Synergistic use of soil microbes and plants to facilitate rehabilitation on gold tailings materials

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PROJECT INFORMATION

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DISCLAIMER

The experimental work conducted and discussed in this dissertation was carried out at the School of Geo and Spatial Sciences (Geology and Soil Science), North-West University, Potchefstroom Campus, South Africa. This study was conducted under the supervision of Pieter W. van Deventer and Jaco Koch. The study represents original work undertaken by the author and has not been previously submitted for degree purpose to any other university. Appropriate acknowledgements have been made in the text where the use of work conducted by other researchers has been included.

Language and style used in this dissertation are in accordance with the requirements of the Applied Soil Ecology Journal.

This dissertation represents a compilation of manuscripts, where each chapter is an individual entity and some repetition between the chapters has been unavoidable. It should be noted that each chapter has its own reference list instead of one comprehensive list appearing at the end of the thesis.

Claudia Schimmer

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ABSTRACT

The rehabilitation of mine tailings put emphasis on the physical/chemical characteristics of tailings storage facilities (TSF) and approaches to alleviate these adverse conditions to ensure plant cover. Minimal attention is given to soil biological properties, both during and after mining operations. This research will exemplify the importance of soil microbial activity as part of rehabilitation specifications and assessment criteria. A combination of chemical, physical and microbiological properties were identified as the major rehabilitation constraints, i.e., pH of 1.7, net acid lime requirement of 300t/ha; low soil enzymatic activity and compost requirement of 65t/ha are amongst the worst. The soil enzymatic activity of the different gold TSFs varied greatly. The enzymatic activity was greater in the voluntarily established grass rhizospheres, with barren TSFs having the lowest enzymatic activity. The low β-glucosidase, urease, dehydrogenase (DHA), acid and alkaline phosphatase enzymatic activities observed at these gold TSFs indicates poor soil quality and soil fertility (insufficient biodegradable organic matter and limited nutrient cycling). A negative association exists between salinity (EC) and DHA (ANOVA r-0.868; $p<0.05$) at the New Machavie TSFs. Salination is an abiotic soil factor, considered hazardous to soil fertility and consequently affect vegetation establishment.

In order to integrate the microbiological components into rehabilitation plans, the synergistic use of soil microbes and plants to facilitate the rehabilitation of various gold TSFs was investigated. This research phase was conducted with the purpose of determining the influence of various bio-stimulants on different mother crop species (Brassicaceae members) survivability and growth in deleterious environments. It was anticipated that the synergistic use of bio-stimulants and mother crop species would improve vegetation establishment and revegetation efficiency. Results indicated that both bio-stimulants and mother crops stimulated soil rhizosphere DHA. Tailings from New Machavie geel TSF with canola/carbohydrates treatment possessing the highest DHA increase (857 INF µg/g/2h), and all un-treated tailings the lowest. Various bio-stimulants significantly increased the mother crop species germination and survival rate (ANOVA $p<0.005$). Results also indicate that rehabilitation is substrate specific, i.e., certain bio-stimulants and different mother crop species performed better on different gold tailings. In conclusion, the study gains novel insights into the use of soil enzymatic activity as a rehabilitation monitoring and assessment criteria indicator. The study also highlights the importance of integrating microbial properties into rehabilitation specifications. Biological factors are vital for soil quality to establish sustainable soil-plant systems.

Keywords: *mine waste environment; enzymatic activity; bio-stimulants; mother crops; plant-microbe synergy; site-specific rehabilitation.*

OPSOMMING

Die rehabilitasie van mynslikdamme lê hoofsaaklik klem op die karakterisering van fisiese/chemiese eienskappe en benaderings om hierdie ongunstige toestande te verlig om sodoende plantvestiging te verseker. Min aandag word aan die grondbiologiese eienskappe gegee word, beide voor en na rehabilitasie. Die navorsing beoog om die belang van mikrobiese aktiwiteit as ‘n rehabilitasie-assesseringsriglyn te beklemtoon. 'n Kombinasie van chemiese, fisiese en mikrobiologiese eienskappe was geïdentifiseer as die primêre rehabilitasie beperkings; pH van 1.7, kalkbehoefte van 300ton/ha, lae ensiematiese aktiwiteit en kompos vereistes van 65ton/ha was die ergste beperkings. Resultate dui aan dat die ensiematiese aktiwiteit van die verskillende goud slikdamme wissel. Die ensimatiese aktiwiteit was hoër in die natuurlike-gevestigde gras rhizosfeer. Terwyl slikdamme met oop gebiede sonder plante, die laagste ensimatiese aktiwiteit besit. Die lae dehidrogenase (DHA), β-glukosidase, urease-, suur- en alkaliese fosfatase ensiematiese aktiwiteite van die goud slikdamme, dui aan dat die mynuitskotgronde swak grondkwaliteit en grondvrugbaarheid het (onvoldoende biologiese-afbreekbare organiese materiaal en beperkte voedingstowwe). By die New Machavie slikdamme kom ‘n negatiewe korrelasie voor tussen versouting (EC) en DHA (ANOVA r-0.868; $p<0.05$). Versouting word beskou as ‘n abiotiese grond eienskap, wat grondvrugbaarheid belemmer en gevolglik plantegroei negatief beïnvloed. Die doeltreffenheid van die sinergisties gebruik van grondmikrobes en plante is ondersoek. Hierdie navorsing is uitgevoer om die effekte van verskillende bio-stimulante op verskillende groei en oorleefbaarheid in mynuitskotgronde te bepaal. Die hipotese stel voor dat die toediening van verskillende bio-stimulante op ‘n verskeidenheid moeder-gewasse (Brassicaceae-lede) plantegroei en plant doeltreffendheid gunstig sal verbeter. Resultate dui aan dat beide die bio-stimulante en die moeder-gewasse die rhizosfeer dehidrogenase positief gestimuleer het. Met die hoogste DHA in slikmateriaal van NM-geel se kanola/koolhidraat behandeling (857 INF µg/g/2h), en die laagste DHA in die onbehandelde mynuitskotgronde. Die verskeidenheid bio-stimulante het die ontkieming en oorlewing van die moeder-gewasse merkwaardig verhoog (ANOVA $p<0.005$). Resultate dui aan dat die bio-stimulante en moeder-gewasse substraat-spesifiek is, dws sekere bio-stimulante en moeder-gewas spesies het beter gedoen op sekere goud mynuitskotgronde. Ten slotte is nuwe insig verwerf ten opsigte van die gebruik van ensiematiese aktiwiteit as 'n spesifikasie vir rehabilitasie asook vir moniterings- en assesseringskriteria riglyne. Verder beklemtoon die studie ook die belangrikheid om mikrobiese eienskappe te intergreer in rehabilitasie planne. Biologiese faktore is noodsaaklik vir die instandhouding van grondkwaliteit en om stabiele grond-plant sisteme te ontwikkel.

Sleutelterme: *myn afval omgewing; ensiematiese aktiwiteit; bio-stimulante; moeder-gewasse; plant-mikrobe sinergie; mynterrein spesifieke rehabilitasie.*

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ABBREVIATIONS/ACRONYMS

µg	microgram
AAS	atomic absorption spectrophotometer
Al	aluminium
AMD	acid mine drainage
AMF	arbuscular mycorrhizal fungi
ANOVA	analysis of variance
C	carbon
%C	organic carbon content
C/N	carbon/nitrogen ratio
Ca	calcium
CEC	cation exchange capacity
CI	chloride
DAFF	Department of Agriculture, Forestry and Fisheries
DEA	Department of Environmental Affairs
DHA	soil dehydrogenase activity
DMR	Department of Mineral Resources
DNA	deoxyribonucleic acid
DPME	Department of Planning, Monitoring and Evaluation
DWA	Department of Water Affairs
EC	electrical conductivity
Eh	redox potential
ESP	exchangeable sodium percentage
HS	humic substances
IAA	indole-3-acetic acid
INF	iodonitrotetrazolium violet-formazan
INT	iodonitrotetrazolium chloride
ISR	induced systemic resistance
K	potassium
KCl	potassium chloride
L	litre
Lreq	lime requirement
Mg	magnesium
N	nitrogen
Na	sodium
NH_4	ammonia
NO_3	nitrate
P	phosphorus
PCA	principal components analysis
PGPM	plant growth promoting microbes
PGPR	plant growth promoting rhizobacteria
PSD	particle size distribution
ROS	reactive oxygen species
S	sulphur
SD	standard deviation
SO_4	sulphate
SOM	soil organic matter
sp.	species (singular)
spp.	species (plural)
THAM	tris (hydroxymethy1)-aminomethane
TSF	tailing storage facility
Tukey's HSD	Tukey's Honestly Significant Difference test
UV-Vis	ultraviolet visible spectroscopy

GLOSSARY

(Adapted from Kumar & Shivay, 2008; du Jardin, 2015; Soil Science Society of America, 2017).

TERMS	DEFINITIONS
Abandoned mine	area formerly used for mining or mineral processing, where closure is incomplete and for which the title holder still exists.
Abiotic	non-living, a physical, meteorological, geological, or chemical aspect of the environment.
Acidification	process associated with atmospheric pollution whereby nutrient bases (calcium, magnesium and potassium) are replaced with acidic elements such as hydrogen and aluminium.
Acid mine drainage (AMD)	mine water that contains free sulfuric acid, mainly due to the weathering of iron pyrites.
Acid-neutralisation capacity(ANC)	sum of all titratable bases in solution, which are available to neutralise inputs of acids.
Acidophilic, acidophilous plants	plants that are adapted to or thriving in an acid soil.
Aggregation	process whereby primary soil particles (sand, silt, clay) are bound together, by natural forces and substances derived from root exudates and microbial activity.
Anthropogenic	occurring because of/or influenced by human activities.
Autotroph	organism capable of utilising CO_2 or carbonates as a sole source of C and obtaining energy for C reduction and biosynthetic processes from radiant energy (photoautotroph or photolithotroph) or oxidation of inorganic substances (chemoautotroph or chemolithotroph).
Biocontrol	process derived from antagonistic interactions between a beneficial organism and a pathogen or parasite that result in the control of a disease or pest.
Biotic	biological organisms, as an animal or plant that influences or affects an ecosystem.
Bio-stimulants	any substance/microorganism applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of its nutrients content.
Calcicole, calciphyte or calciphile plants	plants that require or tolerate considerable amounts of calcium, or are associated with soils rich in calcium.

GLOSSARY (CONTINUED)

TERMS	DEFINITIONS
Ecosystem	dynamic complex of plant, animal and micro-organism communities and their non-living environment interacting as a functional unit.
Ecosystem functions	processes of production and dynamics of resources (organic matter, nutrients, biomass and elements) and energy through systems. A set of ecological processes responsible for providing an environmental good or service.
Edaphic	resulting from or influenced by factors inherent in the soil or other substrates, rather than by climatic factors.
Enzyme	any of numerous proteins that are produced in the cells of living organisms and function as catalysts in the chemical processes of those organisms.
Exudates	low molecular weight metabolites that enter the soil from plant roots and catalysed by soil microbes.
Glucosinolates	class of organic compounds that contain sulphur, nitrogen and a group derived from glucose, which provides pungency, strong smell and bitterness to oils. They occur as secondary metabolites of many plants of the order Brassicales. These are hydrolysed by endogenous myrosinase enzymes released from the parenchymatous cells when crushed.
Heterotrophs	organism able to derive carbon and energy for growth and cell synthesis by utilising organic compounds.
Hyperaccumulator	plant species that accumulates a nutrient or toxic chemical to a very high concentration.
Jarosite	$KFe_3(OH)_6(SO_4)_2$. A pale-yellow potassium iron sulphate mineral.
Microbial biomass	total mass of living microorganisms in a given volume or mass of soil.
Microbial population	sum of living microorganisms in a given volume or mass of soil.
Mine dumps	areas covered with overburden and other waste materials from ore, quarries and smelters, and usually with little/no vegetative cover.
Mother crop	plants grown for the specific purpose of soil management, i.e., utilised primarily for improving soil conditions. Akin to pioneer crop.
Oligotrophic environment	concentration of nutrients available for growth is limited. Nutrient-poor habitats.
Plant growth-promoting rhizobacteria (PGPR)	diverse group of rhizosphere bacteria that impart beneficial effects on plant growth as root colonisers
Pioneer species	first species to colonise an area of disturbance.

GLOSSARY (CONTINUED)

TERMS	DEFINITIONS
Reactive oxygen species	type of unstable molecule that contains oxygen and that easily reacts with other molecules in a cell. Build-up of reactive oxygen species in cells causes damage to DNA, RNA, and proteins, and causes cell death.
Rehabilitation	return of disturbed land to a stable, productive and self-sustaining condition, after taking into account beneficial uses of the site and surrounding land.
Restoration	re-establishment of ecosystem structure and function to an image of its prior near-natural state or replication to a desired reference ecosystem.
Rhizosphere	includes the soil and microorganisms (bacteria, fungi) in the immediate vicinity of a plant's root zone.
Salination	process whereby soluble salts accumulate in the soil environment in very high concentrations.
Siderophores	non-porphyrin metabolite secreted by certain microorganisms that forms a highly stable coordination compound with iron. There are two major types: catecholate and hydroxamate.
Soil amendment	any material such as lime, gypsum, sawdust, compost, animal manures, crop residue or synthetic soil conditioners that is applied to the soil to enhance plant growth. Amendments may contain important fertiliser elements but the term commonly refers to added materials other than those used primarily as fertilisers.
Soil compaction	increasing the soil bulk density, and concomitantly decreasing the soil porosity, by the application of mechanical forces to the soil.
Soil structure	combination or arrangement of primary soil particles into secondary units or peds. The secondary units are characterised on the basis of size, shape, and grade (degree of distinctness).
Sustainability	lies in the dynamic nature of its fundamental components: ecological (spatial and temporal relations, diversity, stability, and resilience; economic (resource distribution and allocation); and social (equity, access, stewardship and institutions).
Tailings storage facility (TSF)	area used to confine tailings; its prime function is to achieve solids settling and improve water quality. It refers to the overall facility, and may include one or more tailings dams.

GLOSSARY (CONTINUED)

TERMS	DEFINITIONS
Thiol	functional group characterised by a sulfhydryl group bonded to a carbon of any hybridisation that is not a carbonyl group carbon.
Trace metal elements (TME)	are sometimes interchangeably used with heavy metals and trace metals. Some controversy exists pertaining to the terminology. van der Perk (2006) stated that "the term heavy metals have no sound terminological and scientific basis", that is "heavy" can vary between researchers, and not all the termed heavy metals are accepted to be heavy metals, some are semimetals and others metalloids (e.g., arsenic and antimony) (Dufuss, 2002). For this dissertation purpose trace metal elements are preferred.
Waste rock	uneconomic rock extracted from the ground during a mining operation to gain access to the ore.

*Soil Science Society of America. 2017. Glossary of soil science terms. https://www.soils.org/publications/soils-glossary# Date of access: 20 Oct. 2017.

*Kumar, D. & Shivay, Y.S. 2008. Glossary of agricultural terms. New Delhi: I.K. International Publishing House.

*du Jardin, P. 2015. Plant biostimulants: definition, concept, main categories and regulation. *Scientia Horticulturae*, 196: 3-14.

PUBLICATIONS ARISING FROM THIS THESIS

Two manuscripts are presented in **Appendix F** and **Appendix G**. Two manuscripts were published in the Applied Soil Ecology Journal.

Schimmer, C. & van Deventer, P.W. 2018? Baseline status of microbial activity on gold tailings facilities in South Africa. *Applied Soil Ecology* (In press).

