shown (ANOSIM: R = 0.05 and p = 0.09).

Table 2.13. ANOSIM showing similarity of microbial communities when compared at the different assessment stages before and after treatment with herbicide treatment. The table displays the R value expressing similarity between *A. mearnsii* bacterial community before and after glyphosate and triclopyr treatment, as well as between *E. camaldulensis* bacterial community before and after fluroxypyr and picloram treatment. In addition, the p-value shows for significant difference from microbial communities before to after herbicide treatment [significance found at $p < 0.05$]

ANOSIM 'R' values:	**R**	**p**
A. mearnsii Glyphosate treated soil	0.11	0.01
A. mearnsii Triclopyr treated soil	0.19	0.00
E. camaldulensis Fluroxypyr and Picloram treated soil	0.05	0.09

4. Discussion

4.1. Effects on soil chemical characteristics

The three herbicides tested showed minor detrimental impacts on the soil properties and processes that were evaluated. Aspects such as electrical conductivity, soil available phosphorus and acid-phosphatase activity remained stable regardless of the type or concentration of herbicide applied. There was, however, an exception for soil pH, soil nitrogen and soil nitrogen mineralization. The soil pH decreased significantly, where 10 % concentrations of the herbicides induced minor reductions, whilst 100 % concentration treatments induced more considerable and significant reductions in the pH levels. Even if the

reduction in pH was only one or a few units, the reduction was consistent across all treatments. One can deduce that the three herbicides including glyphosate (Springbok 360 SL), triclopyr (Garlon 480 EC), and fluroxypyr and picloram (Plenum 160 ME) all have an acidifying effect on fynbos soil. However, as the experiment was only run for 6 weeks, the change may only be short term, and the pH may return to a more basic level beyond the six-week assessment stage. Furthermore, the effect of the herbicide on the soil pH could be a result of the adjuvants included in the mixture of the given herbicide and not necessarily the active ingredient itself. It is therefore possible that the glyphosate, triclopyr and fluroxypyr and picloram active ingredients are not responsible for the acidifying effect observed. It was shown in Howe et al., (2004) and Peixoto, (2005) that the adjuvants in the herbicide tested were more detrimental to soil physicochemical properties such as soil respiration, than the active ingredient itself (i.e. glyphosate).

Soil nitrogen availability furthermore responded significantly to herbicide treatment, especially to that of the glyphosate-based herbicide treatment (Springbok 360 SL) where treatment lead to a significantly higher soil nitrogen level in both experiment 1 and 2 at doses of 10 % or 100 % of the recommended field rate. The total soil available nitrogen increased considerably a month after glyphosate and triclopyr was applied at 100 % concentrations *in situ* (experiment 1). A similar increase in nitrogen with agrochemical treatment has been reported in other studies (Barreiro & Pratt, 1994; Rhoades et al., 2002). Additionally, Reddy et al. (2003) also reported an increase in the soil nitrogen levels when chemical clearing of plants took place yet linked this effect to the decomposing dead root and shoot biomass in the soil. During the time of the plants stress (due to herbicide exposure), the plant could release certain exudates and nutrients into the soil matrix. This has also been reported in Yelenik et al. (2004) where *in situ* total soil nitrogen levels increased with the addition of plant litter and biomass after clearing.

The Plenum 160 ME herbicide (fluroxypyr and picloram emulsion) also showed a significant elevation in soil nitrogen in autumn, but soil nitrogen levels were reduced back to initial pre-treatment levels by week 6 (post 2). It is clear that the given herbicides can affect the nitrogen stocks, with varying degrees. Significant effects were detected for nitrogen mineralization as well. There were slight variabilities, but glyphosate again had the largest impact where nitrogen mineralization increased significantly a week after treatment and thereafter decreased rather aggressively with the 100 % field rate treatment. The triclopyr, and fluroxypyr and picloram treatments showed smaller impacts, yet presented significantly higher nitrogen mineralization rates one week after treatment. The rates did, however, decrease to stable pre-condition levels by week 6 as seen in the glyphosate treatments.

As mentioned above, the remaining soil physicochemical properties including electrical conductivity, soil available phosphorus, and acid-phosphatase activity remained constant and seemed to be unresponsive to treatment of the given herbicides. The electrical conductivity (EC) showed major variability, which did not allow for any conclusions for an effect of the herbicides. Additionally, soil available phosphorus (PO_4^{3-}) was stable throughout the course of the experiments. Both experiment 1 and 2 showed no impacts and the results showed no significant differences between treated and untreated samples. The herbicides also showed limited effects on acid phosphatase activity where controls and treated samples resembled activity rates before herbicide treatment. Neither of the separate treatments seemed to affect rates of acid phosphatase activity. There have been reports of certain herbicide treatments having no effect on soil chemical nutrients or soil chemical processes (Zabaloy et al., 2008). This could be a result of the chemical itself being too weak to induce any changes in the soil, or a result of the herbicide being rapidly and steadily degraded by microbial communities present in the soil (Haney et al., 2000).

4.2. Effects on soil microbial characteristics

Microbial communities showed little to no change when exposed to any of the herbicide treatments. The response of the communities to even 100 % field rate treatments were minor. When assessing the OTUs, bacterial diversity numbers decreased significantly one week after treatment with the 100 % FR treatment of Springbok 360 SL (glyphosate). Thereafter, the microbial OTU numbers increased when assessed six weeks after treatment. This suggests that the glyphosate-based herbicide may have the potential of decreasing OTU numbers, yet communities return to their initial OTU ranges six weeks after treatment. Therefore, one cannot conclude that there are effects persisting longer than six weeks. The same can be said for the other herbicide treatments. OTU numbers and measures of diversity (Shannon's diversity index) revealed no long-lasting effects. If minor changes to the microbial communities did occur a week after treatment, then the community should return to its initial condition by the six-week assessment stage (post 2 assessment stage).

Assessing the similarity between microbial communities before and after treatments revealed that the communities were in fact rather similar, with minor degrees of dissimilarity. Essentially, no microbial community shifts were observed. The lack of change in the communities when faced with herbicide treatment did however, only come from assessing the bacterial communities. Changes in the fungal communities could have occurred. The resistance expressed in these bacterial communities could also have been due to variable conditions occurring in nature. It has been detected that certain microbial groups can be

affected, whilst others thrive (Schulz et al., 1985). Perhaps conducting a more controlled *ex situ* study would reveal more direct impacts of the herbicides on select groups of microorganisms as environmental heterogeneity would then be ruled out of the experiment.

A study conducted by Cole (1976), observed long-term exposure of herbicides to soils in a corn field *in situ* and found that herbicides based on atrazine have no effects on specific microbial communities. Widenfalk et al. (2008) also found no effect on bacterial activity when separate glyphosate and captan herbicides were applied. Moreover, Zabaloy et al. (2012) reported that herbicides applied at ten times the field rate showed no substantial effect on microbial richness, function and activity. The diversity and similarity scores expressed in this chapter presented very little variation or change from before treatment to six weeks after treatment. It is presumed that the herbicides tested, namely, glyphosate, triclopyr, and fluroxypyr and picloram have no adverse effect on the diversity or structure of the bacterial communities in fynbos soil over a short-term period when faced with the doses included in this study. Alternatively, impacts on the soil microbial community could potentially occur within the first few days of treatment and then dissipate by the first week assessment after treatment as reported by Weaver et al. (2007). More studies are required to look at the impacts on short term (hours to days), medium term (weeks) and long term (months to years) assessment stages, especially as it was shown in this study that soil pH is lowered when herbicides are applied, and this in turn can affect microbial structure (Slabbert et al., 2014). No significant effects of herbicides on microbial structure and activity could be detected, but pH could have an effect on a microbial aspect that has not been covered by this study (i.e. dehydrogenase activity and microbial biomass).

4.3. Summary

In summary, the herbicides tested only showed a consistent significant effect on soil pH and partly so for total soil available nitrogen and nitrogen mineralization rates. No convincing effects were exerted on the other soil aspects evaluated, including electrical conductivity, soil phosphorus, and phosphatase activity, given that the application rate remains between 0 and 100 % of the recommended field-rate. The given herbicides could, however, have more conspicuous effects at higher rates (i.e. 10 000 % field rate dose) that are more representative of a product spill or dump *in situ*. Moreover, the effects of the herbicides on the soil microbial community structure and community metabolic potential were minimal. It is essential that we continue testing herbicides and the potential impact they have on the soil microbial communities as well as on the wide array of soil chemical characteristics.

Further, collaborative effects of allelochemical and herbicides remain largely unknown (Weidenhamer and Callaway, 2010). It is recommended that future research studying the impacts of herbicides go deeper into evaluating different herbicides *in situ* in real natural conditions. We should include testing the given herbicides at more and higher concentration levels and for longer periods of time and with more frequent assessment stages to gain a better understanding of the respective herbicides risk assessment over a given period of time.

In situ studies such as this can aid managers and farm owners in understanding the impacts of certain herbicides in nature reserves, urban green areas, and farmlands *in situ*. It is also recommended that consideration is given to evaluating other aspects of the microbial community present in the soil and how they respond to herbicide treatment, e.g. soil fungi. Such aspects can include the identification of the microbial species present in the soil as well as microbial metabolic potential and biomass (Allison & Martiny, 2008; Pratt et al., 1997; Li et al., 1999). Having identified microbial species in the soil before and after herbicide treatment, can to an extent give us an indication of how important genera such as *Desulfovibrio* and *Rhizobia* (which process nutrients in the soil) are affected (Stafford et al., 2005). With this information, we can improve our understanding of how microbial communities and soil processes are affected by certain herbicides and chemical control management schemes.

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CHAPTER 3:

The potential effects of herbicide on Riparian Fynbos soil *ex situ*

1. Introduction

Ex situ studies are important when assessing the potential effects of agrochemicals on various aspects of soil ecosystems and matrices (Carriciolo et al., 2013; van Beelen & Doelman, 1996). The results obtained from *in situ* studies can in some instances have significant variability and consequently not express the true effects of agrochemicals on the ecosystem. The soils assessed in *in situ* experiments could be exposed to disturbances such as digging by macrofauna (e.g. earthworms, porcupine, and aardvark), which can alter the physical condition of the soil (such as the drainage) during the course of the experiment. Additionally, precipitation, run-off and other external environmental factors can cause results to be biased or increase the variability in the results recorded (Fierer & Jackson, 2006). This can be seen in microbial communities where community shifts can occur rather easily with changes in soil moisture (Zogg et al., 1997). Furthermore, fynbos is rather subject to larger amounts of variability when regarding nutrients in the soil. Aspects such as soil texture or soil nitrogen can vary greatly between patches of only a few meters apart (Stock & Lewis, 1986). *Ex situ* microcosm experiments are therefore valuable as it can accomodate minimization of heterogeneity and external factors that could influence certain variables. By means of an *ex situ* experiment, one can simulate certain conditions such as standardized soil moisture and soil temperature which are important for maintaining a stable environment when studying agrochemical effects on various soil microbial and soil chemical aspects.

Ex situ studies have found various inhibitory impacts of agrochemicals such as diquat and glyphosate pesticides on soil physicochemical properties such as soil nutrition as well as root-to-shoot translocation in the soil (Barreiro & Pratt, 1994; Eker et al., 2006). Other *ex situ* studies have however, revealed stimulatory effects on soil nutrient aspects. Haney et al. (2000), recorded significant elevation in carbon and nitrogen mineralization through treatment of a glyphosate-based herbicide. Rhoades et al. (2002), has similarly shown an increase in soil nitrogen when exposed to imazapic herbicide. Soil microbial communities have also been shown to respond significantly to herbicide exposure in *ex situ* experiments. Zhao et al. (2013) recorded adverse non-target effects of atrazine, bromacil, glyphosate, and paraquat herbicide on soil microbial community structure, particularly on microbial predators such as bacterivores and fungivores. Crouzet et al. (2010) also reported a significant shift in genetic structure of bacterial and fungal communities induced by mesotrione herbicide treatments of ten and one

hundred times the recommended field rate. There are however studies that describe herbicide having little to no effect on soils (Hart & Brookes, 1996). Glyphosate in large doses have proven to result in no significant changes in microbial community structure in the rhizosphere (Weaver et al., 2007). Furthermore, *ex situ* studies have reported effects of herbicide on microbial metabolic activities. Caracciolo et al. (2013) reported a negative impact of pesticides such as terbuthylazine and metachlor on bacterial abundance in soil and water. Additionally, a microcosm study expressed carbon metabolic activity with a Biolog-plate microtiter study and reported that a phenylurea herbicide treatment to the soil inhibited metabolic potential of soil microorganisms (El Fantroussi et al., 1999).

This chapter reviews the results obtained from two separate *ex situ* experiments. The first experiment focused primarily on evaluating the metabolic response of bacterial communities to herbicide via carbon substrate utilization. This experiment took place in a nursery and included a microcosm design where plant trays were filled with soil from the *in situ* trial-sites. A micro community of riparian fynbos plants were grown in the plant trays and each tray faced a specific treatment of herbicide. The second experiment involved undisturbed soil-cores collected from the *in situ* trial-sites and stored in an incubation room. Factors such as temperature, light, and soil moisture were regulated and standardized for this experiment. This experiment had a chief aim of assessing the effects of herbicide on soil chemical characteristics as well as soil microbial community structure. The microbial community's response to the different herbicide exposures was assessed via the measure of OTU numbers and a Shannon Diversity-index.

This chapter aims to evaluate the potential of different types and concentrations of herbicide on soil chemical and soil microbial aspects *ex situ*. The concentrations sprayed included 0 % (untreated control), a 10 % FR (simulate under application infield), a 100 % FR (simulating recommended application dosage), and a 1000 % FR (simulating over application or spillage infield). The *ex situ* microcosm experiment discussed in this chapter was particularly important for this study, as the effects observed could be compared to the *in situ* study (discussed in chapter 2) where soil samples were exposed to external environmental conditions and where environmental heterogeneity was greater. Large amounts of variability were found in the results of the *in situ* experiment and this *ex situ* study subsequently meant that variability could be minimized and that the soil samples would be under standardized conditions (DeNoyelles et al., 1982; Roslycky, 1982). The experiments were aimed at answering how soil chemical properties and microbial community structure of riparian fynbos could change over time when treated with certain dosages of conventional herbicides *ex situ*. Further, this chapter discusses the methods and experimental design of the *ex situ* experiments including a

description of the microcosm experimental design. Additionally, the results obtained from these *ex situ* experiments are discussed and reviewed in a discussion.

2. Materials and Methods

2. 1. Experimental design

The experimental design consists of six steps (Fig. 3.1). Step 1 included the selection of an appropriate soil sampling site within a riparian fynbos habitat. Soils were collected at the *A. mearnsii* Alfalfa A and *E. camaldulensis* Alfalfa B site. Step 2 included the collection of soil from the *in situ* trial sites (refer to Chapter 2). Step 3 included the labelling and relevant storage of soil in plant trays for experiment 1 and in soil-cores for experiment 2. Step 4 was the spraying of select herbicide treatments onto the soil-surface of the plant trays and soil-cores *ex situ*. Step 5 included conducting select microbial and soil-chemical analysis of the soils at separate assessment stages including before treatment (pre), one week after treatment (post 1), and six weeks after treatment (post 2).