Zanella, A., Ponge, J.-P., Nold, F., Guercini, S., Rumor, C., Sambo, P., Gobbi, V., Schimmer, C., van Deventer, P.W., Chabaane, C., Mouchard, M.-L. & Garcia, E. 2018? Techno humus systems and recycling of organic wastes. *Applied Soil Ecology* (In press).

CHAPTER 1

INTRODUCTION

"What we know is a drop, what we don't know is an ocean."
–**Isaac Newton**

1.1 Conceptualisation

Mining has been for many years, a vital component of the development of South Africa's economy. Waste generation is a side effect of consumption and production activities within the mining industry and tends to increase with economic advancement. Mining activities have caused severe environmental pollution and ecological degradation in South Africa. The sustainable development of rehabilitation and ecological reconstruction of mine waste sites are the main setbacks in the mining industry. Due to the wide variety of mineral resources mined in South Africa, numerous tailings storage facilities (TSFs) occur throughout the country. In the past, it was common practice to abandon mine sites, once mineral extraction was completed. The Council of Geoscience (2017) identified more than 6 000 derelict and abandoned mines in South Africa. These mining sites were poorly vegetated, exposed and waste minerals remained untreated. Subsequently, rehabilitation of these sites is far from ideal and in many cases completely absent, maintenance is non-existent and the restoration of the mine waste environment abandoned in an uncompleted state. In general, mining activities have a detrimental impact in the mining areas, causing major disruptions in the ecosystem. The ecological disruptions lead to the malfunction of ecosystem components and processes in mining areas, degrading the ecological characteristics and causing environmental damage. Destruction of the landscape, air and water pollution, desertification, and soil quality decline are very common in mining waste environments, consequently, with a considerable reduction in biological populations and diversity. Usually, a decline in soil microorganisms number, diversity and activity, is observed in the mine waste environment (Ge *et al.,* 2008; Labud *et al.,* 2007; Magot, 2005; Maila *et al.,* 2005; 2006; Schloter *et al*., 2003; Torsvik & Øvreås, 2007). One of the major concerns regarding mining is the persistence of detrimental environmental effects even after mining has ceased.

In this thesis, the terms revegetation and phytostabilisation are interchangeably used, with some terms taking preference depending on the reported literature. Soil health and quality are very similar in definition; however, there are definitive distinctions between these two concepts. Soil quality places emphasis the soil's capacity to meet human defined criteria. Whereas, soil health puts emphasis on the soil's ability to sustainably maintain its functions

(Doran, 2002; Doran & Parkin, 1996; Doran & Safley, 1997; Pankhurst *et al*., 1997). For the purpose of this research, the term soil quality will be used, although some interchangeable use of the concepts may occur as per literature being reported.

1.1.1 Rehabilitation background

Mining legislation in South Africa dictates that mine closure requires the rehabilitation of land to a predetermined sustainable post-mining land use capability. Due to greater demand for environmental protection and ecologically sustainable development, mine ecological rehabilitation has become of greater importance. One of the key challenges in mine rehabilitation is the successful establishment of self-sustaining vegetation cover on TSFs and disturbed areas. The majority of mines TSFs are devoid of proper vegetation establishment (Mendez & Maier, 2008a; 2008b; Sheoran *et al.*, 2013). Without the proper rehabilitation mitigation, mining TSFs sites remain barren, due to a combination of physicochemical factors that includes acidic/alkaline pH, trace metal toxicity, weak soil structure, deficient nutrient levels and organic matter, as well as stressed microbial communities (Akala & Lal, 2001; Asensio *et al*., 2013; Barrutia *et al*., 2011; Grandlic *et al*., 2009; Krzaklewski & Pietrzykowski, 2002).

Additionally, different environmental problems may arise depending on the different types of mineral resources and mining methods used. Unfavourable chemical, physical and microbiological characteristics of the mine wastes; in addition to the extreme pH conditions (Alvarenga *et al.*, 2008) and lack of nutrients provide a poor growth substrate for vegetation establishment that is especially critical during the seed development and germination phase (Mains *et al.,* 2006; Munshower, 1994; Tordoff *et al*., 2000; Wong, 2003).

Presently, the standards for successful mine rehabilitation has mainly been limited to physicochemical status, soil erosion and vegetation physiognomies. Traditionally, South African gold mining industry has facilitated TSF's rehabilitation by using 'high-input' grassing methods that include rigorous leaching, liming, fertilisation and irrigation prior to planting pasture grass species (Bradshaw, 1983; Parrotta & Knowles, 1999; 2001; Todd *et al*., 2000). These methods, however, have proven to be ecologically and economically unsustainable (Straker *et al.*, 2008; Weiersbye *et al*., 2006). Poor vegetation cover on South African mine TSFs and a lack of biological norms and standards put question marks behind current practices and specifications. A lot of emphasis have been put on ameliorating mine waste, which allows for immediate revegetation. Current rehabilitation methods are self-restricted, which leads to the establishment of a limited number of plant species (Johnson *et al*., 1994), thus, producing an ecosystem with low diversity, restricted land use potential and wildlife

conservation value (Bradshaw, 1997; Paz-Ferreiro & Fu, 2016). A comprehensive understanding of the environmental problems and the complexity of the ecological process in mine waste environments is necessary, to effectively rehabilitate such an extreme environment.

1.1.1.1 Rehabilitation assessment criteria

As part of the EMP (Environmental Management Plan), a description of methods used to monitor the compliance of the approved rehabilitation plan must be included (van Deventer & Hattingh, 2008). As soon as the rehabilitation plan is complete and vegetation established, the rehabilitation process is evaluated, in order to monitor how similar, the rehabilitated site ecosystem functioning in comparison to a reference undisturbed sites (Sheoran *et al*., 2010). Rehabilitation of derelict mining TSFs are considered to be a very complex process. As such, more than just the presence of vegetative cover must measure rehabilitation success. With the aim of effectively evaluating the soil quality and functionality of ecosystem, several parameters must be considered. Given that, no singular parameter would provide sufficient information on the soil system and ecosystem functionality (Ehrenfeld *et al*., 2005; Sheoran *et al*., 2010). Currently, only above-ground indicators such as soil erosion (Mummey *et al*., 2002b) and vegetation characteristics such as production, cover, diversity, and shrub density (Wick *et al*., 2007) are considered in determining reclamation and rehabilitation success with limited attention to real soil quality indicators i.e. pH, salinity, nutrient status etc. Belowground ecosystem structure and function are rarely included as part of rehabilitation evaluation. A few researchers have indicated the linkage between plant and rhizosphere interactions (Bardgett & van der Putten, 2014; Smalla *et al*., 2001; Wardle *et al*., 2004), particularly plant-soil feedback systems (Eviner & Hawkes, 2008; Harris, 2009; Kardol & Wardle, 2010).

In recent years, various researchers have emphasised the importance of assessing soil microbial community structure, as a parameter for evaluating ecosystem rehabilitation practices on soil quality (Bloem *et al*., 2006; Claassens *et al*., 2006a; Claassens *et al*., 2006b; Claassens *et al*., 2008; Dilly & Munch, 1998; DeGrood *et al*., 2005; Mijangos *et al*., 2009; Mummey *et al*., 2002a; 2002b; Ritz *et al*., 2009; Schloter *et al.,* 2003; Yao *et al*., 2006; Yao *et al.,* 2012).

Soil microorganisms are important ecosystem mediators, promoting appropriate biological activity and improving nutrient availability, soil organic matter (SOM) decomposition and stabilisation, and soil structure (soil aggregate formation) (Crecchio *et al*., 2004; García-Gil *et al*., 2004; Montemurro *et al*., 2006; Pascual *et al*., 1999; Ros *et al*., 2003). The microbial members of soil communities are the most sensitive and rapid indicators of environmental

degradation or land use changes (Hinojosa *et al*., 2004a; Hinojosa *et al*., 2004b; Ye *et al*., 2001; Zhang *et al*., 2006). Subsequently, soil microorganisms are considered sensitive bio-indicators that can be used to monitor soil ecosystem functions in association with physicochemical and biological transformations during ecological rehabilitation of TSFs (Mendez & Maier, 2008a; Wang *et al*., 2012). Soil enzyme activity has been utilised as bio-indicators that reflect the soil quality status of the mine waste environment and its rehabilitation (Alvarenga *et al.*, 2008; Caravaca *et al*., 2003; Izquierdo *et al*., 2005; Mummey *et al*., 2002a). For instance, DHA and hydrolytic enzyme activity, such as phosphatase, urease and β-glucosidase (Alvarenga *et al.*, 2008; Dick, 1992; Gil-Sotres *et al*., 2005; Trasar-Cepeda *et al.,* 1997), reflects the soil microbial activity and represent nutrient cycling processes and organic matter decomposition (Alvarenga *et al.*, 2008; Claassens *et al*., 2008).

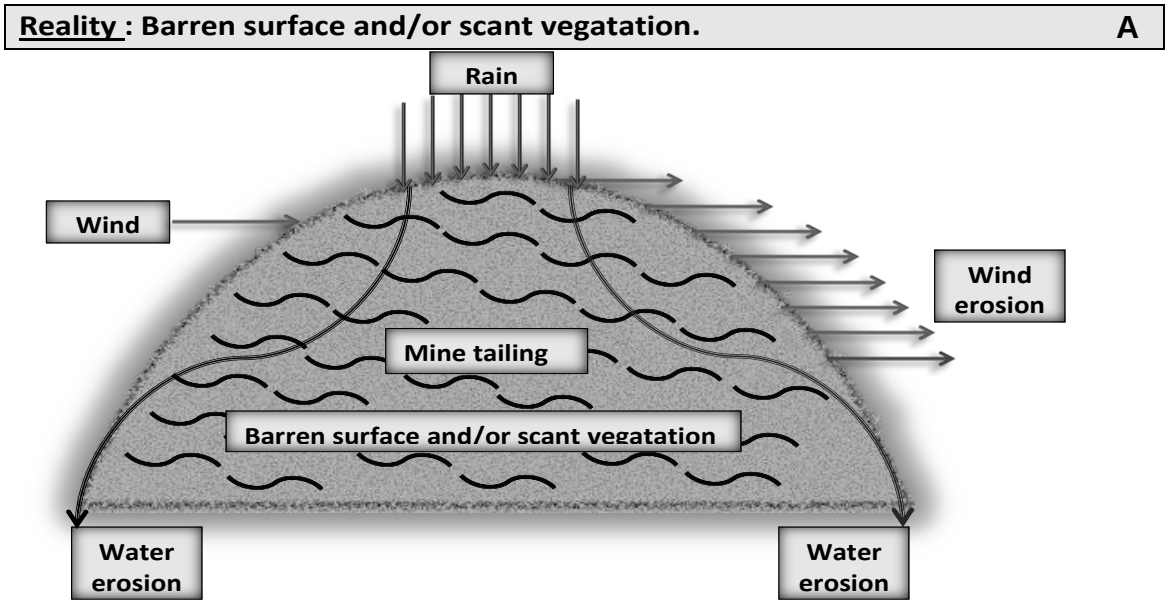
1.2 Problem statement, justification and motivation

Conventional rehabilitation of mine tailings has primarily focused on soil fertility, SOM and plant species selection. However, not one single TSF have obtained a Mine Closure Certificate from the regulating authorities in South Africa because of poor rehabilitation results. In order to re-establish a dynamic, healthy and supportable ecosystem suitable for post-mining land use, an alternative mind-set is required for extreme conditions and additional amelioration is needed to rehabilitate critical ecosystem processes.

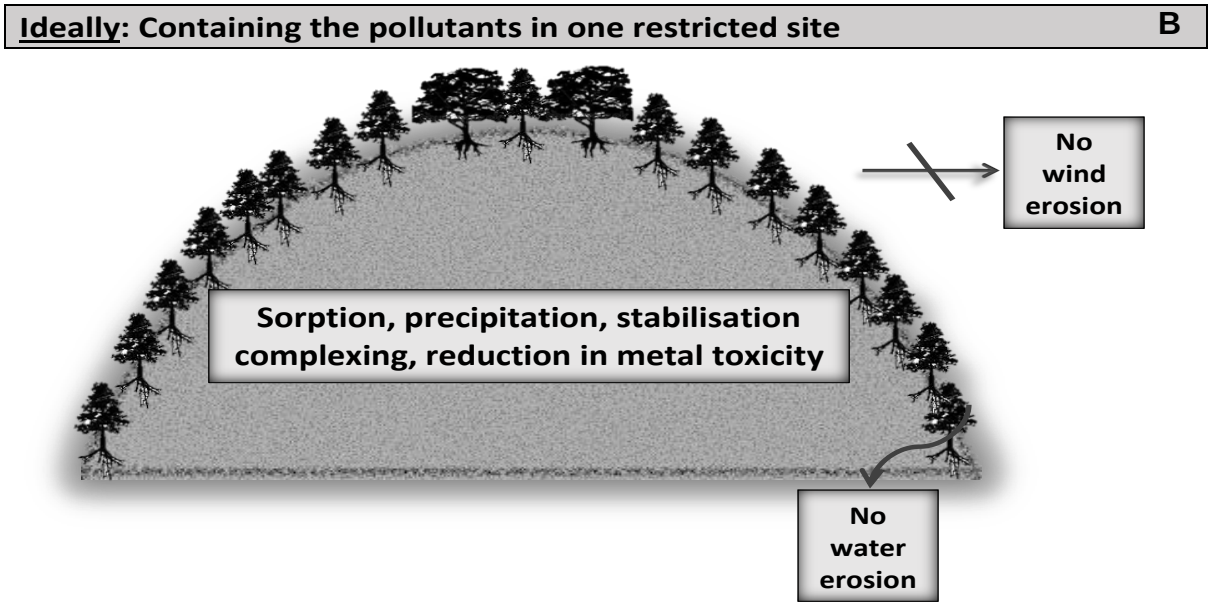
Microorganisms are key components needed for successful rehabilitation, because of their important functional contribution towards nutrient cycling, geochemical alterations, plant establishment, and soil formation. In order to thrive, microbes have the ability to adapt to unreceptive conditions such as high alkalinity/acidity, toxicity and high temperature. Genetically, microbes can acquire a biological resistance against any toxic substance in the environment. Therefore, even if there is a high mortality rate due to unfavourable chemical associated toxicity, some resistant microbes survive and may be cultured for further use. The ability of soil microbes to undergo and maintain functionally and environmentally related mutation and selection may vary.

Plant stress generated by the detrimental effects of the mine waste environment can be countered by enhancing plant defence responses via the microbial-plant feedback system. As a group, microorganisms have the highest ability of all life forms to adapt to extreme and stressful environments. This includes new types of habitats created by anthropogenic activities. Consequently, they can serve as model systems for exploring fundamental ecological principles such as the relationships between diversity and activity of microorganisms and soil environmental conditions. Information on soil microbial diversity and

activity may provide evidence of ecosystem degradation/rehabilitation. Additionally, the mine waste environments microbial communities and activity may provide important information on site-adapted microorganisms and their use as a microbial inoculum. Identified problems, the reality of rehabilitation, expectations and proposed design can be seen in **Figure 1-1.**

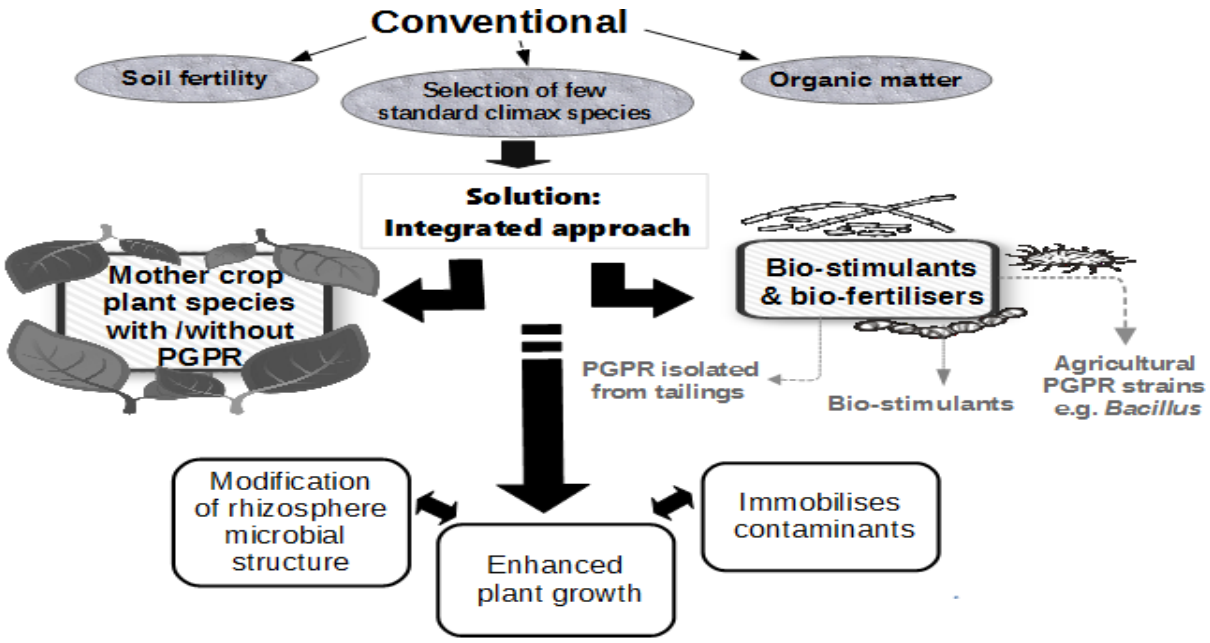


A- Without the proper rehabilitation mitigation, most TSFs remains barren without any natural vegetation establishment over time.



B- Most rehabilitation criteria require the rehabilitation of TSFs to a predetermined post-mine closure state.

Solution: Integration of microbiological properties **C**



C- In order to improve rehabilitation success and to obtain ecological stability, alternative rehabilitation approaches are necessary.

<u>Final restoration</u>: Successional transformation to substrate supporting diversity of native plants close to natural vegetation. **D**

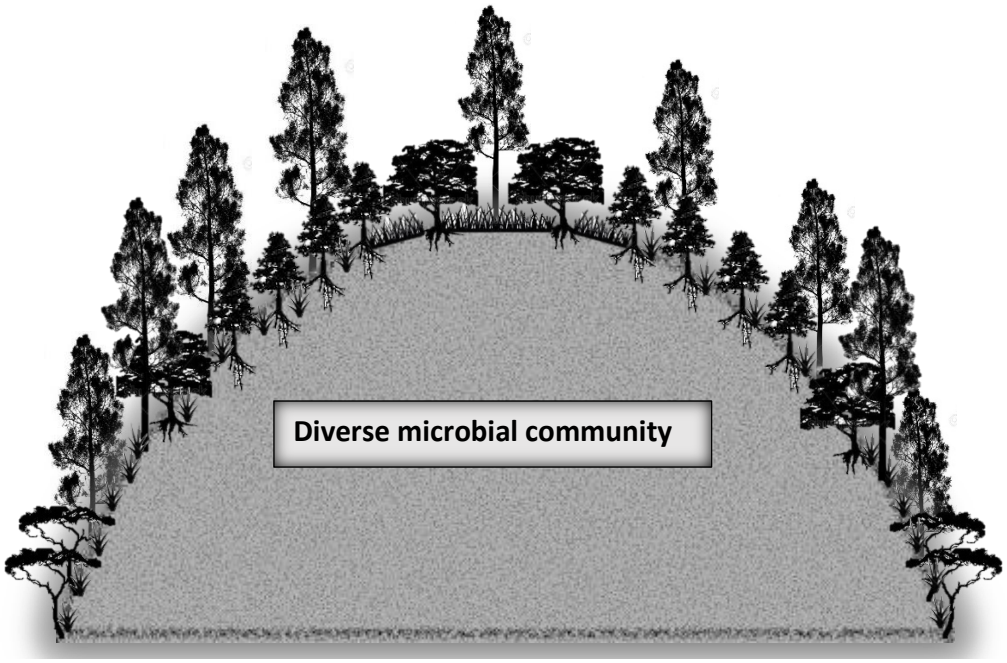


Figure 1-1: Proposed integrated rehabilitation approach versus the conventional approach. Adapted from de-Bashan *et al*. (2012). A- Reality of TSFs, B- Ideal rehabilitation expectation, C- Solution and D- Final rehabilitation.

1.3 Research aims and objectives

The aim of the research project is to identify critical factors in mine rehabilitation (with special emphasis on gold tailings) and the objectives to support the main aim. These aims and objectives can be divided into two objectives or phases:

1. The soil enzymatic activities of different mine tailings and natural soil- a baseline study.

2. Biological amendments and bio-stimulants effect on different mother crop species.

Phase 1 and 2 aims and objectives were researched in order to obtain information on the preliminary status of microbial activity of different tailings and possible solutions to improve microbial activity, thus concentrating on the improvement of plant establishment rather than statistical processing.

The aim of this research phase was to investigate the soil enzymatic status of gold tailings materials, as part of a baseline study to determine whether microbiological properties (i.e., microbial activity) contribute to the overall constraints (including chemical and physical characteristics) of TSF's rehabilitation. This was achieved by setting a number of objectives.

1. Firstly, by identifying the soil enzymatic activities of different gold mining TSFs sites in order to determine whether the deficit biodegradable organic matter and limited nutrient cycling contribute towards low microbial activity.

2. Secondly, comparing the microbial activities in different mine tailings, to those of natural soils, to determine the degree of biological degradation present in mine tailings. The microbial activity present in the rhizosphere of selected grass species growing on different TSFs was also investigated, in order to gain an understanding of self-established plant rhizosphere microbial activity of TSFs.

3. The chemical and physical constraints present in the gold mine TSFs will not only negatively influence the enzymatic activities of untreated barren TSFs but also naturally established plant rhizospheric enzymatic activities.

It was hypothesised that the mine waste environment would possess a low soil enzymatic activity compared to natural soils. In addition, it was also hypothesised that the chemical and physical properties of the tailings would influence the microbial activities of various TSFs. Consequently, low microbial activity would persist even in the rhizosphere of untreated naturally established vegetation.

The second aim of this study was to explore various means of improving the revegetation potential of gold TSFs. In order to achieve this, a number of objectives were set.

1. Firstly, to identify various biological amendments, bio-stimulants and bacterial inoculants that will improve the synergistic use of soil microbes and plants. With the expectations were that these bio-stimulants would improve the DHA in various tailings materials.

2. A second, related objective was to identify different mother crop species, particularly species that can tolerate and successfully establish on gold tailings materials.

3. Lastly, research was conducted to determine the effects of various bio-stimulants on different mother crop species survivability and growth in deleterious environments, in the anticipation that these could improve vegetation recovery and subsequently revegetation efficiency.

The mother crop species utilised for pioneer species purposes, and their responses to bio-stimulant treatments were investigated to determine if these treatments would support their survival. It was hypothesised that bio-stimulants application can influence soil microbial enzymatic activity, thus positively stimulate plant growth and survivability. Secondly, it was hypothesised that the synergistic use of mother crop species and bio-stimulants effects would be substrate specific. Refer to **Chapter 4** and **Chapter 5** for detailed aims of objectives for each of the individual phases.

It should be noted that each chapter has its own reference list instead of one comprehensive list appearing at the end of the thesis. Two manuscripts are presented in **Appendix F** and **Appendix G**. Two manuscripts were published in the Applied Soil Ecology Journal.

Schimmer, C. & van Deventer, P.W. 2018? Baseline status of microbial activity on gold tailings facilities in South Africa. *Applied Soil Ecology* (In press).

Zanella, A., Ponge, J.-P., Nold, F., Guercini, S., Rumor, C., Sambo, P., Gobbi, V., Schimmer, C., van Deventer, P.W., Chabaane, C., Mouchard, M.-L. & Garcia, E. 2018? Techno humus systems and recycling of organic wastes. *Applied Soil Ecology* (In press).

1.4 Thesis structure and content

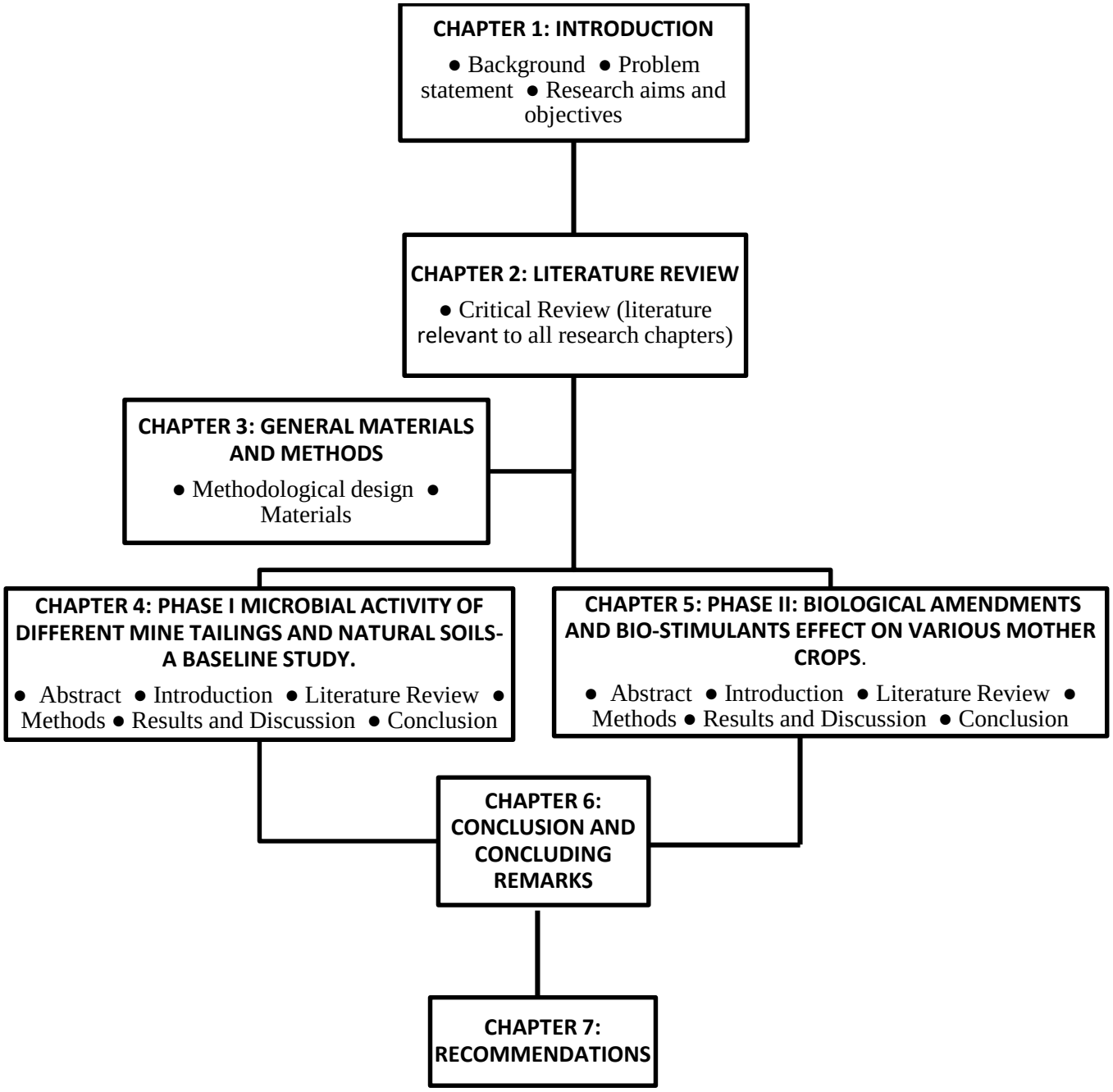


Figure 1-2: Schematic representation of dissertation structure.

- **CHAPTER 2** - Literature review.

Directly after the introduction, a comprehensive **literature review** commences. Detailing research subject associated with plant-soil, plant-microbe feedback system, soil quality and health, microbial status of disturbed land, and microbiological soil quality parameters.

- **CHAPTER 3** - General materials and methods.

This chapter outlines the **general materials methods and approaches** used throughout the study and summarises the research design detailing field, laboratory, procedures, material and methods used, and statistical analyses performed.

- **CHAPTER 4** - Microbial activities of different mine tailings and natural soils- a baseline study.

This chapter describes the **soil enzymatic activities** of different mine tailings and natural soils as part of **a baseline study** to determine the microbiological properties of tailings materials. This chapter also contains a description of mine sites, chemical and physical properties of the various tailings materials used during this research.

- **CHAPTER 5** - Biological amendments and bio-stimulants effect on different mother crop species.

This chapter describes the effect of applying different bio-stimulants to gold tailings materials in order to improve the vegetation establishment. Different bio-stimulant categories were applied to the tailings materials to improve microbial activity by either improving the native microbial community or introducing agricultural-cultivated strains thus changing microbial community dynamics. DHA was assayed as an indicator of the overall microbial activities of the various tailings materials. Additionally, this chapter describes the **synergistic effects of bio-stimulant and mother crop species** selection on the DHA of different gold tailings materials. Plant performance was assessed by means of seed germination, plant survival and growth rate of seedlings.

- **CHAPTER 6** - Conclusion and concluding remarks.

Chapter 6 a collective conclusion for Chapter 4 and Chapter 5 are included in this chapter, which integrates and summarises the results obtained Chapter 4 and Chapter 5.

- **CHAPTER 7** - Recommendation and future studies.

Chapter 7 describes future studies, and recommendation that can improve research quality and further improve rehabilitation approaches.

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CHAPTER 2

LITERATURE REVIEW

"The scientist is not a person who gives the right answers; he's one who asks the right questions."

–**Claude Lévi-Strauss**

The literature review chapter comprises of research pertaining to the complexity of soil, plant-soil and microbial interactions and the influence of tailings properties on both plant and microbiological characteristics. For individual literature reviews, pertaining to specific research aims and objectives, refer to **Chapter 4** and **Chapter 5**.

2.1 Soil-rhizosphere-plant continuum

Within the soil-rhizosphere-plant system, equilibria are regulated by the chemical properties (e.g., nutrient status, pH and redox potential (Eh), physical characteristics (e.g., temperature, soil moisture and soil structure) and biological characteristics (e.g., nature of SOM and microbial diversity and activity) (Cardoso *et al*., 2013). Soils host a complex interaction between the soil physical and chemical properties, soil organisms and plant rhizosphere, for examples of this interconnection refer to **Figure 2-1**.

Soil biological properties are interconnected with the soil physical and chemical properties; e.g., chemical properties affect the microbial activity such as SOM or pH, which sequentially perform relevant activities in carbon (C) and nutrient cycling. For example, microbial mineralisation activity decreases SOM and increases soil C (Delgado & Gómez, 2016), see interaction in **Figure 2-1**. Comprehending the complex interrelationships between biological, physical and chemical components can be attained by examining the origin of the soil processes and their consequential fate (Schjønning *et al*., 2004).