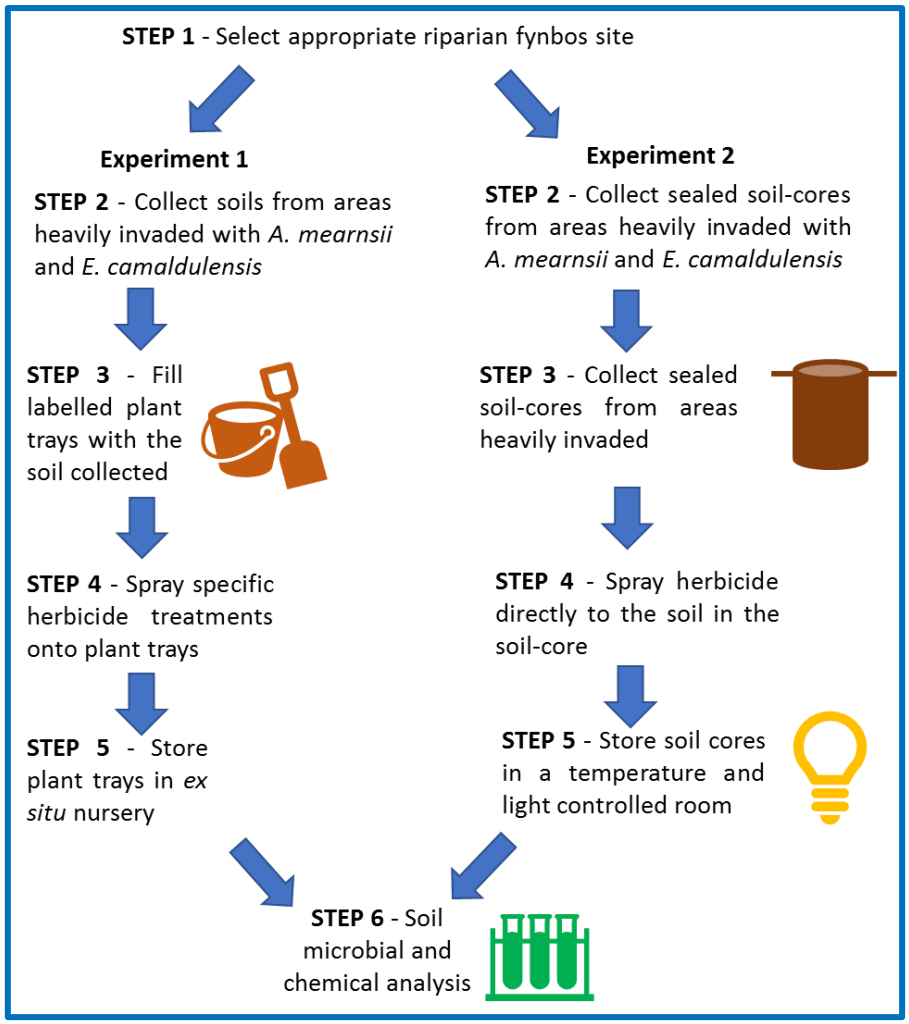


Figure. 3.1. A flow diagram displaying the experimental procedure of this *ex situ* study. The study consists of two separate *ex situ* experiments including a plant tray microcosm experiment and a soil-core microcosm experiment. Soils are collected at select trial sites and submitted to herbicide exposure *ex situ* under simulated conditions. Soil chemical and microbial analysis are done at specific assessment stage.

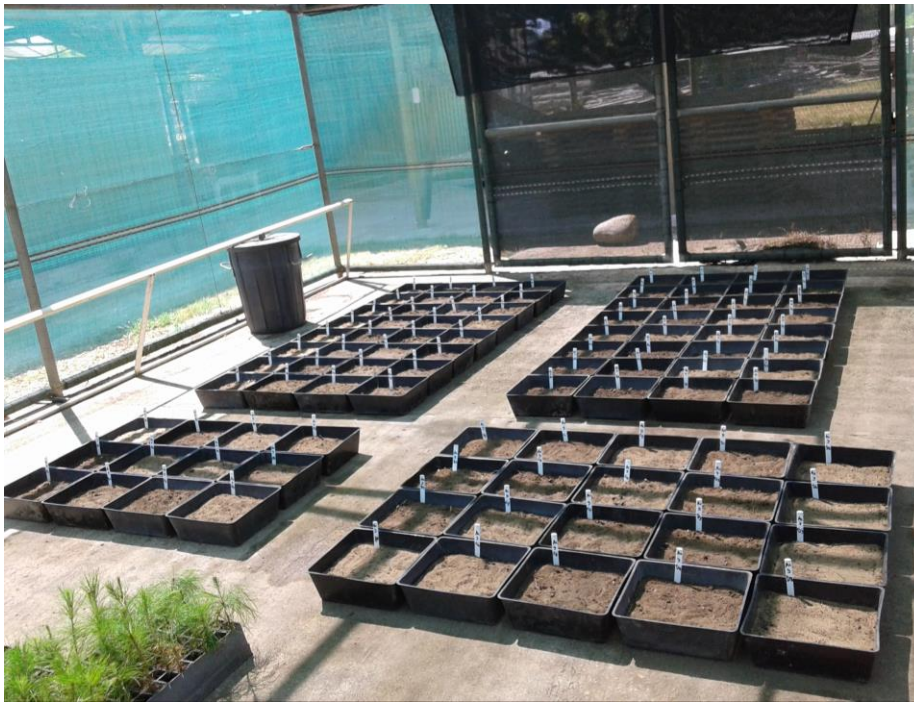
2.1.1. Localities and sampling sites

The *ex situ* experiments took place at facilities of Stellenbosch University. Soil was collected in bulk quantities from the Alfalfa A and Alfalfa B trial sites in areas where *A. mearnsii* and *E. camaldulensis* were heavily populated. Experiment 1 included collected soils being sieved (5 mm sieve) and then transferred to plant trays (with dimensions: 32 cm x 28 cm x 8 cm) at the nursery of the Forestry Department of Stellenbosch University. As for experiment 2, the soils were collected in undisturbed soil-cores (10 cm diameter) which were sealed with parafilm. These soil-cores were placed in a temperature and light controlled room in the Conservation Ecology and Entomology Department at Stellenbosch University.

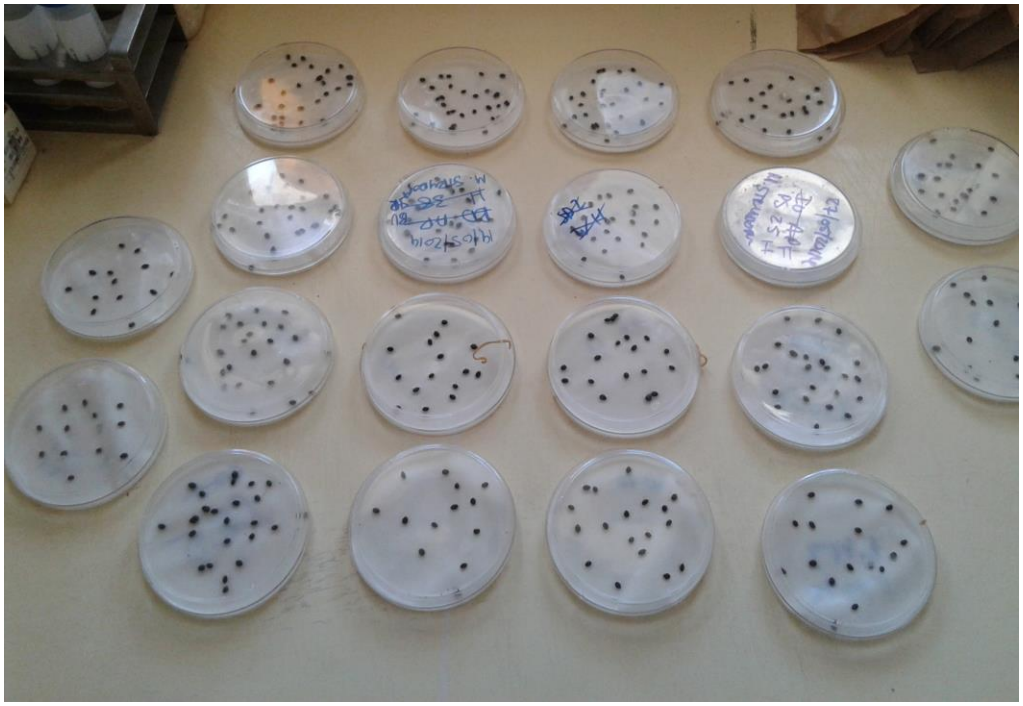
2.1.2. Experimental conditions

Experiment 1

The experiment took place in the autumn of 2016 and was carried out in the Forestry Department nursery of Stellenbosch University. The experiment involved a microcosm approach to evaluating the effects of herbicides on soils. Plant trays were filled with soils that are characteristic of common riparian fynbos habitats found along the Breede River and Wit River. A set number of trays were filled with soils collected in the immediate vicinity of *Acacia mearnsii* trees (found at the Alfalfa A site) whilst the other trays were filled with soils collected from around *Eucalyptus camaldulensis* trees (found at the Alfalfa B site). Some species' seed were sown in the plant-trays and left to grow alongside the seeds already found in the seedbank of the collected soil. *Acacia mearnsii* and *Eucalyptus camaldulensis* seeds were sown separately in respective plant trays and would be treated with herbicide in the plant tray once they have grown to a sapling stage. Other species' seeds that were planted include *Metrosideros angustifolia*, *Sersia pyroides*, *Brabejum stellatifolium* and *Acacia karoo*. In this way, each tray could simulate a micro-community of an invaded riparian fynbos system (Fig. 3.2). Once the seeds germinated and grew out to a seedling stage, the seedlings as well as the soil surface was sprayed with a specific type and concentration of herbicide (Table 3.1). The herbicides were applied with a 16 litre knapsack with a solid cone nozzle at an approximate pressure of 200 kPa.



(i)



(ii)

(iii) (iv)

Figure. 3.2. (i) A picture of the plant trays filled with soil medium obtained from riparian fynbos habitats *in situ*. The plant trays are placed in the nursery and await herbicide treatment once the planted seeds have germinated (ii) Seeds are treated in smoke disc solution to increase efficacy of germination once sown into respective plant trays. (iii) Picture of a grown seedling community shown before facing the given herbicide treatment. (iv) Micro plant community dying one month after herbicide treatment into plant tray.

Experiment 2

This experiment took place in the summer months of 2016. The experiment started with undisturbed soil-cores which were obtained from the Alfalfa A and Alfalfa B sites. PVC tubes, 10 cm diameter and 15 cm in length were hammered into the soil and carefully removed to preserve the structural integrity of the soil core, whereafter it was sealed with PVC caps underneath to make a soil-core container. Parafilm was cast over each soil-core top to regulate the soil moisture content in each container over time (Roslycky, 1982). The soil cores were incubated in a temperature and light controlled room where it could be unaffected by the varying light intensity and fluctuations in temperature and other climatic conditions (Fig. 3.3). The soil cores were watered weekly with a set volume of water calculated from a soil-moisture content analysis. The aim was to minimize effects of other external factors such as rain or fluctuating soil temperature. Herbicides were sprayed directly onto the soil surface with a knapsack sprayer with a solid cone nozzle at an approximate pressure of 200 kPa. The herbicides tested and the concentrations (field rates) are shown below (Table 3.1).

(i) (ii)

Figure. 3.3. Photographs of the soil-cores placed in an incubation room. The PVC soil-cores were sealed with parafilm as to prevent any rapid drying of the soil. The soil-cores were labelled with the date of treatment and the type of treatment.

Table 3.1. The field rate of the different herbicide treatments is displayed for *ex situ* experiments 1 and 2. Each of the herbicides are applied at 4 different rates including, Untreated control which is 0 % FR (field rate), 10 % FR, 100 % FR, and 1000 % FR. The soil type (*A. mearnsii* invaded and *E. camaldulensis* invaded soil) along with the respective active ingredients involved are shown including, glyphosate, triclopyr, and fluroxypyr and picloram.

Target soil:	Treatment FR (field rate):	Active ingredient:
Soil from *Acacia mearnsii* invaded areas	Garlon 0.75 l/ ha: • 0 % FR • 10 % FR • 100 % FR • 1000 % FR	Triclopyr (as butoxy ethyl ester) 480 g/L EC (emulsifiable concentrate)
	Springbok 4.5 l/ ha: • 0 % FR • 10 % FR • 100 % FR • 1000 % FR	Glyphosate (as isopropylamine salt) 360 g/L SL (soluble concentrate)
Soil from *Eucalyptus camaldulensis* invaded areas	Plenum 9 l/ ha: • 0 % FR • 10 % FR • 100 % FR • 1000 % FR	Fluroxypyr + Picloram 80 g/L ME (micro-emulsion)

2.1.3. Sampling and storage methods

Soil samples for analysis were taken directly from the plant trays (Experiment 1) and sealed soil cores (Experiment 2). The soil samples were taken to a depth of 4 cm with a sterile scoop and placed into labelled Ziploc-bags. The bagged soil samples were placed into refrigeration at 4˚C within two hours of sampling to preserve its natural soil-properties. Soil sampling took place at two separate assessment stages including 'Pre' (before treatment) and 'Post' (a month after treatment) for experiment 1 (the nursery experiment). For experiment 2, the soils were sampled at three separate assessment stages namely, 'Pre' (before treatment), 'Post 1' (one week after treatment), and 'Post 2' (six weeks after treatment). All soil chemical and microbial analysis were done within 48 hours of collection as sensitive properties of the soil (such as nitrogen mineralization or microbial biomass) could change over-time in the refrigerated conditions.

2.2. Soil chemical analysis

2.2.1. pH

Soil pH was determined using a HI-8424 pH meter. Soil samples were sieved (to 2 mm) and weighed (to 10 g) and thereafter mixed into solution with distilled water (Robertson et al., 1999). Soil particles were left to settle before pH was recorded to one decimal place.

2.2.2. Electrical conductivity

Electrical conductivity was recorded with a HI-8733 conductivity meter. Soil samples were sieved (to 2 mm) and weighed (to 10 g) and mixed into solution with distilled water. The conductivity meter was calibrated, and recordings were taken to one decimal place after soil settled in the solution.