Plant communities play a key role in the determination of soil organism and community structure (Frouz *et al*., 2001; Frouz *et al*., 2008; Frouz *et al*., 2013; Milcu *et al*., 2006; Spehn *et al*., 2000; Zhang *et al*., 2011). Plant community affects the belowground subsystems, via plant-soil feedback systems. These plant-soil interactions are major drivers of vegetation diversity and functioning of ecosystems (Packer & Clay, 2000; Petermann *et al*., 2008; van der Putten, 2003; Wardle *et al*., 2004). For example, soil organisms can influence plant community structure either indirectly, by influencing the soil environment, which plants are reliant on or directly via rhizosphere interaction (Frouz *et al*., 2008).

According to De Deyn *et al*. (2003) and Kardol *et al*. (2007), a positive feedback mechanism exists between a beneficial soil community and plant succession. In their findings, soil organisms positively influenced plant succession and late successional plant growth. In addition, a negative feedback exists between soil pathogens and early succession plant growth (Bezemer *et al*., 2006; de Deyn *et al*., 2003; Ehrenfeld *et al*., 2005; Kardol *et al*., 2006; Kardol *et al*., 2007; van der Putten *et al*., 1993; van Schoor, 2009). Fundamentally, two major soil biota-plant pathways can be characterised (Wardle *et al*., 2004). In the first pathway, soil organisms modify soil properties, i.e., via bioturbation and soil litter decomposition. The second pathway includes rhizosphere interactions (i.e., soil organisms interact directly with the roots). These plant rhizosphere-microorganism interactions perform a key role in nutrient cycling and ecosystem functioning (Berg & Smalla, 2009; Mendes *et al*., 2011; van Schoor, 2009).

It has been documented that plant species composition (Kowalchuk *et al*., 2002; Wieland *et al*., 2001) or soil type (Berg & Smalla, 2009; da Silva *et al*., 2003; Salles *et al*., 2004) are the dominant factors influencing rhizosphere microbial community composition (Berg & Smalla, 2009; Li *et al*., 2011). Different soil types have specific microbial communities, as the distinct particle size distribution (PSD), pH, moisture-holding capacity and physicochemical characteristics of soil types influence soil microbial communities (Fierer & Jackson, 2006; Garbeva *et al*., 2004).

In order to understand the impact of microorganisms on plant development in the mine waste environment, fundamental knowledge of the soil-rhizosphere-plant feedback system is necessary, specifically microbes associated with the plant rhizosphere. The mine waste environment presents an opportunity to research the ecological effects of extreme edaphic conditions on vegetation establishment and their subsequent rhizosphere soil microbial community structure and activity. These rhizospheric soil microbial communities are essential for the plant species succession and survival on mine tailings (de la Iglesia *et al*., 2006; Grandlic *et al*., 2008; Grandlic *et al*., 2009; Li *et al*., 2011; Mendez *et al*., 2007; Mendez & Maier, 2008a; 2008b; Solís-Domínguez *et al*., 2011). The rhizosphere is considered as the soil region that is influenced by plant roots and containing a characteristically high microbial activity (Wang *et al*., 2007).

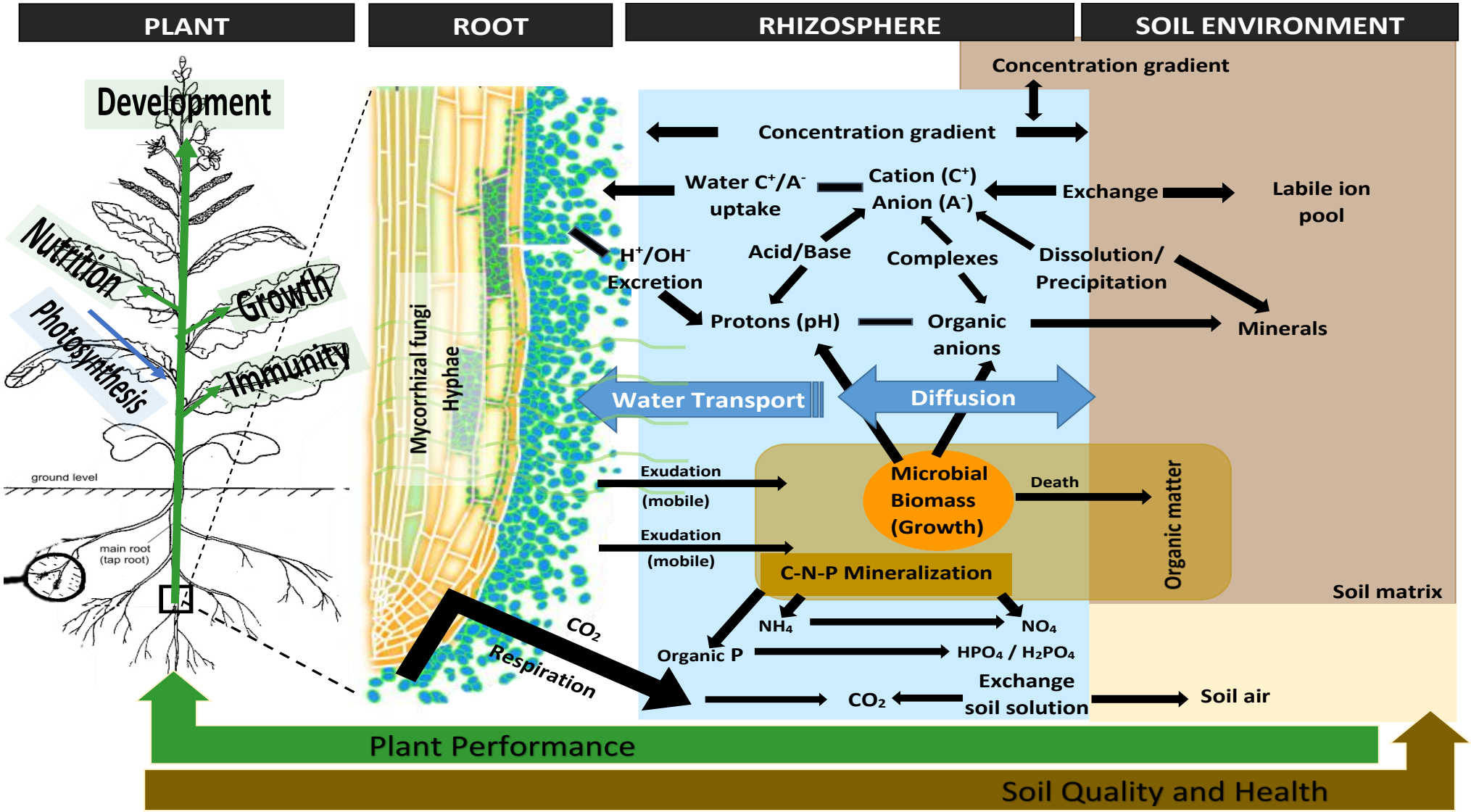

Figure 2-1: **The soil-rhizosphere-plant continuum illustrating the complex interaction between the soil physical and chemical properties, rhizosphere soil organisms and plant roots (adapted from Bhaduri *et al*., 2015; Fageria & Stone, 2006; Pieterse *et al*., 2016).**

2.1.1 Edaphic factors influencing the mine waste environments soil-rhizosphere-plant continuum

Mine waste has numerous restrictions, affecting their future revegetation establishment and development into natural soils, which includes extreme pH, high concentrations of metals and low organic matter (Akala & Lal, 2001; Asensio *et al*., 2013; Barrutia *et al*., 2011; Krzaklewski & Pietrzykoski, 2002). Mine wastes, particularly mine tailings, can be a hostile environment for plant introduction and growth (Mains *et al*., 2006; Munshower, 1994; Tordoff *et al*., 2000; Wong, 2003). Mining areas vary depending on the different types of mineral resources and mining methods, which may lead to different environmental problems. Apart from topographical features such as slope gradients and aspects, the main factors that require consideration to ensure successful vegetation establishment and plant growth on mine TSFs are as follows.

2.1.1.1 Low moisture status

Due to semi-arid dryland conditions of most South African gold mining sites, the only humidity comes from the annual rain (Fierer & Schimel, 2002; Mendez & Maier, 2008a). Moisture content in mine dumps are an ever-changing parameter which is affected by the height of the gold mine dumps, amount of soil organic matter and texture (Sheoran *et al.,* 2010), as well as the moisture retention capacity of the gold mine waste (Donahue *et al*., 1990; Mendez & Maier, 2008a; 2008b). Soil moisture primarily controls plant ecology characteristics, i.e., from individual plant performance to community dynamics (Gurevitch *et al*., 2002). Soil microbial growth and activity similarly rely on soil moisture, sequentially, producing major effects on plant residue decomposition and nutrient cycling. Microbial biomass, activity and nitrification decline with an accumulative number of dry and rewetting cycles (Mikha *et al*., 2005; Nelson *et al*., 1996; Wu & Brookes, 2005).

2.1.1.2 Extreme soil temperatures

TSFs possess extreme and widely fluctuating temperatures, predominantly on the top surface of bare TSFs. These widely fluctuating temperatures inhibit microbial growth and negatively influence sensitive organism survival (Visser, 1983; Xu *et al.*, 2017). Temperature is considered a primary factor that affects plant development rate (Hatfield & Prueger, 2015). Plant's responses to temperature vary among plant species throughout their life cycle and are primarily phenological responses, i.e., stages of plant development. Vegetation positively influences soil temperature chiefly via a combination of shading by litter and aboveground biomass (Gates, 1980; Hillel, 1998; Raich & Tufekcioglu, 2000).

2.1.1.3 Soil chemical and physical characteristics

1) Poor physical characteristics

Soil physical structure positively influences vegetation growth and establishment (Passioura, 1991). Mine wastes are known to have low soil structure development, mainly due to the PSD, SOM and mineralogy. That is intervened by a variety of other related soil physical characteristics including those that relate to moisture availability. Soil texture determines the long-term water retention behaviour of the mine tailings, in particular, the location and connectivity of hydrated microhabitats (Carson *et al*., 2010; Zhou *et al*., 2002), consequently, the microbial community structure and activity are indirectly negatively influenced by the low moisture-holding capacity of the mine tailings. Due to mine tailings physical properties that results in compaction, i.e., reduced water infiltration rate and reduced soil air permeability, also influence the chemical properties (Nawaz *et al*., 2013). Compaction of mine tailings on steep slopes results in enhanced runoff and ultimately an increase in soil erosion. Low soil structure development of mine tailings negatively impacts both plant growth and community structure (Burke *et al*., 1998; Ishaq *et al*., 2001; Kozlowski, 1999; Saqib *et al*., 2004; Sheoran *et al*., 2010). Usually, compaction decreases root length, root penetration, and rooting depth (Glinski & Lipiec, 1990; Kristoffersen & Riley, 2005; Nawaz *et al*., 2013).

2) Poor chemical characteristics

a) Nutrient deficiency

Gold tailings materials are particularly low in plant-available nutrients. The three major macronutrients, N, P, and K, are usually deficient in gold tailings (Coppin & Bradshaw, 1983; Sheoran *et al*., 2010). Consequently, additional nutrient inputs are required by means of fertiliser applications plant community establishment and sustainability (Sheoran *et al*., 2010). Microorganisms have a major impact on the elements nutrient cycling, most of which are fundamental for the growth of living organisms and plants (Brady & Weil, 2002). Nitrates (nitrification), sulphates (S-oxidation) and phosphates (P-mineralisation) are present in soils primarily due to the action of microorganisms (French *et al*., 2009). Nutrient compounds transformation to plant-available forms is highly reliant on the microbial oxidation of these compounds. Given that mine tailings are deprived of key nutrients such as N and P, it is essential to ascertain the roles of symbiotic microorganisms (i.e., N-fixing bacteria and AMF) in recovering nutrient status of plants used to inhabit mine wastes (Sheoran *et al*., 2010; Visser, 1983). Essential mineral elements are particularly important for plant growth. In the absence of these elements, the plants are unable to complete their life cycles (Marschner, 2012).

b) Low levels of organic matter

Gold tailings are characterised by very low organic matter (<0.1%). Organic matter affects both the chemical and physical properties of soil and its overall health (Akala & Lal, 2001; Conesa *et al*., 2006; Ottenhof *et al*., 2007; Zanuzzi *et al*., 2009). In addition, SOM also influences the soil structure; moisture-holding capacity; soil organism's diversity and activity, and nutrient availability. Furthermore, it also influences the effects of chemical amendments, fertilisers, pesticides and herbicides (FAO, 2017).

Organic matter decay provides two functions for soil microorganisms, providing energy for growth and supplying carbon for the formation of new cells. Soil microbes require a regular supply of active SOM in the soil to thrive (Hoorman, 2010). The role of organic amendments in improving chemical, physical and biological properties of degraded and anthropogenic soils is well documented (Pascual *et al*., 1999; Stewart *et al*., 2000). The role of SOM has been the most widely examined and cited source concerning the plant-soil feedback (Aerts, 1997; Aerts *et al*., 2003; Ågren & Bosatta, 1996; Berendse, 1998; Chapin, 2003; Cornelissen, 1996; Hobbie, 2015; Scott & Binkley, 2002; van Breeman, 1998).

c) Acidification and acid mine drainage

As gold mine tailings are generated, pyrite and other sulphide minerals are exposed to an increased amount of oxygen creating a favourable environment for sulphide-oxidising microbes (Ledin & Pedersen, 1996). Combined with the oxygen and water the microorganisms further oxidise the pyrite, resulting in the production of sulphuric acid, consequently, lowering the pH of the gold tailings (Alpers *et al*., 2003; Dold & Fontbonte, 2002). The acidic water formed under saturated conditions can solubilise metals, resulting in metal toxicity (Dold, 2010; Edwards *et al*., 2000; Jamieson, 2011; Walder & Chavez, 1995). Acid mine drainage (AMD) is the common and widespread environmental problem associated with pyrite-rich mine sites (Blowes *et al*., 2003; Doupé & Lymbery, 2005; Kalin *et al*., 2006; Klapper & Geller, 2002; McCullough & Lund, 2006; McDonald *et al*., 2006; Saarinen *et al*., 2013). Addition of alkali-chemicals interacts with the acidic tailings, reducing the release of acid, thus precipitating Fe and other trace metal elements. The major disadvantage of this method is that it does not prevent the re-acidification after a few years.

Soil pH has a negative effect on all soil properties (i.e., physical, chemical and biological) (Brady & Weil, 2002). For example, pH strongly influences abiotic factors, such as organic C solubility (Andersson *et al*., 2000; Kemmitt *et al*., 2005; Kemmitt *et al*., 2006; Wardle, 1992), nutrient availability (Aciego Pietri & Brookes, 2008; Kemmitt *et al*., 2005; Kemmitt *et al*., 2006), and the solubility of trace metal elements e.g., increases the bioavailability of aluminium (Al) with

decreased pH (Andersson *et al*., 2000; Firestone *et al*., 1983; Flis *et al*., 1993). In addition, soil pH also affects biotic factors, such as microbial activity and microbial community structure (Aciego Pietri & Brookes, 2008; Anderson, 1998; Marstorp *et al*., 2000; Bååth & Anderson, 2003; Rousk *et al*., 2009; Zelles, 1999).

Substrate pH also influences plant growth primarily via its effects on the chemical solubility of nutrients and toxic metals. Plant communities are distinguished along a pH gradient, specifically at extreme pH values, i.e., acidophilic plant (pH < 5) and calciphyte plant (pH > 7) communities (Ehrenfeld *et al*., 2005). It is also commonly accepted that the optimum plant nutrient availability and minimum nutrient toxicity are at a pH of around 6.5 (Harris *et al*., 1996).

d) Trace element contamination

Trace elements are naturally-occurring elements located throughout the earth's crust. The majority of trace metal element contamination can be attributed to anthropogenic activities such as mining and smelting operations, industrial effluents, urban runoff, sewage discharge, agricultural pest control agents (Goyer, 2001; He *et al*., 2005; Herawati *et al*., 2000; Morais *et al*., 2012). For this research purposes, the term trace metal elements will be used to refer to both metals and metalloids. Trace metal elements include potently toxic metals (e.g., lead/Pb and nickel/Ni), essential metals (e.g., zinc/Zn, copper/Cu and cobalt/Co), medicinal metals (e.g., platinum/Pt and bismuth/Bi), toxic metalloids (e.g., arsenic/As and antimony/Sb) (Tokar *et al*., 2010). In the mine waste environment, two routes regulate the release of trace metal elements into the environment. The first route comprises of an accelerated natural weathering of the pulverised waste, which oxidises the sulphide minerals, thus liberating the trace metal elements (Nordstrom & Alpers, 1999). The second process is facilitated by AMD. The waste rock and tailings are easily dissolved by the acid mine drainage water, thus mobilising metals such as Fe, Al, As, Pb, cadmium (Cd), and mercury (Hg) (Krzaklewski & Pietrzykowski, 2002; Mendez & Maier, 2008a; 2008b). Gold mine tailings are known to have high potently toxic trace metal element concentrations of Pb, Cu, Zn, Co, As, Cd, Hg and Ni (da Silva *et al*., 2004; Fashola *et al*., 2016).

Trace metal elements play a key role in metabolic and physiological processes of plants, humans and microorganisms. Elevated levels of trace metal elements in gold mine tailings greatly influence microbial diversity, population size, and overall microbial activity (Bamborough & Cummings, 2009; Gleeson *et al*., 2006; Hinojosa *et al*., 2005; Joynt *et al*., 2006; Li *et al*., 2006; Li *et al.*, 2011; Liao & Xie, 2007; Obbard, 2001). Trace metal elements have a negative effect on soil microbial growth, metabolism and morphology as a consequence of functional disturbance,

destruction of cell membrane integrity or protein denaturation (Chakravarty & Banerjee, 2008; Collins & Stotzky, 1996; Šmejkalová *et al*., 2003; Westcott, 2011).

Plants require certain trace elements for their growth and maintenance; however, excessive trace metal element concentrations can be toxic to plants. The plant mechanism which enables a plant to accumulate essential trace elements also allows the accumulation of other non-essential trace metals (Djingova & Kuleff, 2000). High concentrations of trace elements disrupt the plant cellular structure, metabolic and transport processes (Sharma & Dietz, 2009; Tripathi *et al*., 2015). A direct response of plants upon exposure to high trace metal concentration is to generate reactive oxygen species (ROS) (Assche & Clijsters, 1990; Jadia & Fulekar, 1999). Additionally, some trace metal elements have an indirect effect on plants, through the generation of ROS directly via the Haber-Weiss reactions or the overproduction of ROS, resulting in trace metal element toxicity (Hossain *et al*., 2012; Mithöfer *et al*., 2004; Wojtaszek, 1997; Yadav, 2010). Despite this, certain plants have acquired a potential mechanism to combat adverse environmental trace element toxicity, responses involve the immobilisation, and exclusion, chelation, and metal ion compartmentalisation of toxic trace metal elements (Ovečka & Takáč, 2014; Pattanayak *et al*., 2014; Yadav, 2010). In addition, certain plants are able to generate low molecular weight thiols that display a high affinity for toxic trace metal elements (Bricker *et al.*, 2001).

e) Salinity

Salinity is commonly associated with semi-arid and arid climates. Salt accumulation occurs when the evaporation rates exceed rainfall (Munshower, 1994). Salinity causes osmotic stress and ionic toxicity that negatively influences plant growth and microbial community structure and activity. The influences of soil salinity on microbial diversity and activity can be assigned to two primary mechanisms, namely, the osmotic effects and specific ion effects (Yan *et al*., 2015). Several researcher's findings have demonstrated that salinity reduces microbial activity, microbial biomass and alters microbial community structure (Andronov *et al*., 2012; Batra & Manna, 1997; Chowdhury *et al*., 2011; Gennari *et al*., 2007; Rietz & Haynes, 2003; Rousk *et al*., 2011; Setia *et al*., 2010; Yan *et al*., 2015). Salinity decreases microbial biomass mainly due to the osmotic stress that causes cell drying and lysis (Batra & Manna, 1997; Pathak & Rao, 1998; Rietz & Haynes, 2003; Sarig *et al*., 1996; Sarig & Steinberger, 1994; Yan *et al*., 2015; Yuan *et al*., 2007).

Salt-stress adversely influences germination, plant growth, and reproduction by disturbing the plant physiological processes. Salt-stress on physiological processes such as respiration, transpiration, photosynthesis, enzymes functioning, hormones abscisic acid (ABA), stress on ethylene upregulation, and ROS generation are hampered (Akbarimoghaddam *et al*., 2011;

Barkla & Pantoja, 2011; Kang *et al*., 2014; Nawaz *et al*., 2010; Neocleous *et al*., 2014; Paul, 2012; Shibli *et al*., 2007; Singh & Chatrath, 2001; Singh *et al*., 2011).

2.1.1.4 Stressed microbial communities

The microbiology and diversity of the acid mine environment have been well documented (e.g., Baker & Banfield, 2003; Bond *et al*., 2000; Johnson, 2007; Johnson & Hallberg, 2008; Jones *et al*., 2012; Pronk *et al*., 1992; Ziegler *et al*., 2013). In the gold mine waste environment, the chemolithotrophic acidophilic species are well-documented microbes associated with the bio-oxidation of sulphide-bearing minerals, most notably the Fe/S-oxidising bacteria *Acidithiobacillus ferrooxidans* (Karavaiko *et al*., 2003; Waltenbury *et al*., 2005). Heterotrophic microorganisms have also been isolated in extremely acidic mine waste. These heterotrophic species are highly adapted, scavenging C originating from chemolithotrophic acidophiles leakage or lysis products. A symbiotic relationship exists between the heterotrophic species and certain autotrophic species in the AMD ecosystem, i.e., the autotrophs depend on co-existing with the heterotrophs (removes organic compounds that are toxic to the autotrophs) (Baker & Banfield, 2003; Plante, 2007).

Gold mining waste environment is characterised by extreme edaphic conditions, e.g., lack of water, presence of toxic elements, low nutrient availability, highly fluctuating temperature and high salinity and acidic pH (Bradshaw, 1997; Bradshaw, 2000; Edwards *et al*., 2000; Fierer *et al*., 2003; Lear *et al*., 2009; Liao & Xie, 2007; Mendez & Maier, 2007; 2008a; Visser, 1983). These edaphic factors constraints the biological activity, consequently organism diversity recovery in these environments are incredibly slow (Bloem & Breure, 2003; Elhottová *et al*., 2006; Frouz *et a*l., 2001; Gould *et al*., 1996; Urbanová *et al*., 2011).

The mine waste environment exerts acute/chronic stresses on the soil ecosystems and evolutionary pressure on soil organism diversity, communities and ecosystem functioning (Freedman, 2015; Tobor-Kapton *et al*., 2006). These stresses result in soil organism's energy budget alteration. Stress produces an additional load on the energy budget and an increase in maintenance costs (Hoffmann & Parsons, 1997; Sibly & Calow, 1989). High level of stress causes a decrease in microbial diversity, whilst sensitive species are not able to successfully tolerate these specific stress factors (Giller *et al*., 1998; Kozdrój & van Elsas, 2001; Kurek & Bollag, 2004). Because of lowered competition and easier resource access, more resistant species become abundant; consequently, microbial populations in tailings are commonly small and restricted in diversity (Ritcey, 1989).

In terms of plant-microbe interaction, a number of mine tailing rehabilitation studies have emphasised a sound association between stable plant community establishment and soil

microbiota abundance and composition (Grandlic, 2008; Grandlic *et al*., 2008; Grandlic *et al*., 2009; de la Iglesia *et al*., 2006; Li *et al*., 2011; Londry & Sherriff, 2005; Mendez *et al.*, 2007; Mendez & Maier, 2008a; 2008b; Mummey *et al*., 2002a; Schippers *et al*., 2000; Solís-Domínguez *et al*., 2011; Wielinga *et al*., 1999). Whilst, a large population of autotrophic Fe- and S-oxidising bacteria are associated with high plant mortality rates in acidic mine tailings with limited acid-neutralising potential, whilst a rise in neutrophilic heterotrophic bacteria has been positively associated with vegetation establishment (Grandlic, 2008; Londry & Sherriff, 2005; Mendez, 2007; Mendez *et al*., 2007; Mendez & Maier, 2008a; 2008b; Mendez *et al*., 2008; Rosario *et al*., 2007).

2.2 Concepts of ecosystem disturbance and stability, resistance and resilience

Ecological communities can be affected either by stress (harsh conditions, e.g., low pH, trace metal toxicity) or by disturbance (e.g., wildfire, cultivation) (Wardle & Giller, 1996). According to Griffiths *et al*. (2000) and Griffiths *et al*. (2001), a stable system can tolerate disturbances and maintain a normal state or steady state (Shade *et al*., 2012). The soil ecosystems react to disturbance via two mechanisms, i.e., resistance and resilience (Fisher & Grimm, 1991; Holling, 1973; Pimm, 1991). The collective consequences of these two concepts regulate ecosystem stability (Lake, 2013; Shade *et al*., 2012). Resistance is considered to be the systems inherent capacity to tolerate disturbance (Griffiths *et al*., 2001; Lake, 2013; Lal, 1994). While resilience can be described as the ability of a system to recover after being disturbed/stressed (Aarts & Nienhuis, 1999; Seybold *et al*., 1999; Pimm, 1984). As a result, soil resilience can be described as the soil capability to recover its functional and structural integrity when disturbed (Abdel Kawy & Ali, 2012; Eswaran, 1994; Lal, 1997). Consequently, it describes the extent to which the system is capable of self-organisation, learning and adaptation (Gunderson & Holling, 2002; Walker *et al*., 2004).

Two categories of resilience can be defined, i.e., engineering and ecological resilience. Engineering resilience described as a system that behaves like an engineering material, which shows displacement and recovery regarding its pre-disturbance state or a new stable state. Ecological resilience is considered the amount of disturbance needed to shift the system from one stable state to another alternate stable state (refer to **Figure 2-2**; Gunderson, 2000; Holling, 1973). Resilient ecosystems can occasionally disappear or reappear at an alternative location. Ecosystem resilience is considered an important indicator of the ecological sustainability (Neumayer, 1998; Sutherland *et al*., 2006). A longer period for stability recovery after disturbance is indicative of loss of resilience (Ludwig *et al*., 1997).

Ecosystem stability recovers with the increase in resistance and resilience. Within the ecological stability concept, two contrasting theories exist. That is, non-stressed systems are more stable

due to a larger resource positioning, thus non-stressed systems maintain their function in the event of stress (Aarts & Nienhuis, 1999; Griffiths *et al*., 2001; Loreau, 2000). The other theory predicts that stressed systems are more stable, as a result of stress, the system gain capabilities (e.g., adaptation) to handle stress, thus maintaining system functioning (Griffiths *et al*., 2001; Odum, 1981).

Soil resilience and stability form part of broader concepts of soil health and quality, which represents the overall state of a soil. Soil health and quality definitions are considered very similar; however, some distinctions can be made. For instance, soil quality mostly focuses on the soil's capacity to meet human defined criteria, whereas soil health concentrates on the soil's sustained capacity to maintain its functions (Doran, 2002; Doran & Parkin, 1996; Doran & Safley, 1997; Pankhurst *et al*., 1997).

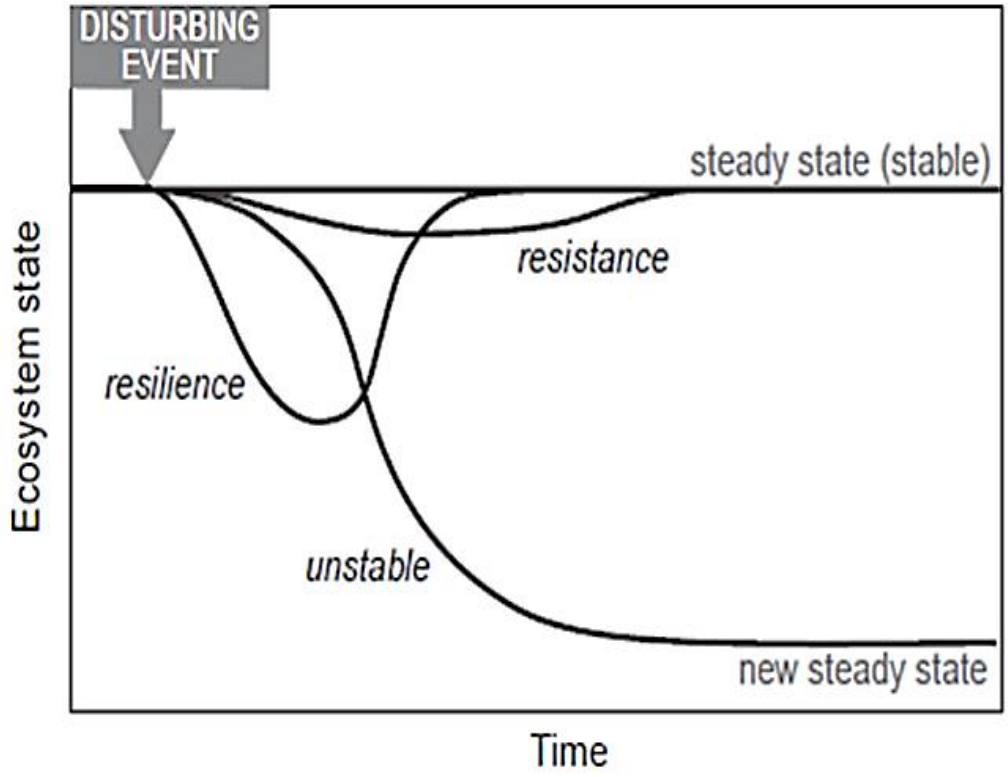

Figure 2-2: Ecosystem response to disturbance: resistance and resilience concepts of ecosystem recovery (adapted from Aber & Melillo, 1999).

Disturbance described as a long-term disruption of a constant or steady state. In which the system is unable to return to a steady state via resistance or resilience (Holling, 1973; Mitchell *et al*., 2000). Consequently, the system is unable to revert to the initial system state, at least not in short period of time; as a result, the system can attain an alternative state, which may be an alternative new steady state (Lake, 2013).

Fundamentally, disturbances are considered events that either/or:

- Changes the immediate environment and consequently influences the faunal and flora diversity and community structure.
- Directly changes the faunal and flora diversity and community structure (Shade *et al*., 2012).

Although, these concepts are clear in principle, in reality, a combination of these disturbances may exist and subsequently, organism's responses may vary (Glasby & Underwood, 1996; Shade *et al*., 2012). Stress can be considered an environmental condition (chemical, physical and biological) that permanently negatively affects fauna and flora biodiversity (Degens *et al*., 2001; Giller *et al*., 1998). Whilst disturbance is described as an event that is confined by time; resulting in a short/permanent impact on organism diversity.