2.2.3. Total available soil nitrogen

Total soil available nitrogen as determined via a summation of soil available nitrate, NO_3^-, and soil available ammonium, NH_4^+, to units measured in µg $N.g^{-1}$. Nitrate was determined spectrophotometrically using a Thermo Genesys 20 Spectrophotometer at an asorbance of 410 nm (Keeney, 1982). Ammonium was determined spectrophotometrically using a Thermo Genesys 20 Spectrophotometer at an absorance of 655 nm (Buurman et al., 1996).

2.2.4. Nitrogen mineralization

Nitrogen mineralization was determined by measuring the daily rate at which ammonium was mineralized. Absorbance was measured before and 7 days after being mixed with the appropriate reagents, and an average daily rate was calculated in units of $N\text{-}\mu g.g^{-1}.day^{-2}$ (Schomberg et al., 2009).

2.2.5. Soil available phosphorus

Soil available inorganic phosphorus, PO_4^{3-}, was determined using the Bray no.2 method (Tabatabai, 1982). Soil samples were sieved (2 mm) and weighed (6.7 g) before being mixed with the relevant reagents. After allowing complete solution with reagents and filtration with Whatman no.40 filter paper, the absorbance was read with a Thermo Genesys 20 Spectrophotometer at 660 nm and determined in units of ppm.

2.2.6. Acid phosphatase activity

Soil acid phosphatase activity was determined spectrophotometrically. Soil samples were sieved (to 2 mm), weighed (to 1 g) and mixed into solution with the appropriate reagents (Tabatabai & Bremner, 1969). A fter filtration of the solution through no. 12 filter paper a Thermo Genesys 20 Spectrophotometer was used to read absorbance at 402 nm and determined to units of µg-p-NP.g^{-1}.

2.3. Soil microbial analysis

2.3.1. DNA extraction

The Zymo DNA extraction method was used to isolate DNA from soil samples (refer to Chapter 2).

2.3.2. Gel electrophoresis

Gel electrophoresis was performed to check for the prescence of DNA from the DNA solution generated in the DNA extraction process. A 1 %-agarose gel was used, stained with 0.05 µg/ml EtBr. The EtBr binds to the DNA and allows for the DNA to be visulaised under UV light.

2.3.3. Automated Ribosomal Intergenic Spacer Analysis (ARISA)

The ARISA method is based on PCR. The presence of DNA was confirmed on an 1% agarose gel stained with Ethidium Bromide (EtBr) under UV light. Furthermore, peak height was preferred over peak size (refer to Chapter 2).

2.3.4. Microbial Community Metabolic Activity

Biolog EcoPlate™ (BIOLOG, Inc. California, USA) were used to measure the metabolic capacities of the bacterial communities treated with herbicides. Biolog-plates are microtiter eco-plates containing wells with various carbon substrates containing a colourless tetrazolium dye. If the carbon substrates are metabolized by the microbes, colour development is induced via formazan production. The wells of each plate were inoculated with a 100 µl soil dilution and left to incubate at a temperature of 25˚C. The soil dilution is a slurry made up of a diluent with a particular mass of the respective soil sample to produce 10^5 colony forming units (cfu's) per 100 µl volume of the mixture.

As mentioned above, 100 µl volumes of the 'slurry' were pipetted into the wells of the eco-plates and incubated. The incubation induced metabolic activity for the microbes present in

each well, and the utilization of the respective carbon substrate was determined by measuring the degree of colour development. The colour development was measured spectrophotometrically with a microplate reader at an absorbance of 590 nm. Measurements were taken after 0, 24, 72, 120, and 168 hours of incubation. The recordings were used to calculate average well colour development (AWCD), which was collated to draw a community level physiological profile showing for metabolic potential of the microbes present in the soils treated with the different herbicide treatments.

2.4. Statistical analysis

All statistical analyses were done using Excel (2016), Statistica (version. 12) and 'R' statistic. The distribution of the data was tested through a Shapiro-Wilks test. This test gave the normality of the data-set based around significance values ($p < 0.05$ is significant and hence non-parametric, whilst $p > 0.05$ is parametric). If the data revealed a non-parametric distribution, then a Kruskal-Wallis Analysis of Variance (ANOVA) was conducted. An ANOVA was done to test for any significant differences between the different herbicide treatments (i.e. 0 % (non-treated soils) compared to soils treated with 10 % or 100 % of the herbicide dosage). Furthermore, if data proved to be parametric then a One-Way ANOVA was conducted. Additionally, an Analysis of Similarity (ANOSIM) was used to evaluate the degree of similarity between microbial communities before and after herbicide treatment as well as comparing the control sample communities to the communities found in the samples that were treated with 10 % or 100 % of the herbicide dosage.

3. Results

3.1. *Ex situ* experiment 1

Ex situ experiment 1 was conducted in autumn in the Forestry Department nursery of Stellenbosch University. Plant trays were filled with soil from the *in situ* trial-sites namely Alfalfa A and Alfalfa B and seeds of *A. mearnsii, E. camaldulensis* and various native plant species were germinated in the plant trays. The seedling plants and soils in the trays were directly treated with herbicides. A set number of replicate plant trays were filled with soil from *A. mearnsii* invaded areas, while other trays were filled with soil from *E. camaldulensis* invaded areas. The *A. mearnsii* plant trays were treated with two separate herbicides including glyphosate and triclopyr, whilst the *E. camaldulensis* plant trays were treated with one herbicide including a fluroxypyr and picloram emulsion. The results of *ex situ* experiment 1 include an assessment of total soil available nitrogen and nitrogen mineralisation from before

(pre) to after (post) specified herbicide treatments. Microbial metabolic potential is also reviewed and compared to microbial community structure in experiment 2 to compare potential herbicide effects on microbial function to effects on microbial community structure.

3.1.1. Soil chemical

3.1.1.1. Total soil available nitrogen

Total soil available nitrogen remained unaffected in *A. mearnsii* glyphosate and triclopyr treated soils (Fig. 3.4 (i) and (ii) respectively).

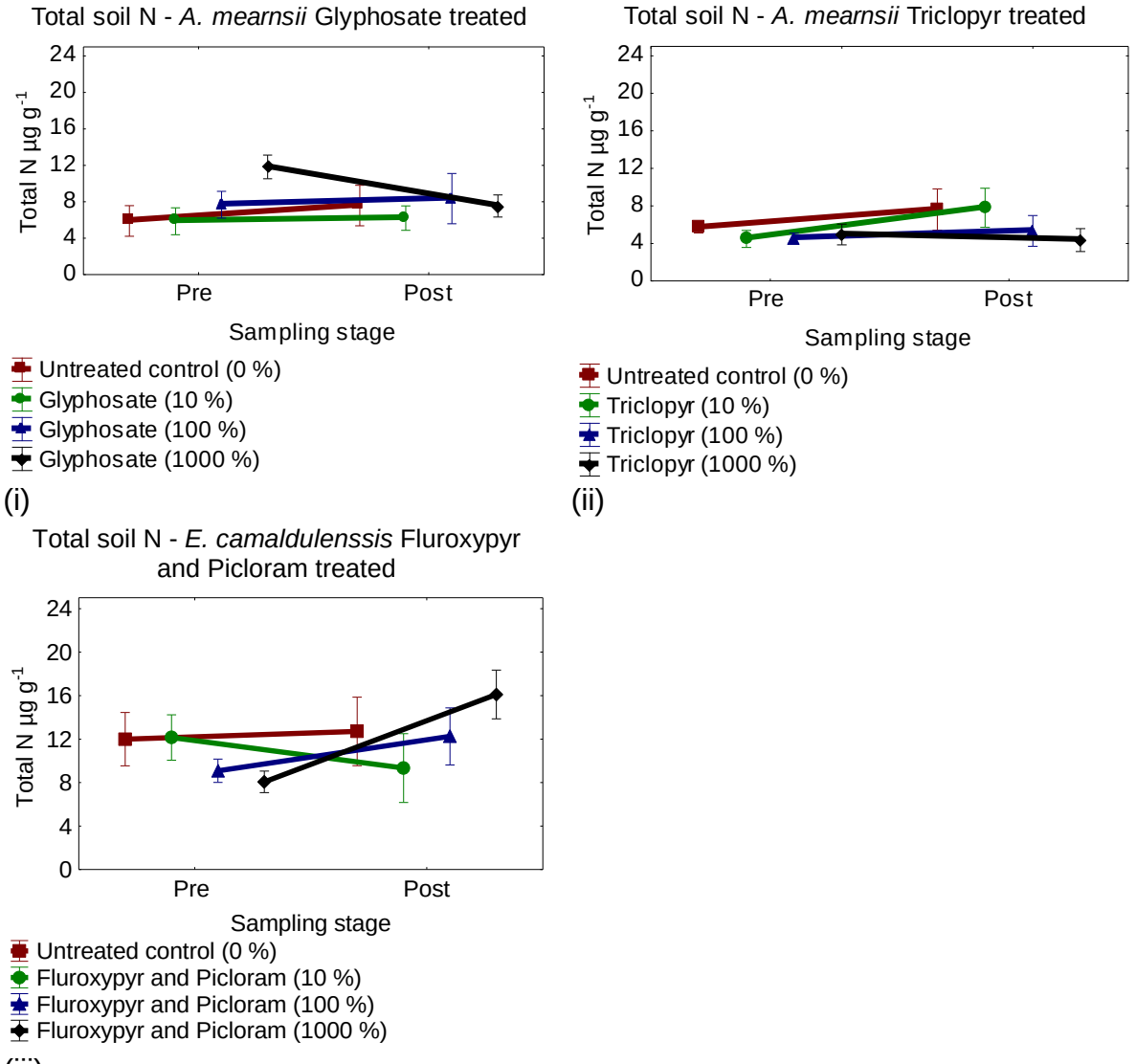


Figure. 3.4. The total soil available nitrogen expressed in units of N-µg g^{-1} for *A. mearnsii* soil treated with (i) glyphosate and (ii) triclopyr, as well as for *E. camaldulensis* soil treated with (iii) fluroxypyr and picloram. Mean total soil available nitrogen shown for herbicide treatments at concentrations of 10 %, 100 %, 1000 % of the recommended field rate, as well as an untreated control (0 % of the field rate), at assessment stages including before treatment (pre) and one month after treatment (post).

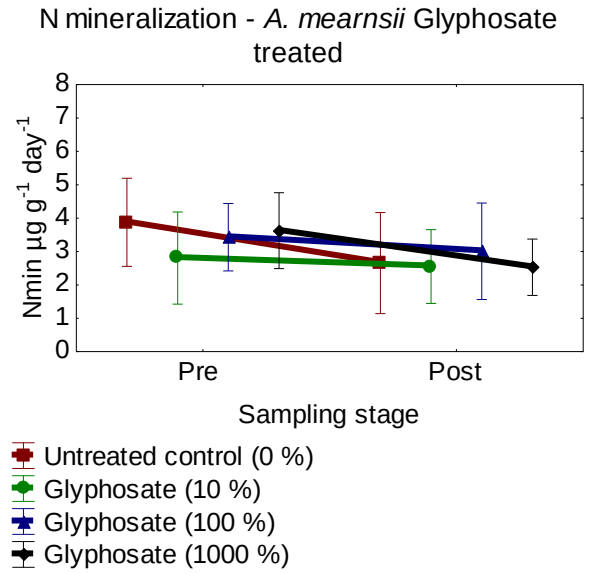
The total soil available nitrogen levels revealed no significant effects of the treatments when assessed from before treatment to one month after treatment (Kruskal-Wallis ANOVA: $p > 0.05$). The *E. camaldulensis* soils treated with the fluroxypyr and piclroam did, however, show a significant increase from before to one month after treatment with the 1000 % field rate dose (Kruskal-Wallis ANOVA: $H = 5.34$; $p = 0.02$).

Table 3.2. Kruskal-Wallis ANOVA showing differences in total soil available nitrogen between pre, post 1, and post 2 when treated with a given herbicide dose. Significance at $p<0.05$ ($n = 16$).

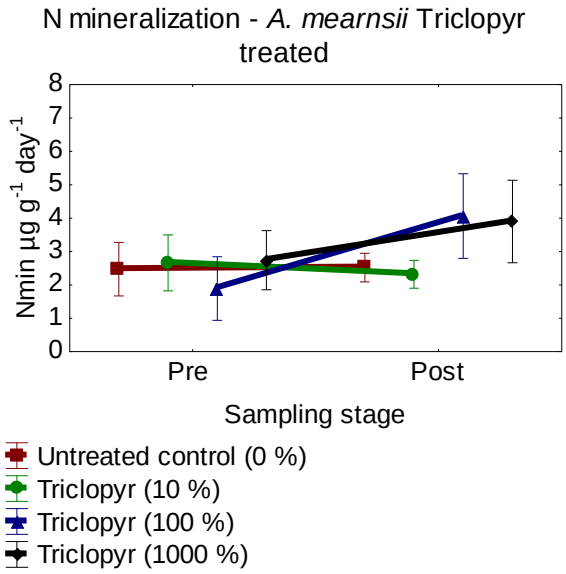
Alfalfa A, *A. mearnsii*						**Alfalfa B, *E. camaldulensis***		
Treatment	**H**	**p**	**Treatment**	**H**	**p**	**Treatment**	**H**	**p**
Untreated control (0 %)	0,89	0,34	Untreated control (0 %)	0,01	0,92	Untreated control (0 %)	0,01	0,92
Glyphosate (10 %)	0,71	0,40	Triclopyr (10 %)	1,59	0,21	Fluroxypyr/ Picloram (10%)	1,33	0,25
Glyphosate (100 %)	0,28	0,60	Triclopyr (100 %)	1,33	0,25	Fluroxypyr/ Picloram (100%)	0,40	0,53
Glyphosate (1000 %)	3,57	0,06	Triclopyr (1000 %)	1,33	0,25	Fluroxypyr/ Picloram (1000%)	5,34	0,02

3.1.1.2. Nitrogen mineralization

The nitrogen mineralization rates of soils treated with glyphosate herbicide showed no significant effects when comparing before and after treatments (Table 3.3). The triclopyr herbicide did, however, significantly increase nitrogen mineralization rates at the 100 % field rate (Kruskal-Wallis ANOVA: $H = 5.83$; $p = 0.02$). Furthermore, the fluroxypyr and picloram treatment of *E. camaldulensis* treatments expressed no significant effects on nitrogen mineralization which ranged between 2 and 5 $\mu g\ g^{-1}\ day^{-2}$ (Fig. 3.5).



(i)



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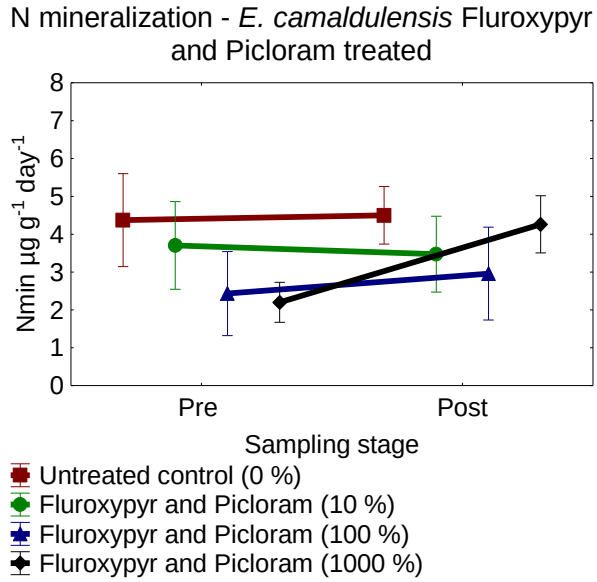


(iii)

Figure. 3.5. The nitrogen mineralisation expressed in units of µg g^{-1} day^{-2} for *A. mearnsii* soil treated with (i) Glyphosate and (ii) Triclopyr, as well as for *E. camaldulensis* soil treated with (iii) Fluroxypyr and picloram. Mean total soil available nitrogen shown for herbicide treatments at concentrations of 10 % , 100 %, 1000 % of the recommended field rate, as well as an untreated control (0 % of the field rate), at assessment stages including before treatment (pre) and one month after treatment (post).

Table 3.3. Kruskal-Wallis ANOVA showing differences in nitrogen mineralisation between pre, post 1, and post 2 when treated with a given herbicide dose. Significance at p<0.05 (n = 16).

Alfalfa A, *A. mearnsii*						**Alfalfa B, *E. camaldulensis***		
Treatment	**H**	**p**	**Treatment**	**H**	**p**	**Treatment**	**H**	**p**
Untreated control (0 %)	1,59	0,21	Untreated control (0 %)	0,89	0,35	Untreated control (0 %)	0,18	0,67
Glyphosate (10 %)	0,01	0,92	Triclopyr (10 %)	0,04	0,83	Fluroxypyr/ Picloram (10%)	0,01	0,92
Glyphosate (100 %)	0,89	0,35	Triclopyr (100 %)	5,83	0,02	Fluroxypyr/ Picloram (100%)	0,54	0,46
Glyphosate (1000 %)	0,40	0,53	Triclopyr (1000 %)	0,01	0,92	Fluroxypyr/ Picloram (1000%)	3,98	0,05

3.1.2. Soil microbiology

3.1.2.1. Microbial metabolism – Average Well Colour Development (AWCD)

A. mearnsii glyphosate treated

The glyphosate treated soils showed no considerable effects of herbicide application on bacterial metabolic potential over time. Absorbance values elevated with time with no detectable inhibitory effects after the 10 %, 100 %, and 1000 % glyphosate field rate treatments (Fig. 3.6).

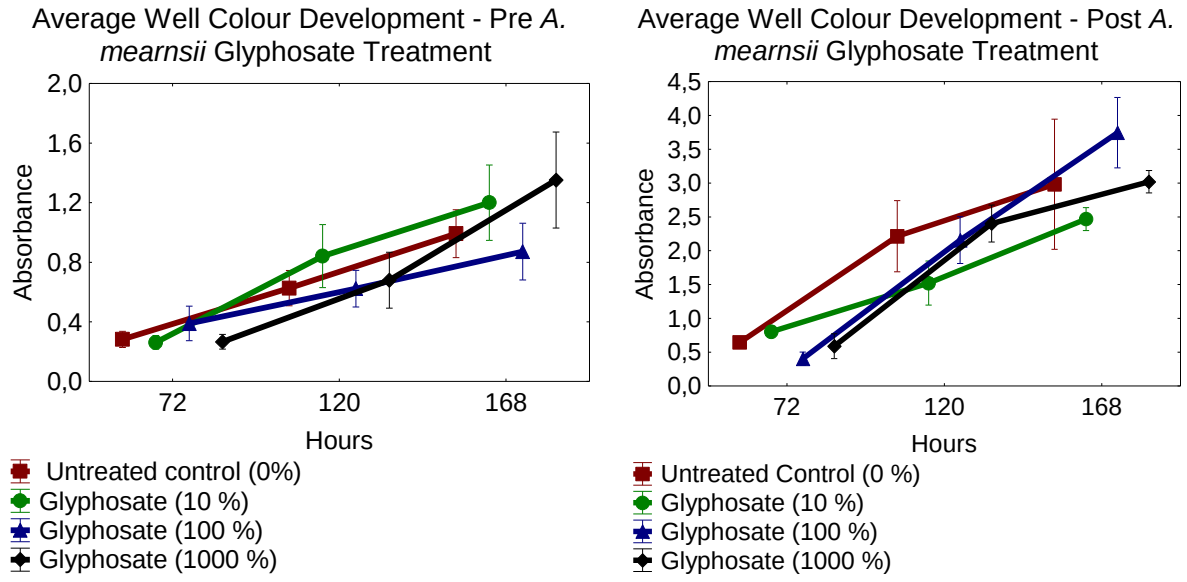


Figure. 3.6. The average well colour development (AWCD) is shown for the *A. mearnsii soils* treated with the glyphosate-based herbicide, at concentrations of 0 %, 10 %, 100 %, and 1000 % of the recommended field rate. Mean AWCD and standard error of the mean are displayed (i) before herbicide treatment (pre) and (ii) one month after treatment (post).

Despite no inhibitory effects, a Kruskal-Wallis ANOVA did reveal that there was a significant increase in average well colour development from before to after treatment for all doses including the untreated control (Table 3.4). Absorbance increased from pre to post with a mean absorbance of 1.3 to 3.5 at hour 168 across all treatments respectively. This significant increase in microbial metabolism was not induced by herbicide as the untreated control behaved similar to *A. mearnsii* soils that were treated with the 10 %, 100 % and 1000 % glyphosate field rate doses (Fig. 3.6).

Table 3.4. Kruskal-Wallis ANOVA showing differences in average well colour development between before treatment (pre) and one month after treatment (post) in *A. mearnsii* soils faced with a given glyphosate dose. Significance at $p < 0.05$ (n = 18).

***A. mearnsii* Glyphosate treated**		
Treatment	**H**	**p**
Untreated control (0 %)	15,13	0,01
Glyphosate (10 %)	14,29	0,01
Glyphosate (100 %)	14,85	0,01
Glyphosate (1000 %)	14,75	0,01

A. mearnsii triclopyr treated

Acacia mearnsii soils treated with triclopyr showed no inhibitory effects on the microbial metabolic potential. The absorbance values displayed a uniform increase with time expressing no considerable inhibitory effects on microbial metabolic activity (Fig. 3.7).

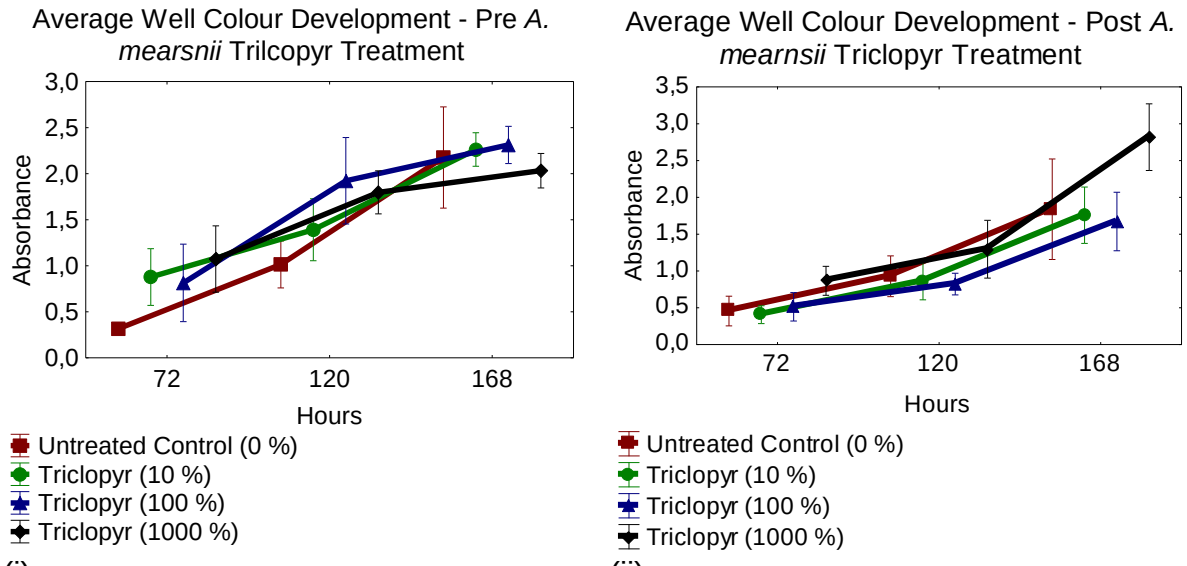


Figure. 3.7. The average well colour development (AWCD) is shown for the *A. mearnsii* soils treated with the triclopyr-based herbicide, at concentrations of 0 %, 10 %, 100 %, and 1000 % of the recommended field rate. Mean AWCD and standard error of the mean are displayed (i) before herbicide treatment (pre) and (ii) one month after treatment (post).

A Kruskal-Wallis ANOVA revealed a significant difference in AWCD from before to after treatment, even for the untreated control soil (Table 3.5). Despite this, no extensive effects could be detected and the microorganisms in the *A. mearnsii* soils continued to display an increase in AWCD over time reaching a mean absorbance of 2.5 before treatment (pre) to 2.7 after treatment (post) at hour 168 across all treatments (Fig. 3.7).

Table 3.5. Kruskal-Wallis ANOVA showing differences in average well colour development between before treatment (pre) and one month after treatment (post) in *A. mearnsii* soils faced with the given triclopyr dose. Significance at $p < 0.05$ (n = 18).

***A. mearnsii* Triclopyr treated**		
Treatment	**H**	**p**
Untreated control (0 %)	11,50	0,04
Triclopyr (10 %)	11,48	0,04
Triclopyr (100 %)	11,43	0,04
Triclopyr (1000 %)	12,30	0,03

E. camaldulensis fluroxypyr and picloram treated

Eucalyptus camaldulensis soils treated with the fluroxypyr and picloram emulsion displayed no extensive effects on microbial metabolic potential. Absorbance values increased steadily with time and showed for no considerable inhibitory effects on average well colour development (AWCD) from before (pre) to one month after treatment (post) (Fig. 3.8).

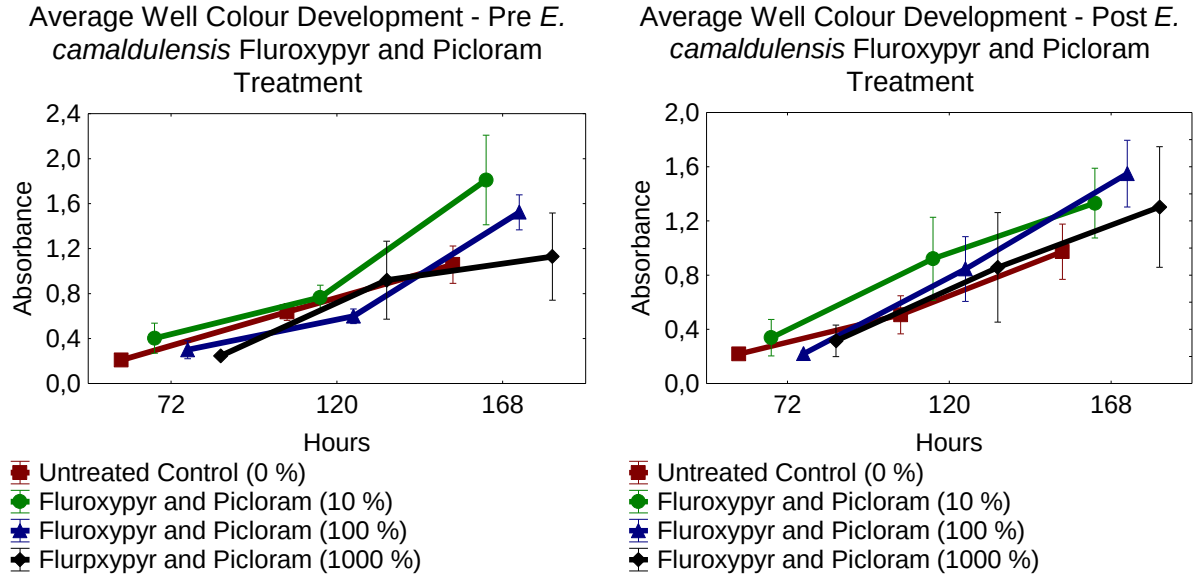


Figure. 3.8. The average well colour development (AWCD) is shown for the *E. camaldulensis* soils treated with the fluroxypyr and picloram-based herbicide, at concentrations of 0 %, 10 %, 100 %, and 1000 % of the recommended field rate. Mean AWCD and standard error of the mean are displayed (i) before herbicide treatment (pre) and (ii) one month after treatment (post).

A Kruskal-Wallis ANOVA revealed a significant difference in *E. camaldulensis* soils from before to one month after for the 10 % and 100 % treatments as well as for the untreated control (Table 3.6).

Table 3.6. Kruskal-Wallis ANOVA showing differences in average well colour development between before treatment (pre) and one month after treatment (post) in *E. camaldulensis* soils faced with the given fluroxypyr and picloram dose. Significance at $p < 0.05$ ($n = 18$).

***E. camaldulensis* Fluroxypyr and Picloram treated**		
Treatment	**H**	**p**
Untreated control (0 %)	15,01	0,01
Fluroxypyr and Picloram (10 %)	11,81	0,04
Fluroxypyr and Picloram (100 %)	14,76	0,01
Fluroxypyr and Picloram (1000 %)	8,58	0,13

3.2. *Ex situ* experiment 2

Ex situ experiment 2 was conducted in summer in a temperature and light controlled room at the Department of Conservation Ecology and Entomology of Stellenbosch University. Soil cores were taken from the *in situ* trial sites where a set number of soil cores were filled with soil from *A. mearnsii* invaded areas, while other soil cores were filled with soil from *E. camaldulensis* invaded areas. The *A. mearnsii* soil cores were treated with two separate herbicides including glyphosate and triclopyr, whilst the *E. camaldulensis* soil cores were treated with one herbicide, a fluroxypyr and picloram emulsion. The results of *ex situ* experiment 2 include an assessment of various soil chemical aspects including soil-pH, electrical conductivity, total soil available nitrogen, nitrogen mineralisation, soil available phosphorus, acid phosphatase activity. The assessment of microbial aspects was included and involved measures of bacterial comminty structure. Both soil chemical and soil microbial aspects were assessed before (pre), one week after (post 1), and six weeks after (post 2) specified herbicide applications.