2.3 Vegetation establishment and the mine waste environment

Several chemical and physical rehabilitation techniques exist to enhance TSF's stability. Nonetheless, mine waste revegetation is relatively popular due to its simplistic, cost-effective and aesthetically pleasing design (Alvarenga *et al.*, 2008; Bradshaw, 2000; de Souza *et al*., 1999; Mench *et al*., 2003; Mendez & Maier, 2008a; 2008b; Pérez-de-Mora *et al*., 2006; Vangronsveld *et al.*, 1995).

Revegetation of mine tailings is one of the most common mining rehabilitation practices managed globally. As part of the mining site remediation strategy, revegetation techniques aim at the permanent plant cover establishment, for the purpose of stabilising and enhancing aesthetic value. Revegetation of land devastated by mining activities includes extensive ecosystem reconstruction, equivalent to primary succession initial stages succeeding major disturbances, but comprising of shorter periods (Bradshaw, 1983; 1997; Huang *et al*., 2012; Walker & del Moral, 2003). The tailings surface can be a hostile environment for plant establishment whilst tailings are biologically sterile with virtually no microbial activity. Furthermore, the tailings have a high drainage and evaporation rate, poor plant nutrient availability, low organic content and sub-optimal pH for plant growth (Alvarenga *et al.*, 2008; Grandlic, 2008; Johnson, 2003; Mains *et al.,* 2006; Mendez & Maier, 2008a; Munshower, 1994; Pilon-Smits, 2005; Tordoff *et al*., 2000; Wong, 2003). The tailings materials also have elevated levels of potentially toxic trace metal and metalloids (Conesa *et al*., 2007; Wang *et al*., 2009) that can inhibit plant growth (Baroni *et al.,* 2004; Bruce *et al.,* 2003; Oancca *et al*., 2005). Trace metal elements in tailings affect several physiological, biochemical and growth processes in plants (Fernandes & Henriques, 1991; Reeves & Baker, 2000). Due to these shortcomings, natural plant colonisation on mine tailings

can be extremely slow (Bradshaw, 2000; Conesa *et al*., 2007; Ellery & Walker, 1986). All these issues, in order for successful plant establishment to take place, need addressing.

The revegetation of gold mine TSFs is said to reduce acid production by affecting quite a few plant-soil processes (Sobek *et al*., 1990), including

- Root system competes with the acid-producing bacteria for oxygen and moisture.
- Beneficial heterotrophic soil bacterial and fungal populations use additional oxygen and forms organic acids that may inhibit *Thiobacillus ferrooxidans.*
- Carbon dioxide (CO_2) in the tailings increase through plant root respiration and heterotrophic bacterial activity, resulting in the formation of an unfavourable microenvironment for *T. ferrooxidans* (Ledin & Pedersen, 1996). Suppresing the growth *T. ferrooxidans.*
- The established vegetation also stabilised the cover, thus controlling dust emission, reducing wind erosion and deflation, water erosion and seepage (Grantz *et al*., 1998; Gyssels *et al.,* 2005; Kort *et al*., 1998).
- Vegetation establishment also amends the gold tailings chemical and biological characteristics by improving nutrient cycling, soil structure, organic matter content (%C) and microbial activity (Arienzo *et al.,* 2004; Asensio *et al.*, 2013).

Vegetation establishment on new ecosystems or disturbed systems, including mining waste environment, should yield a self-sustaining community that is dynamic and able to change as the rehabilitated site ages over time. Ultimately, mine rehabilitation aims for soil ecosystem stability and sustainability that will lead to mine closure. Mine rehabilitation success means the extent of alteration, in the tailings characteristics towards characteristics of natural soil. Characteristics include detritus accumulation and decomposition, organic matter, %C and organic nitrogen (%N), and root proliferation, all these properties are strongly affected by soil microbial activity (Sopper, 1992). Several studies have postulated that root-associated microbes may be essential to facilitate tailings plant establishment by improving plant biomass and development and can serve as bio-indicators of revegetation status (Grandlic *et al*., 2008; Ma *et al.*, 2011; Mendez & Maier, 2008a; Solís-Domínguez *et al*., 2011; Valentín-Vargas *et al*., 2014; Weyens *et al.*, 2009).

Symbiotic relationships between plants and microbes in the rhizosphere provide plants with a better likelihood of being valued for rehabilitation purposes. Plant growth-promoting rhizobacteria (PGPR) and other bio-stimulants have prospective capabilities to counter the problems that constrain proper mine rehabilitation, and hence have attracted considerable attention (Dary *et al*.,

2010; Glick, 2003; Grandlic *et al.*, 2008; Zhuang *et al*., 2007). Phytostabilisation aims to successfully establish a vegetation cover that reduces erosion and immobilises pollutants but also achieve plant successional diversity associated with ecosystem stability and resilience (Mendez *et al.*, 2008b; Mummey *et al*., 2002b).

Numerous mine tailings rehabilitation studies have emphasised the strong correlation between the stable plant community establishment, soil microorganism diversity and richness (de la Iglesia *et al*., 2006; Londry & Sherriff, 2005; Mendez *et al*., 2008a; Mummey *et al*., 2002a; 2002b; Schippers *et al.*, 2000; Wielinga *et al*., 1999). Specific microbial communities dominate barren tailings, for example, extremely acidic mine tailings are usually associated with high quantities autotrophic iron (Fe)- and sulphur (S)-oxidising bacteria, that are related to a high vegetation mortality rate (Grandlic, 2008). While a shift in microbial structure and diversity occurs during phytostabilisation, with an increase in neutrophilic heterotrophic bacteria, indicating a positive correlation with plant establishment (Grandlic, 2008; Londry & Sherriff, 2005; Mendez, 2007; Mendez *et al*., 2007; Rosario *et al*., 2007; Schippers *et al*., 2000). Valentín-Vargas *et al.* (2014), stated that a knowledge gap exists regarding the long-term microbial communities spatial and temporal changes during revegetation of TSFs. More precisely, how microbial communities react to rehabilitation treatments, influence trace metal stabilisation, plant establishment success (Valentín-Vargas *et al*., 2014).

2.3.1 Methods used for rehabilitating mining waste environment

The revegetation of the mine waste environment can be described according to Johnson *et al.* (1994), via three different philosophies (i) ameliorative (ii) adaptive and (iii) agricultural. Mine waste characteristics determine which approach is most suited.

- Ameliorative approach involves chemical and physical alteration of the growth substrate, to specifications that will improve plant germination and growth. Using fertilisers, organic matter, and/or liming will improve the chemical and physical nature of the mine waste. In this approach, rather than tolerated, the potential toxicity is avoided or diluted by using some form of covering system (Bellitto *et al.*, 1999; Johnson *et al.*, 1994; Maiti, 2013; Odum *et al.*, 2000).
- Adaptive approach accentuates the selection of suitable species, subspecies, cultivars, and ecotypes to combat the toxicity of the waste by direct seeding. This involves identifying, specifying and establishing plants, which are ecotype differentiated or adapted and tolerant of the mine waste environment (Bellitto *et al.*, 1999; Johnson *et al.*, 1994; Maiti, 2013; Odum *et al.*, 2000).

- Agricultural or forestry approach concentrates on standard methods of replacing the topsoil, fertilising and planting typical rehabilitation species to the disturbed land. However, topsoil is not always available or usable and importing suitable soils typically requires long, uneconomic haul distance (Bellitto *et al*., 1999; Maiti, 2013; Odum *et al.*, 2000).

Therefore, the ameliorative and adaptive approaches, which directly vegetates mines wastes without topsoil, are often desirable. Adaptive and ameliorative approaches are used in juxtaposition with one another or as separate approaches (Bellitto *et al.*, 1999; Johnson *et al.*, 1994; Maiti, 2013; Odum *et al.*, 2000). Quite a few approaches for plant-assisted growth in the mine waste environment exist within these approaches (Alvarenga *et al.*, 2008; Mendez *et al*., 2007; Mendez & Maier, 2008a; 2008b; Tordoff *et al.*, 2000; Valentín-Vargas *et al*., 2014 Wong, 2003).

2.3.2 Species used in revegetation rehabilitation techniques

Plant species selection is of importance (Alvarenga *et al.*, 2008; Arienzo *et al*., 2004; Bradshaw, 1997; Bradshaw & Hüttl, 2001; Conesa *et al*., 2007; Rizzi *et al*., 2004; Tordoff *et al*., 2000). Suitable species should have the ability to tolerate metal toxicity and poor fertility (Bradshaw, 1983; Bradshaw & Hüttl, 2001). Selection of suitable plant species is site-specific and affected by the local climate. Under dryland conditions, the revegetation of mine tailings necessitates plants that are salt-, metal-, drought tolerant (Grandlic, 2008) and does not accumulate trace metal contaminants in plant's shoot tissue (Mendez & Maier, 2008b; Solís-Domínguez *et al*., 2012). Preferably, indigenous, or locally acclimatised plants are considered the most suitable as they are adapted to semi-arid and arid environments (Conesa & Schulin, 2010; Mendez & Maier, 2008b). Revegetation usually includes the seeding of tolerant genotypes with the addition of compost to facilitate the revegetation of the TSFs. Using sensitive plant species is not viable under these conditions (Tordoff *et al*., 2000) and hence, selection of suitable plants is critical.

Pioneer species are frequently chosen based on their capability to grow in disturbed areas, inhabiting areas that are barren of plant life. Pioneer species possess the potential for improving degraded land, both in arid (Zhang *et al*., 2001), and polluted (Lei & Duan, 2008) environments. According to Zhang *et al*. (2001), the selection and disposition of pioneer plants improved the landscape and ecological conditions of pollution-associated degraded land. Furthermore, these pioneer species also play an essential role in supplying detritus and soil organic matter in the soil (Lei & Duan, 2008) that may help facilitate the creation of suitable conditions for succession and emergence of other plant species.

2.4 Microorganisms in mine waste and microbial-assisted rehabilitation

Various studies have signified the significance of soil microbial communities structure and their role in the successful plant establishment and growth (Ehrenfeld *et al*., 2005; Jeffries *et al.,* 2003; Kulmatiski *et al*., 2008; Requena *et al*., 2001). The problem, however, arises in the fact that the extreme soil conditions of mine tailings usually have deleterious effect on soil microbial diversity and microbial activity (Frouz *et al*., 2001; Liao & Xie, 2007; van Schoor, 2009). Several researchers have stated that mine rehabilitation can be lengthy due to limited microbial functional groups and activity and restricted plant growth (Jha & Singh, 1991; Mummey *et al*., 2002a; Nath, 2004; Visser *et al*., 1979; Visser *et al*., 1983).

Two alternative methods exist to facilitate revegetation of TSFs. The first approach is a popular approach that is widely used, in this method a great amount of soil organic amendments are used, including compost, biosolids and water (Chiu *et al*., 2006; Grandlic, 2008). Soil amendments have been widely used to facilitate TSF's stabilisation and assist soil biogeochemical development before vegetation establishment (Li & Huang, 2014). The ecological consequences of soil amendments on microbial community structure and microbial activity in mine rehabilitation and mine waste environment, however, are poorly understood (Li *et al*., 2017). The second method suggests the use of bio-stimulants and microbial inoculants to support plant establishment and development on TSFs.

Various researchers have proposed the use of microbial inoculants with plant growth promoting rhizomicroorganisms (i.e., rhizobacteria and beneficial fungi) to support plant growth on tailings (Grandlic *et al*., 2008; Mendez & Maier, 2008a; Mendez *et al*., 2008; Petrisor *et al*., 2004; Solís-Domínguez *et al*., 2011; Vivas *et al*., 2006; Zhuang *et al*., 2007).

Two PGPR isolation and inoculation techniques exist

- The first proposes the isolation and cultivation of native PGPR from plants naturally established on the tailings. With this technique site-adapted resilient microbes are cultivated ensuring a higher compatibility to the tailings materials; this technique has been successfully tested (de-Bashan *et al.,* 2012; Grandlic *et al*., 2008; Grandlic *et al*., 2009). Microorganisms in native tailings from local plant communities have the ability to tolerate in situ edaphic and climatic conditions with the same phyla reported in the rhizosphere of different plants (Li & Huang, 2014; Parraga-Aguado *et al*., 2013; Uroz *et al*., 2014). One disadvantage to this technique is the fact that it is a labour-intensive procedure.
- The second proposes the use of well-tested agricultural PGPR. This technique makes use of commercially available strains of PGPR to assist plant germination and growth on TSFs.

For example, agricultural strains of *Bacillus* used to promote crop cultivation in agriculture, assisting with plant growth on TSFs. In addition, *Bacillus* strains have been isolated in mine tailings materials (de-Bashan *et al.,* 2012; Tsuruta, 2007; Vijaya *et al*., 2011; Vijayalakshmi & Wu *et al*., 2006; Zhang *et al.,* 2007). One drawback of using this approach is the fact that the microbial strains used for agricultural purposes might not be site-adapted and have a low resilience to stressful environments, i.e., extreme pH variations, low nutrient status, steep slopes with severe windy conditions etc.

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CHAPTER 3

GENERAL METHODS AND MATERIALS.

"**The scientist does not study nature because it is useful to do so. He studies it because he takes pleasure in it, and he takes pleasure in it because it is beautiful. If nature were not beautiful it would not be worth knowing, and life would not be worth living.** I am not speaking, of course, of the beauty which strikes the senses, of the beauty of qualities and appearances. I am far from despising this, but it has nothing to do with science. What I mean is that more intimate beauty which comes from the harmonious order of its parts, and which a pure intelligence can grasp."

—**Henri Poincaré**, **Science and Method**

This chapter describes the general method design for this research, as well as recurring methods that were conducted in the research phases. Refer to relevant chapters, for detailed descriptions of methods and materials for each phase. In order to get a comprehensive study on the microbial activities as an integrated part of mine rehabilitation, the research entails both microbial activity baseline TSF's studies and nursery trials to improve the microbial activity.

The study, conducted during 2015 to 2017, entailed two phases.

- **Phase 1** Investigate soil enzymatic activities on various New Machavie gold TSFs, in order to acquire a comparative baseline study. To identify the soil enzymatic activity status of various other tailings materials compared to natural soils. Eleven mine waste materials and various natural soils were selected for this research phase.
- **Phase 2** Investigate methods of improving the revegetation of gold tailings materials, by means of bio-stimulants and selected mother crop species, were investigated. This phase entailed a nursery trial study, with various biological amendments and bio-stimulants applied to several mother crop species. The plant performance of the different treatments was evaluated in three different gold tailings materials and one control soil, as well as the subsequent changes in DHA.

A summary of each research phase can be seen in **Table 3-1**. For a detailed description of the different tailings materials used during Phase 1 and Phase 2 refer to **Chapter 4** site description.

Each chapter in this dissertation is an individual entity, consequently some repetition may occur. Gold TSFs' naming is different in Chapter 4 and Chapter 5, for similarities in TSFs naming, refer to **Appendix A-2**.

3.1 Research design

Table 3-1: Summary of the multi-phase layout of the research.

A- Baseline DHA titration.

Phase I- Microbial activity of different mine tailings and natural soils.

1. DHA of different mine TSFs and natural soils.
2. Soil enzymatic activities of different gold tailings
3. Physical and chemical characteristics of mine tailings and natural soils.
4. Correlate microbial activity with physical and chemical properties.

B- Biostimulant preparation.

C- Mother crop establishment.

Phase 2- Biological amendments and bio-stimulants effect on various mother crops.