3.2.1. Soil chemical

3.2.1.1. pH

All three herbicide products had an acidifying effect on the soil. In *A. mearnsii* glyphosate soils, the pH dropped significantly with as much as 0.8 units (Fig. 3.9 (i)). In addition, a Kruskal-Wallis ANOVA revealed that all glyphosate treatments including 10 %, 100 %, and 1000 % field rates induced a significantly lower pH from before treatment to one week after treatment and further to six weeks after treatment (Table 3.7). A similar trend was detected

for the triclopyr treatments including the 100 % and 1000 % field rate doses (Kruskal-Wallis ANOVA: H = 9.84; p = 0.01 and H = 10.15; p = 0.01 respectively). *E. camaldulensis* soils treated with fluroxypyr and picloram similarly significantly reduced pH with 10 %, 100 % and 1000 % field rate doses (Table 3.7). The pH in the *E. camaldulensis* soils dropped from an average of 6.2 to a low of 5.3 (Fig. 3.9 (ii)).

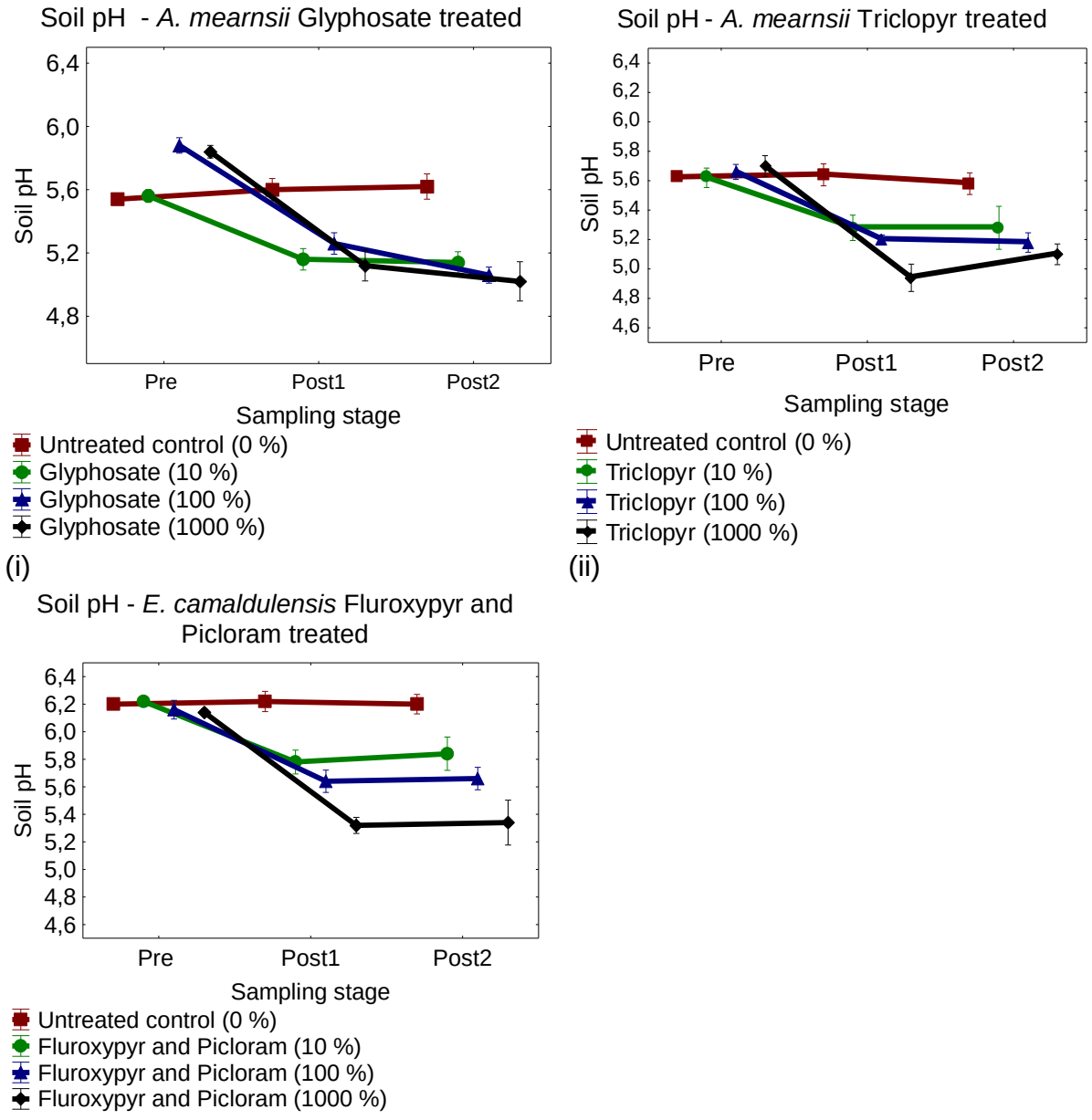


Figure. 3.9. The mean pH is expressed for the *A. mearnsii* soil treated *ex situ* with (i) glyphosate, and (ii) triclopyr, as well as for the *E. camaldulensis* soil treated *ex situ* with (iii) fluroxypyr and picloram. The graph shows soil pH when treated with herbicides at concentrations of 10 %, 100 %, 1000 % of the recommended field rate, as well as an untreated control (0 % of the field rate), at assessment stages including before treatment (pre) and one week after treatment (post 1), and six weeks after treatment (post 2).

Table 3.7. Kruskal-Wallis ANOVA showing differences in soil pH between pre, post 1, and post 2 when treated with a given herbicide dose. Significance at $p<0.05$ (n = 15).

Alfalfa A, *A. mearnsii*						**Alfalfa B, *E. camaldulensis***		
Treatment	**H**	**p**	**Treatment**	**H**	**p**	**Treatment**	**H**	**p**
Untreated control (0 %)	0,42	0,81	Untreated control (0 %)	0,60	0,74	Untreated control (0 %)	0,15	0,93
Glyphosate (10 %)	9,64	0,01	Triclopyr (10 %)	5,90	0,05	Fluroxypyr/ Picloram (10%)	9,35	0,01
Glyphosate (100 %)	11,42	0,00	Triclopyr (100 %)	9,84	0,01	Fluroxypyr/ Picloram (100%)	9,57	0,01
Glyphosate (1000 %)	9,78	0,01	Triclopyr (1000 %)	10,15	0,01	Fluroxypyr/ Picloram (1000%)	9,57	0,01

3.2.1.2. Electrical conductivity

Electrical conductivity remained stable from before to six weeks after herbicide treatment. In *A. mearnsii* soils treated with glyphosate the electrical conductivity ranged between 0.09 and 0.20 mS. The *A. mearnsii* soils treated with triclopyr showed a range between 0.09 and 0.16 mS. The *E. camaldulensis* soils treated with the fluroxypyr and picloram maintained a range between 0.07 and 0.15 mS (Fig. 3.10). All herbicides tested therefore seem to have had no effect on soil electrical conductivity as figures expressed by treated soils were similar to untreated control soils.

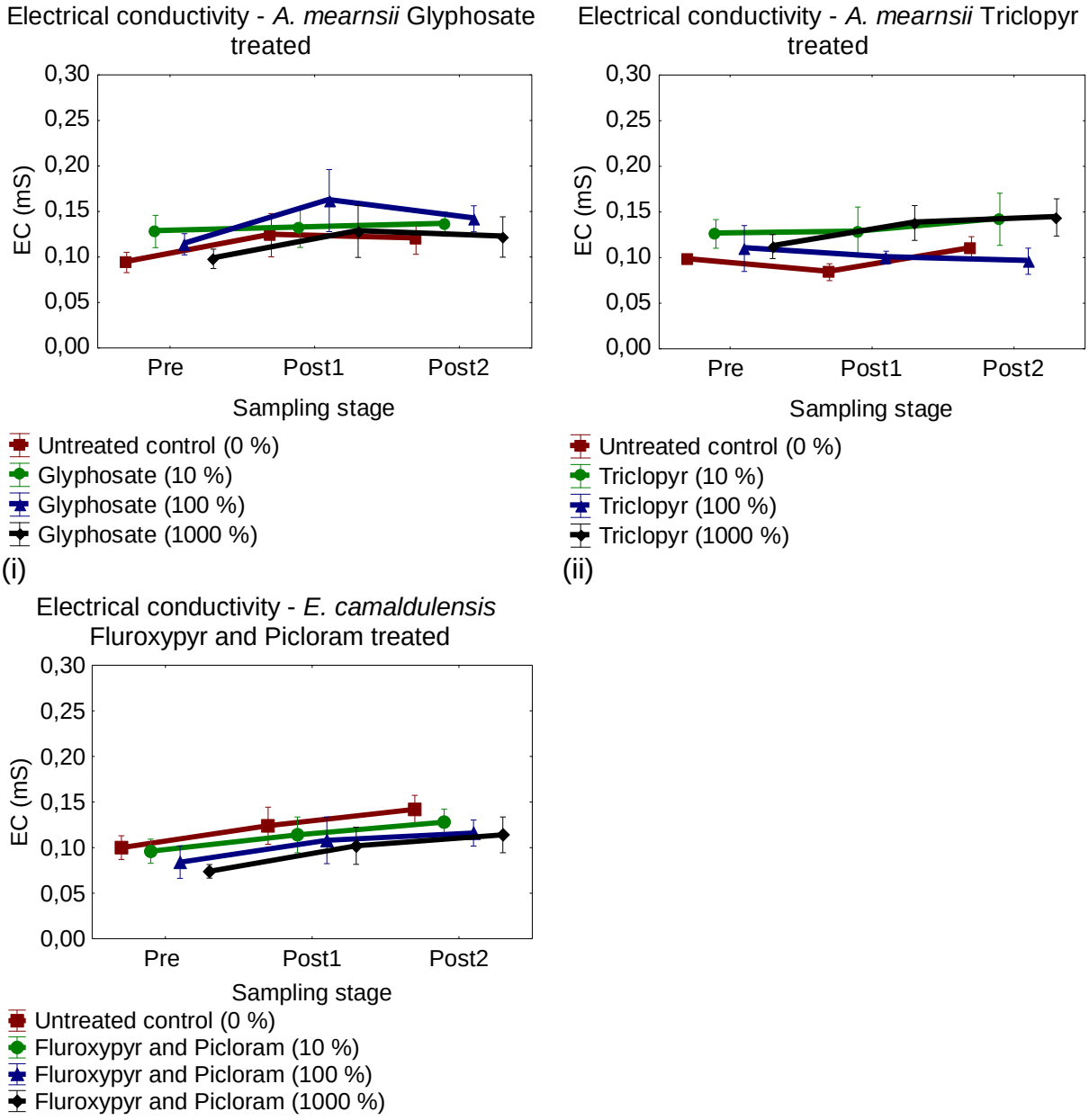


Figure. 3.10. The mean electrical conductivity is expressed in microSiemens (mS) for the *A. mearnsii* soil treated *ex situ* with (i) glyphosate, and (ii) triclopyr, as well as for the *E. camaldulensis* soil treated *ex situ* with (iii) fluroxypyr and picloram. The graph shows for soil electrical conductivity when treated with herbicides at concentrations of 10 %, 100 %, 1000 % of the recommended field rate, as well as an untreated control (0 % of the field rate), at assessment stages including before treatment (Pre) and one week after treatment (Post 1), and six weeks after treatment (Post 2).

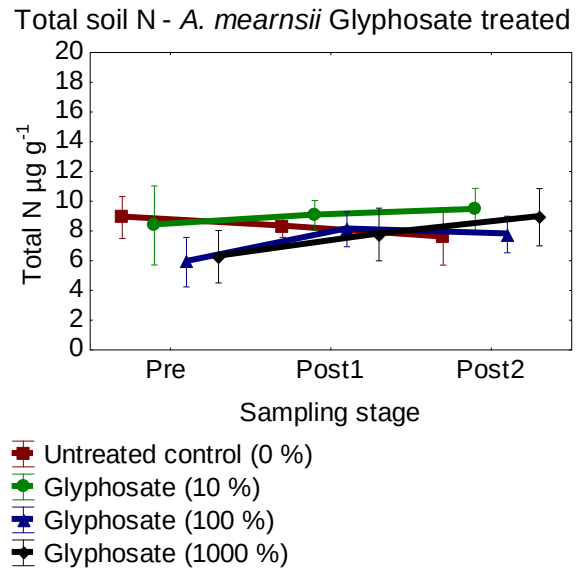
Furthermore, a Kruskal-Wallis ANOVA displayed no significant effects of either herbicide on soil electrical conductivity (Table 3.8).

Table 3.8. Kruskal-Wallis ANOVA showing differences in soil electrical conductivity between pre, post 1, and post 2 when treated with a given herbicide dose. Significance at p<0.05 (n = 15).

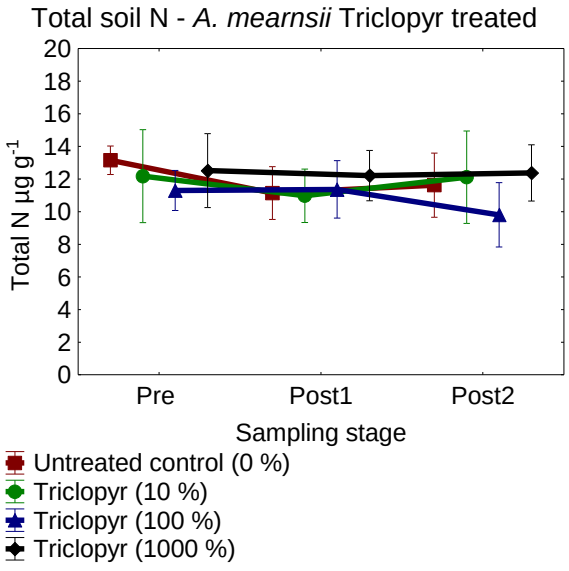
Alfalfa A, *A. mearnsii*						**Alfalfa B, *E. camaldulensis***		
Treatment	**H**	**p**	**Treatment**	**H**	**p**	**Treatment**	**H**	**p**
Untreated control (0 %)	1,88	0,39	Untreated control (0 %)	3,41	0,18	Untreated control (0 %)	3,57	0,17
Glyphosate (10 %)	0,07	0,97	Triclopyr (10 %)	0,83	0,66	Fluroxypyr/ Picloram (10%)	2,03	0,36
Glyphosate (100 %)	1,57	0,45	Triclopyr (100 %)	0,44	0,80	Fluroxypyr/ Picloram (100%)	1,83	0,40
Glyphosate (1000 %)	0,98	0,61	Triclopyr (1000 %)	1,98	0,37	Fluroxypyr/ Picloram (1000%)	4,47	0,11

3.2.1.3. Total available soil nitrogen

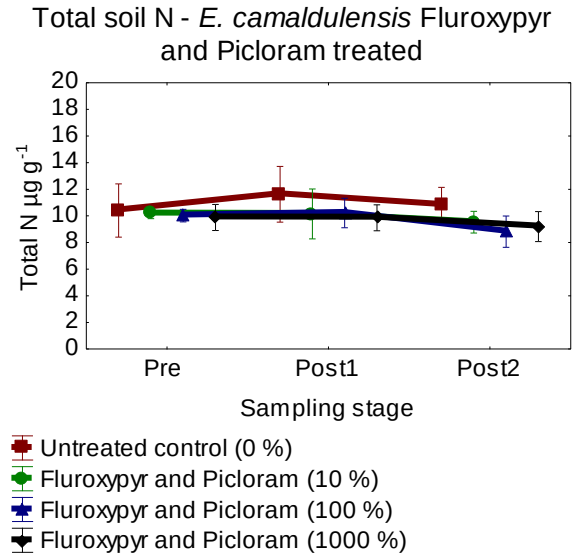
The total soil available nitrogen in soils treated with herbicide showed no considerable effects across all treatments. The soils treated with 10 %, 100 % and 1000 % field rates revealed similar ranges in total soil available nitrogen as seen for the untreated control soils (Fig. 3.11). *Acacia mearnsii* soils treated with glyphosate ranged between 8 and 14 µg N g^{-1} from before to six weeks after treatment irrespective of the concentration of field rate dosage (Fig. 3.11 (i)). In addition, *A. mearnsii* soils treated with triclopyr ranged between 9 and 16 µg N g^{-1} (Fig. 3.11 (ii)) and *E. camaldulensis* soils treated with fluroxypyr and picloram ranged between 8 and 14 µg N g^{-1} (Fig. 3.11 (iii)).



(i)



(ii)



(iii)

Figure. 3.11. The mean total available soil nitrogen is expressed in µg N g^{-1} for the *A. mearnsii* soil treated *ex situ* with (i) glyphosate, and (ii) triclopyr, as well as for the *E. camaldulensis* soil treated *ex situ* with (iii) fluroxypyr and picloram. The graph shows total soil aviable nitrogen when treated with herbicides at concentrations of 10 %, 100 %, 1000 % of the recommended field rate, as well as an untreated control (0 % of the field rate), at assessment stages including before treatment (Pre) and one week after treatment (Post 1), and six weeks after treatment (Post 2).

Additionally, a Kruskal-Wallis revelead no significant impact of either herbicide treatment on total soil available nitrogen (Table 3.9).

Table 3.9. Kruskal-Wallis ANOVA showing differences in total soil available nitrogen between pre, post 1, and post 2 when treated with a given herbicide dose. Significance at $p<0.05$ (n = 15).

Alfalfa A, *A. mearnsii*						**Alfalfa B, *E. camaldulensis***		
Treatment	**H**	**p**	**Treatment**	**H**	**p**	**Treatment**	**H**	**p**
Untreated control (0 %)	0,23	0,80	Untreated control (0 %)	0,46	0,64	Untreated control (0 %)	0,11	0,89
Glyphosate (10 %)	0,08	0,92	Triclopyr (10 %)	0,07	0,93	Fluroxypyr/ Picloram (10%)	0,09	0,91
Glyphosate (100 %)	0,74	0,50	Triclopyr (100 %)	0,28	0,76	Fluroxypyr/ Picloram (100%)	0,61	0,56
Glyphosate (1000 %)	0,54	0,60	Triclopyr (1000 %)	0,01	0,99	Fluroxypyr/ Picloram (1000%)	0,14	0,87

3.2.1.4. Nitrogen mineralization

Nitrogen mineralization remained stable from before herbicide treatment to six weeks after treatment. All herbicide products seemed to have no effect on nitrogen mineralization and both *A. mearnsii* and *E. camaldulensis* soils expressed large amounts of variability (Fig. 3.12). *A. mearnsii* soils treated with glyphosate showed mean mineralization rates between 0.5 and 3 NH_4^+ g^{-1} day^{-1}, whilst *A. mearnsii* soils treated with triclopyr showed mean mineralization rates between 0.5 and 2 NH_4^+ g^{-1} day^{-1}. The *E. camaldulensis* soils treated with fluroxypyr and picloram showed mineralization rates ranging between 0.5 and 3 NH_4^+ g^{-1} day^{-1}.

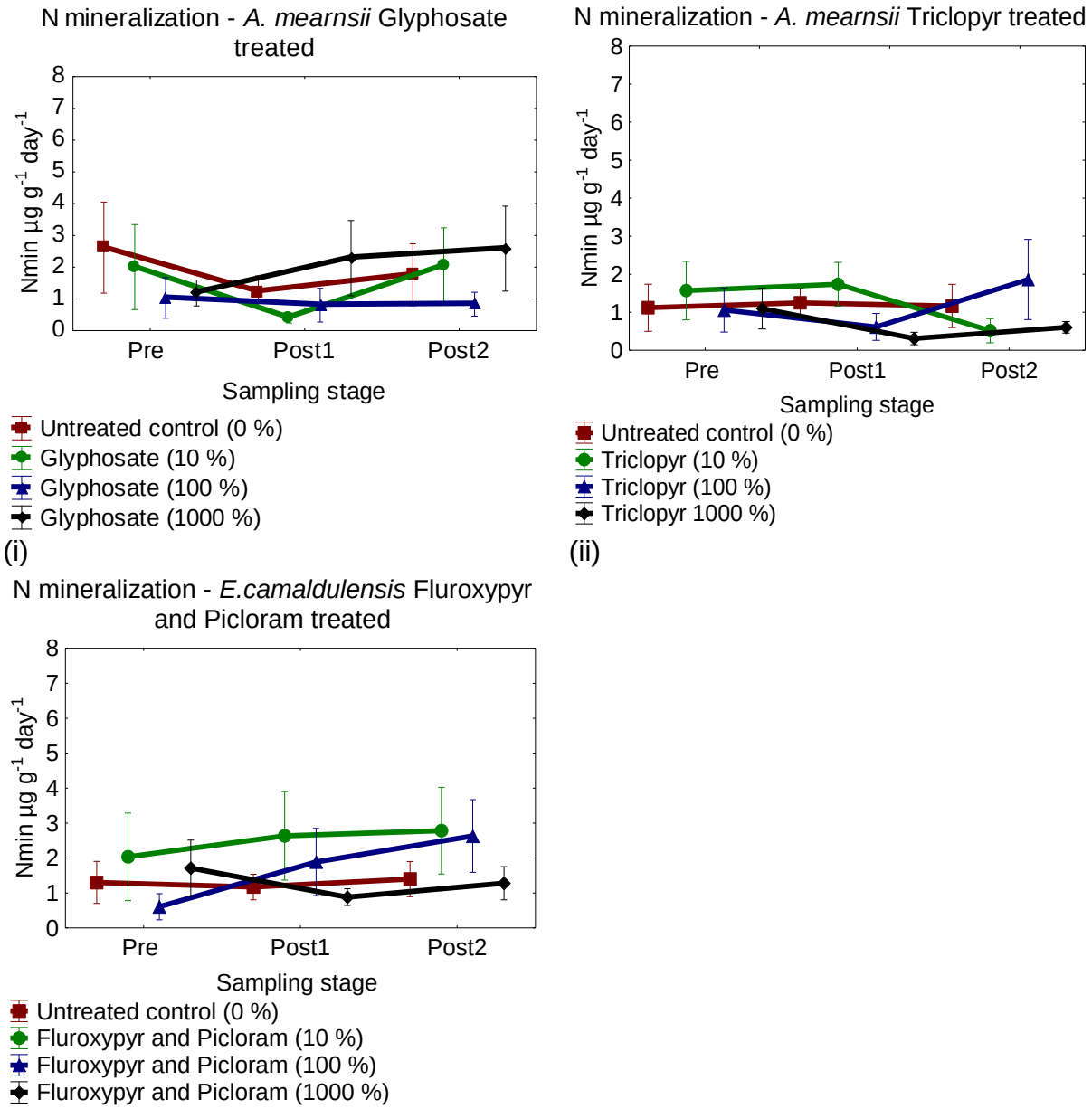


Figure. 3.12. The mean nitrogen mineralization is expressed in NH_4^+ g^{-1} day^{-2} for the *A. mearnsii* soil treated *ex situ* with (i) glyphosate, and (ii) triclopyr, as well as for the *E. camaldulensis* soil treated *ex situ* with (iii) fluroxypyr and picloram. The graph shows nitrogen mineralization levels when treated with herbicides at concentrations of 10 %, 100 %, 1000 % of the recommended field rate, as well as an untreated control (0 % of the field rate), at assessment stages including before treatment (Pre) and one week after treatment (Post 1), and six weeks after treatment (Post 2).

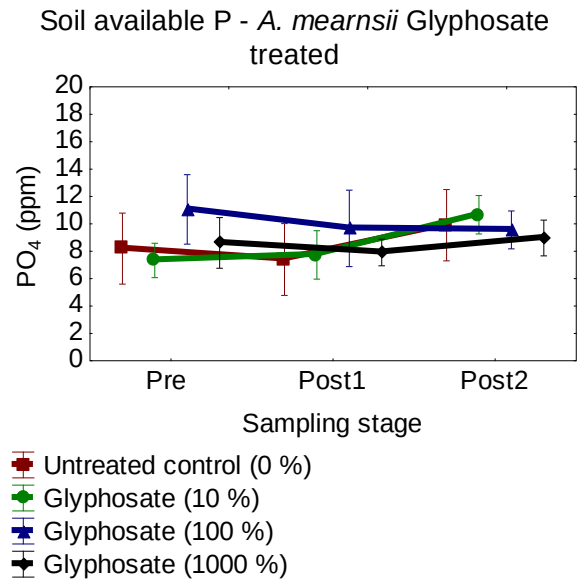
In addition, a Kruskal-Wallis ANOVA revealed no significant differences in mineralization rates across all treatments including glyphosate, triclopyr and fluroxypyr and picloram at the 10 %, 100 % and 1000 % field rates (Table 3.10).

Table 3.10. Kruskal-Wallis ANOVA showing differences in nitrogen mineralisation between pre, post 1, and post 2 when treated with a given herbicide dose. Significance at $p<0.05$ (n = 15).

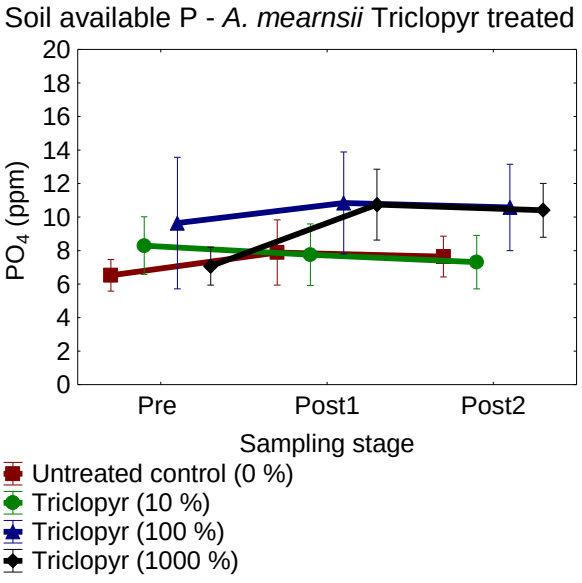
Alfalfa A, *A. mearnsii*						**Alfalfa B, *E. camaldulensis***		
Treatment	**H**	**p**	**Treatment**	**H**	**p**	**Treatment**	**H**	**p**
Untreated control (0 %)	0,56	0,76	Untreated control (0 %)	0,38	0,76	Untreated control (0 %)	0,14	0,93
Glyphosate (10 %)	4,25	0,12	Triclopyr (10 %)	3,71	0,12	Fluroxypyr/ Picloram (10%)	0,50	0,78
Glyphosate (100 %)	0,62	0,73	Triclopyr (100 %)	1,12	0,73	Fluroxypyr/ Picloram (100%)	2,90	0,23
Glyphosate (1000 %)	0,06	0,97	Triclopyr (1000 %)	2,06	0,97	Fluroxypyr/ Picloram (1000%)	0,37	0,83

3.2.1.5. Soil available phosphorus

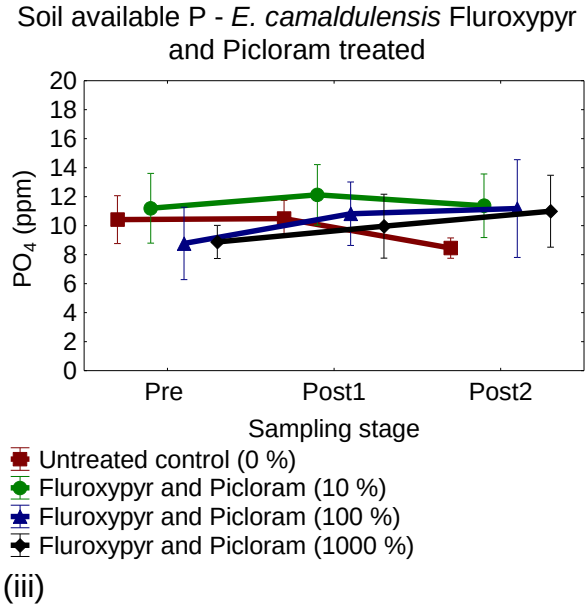
Soil available phosphorus showed no significant changes from before to after herbicide treatment when comparing phosphorus levels of before (pre) to one week after (post 1) and six weeks after treatment (post 2). The soil available phosphorus in both *A. mearnsii* soils which were treated with glyphosate and triclopyr show for a range of 6 to 14 ppm irrespective of field rate dose (Fig. 3.13 (i) and (ii) respectively). The *E. camaldulensis* soils treated with fluroxypyr and picloram similarly expressed a stable range of 6 to 14 ppm (Fig. 3.13 (iii)).



(i)



(ii)



(iii)

Figure. 3.13. The mean soil available phosphorus is expressed in ppm for the *A. mearnsii* soil treated *ex situ* with (i) glyphosate, and (ii) triclopyr, as well as for the *E. camaldulensis* soil treated *ex situ* with (iii) fluroxypyr and picloram. The graph shows fr soil available phosphorus when treated with herbicides at concentrations of 10 %, 100 %, 1000 % of the recommended field rate, as well as an untreated control (0 % of the field rate), at assessment stages including before treatment (Pre) and one week after treatment (Post 1), and six weeks after treatment (Post 2).

A Kruskal-Wallis ANOVA revealed no significant differences in soil available phosphorus across all herbicide treatments (Table 3.11).

Table 3.11. Kruskal-Wallis ANOVA showing differences in soil available phosphorus between pre, post 1, and post 2 when treated with a given herbicide dose. Significance at $p<0.05$ (n = 15).