1. 6x amendments includes: no amendments (control), K-humate, amino acids and metabolites, carbohydrate derivatives, beneficial microbes, and mixture.
2. DHA evaluated for untreated, biostimulants and other biological amendments.
3. Performance correlation between different bio-stimulants.
4. 4x members of Brassicaceae: rape, kale replaced by radish and canola, ryegrass (control) and no plants.
5. Plant performance monitoring including germination rate, mortality and survivability.
6. DHA after plant growth season was completed.
7. Correlation between plant species performance and DHA.

The substrates, bio-stimulants and mother crops used for each phase are explained in **Figure 3-1.**

3.1.1 Detailed research design and materials

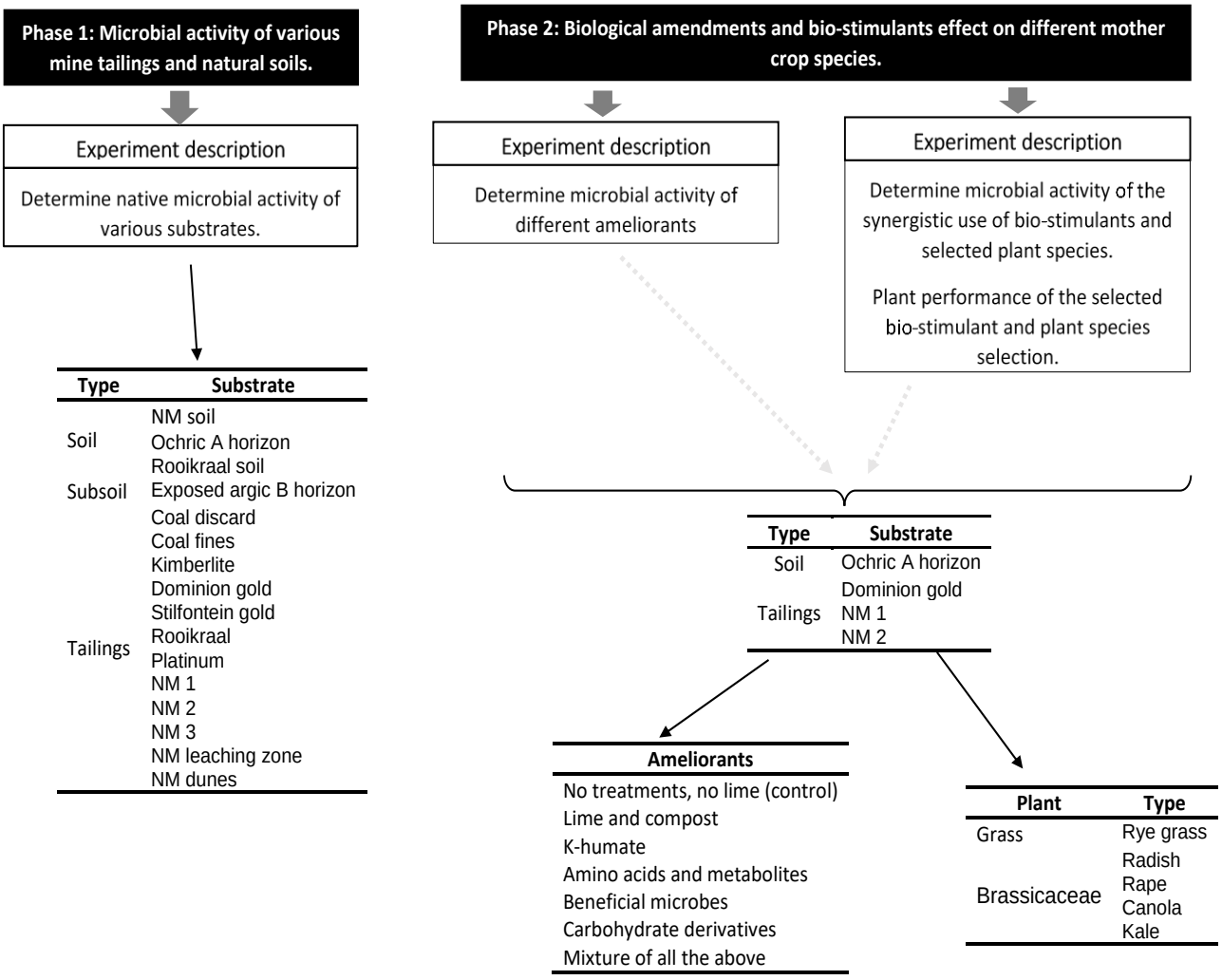

Figure 3-1: Nursery and baseline research design.

3.1.2 Baseline study

A baseline study was conducted, in order to get a comprehensive understanding of the soil enzymatic activities status in different TSFs. This study used DHA as an indicator, measured from barren TSFs sites and naturally vegetated rhizospheric zones. Detailed DHA sampling was conducted on New Machavies gold mine waste area (on top of TSFs, leaching zones, coppice dunes and footprints). The enzymatic activities (urease, β-glucosidase, acid phosphatase and alkaline phosphatase) of various gold tailings materials were also determined. DHA of other types of tailings materials, such as kimberlite, platinum, etc. as well as a range of natural soils was also determined. Refer to **Chapter 4** for detailed methods used.

3.1.3 Nursery trial experimental set up

A 24-month nursery pot trial was conducted to evaluate the effects of the different biological amendments and bio-stimulants on various mother crop species performance. The physical, chemical and biological parameters were evaluated during plant establishment in amended gold mine tailings. The experiment was prearranged in a completely randomised design comprising of four replicates in each of the bio-stimulant/mother crop combinations. Three gold tailings materials were chosen to represent gold tailings with a broad degree of acidity and lime requirements. An ochric red sandy soil was selected as control growth substrate. The control soil provided a homogenous seed-friendly growth substrate that possesses a more supportive soil condition. The experiment consisted of seven treatments and monitored for 2 years under irrigated nursery conditions (**Figure 3-2**). The experiment was carried out in 20L potting bags, consisting of four replicates of six different treatments (includes organic amendments, inorganic amendments and untreated controls). After 21 days of bio-stimulant application, four replica samples from each bio-stimulant were sampled for DHA assays. Mother crop establishment consisted out of four Brassicaceae family species, ryegrass and no plants. Nursery trial pots without any bio-stimulants or plants served as control. The trial was subjected to natural conditions and was irrigated regularly. Germination monitoring commenced seven days after the seed was sown and seedling emergence was recorded. A germination average was calculated from the sum of the four replicates per seed batch. Refer to **Chapter 5** for detailed methods used during the nursery phase.

Figure 3-2: Nursery pot trials with randomised treatment combinations.

3.1.3.1 Lime, fertiliser and compost requirements

The gold tailings selected for this research were sampled from the different mining sites and homogenised from the oxidised surface layer. The substrate for the experiments was collected to represent the heterogeneity of the tailing dam surface. The net acid potential and lime requirements analyses were done by GeoLab (Grond- en Omgewingslaboratorium), in accordance with standard methods stipulated Bloem (2017) and Usher *et al*. (2003). This method consists of two components:

- Active acidity (titratable acidity), that provides the lime requirement that is needed to raise soil pH(KCl) to neutral.
- Latent acidity (lime requirement[1] as per lime requirement results in **Chapter 4**), that relates to the acidity caused by future pyrite oxidation (Bloem, 2017). Method for latent acidity measurement was done in accordance to Usher *et al*. (2003).

For the gold tailings materials acid-base account and liming requirements, refer to **Table 4.3.** For successful establishment of mother crop species, i.e., Phase 2, the gold tailings materials were neutralised using dolomitic lime. The neutralisation period for the different gold tailings materials is dependent on the unique physicochemical characteristics. As such, the neutralisation of the gold tailings took place seven weeks prior to Phase 2 mother crop species establishment. For the neutralisation period (weekly change in pH) of each gold tailings materials, refer to **Appendix A-1.** As part of the standard rehabilitation protocol for vegetation establishment, the gold tailings were treated with organic amendments and fertiliser. Organic amendment was used in the form of compost. **Table 4.3** provides the compost requirement for the different tailings materials used. Fertilisation using inorganic fertilisers is a common method used to promote plant growth. Standard NPK fertilisers were applied using 5:3:4(33) N:P:K, and LAN (28) for the individual species. For fertiliser requirements for the mother crop species, refer to **Appendix A-3.**

3.2 Growth substrate analyses

Physical and chemical characteristics of the different growth substrates were analysed by Eco-Analytica®, an independent laboratory, using standard methods (Non-Affiliated Soil Analysis Work Committee, 1990; Soil and Plant Analysis Council, 1999). The first sampling was done during February 2016; both undisturbed control samples and mine tailings composites were taken of each substrate using a hand-operated auger.

For each of the chemical and physical analysis, appropriate pre-treatment requirements were met. Substrate samples were dried, sieved <2mm and analysed in accordance to standard

methods (Non-Affiliated Soil Analysis Work Committee, 1990). The chemical and physical analyses include the following:

1. Nutrient status of composite sample, exchangeable and active pH, electrical conductivity (EC), exchangeable cations (**Table 4-4**).
2. Anions (Cl^-, NO_3^- and SO_4^{2-}) from a saturated paste extract and cations (Ca^{2+}, Mg^{2+}, K^+ and Na^{2+}), cation exchange capacity (CEC) (**Table 4-4**).
3. Particle size distribution (**Table 4-2**).
4. Lime, acid-base account and compost requirement (**Table 4-3**).

In accordance with The Non-Affiliated Soil Analysis Work Committee (1990), and Soil and Plant Analysis Council (1999), the methods for the chemical and physical analyses can be summarised as follows.

- Extractable phosphorus was analysed using the P-Bray 1 extraction method, the phosphate content results were obtained by using an Ultraviolet-Visible Spectroscopy (UV-Vis).
- Exchangeable cations (Ca^{2+}, Mg^{2+}, K^+ and Na^{2+}) were determined by leaching method using ammonium acetate (1 mol/dm^3 and pH buffered at 7.0). With the use of an Atomic Absorption Spectrophotometer (AAS) results were obtained.
- Both exchangeable acidity pH(KCl) and actual acidity pH(H_2O) were determined by means of an electrometric method using a calibrated pH/EC multi-meter. The pH was determined using a ratio of 1:2.5 substrate samples to de-ionised water/KCl suspension on a mass basis.
- Electrical conductivity (EC) and pH values were determined simultaneously by means of saturated substrate paste with a calibrated pH/EC multi-meter.
- Soluble anions (SO_4-S, NO_3-N, Cl, F, and PO_4-P) were determined from saturated substrate paste, using an ion chromatograph.
- The CEC was analysed using the sodium acetate (1 mol/dm^3 and pH buffered at 7.0) method and subsequently calculated.
- Extractable and exchangeable micro-nutrient elements were quantified by means of a 0.02 mol/dm^3 $(NH_4)_2$ EDTA.H_2O solution and measured by making use of AAS.
- The %N, %C and %S was measured using the LECO approach.

- Total trace element concentrations were extracted using the HNO_3/H_2O_2 method (USEPA 3050b acid digestion) and measured via inductively coupled plasma mass spectrometry (ICP-MS).
- Quantification of particle size distribution of the substrate samples was conducted based on a method advocated by the Non-Affiliated Soil Analysis Work Committee (1990).

3.3 Enzyme activity

Eco-Analytica® assayed soil enzymatic activity, using appropriate standard methods unless otherwise stipulated (e.g., **Chapter 5**). For all enzymes assayed controls were done by substrate addition after incubation period, i.e., blank controls (Alef & Nannipieri, 1995). Soil moisture content was determined after air-drying substrate samples. **Chapter 4** provides detailed methods used to assay β-glucosidase, urease, acid phosphatase and alkaline phosphatase. Soil enzymatic activity was assayed in accordance to Alef and Nannipieri (1995); Dick *et al*. (1996) and Tabatabai (2000).

- Acid phosphatase (orthophosphoric monoester phosphohydrolase, buffered to pH 6.5) and alkaline phosphatase (orthophosphoric monoester phosphohydrolase, buffered to pH 11.0).
- Dehydrogenase activity (DHA).
- β-glucosidase (β-D-glucoside glucohydrolase).
- Urease (urea amidohydrolase) (Claassens, 2003; Claassens *et al*., 2008).

Soil dehydrogenase activity

DHA was determined with the substrate with iodonitrotetrazolium violet (INT) that is based on the method by von Mersi and Schinner (1991), refer to by Alef and Nannipieri (1995).

- Samples were mixed with [2-(p-iodophenyl)-3-(phenyl)-5-phenyl tetrazolium chloride] (INT) solution and incubated for 2hrs at 40°C.
- Reduced iodonitrotetrazolium formazan (INTF) was extracted with N, N-dimethylformamide and ethanol.
- Field-moist samples (1.0g) was measured into screw-cap Erlenmeyer flasks with 1.5ml Tris (hydroxymethyl)-aminomethane buffer and 2ml iodonitrotetrazolium chloride (INT) (0.5g/ml 2%(v/v) N,N'-dimethylformamide), and incubated in the dark for 2hrs at 40°C.
- Control substrate samples were prepared by sterilisation (1.0g samples, autoclaved at 121°C for 20min).

- After the 2hrs incubation period, samples were mixed with a 10ml extracting solution (N, N-dimethylformamide/ethanol in a 1:1 ratio).
- In order to extract the devolved INTF, the samples were kept in the dark for 1hrs, shaken at 20-minute intervals, and then the solution was filtered.
- The soil suspension was filtered and the filtrate (**Figure 3-3**) was measured spectrophotometrically at 464nm using the extracting solution as a blank.
- Pipetting 0, 1, 2, and 5ml of INF solution into test tubes and adding 13.5ml of extraction solution to each test tube prepared a calibration curve. Calibration concentrations were: 0, 100, 200, and 500µg INF per test (van Coller, 2011).
- The control reading was subtracted from the sample readings and compared with INTF standards
- As part of DHA determination, field-moist samples moisture was determined using the dry weight method. The method is based on removing soil moisture by oven drying the soil sample until the weight remains constant (von Mersi & Schinner, 1991).

Figure 3-3: Titration of DHA assays. The more orange the filtrates colour, the higher the DHA activity. A- low DHA; B-intermediar DHA; C- high DHA and D- control. The titrate collected as seen in Figure 3-3 are spectrophotometrically measured.

Soil dehydrogenase activity is expressed as g INF/g dry weight/2h and calculated as follows:

$$INF\left(\mu g\ g^{-1} dwt\ 2h^{-1}\right) = \frac{S_1 - S_2}{dwt}$$

Urease activity

Urease hydrolysing activity was assayed by the method of Kandeler and Gerber (1988), as described by Alef and Nannipieri (1995).

- The method is based on the release of ammonia after the soil samples were incubated at 37˚C with a urea solution for 2h, and colourimetric determined.
- Air-dried soil (5g) was mixed with 2.5ml urea solution and 20ml borate buffer and incubated at 37°C for 2h.
- After the incubation, 30ml of 1M potassium chloride solution was added and the flask was shaken for 30 min.
- After filtering the soil suspension through Whatman no.2 filter paper, the filtrates were measured spectrophotometrically using the extracting solution as a blank.
- Controls were prepared with 2.5ml distilled water and the urea solution was added at the end of the incubation, immediately before the addition of the potassium chloride solution.
- Soil moisture content was measured after air drying substrate samples (Alef & Nannipieri, 1995; Claassens *et al*., 2008).
- Urease activity was expressed as µg NH_4-N/g dry weight/2h.