Alfalfa A, *A. mearnsii*						**Alfalfa B, *E. camaldulensis***		
Treatment	**H**	**p**	**Treatment**	**H**	**p**	**Treatment**	**H**	**p**
Untreated control (0 %)	0,42	0,81	Untreated control (0 %)	1,26	0,53	Untreated control (0 %)	2,54	0,28
Glyphosate (10 %)	3,02	0,22	Triclopyr (10 %)	0,26	0,88	Fluroxypyr/ Picloram (10%)	0,18	0,91
Glyphosate (100 %)	0,26	0,88	Triclopyr (100 %)	0,06	0,97	Fluroxypyr/ Picloram (100%)	1,62	0,44
Glyphosate (1000 %)	0,74	0,69	Triclopyr (1000 %)	2,96	0,23	Fluroxypyr/ Picloram (1000%)	0,38	0,83

3.2.1.6. Acid phosphatase activity

No considerable effects of either of the three herbicides could be observed for acid phosphatase activity (Fig. 3.14). There was also large amounts of variation, but no significant differences were detected in acid phosphatase activity of soils treated with 10 %, 100 % or 1000 % field rates when comparing before to six weeks after treatment (Table 3.12).

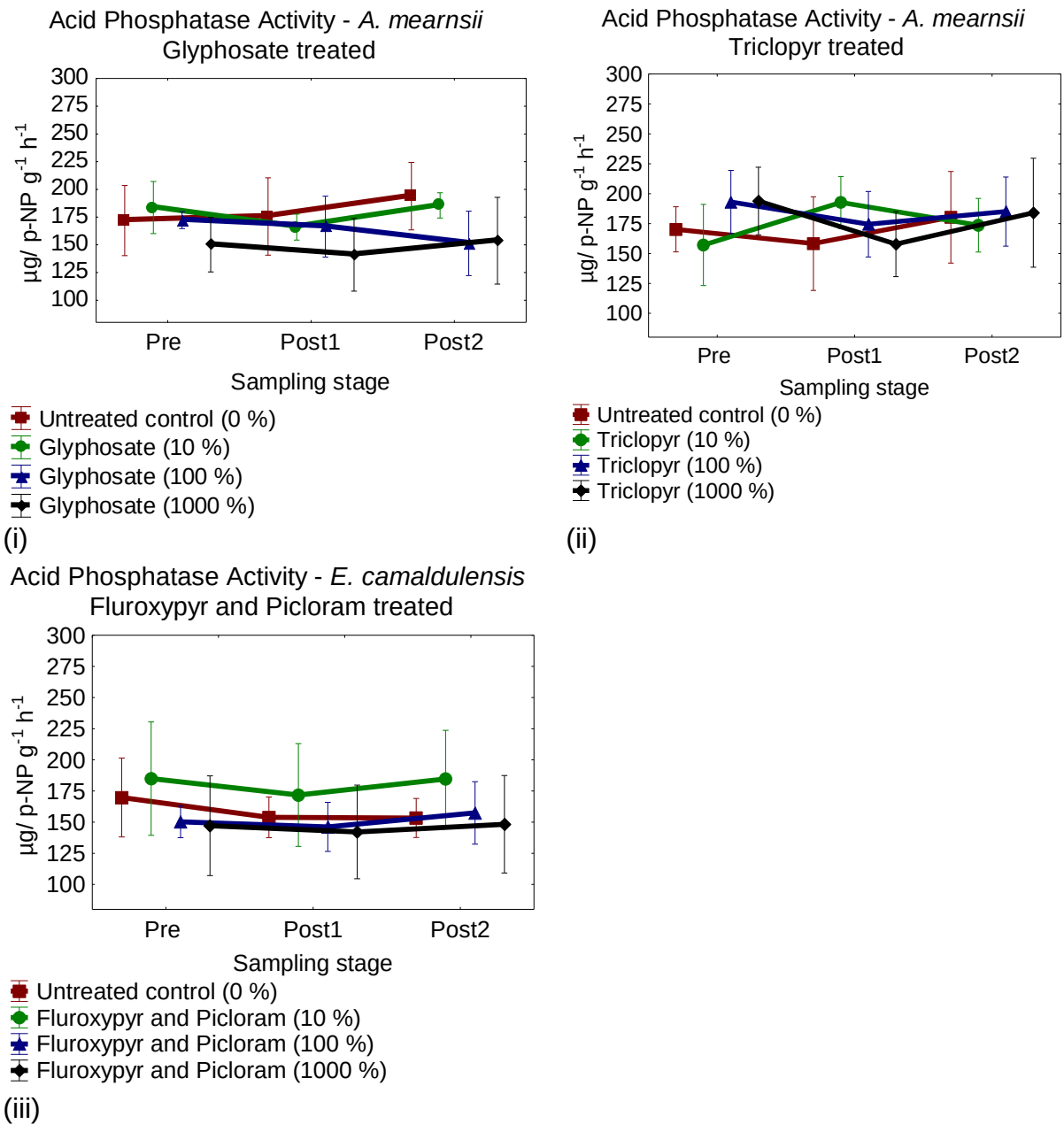


Figure. 3.14. The mean phosphatase activity is expressed in µg *p*-NP g^{-1} dry soil for the *A. mearnsii* soil treated *ex situ* with (i) glyphosate, and (ii) triclopyr, as well as for the *E. camaldulensis* soil treated *ex situ* with (iii) fluroxypyr and picloram. The graph shows acid phosphatase activity when treated with herbicides at concentrations of 10 %, 100 %, 1000 % of the recommended field rate, as well as an untreated control (0 % of the field rate), at assessment stages including before treatment (Pre) and one week after treatment (Post 1), and six weeks after treatment (Post 2).

Table 3.12. Kruskal-Wallis ANOVA showing differences in acid phosphatase activity between pre, post 1, and post 2 when treated with a given herbicide dose. Significance at $p<0.05$ (n = 15).

Alfalfa A, *A. mearnsii*						**Alfalfa B, *E. camaldulensis***		
Treatment	**H**	**p**	**Treatment**	**H**	**p**	**Treatment**	**H**	**p**
Untreated control (0 %)	0,72	0,70	Untreated control (0 %)	0,42	0,81	Untreated control (0 %)	0,00	1,00
Glyphosate (10 %)	1,04	0,59	Triclopyr (10 %)	1,82	0,40	Fluroxypyr/ Picloram (10%)	0,14	0,93
Glyphosate (100 %)	0,62	0,73	Triclopyr (100 %)	0,26	0,88	Fluroxypyr/ Picloram (100%)	1,14	0,57
Glyphosate (1000 %)	0,24	0,89	Triclopyr (1000 %)	0,86	0,65	Fluroxypyr/ Picloram (1000%)	0,14	0,93

3.2.2. Soil microbiology

3.2.2.1. Shannon diversity index (H')

The OTUs, however variable, showed no effects from the glyphosate treatments (Table 3.13 (i)). The standard error values were as high as 22.10 expressing high amounts of variability. A Kruskal-Wallis ANOVA revealed no significant effects of the glyphosate treatments on the microbial community structure.

Table. 3.13. The Shannon diversity values and number of OTUs for bacterial communities in soils treated with herbicide are shown expressing the mean and relative standard error. The herbicides were applied at four different concentrations including 0 % (untreated control), 10 %, 100 % and 1000 % of the recommended field rate.

(i) Glyphosate		
Pre	**Shannons-diversity index:**	**Number of OTUs:**
0 %	1.78 ± 0.01	59.33 ± 4.11
10 %	1.91 ± 0.08	50.00 ± 2.16
100 %	2.00 ± 0.09	26.33 ± 1.70
1000 %	1.88 ± 0.08	37.00 ± 18.06
Post 1	**Shannons-diversity index:**	**Number of OTUs:**
0 %	1.80 ± 0.02	57.00 ± 4.32
10 %	1.96 ± 0.14	47.00 ± 22.63
100 %	1.96 ± 0.09	18.67 ± 0.47
1000 %	1.97 ± 0.03	22.67 ± 3.40
Post 2	**Shannons-diversity index:**	**Number of OTUs:**
0 %	1.91 ± 0.07	41.33 ± 6.55
10 %	1.88 ± 0.11	32.33 ± 10.62
100 %	1.85 ± 0.06	48.67 ± 9.81
1000 %	1.85 ± 0.06	52.00 ± 7.79
(ii) Triclopyr		
Pre	**Shannons-diversity index:**	**Number of OTUs:**
0 %	1.87 ± 0.03	47.00 ± 2.83
10 %	1.88 ± 0.01	41.33 ± 13.07
100 %	1.93 ± 0.06	21.67 ± 8.18
1000 %	1.90 ± 0.06	40.00 ± 16.31
Post 1	**Shannons-diversity index:**	**Number of OTUs:**
0 %	1.94 ± 0.07	54.33 ± 4.64
10 %	1.96 ± 0.03	30.33 ± 13.89
100 %	1.87 ± 0.01	41.00 ± 13.59
1000 %	1.97 ± 0.09	31.00 ± 14.99
Post 2	**Shannons-diversity index:**	**Number of OTUs:**
0 %	1.86 ± 0.00	55.33 ± 4.03
10 %	1.94 ± 0.10	38.33 ± 3.30
100 %	1.88 ± 0.02	37.33 ± 10.21
1000 %	1.92 ± 0.07	57.67 ± 2.05
(iii) **Fluroxypyr and Picloram**		
Pre	**Shannons-diversity index:**	**Number of OTUs:**
0 %	2.04 ± 0.16	54.00 ± 2.16
10 %	2.05 ± 0.16	53.67 ± 0.47
100 %	2.04 ± 0.16	33.33 ± 14.27
1000 %	2.16 ± 0.02	21.33 ± 3.09
Post 1	**Shannons-diversity index:**	**Number of OTUs:**
0 %	1.95 ± 0.02	51.00 ± 4.32
10 %	2.16 ± 0.09	48.00 ± 9.09
100 %	2.23 ± 0.04	28.33 ± 11.26
1000 %	2.06 ± 0.15	18.00 ± 5.66
Post 2	**Shannons-diversity index:**	**Number of OTUs:**
0 %	1.96 ± 0.02	49.67 ± 4.11
10 %	2.12 ± 0.10	37.00 ± 7.26
100 %	1.95 ± 0.02	52.67 ± 4.92
1000 %	2.13 ± 0.08	30.00 ± 7.12

The triclopyr based herbicide similarly showed minor effects, but a One-Way ANOVA did show a significant difference in the number of OTUs between the different concentration levels at

post 2 (6 weeks after treatment) (Table 3.14). In addition, it was detected that the 10 % level samples had a considerably lower number of OTUs after treatment (Table 3.13 (ii)). The diversity levels similarly seemed unaffected by triclopyr treatment. Diversity values showed no changes or effects of the herbicide on the bacterial community even six weeks after the given fluroxypyr and picloram treatments (Table 3.13 (iii)). The OTU numbers did, however, show slight variations with major deviation from the mean as high as 14.27 in the pre samples which resulted in no consistent impact.

Table. 3.14. One-Way ANOVA of Shannon diversity for the soils treated with the herbicides including glyphosate, triclopyr, and fluroxypyr and picloram at the different assessment stages of pre (before), post 1 (one week after), and post 2 (six weeks after). Significance at $p < 0.05$; df = 12

Assessment stage	**Glyphosate *A. mearnsii***		**Triclopyr *A. mearnsii***		**Fluroxypyr and Picloram *E. camaldulensis***	
	F	**p**	**F**	**p**	**F**	**p**
Pre	4,79	0,03	1,89	0,21	9,47	0,01
Post 1	5,11	0,03	1,62	0,26	7,70	0,01
Post 2	1,95	0,20	6,92	0,01	6,30	0,02

3.2.2.2. Analysis of Similarity

The microbial community structure was not affected by any of the herbicide treatments. An ANOSIM revealed that untreated soils and soils treated with glyphosate were in fact highly similar in community structure ($R < 0.15$). There appeared to be no shift in community structure from pre to post 1 to post 2, with no observable clustering of sample groups (Fig. 3.15 (i)). An ANOSIM revealed that the triclopyr herbicide expressed similar results with an even lower R-value of 0.07 showing very little dissimilarity between communities (Table 3.15). The fluroxypyr and picloram emulsion similarly showed no effects and the ANOSIM revealed low amounts of dissimilarity ($R = 0.15$) and no observable shifts in the bacterial community's structure from before to one week or six weeks after herbicide treatment. There were no observable effects of any herbicide on the microbial community structure.

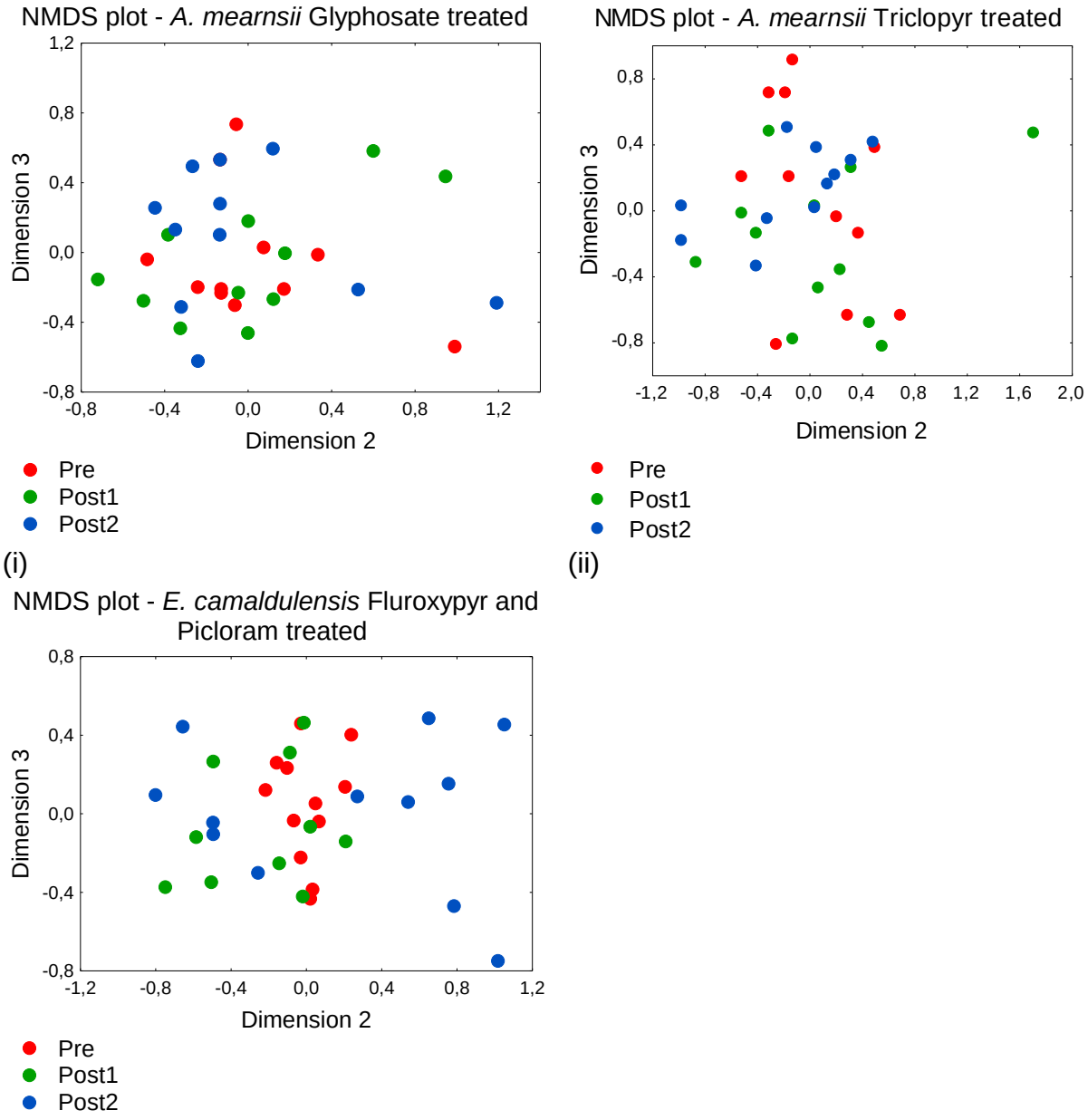


Figure. 3.15. Non-metric dimensional scaling (NMDS) plots for the microbial community's present in the soils treated with the herbicides. The NMDS plots are shown for (i) glyphosate (Springbok 360 SL) treated soils, (ii) triclopyr (Garlon 480 EC) treated soils, (iii) fluroxypyr and picloram (Plenum 160 ME) treated soils. Microbial communities' structure is compared at different assessment stages, namely pre, post 1, and post 2.

Table 3.15. ANOSIM showing similarity of microbial communities when compared at the different assessment stages before and after herbicide treatment. The table displays the R value expressing similarity between *A. mearnsii* bacterial community before and after glyphosate and triclopyr treatment, as well as between *E. camaldulensis* bacterial community before and after fluroxypyr and picloram treatment. In addition, the p-value shows for significant difference from microbial communities before to after herbicide treatment [significance found at $p < 0.05$]

ANOSIM 'R' values:	**R**	**p**
Springbok 360 SL (glyphosate)	0.10	0.04
Garlon 480 EC (triclopyr)	0.07	0.04
Plenum 160 ME (fluroxypyr and picloram)	0.15	0.01

4. Discussion

4.1. Effects on soil chemical characteristics

The three herbicides tested showed little effect on the various soil chemical aspects studied. Aspects such as electrical conductivity, soil available phosphorus and acid-phosphatase activity remained stable regardless of the type of herbicide applied. Even herbicide treatments of a 1000 % concentration of the recommended field rate showed no significant effects on the said soil aspects when looked at *in situ* or *ex situ,* which was in a more controlled and homogenous environment. Although few studies have looked at the effect of herbicides on the soil chemical characteristics, there is prior research that similarly displays little to no effect of herbicides on many soil nutrient levels in the soil or on microbial activity in the soil (Weidenhamer & Callaway, 2010).

Furthermore, soil pH decreased consistently with the application of either of the three herbicides. The pH was significantly lower a week after and six weeks after the given herbicide treatment took place (with no difference between the two latter dates). The stronger concentration treatments (100 % and 1000 %) seemed to decrease the pH more than that of the weaker concentrations (10 %). One can evidently conclude that the three herbicides, based on glyphosate, triclopyr, and fluroxypyr and picloram as active ingredients all have an acidifying effect on the soil depending on the strength of the dose. One cannot conclude that these effects on the soil pH are permanent as the experiment only took place over a six-week period. The pH could return to a more alkaline pH subsequent to the six weeks period after the initial herbicide treatment. The other soil aspects evaluated in this study (*ex situ*) was generally unaffected by herbicide treatments even as high as 1000 % of the recommended field rate. This is likely the result of microbial communities readily processing the active ingredients and additives of the herbicide sprayed directly into the soil matrix. The *in situ* study (Chapter 2) interestingly showed some affect of the herbicides on total soil available nitrogen.

The *ex situ* study, however, shows no such impact, and this could suggest that there are environmental factors such as veld-fire or large nitrogen fixing plants *in situ* that is contributing to changes in nitrogen levels over time (Stock & Lewis, 1986; Stock et al., 1995).

4.2. Effects on soil microbial characteristics

The microbial metabolic activity in the soil showed no changes when treated with a given herbicide. The results of the *ex situ* experiment 2 displayed a steady metabolic potential of the bacterial communities throughout the four weeks after any of the herbicide applications. This has been observed in Crouzet et al. (2010) and Wardle & Parkinson (1990), where the herbicides tested induced no significant or adverse effects on the microbial biomass and microbial activity. Zabaloy et al. (2012) reported that even herbicides applied at ten times the field rate showed no substantial effects on aspects of microbial richness, function and activity. Widenfalk et al. (2008) also showed that no effects on bacterial activity were found when different herbicides including glyphosate and captan were applied. Another microbial aspect studied included the bacterial community structure *in situ* and *ex situ* (experiment 2). The results showed that little to no effect was induced by the different herbicide treatments even at the 100 % and 1000 % field rate, similarly to the microbial metabolic potential as mentioned above.

The diversity scores as well as the similarity scores presented very little variability or change when comparing the communities from before herbicide treatment to a week or six weeks thereafter. It seems as though the herbicides tested have no adverse effects on the diversity of the bacterial communities in the soil when applied. Additionally, no dissimilarity between the pre and post microbial groups were shown when assessing the community structure as no clustering or shifts in the community structure were evident. Weaver et al. (2007) similarly reported minimal effects of herbicides on the community structure of microorganisms. It appears that the given herbicides have no effect on the microbial community structure and microbial communities' metabolic potential. If there are any impacts on these aspects of the soil microbial community within the treatment period, then it could only potentially occur within the first few days of treatment and then dissipate by the first week after treatment when the first assessment stage was due. The microbial community seems to retain its original state by the first week after herbicide treatment.

These results do not preclude medium and longer term impacts on the soil microbial community and soil properties spanning over several months or years (Busse et al., 2001; Seghers et al., 2003). This is especially the case as the soil pH was changed significantly after application of all three herbicides. Slabbert et al. (2014) found that soil microbial

community composition in riparian fynbos soils were closely related to soil pH. This could mean that if pH significantly decreased after herbicide treatment and remained significantly lower for extended periods of time, that the present microbes could be compromised at a later stage than was evaluated in this shorter-term study. The soil pH treated with either of the herbicides remained significantly lower even six weeks after treatment (post 2 assessment stage). In addition, one cannot conclude whether the treated soil ever returns to its original pH or whether the reduced pH will persist longer than six weeks and subsequently induce impacts on soil microorganisms.

4.3. Summary

In summary, the herbicides tested only showed a consistent significant effect on the soil pH levels *ex situ*. No convincing effects were exerted on the other soil aspects covered, including electrical conductivity, soil phosphorus, and phosphatase activity. Moreover, the effects of the herbicides on the soil microbial community structure and community metabolic potential were minimal. When comparing soil microbial metabolic potential to bacterial community structure one can observe no affect on the soil microorganisms. Metabolic activity as a metric of microbial function and bacterial community structure as a metric of microbial community structure both seem largely unaffected by either of the herbicides tested. It is, however, necessary that we continue testing for herbicides and the potential impact they could have on the soil microbial communities as well as on the soil chemical characteristics.

It is recommended that future research studying the impacts of herbicides delve into evaluating different herbicides containing different active ingredients and adjuvants. Doing *ex situ* studies in simulated microcosm conditions also means that we can test soil chemical and soil microbial responses to herbicide exposure in a variety of conditions. We should include testing the given herbicides at more concentration levels and for longer periods of time and consider more frequent assessment stages in order to gain a better understanding of the respective herbicides risk assessment. It is also recommended that we focus on covering other aspects of the microbial community present in the soil and how they respond to herbicide treatment.

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CHAPTER 4

Conclusions and future recommendations

1. Main findings

Soil pH dropped consistently in both *in situ* and *ex situ* experiments when any of the three herbicides, Springbok 360 SL, Garlon 480 EC and Plenum 160 ME, were applied. Based on this study it is clear that all three herbicides have an acidifying effect on soil of riparian fynbos habitats. A reduced pH could suggest other indirect effects over an extended period of time which begs for further research on herbicide and pH-related effects on the soil. Additionally, the observed acidity effects could persist longer than the six-week assessment period. Future studies should consider a long-term study with assessment intervals that span over several months. Total soil available nitrogen also seemed to increase somewhat with glyphosate-based herbicide treatment in *A. mearnsii* sites, but only in the *in-situ* experiments. This could indicate that an external factor in the *in situ* environment is contributing to the soil nitrogen levels and that it is not solely the herbicide increasing the total soil available nitrogen (Domsch et al., 1983).

This study further revealed that there are no significant effects of the herbicide treatments on other evaluated soil chemical aspects such as electrical conductivity, nitrogen mineralization, soil available phosphorus and acid phosphatase activity. Soil available phosphorus and acid phosphatase activity particularly remained unaffected and stable throughout the six-week assessment period. Other researchers such as Naude (2012), have recorded similar ranges for these aspects also including electrical conductivity, and nitrogen mineralization. The above-mentioned aspects evaluated in this study showed that little to no change occurred even when treatment of 10 times the field rate (1000 % field rate dose) was applied. This could suggest that despite effects on pH and nitrogen, these herbicides do not have major impacts on the soil environment when regarding the chemical structure of the soil, at least in the short term. Other soil chemical aspects not included in this study such as SIR (substrate induced respiration), DHA (dehydrogenase activity) and CEC (cation exchange capacity) could be assessed in future studies for a more comprehensive analysis of herbicide effects.

Similarly, soil bacterial communities seemed unaffected when assessing the community structure (community richness and evenness) as well as microbial function (metabolic capacity in the face of certain herbicide treatments) over time. Bacterial communities may be able to assimilate xenobiotic compounds (from the herbicide) and utilize the product as energy. Certain microbial groups have also been recorded to dominate over other genera or species

when exposed to herbicide and hence alter the community structure in the soil while also maintaining a stable level of microbial biomass. Although the microbial groups show no observable effects, the herbicide could result in one microbial group becoming dominant over others. The species or genera present might therefore change, but the roles performed by certain microbes may still be fulfilled in the soil matrix and hence result in no observable detrimental or inhibitory effects to the soil matrix. Shade et al. (2012) and Allison & Martiny (2008), have also proven that microbes are rather resilient in the face of chemical toxicant exposure. There is no doubt however, that research on microbes over the long term is few and far between. Experiments testing the extended exposure to treatment of chemical agricultural remedies could show for long term effects on given microbial groups.

2. Implications for management

With the observed effects of the conventional herbicides tested, users of herbicides should be especially conscious of the acidity of the soil where the invasive aliens are being cleared. Being wary of the local environments' soil pH is important and users of these herbicides should consider that the acidity of the soil may be significantly increased when applying herbicide. A pH level of only a few units lower than usual could be a significant disturbance to local flora and fauna alike. A reduction in the pH may significantly reduce the abundance and diversity of certain bacteria, protozoans and other microorganisms and subsequently fail to fulfill their specified roles in the ecosystem (Fierer & Jackson, 2006). Essentially, a soil pH level too low may render the soil matrix toxic or otherwise inhospitable. Additionally, microbial organisms, soil nutrients and soil cations could be greatly altered by a lowered pH and could impede a clearing operation or rehabilitation programme in the long term.

Farm owners must be cautious of the active ingredients as well as adjuvants used, as the herbicides even at low doses could harm crops for more than one season and the health of cattle and other livestock could be at risk. It is paramount that soil tests are done, and that the unique environment of the farm be considered before applying herbicides. Furthermore, nature reserve managers and managers of green urban spaces should always be conscious of the type of herbicide intended for use against the target alien invasive plant species. Using the wrong herbicide or using the incorrect treatment methodology could mean large-scale damage to the ecosystem. Toxicity to other flora and fauna must also be accounted for, especially species that are heavily dependent on local soil conditions.

Perhaps, studies looking into the important microbial genera present in fynbos and farms of the greater Cape Region could show which microbial properties herbicide users should consider in order to minimize impacts. Moreover, continuous tests in various environments with different herbicides should be conducted. Fynbos harbors habitats of much heterogeneity meaning that a certain herbicide may affect one habitat patch to a lesser extent while affecting a different patch to a greater extent. We therefore require continuous and consistent testing to have a record of possible herbicide effects on the greater Fynbos biome.

3. Recommendations and future research

It is essential that further research be conducted to better understand the effects that agricultural remedies may or may not have on natural ecosystems and farm environments. Soil is an invaluable resource when regarding ecosystems and the dynamic of nutrients and biotic organisms. The effects of certain herbicides on fynbos and riparian fynbos alike cannot be overlooked. This short-term study serves as a 'snap-shot study' where the effects of herbicides were shown to be rather minuscule over a six-week period. A longer-term study might reveal effects that were not otherwise observed in this study. The current study additionally included the most commonly used herbicides used on *A. mearnsii* and *E. camaldulensis*. Future research should include assessing other herbicides with different active ingredients used to combat other common invasive alien plants species in South Africa such as *Prospois glandulosa, Stoebe vulgaris, Populus canescens*, and *Solanum mauritianum*.

It is also important that any signs of phyto-toxicity on indigenous plant species be recorded so that farm owners and reserve managers are aware of the indigenous plant species that may be affected by any given herbicide. It is vital to study how native and alien plants fare in the face of herbicide treatment to enhance long term rehabilitation of a fynbos or riparian fynbos site. As mentioned before, studying the potential effects of herbicide on other soil chemical aspects could supplement one's understanding of the extent of damage that a given chemical herbicide could have. Such aspects include SIR, DHA, FDA, soil carbon and cations and CEC. It is also recommended that these aspects are studied when treated with various concentration levels. Herbicide operators could mix and apply herbicides incorrectly and this needs to be simulated in experiments. This study looked at 10 % field rate doses simulating under application, whilst 100 % FR doses simulated correct mixing and spraying according to the registered product label. A 1000 % FR dose was also included in this study and this

simulated over spraying, mixing herbicide too strong or spilling infield. It is importamt that these various field rate doses are tested, and the respective effects recorded.

Furthermore, future studies should assess even more aspects of microbial responses to herbicides. One can include microbial species identification to distinguish the species and genera present and determine to an extent how greatly they are affected by the respective chemical clearing methodology used. This will help in expressing which microbial groups become dominant or recessive when exposed to a certain chemical or xenobiotic-compound. Additional analyses can also include aspects, such as the response of the microbial biomass to a log dose treatment of a given herbicide. Wardle & Parkinson (1990); Perucci & Scarponi (1994), and Perucci et al. (2000), are of a few studies evaluating microbial biomass responses to herbicides and the results captured in these reviews are valuable. There is a definite research gap in the study of agrochemical non-target environmental effects and researchers should explore using agricultural remedies in the most ecologically conscious and beneficial way so that damage and pollution is minimized. A sustainable future will not allow unfavorable or irresponsible usage of herbicides and other agrochemicals. The correct chemical products need to be applied using the most precise methodology. The most environmentally compatible means of alien clearing and farming is fundamental for our future and future generations to come.

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