β-glucosidase activity, phosphatase activity

β-glucosidase acid phosphatase and alkaline phosphatase activity were determined based on *p*-nitrophenol release and spectrophotometric detection (Tabatabai, 1982; Tabatabai, 2000; Tabatabai & Bremner, 1969). This method is based on the release of p-nitrophenol after cleaving to an artificial substrate (and *p*-nitrophenyl glucoside and *p*-nitrophenyl phosphate, respectively) (Alef & Nannipieri, 1995; Dick *et al*., 1996). •Acid phosphatase, alkaline phosphatase activity and β-glucosidase, are expressed as µg *p*-nitrophenol/g dry weight/h (Claassens, 2003; Claassens et al., 2008).

- For the β-glucosidase assay,1g soil (air dried) was placed in a 50ml Erlenmeyer flask and incubated for 1h at 37°C with 0.25ml toluene, 4ml modified universal buffer (pH 6.0) and 1ml *p*-nitrophenyl-*p*-*D*-glucoside.
- The reaction was terminated by the addition of 1ml 0.5M calcium chloride and 4ml 0.1M THAM buffer (pH 12.0).

- Controls were performed by adding substrate immrdiatcly after incubation, before the addition of calcium chloride and THAM buffer.
- The soil suspension was filteredthrough Whatman no. 2 filter paper and the absorbance of the filtrate was measured at 410nm.
- Phosphatase activity was assayed different from the above only in the choice of buffer. Modified universal buffers, pH 6.5 and pH 11.0 were used for acid and alkaline phosphomonoesterase, respectively.
- Soil moisture content was measured after air drying substrate samples (Alef & Nannipieri, 1995; Claassens *et al*., 2008).

3.4 Statistical analysis

Results of particle size, nutrient status and other chemical parameters were averaged and standard deviations of the means calculated. Chemical analyses data were used to determine lime requirements and fertiliser (N:P:K) requirements. Both the chemical and physical data were used as background data for the other phases.

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CHAPTER 4

PHASE ONE

MICROBIAL ACTIVITY OF DIFFERENT MINE TAILINGS AND NATURAL SOILS - A BASELINE STUDY

"I do not know what I may appear to the world, but to myself I seem to have been only like a girl playing on the seashore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me."

–**Isaac Newton**

Abstract

In many ways, South Africa's landscape has been dominated by mining, given that, for so many years this sector has been the mainstay of the South African economy. As such the environmental impacts of these mining activities are one of the greatest globally. Exploring the microbial enzymatic activity in gold tailings disposal environments, associated with different gold-bearing geological and lithological units, provides an opportunity to differentiate the impact of mining on associated ecosystems. The aim of this research is to exemplify the importance of soil enzymatic characteristics as part of mine TSF's rehabilitation assessment criteria. Soil enzymatic and physicochemical analyses properties were analysed by standard methods and the result emphasised that these mine tailings materials possess poor microbial enzymatic activities. The microbial activity of the different gold TSFs varied greatly. The baseline microbial activity levels of these tailings are dependent on the specific chemical, physical and biological parameters of the tailings materials. Generally, the soil enzymatic activity on the surface/rhizosphere zone was greater in the partially naturally vegetated tailings. In contrast and as anticipated, the barren tailings, associated with a more acidic pH, had the lowest soil enzymatic activity. Analytical results indicated, low soil β-glucosidase, urease, dehydrogenase, acid- and alkaline phosphatase enzymatic activities observed at these tailings sites, signifying that gold tailings materials possess poor microbiological enzymatic characteristics. Mine waste environments are devoid of true soil characters, deprived of macro and micro-nutrients, possesses an acidic pH with elevated concentrations of trace metal elements, and low microbial enzymatic activity. Consequently, it can be postulated that tailings materials quality and fertility of these sites are poor, because of inadequate microbial activity, due to insufficient biodegradable organic matter and incomplete nutrient cycling. Resulting in a high degree of degraded substrate, which not only affects the tailings materials' chemical and physical characteristic, but also the microbiological characteristics. It is therefore clear that in extreme environments, such as TSFs, the contribution that microbes make to the ecosystems functioning is vastly underrated. To conclude, the study

provides a microbial baseline status for different gold mine TSFs that will provide a platform for future investigations.

Keywords: *mine waste environment, microbial activity, ecosystem functioning, rehabilitation.*

4.1 Conceptualisation

The concept for a baseline status of a specific study or project is to make use of known data or values pertaining to such study or project, the relevant data can then be used for comparison purposes. In relative new or future research projects it is essential to establish a database of data or values that can be used to create the baseline status. In research projects where variations in the qualitative data are present, it is crucial to include this data for baseline status results. Although baseline data in natural sciences are absolute data, it is used rather in a relative sense associated with new research data. Numerous data sets exist for agricultural/natural soils, although microbial activity is not always well documented in these data sets.

In South Africa, data sets for mine tailings are scarce especially with regard to microbial activity. Datasets for gold mining tailings are almost non-existent, specifically as microbial activity in gold mine tailings is not a very common topic of discussion in natural sciences.

The focus of this report and data discussion is to establish a database and baseline status for soil enzymatic activity in gold mine TSFs. A condensed version of **Chapter 4** was published in the Applied Soil Ecology Journal; refer to **Appendix F** (Zanella *et al*., 2018) and **Appendix G** (Schimmer & van Deventer, 2018).

4.1.1 Introduction

In South Africa, TSFs are synonymous with environmental contamination, erosion and most are extremely difficult to rehabilitate. These mine tailings have various constraints (refer to **Chapter 2** for a detailed discussion) that prevent successful rehabilitation such as:

- Low nutrient status.
- Poor physical characteristics.
- Extremely acidic pH.
- Poor moisture-holding capacity.
- High salinity.
- Low soil microbial activity and microbial diversity.

Gold mine TSFs have poor physical, chemical and biological characteristics are responsible for very hostile conditions and in total a very low soil quality status. TSFs are hostile environments

and without appropriate rehabilitation for the establishment of plants and soil organisms, TSFs will forever be referred to as harsh environments. Soil organisms positively contribute to the modification of soil structure, creating new ecosystems and contribute mainly to plant germination, root development, and many other plant physiological activities.

Gold has been mined in South Africa for more than a century and as a result of poor mine rehabilitation performances, not a single gold TSF has received a mine closure certificate. More disturbing is the fact that South Africa has around six thousand discarded and derelict mines (Council of Geoscience, 2017).

Mine closure certificates are rarely issued. Difficulties arise from the fact that too many government departments are involved in mine closure (i.e., DMR, Department of Water Affairs (DWA) and DEA). DMR is reluctant to issue mine closure certificates as the mine companies environmental liabilities are transferred to the State. The mining companies are also reluctant to apply for mine closure, as the mining company cannot re-mine after closure (Genesis Analytics and Digby Wells Environmental Report for DMR/DEA/DPME, 2015; Milaras *et al*., 2014). The amount of 575 mine closure applications were under assessment during 2013 - 2014 of which only 159 were issued (Genesis Analytics and Digby Wells Environmental Report for Department of Mineral Resources (DMR)/Department of Environmental Affairs DEA/Department of Planning, Monitoring and Evaluation DPME, 2015).

4.1.1.1 Research question and motivation

Poor vegetation cover of rehabilitated gold mine TSFs and a lack of biological norms and standards put question marks behind current practices and specifications. A major lack of available literature on soil quality, specifically soil microbial activity on gold mines that have been rehabilitated, also contribute to the ambiguous specifications of rehabilitation projects.

Questions that evolved from the above mentioned are as follows:

- To what extent does soil microbial activity contribute to soil quality in mine rehabilitation?
- Is it possible to distinguish between microbial activity in natural soils and anthropogenic mine soils?
- Is there a difference in soil microbial activity between different anthropogenic mine soils?
- Lastly, is it worth spending time and resources on microbial activity investigations and research relating to soil quality in mine tailings rehabilitation?

4.1.1.2 Aims and objectives

This chapter will outline the baseline status of microbial activity of a few gold TSFs and compare it to natural soils and to a limited extent to other types of mine tailings. It will also emphasise the importance of quantifying soil microbial activity. This chapter is part of a larger research project which is undertaken to differentiate the soil quality of different tailings materials and to develop a framework to improve soil microbial activity and soil quality in mine rehabilitation.

The objectives of this chapter can be summarised as follows:

- Determine the soil enzymatic activity of gold TSFs and other mine tailings,
- Compare the data with data from natural soils,
- Demonstrate the degree of degradation present of different TSFs,
- Ultimately demonstrate the importance of microorganisms as a factor that needs to be taken into consideration when rehabilitating mine waste environments.

Emphasis is placed on the degree of differences in soil enzymatic activity of various gold mining TSFs, and to relate the microbial activities to the physical and chemical conditions of the tailings, as well as the performance level of vegetation projects.

4.1.2 Background

Abandoned pyrite-rich gold TSFs pose numerous threats to the environment and requires rehabilitation according to legislation. In the South-African rehabilitation industry, the establishment of vegetation is common practice. The primary aim of mine rehabilitation is to create surface stability and secondary to restore and re-establish a sustainable, functional ecosystem. One of the key challenges in mine rehabilitation is the successful establishment of a self-sustaining vegetative cover on the TSFs and disturbed areas. Presently, the standards for successful mine rehabilitation has mainly been constrained to physicochemical status, soil erosion and vegetation physiognomies. These environmental stresses induce profound changes and disrupting the functional stability of the microbial community. Criteria used to determine successful rehabilitation necessitate the integration of ecological principles. Present-day rehabilitation methods use narrow criteria sets for a few selected climax species and definite chemical characteristics that concentrate primarily on soil fertility, SOM and pH. Rehabilitation results proved that current criteria parameters to be totally inadequate.

Microorganisms influence most of the criteria used to determine rehabilitation success, yet they are not specifically included in any assessment criteria. It is widely known that microbial communities control essential ecosystem processes, yet microorganisms largely remain unrecognised in mining reclamation and rehabilitation. Microorganisms mediate important

ecosystem functions in the soil and thus the recovery of the soil microbial community is a critical step in achieving the goal of sustainable and beneficial tailings rehabilitation. In order to get a comprehensive understanding of mine TSF's environments and its rehabilitation, microbial processes need to be included in hypotheses, models, and interpretation of findings (van Deventer & Koch, 2016).

4.1.2.1 Mine waste characterisation

Research is necessary to study the mine TSF's environment to get a better understanding and background on mine rehabilitation and specifically on soil quality and microbial activity. Mine tailings characteristics, as well as a comparison between anthropogenic mine soils and natural soils, and microbial activity in these two substrates are discussed briefly.

South Africa is considered one of the most important mining countries in the world and produces large amounts of mine waste. Mine waste generation at different mines varies considerably in their properties due to the differences in the mineralogical composition of the mined ore/rock, distinctive mining method and metallurgical processing techniques.

Mine waste can be divided into two groups:

(i) Mine tailings, generated when processing the ore, and (ii) waste rock produced when uncovering the ore body (Dold, 2010; Ledin & Pedersen, 1996; Lottermoser, 2010).

Mine tailings are generally considered an environmental threat due to their impacts on air quality, groundwater quality, aesthetics and land use (Plumlee & Morman, 2011; Ritcey, 1989). The physical, chemical, mineralogical and microbiological aspects of the tailings will determine its impact on ecosystem functioning and stability (Ledin & Pedersen, 1996). Waste generation is interconnected to resources consumption and production activities within the mining industry and tends to increase with economic advancement. During the mining and processing of ore bodies, large quantities of overburden waste are generated. These materials are often the source of potential trace metal toxicity and pollution in the local environment, as a result of wind erosion (dust dispersal) and leaching of the mine waste products into water sources. Acidic mine waste contains large amounts of iron-sulphide minerals, such as pyrite and pyrrhotite (Dold, 2010) and associated with these iron-sulphide minerals are trace metal elements such as Cu, As, Ni, Pb, U, Cd, etc. The exposed TSF's oxidises the pyrite-containing tailings materials, consequently producing sulphuric acid and causing a highly acidic environment. Under these acidic conditions, the metal sulphides react to the sulphuric acid, leaching high concentrations of potentially toxic trace metal elements (e.g., Cu, Zn, Pb and Cd) into the environment (Dold, 2010; Jamieson, 2011; Walder & Chavez, 1995). AMD is the most frequent and widespread environmental problem

associated with gold and coal mining (Blowes *et al.*, 2003). Due to the oxidation of sulphide minerals and the formation of sulphuric acid, exposed gold tailings can possess a pH that ranges between 1.5 to 3.5 and lime requirement of as high as 1300 tonnes/ha (Alpers *et al*., 2003; Dold & Fontbonte, 2002). Most acidic mine wastes and tailings contain around 1-50g/kg potential toxic trace metal elements (e.g., Cu, Zn, Pb and Cd) (Boulet & Larocque, 1998; Walder & Chavez, 1995; Wong *et al*., 1998).

TSFs normally contain no SOM or macro-nutrients (Akala & Lal, 2001; Asensio *et al*., 2013; Barrutia *et al*., 2011; Krzaklewski & Pietrzykowski, 2002). Consequently, tailings are typified as having no soil aggregate structure and incline to have severely strained heterotrophic microbial communities (Mendez *et al*., 2007; Southam & Beveridge, 1992). As a result, the microbial community structure within the tailings tends to have a low species richness and species diversity and C utilisation diversity compared to natural soils (Moynahan *et al*., 2002). The microbial community that dominates most mine tailings tend to be autotrophic Fe- and S-oxidising bacteria. These microorganisms are associated with plant mortality in acidic tailings (Londry, 2005; Mendez & Maier, 2008; Schippers *et al*., 2000). Other threats that are associated with the actual mine tailings materials include difficulties to re-establish vegetation in the area, due to a combination of factors that includes: acidic pH, trace metal toxicity, poor soil aggregation, nutrient deficiency, low SOM content and stressed microbial communities (Grandlic *et al.*, 2009; Ledin & Pedersen, 1996; Munshower, 1994; Nordstrom & Alpers, 1999; Wong, *et al*., 1998).

Classification of mine tailings is included in many classification systems, i.e., Technosol in WRB, Inceptisols in Soil Taxonomy and “Technosol covering undisturbed natural soil - Human induced Technosol transported as a cover over a recognisable natural soil” in South Africa (van Deventer, 2016).

4.1.2.2 Natural soils and mine tailings

Some similarities exist between natural soils and mine tailings materials, for instance, both natural soils and mine tailings are subjected to pedogenesis i.e., oxidation, reduction, weathering and illuviation (van Deventer & Koch, 2016).

When comparing mine tailings characteristics to those of natural soils, distinct differences exist. The particle size of natural soils usually varies between sand- and clay-fractions (2.00mm - 0.002mm), whilst tailings material are uniform, having more than 80% fine sand and or silt. In some cases, the particle size may be similar; however, the particle-form differs. Tailings materials clay-fraction rarely contains any 1:1 or 2:1 clay minerals, as it comprises of finely crushed rock (>0.002mm). Subsequently, the clay fraction of tailings may carry no charge, resulting in the cation

exchange capacity being low, except for kimberlite tailings (Jones & Haynes, 2011; Lottermoser, 2010; van Deventer & Hattingh, 2004).

In the majority of natural soils, saline and acid conditions tend not to appear simultaneously. As saline soils have a pH above 7, however below pH 7 free-salts are absent. Free salts are indicative that all the exchange complex positions of the natural soil are filled and subsequently excess salts will not dissociate but will rather be present as free salts. In the case of tailings materials, the exchange complex is absent (lack of normal clay-fraction) therefore free salts can exist even in low pH conditions. In terms of pH, the tailings materials pool of acidity does not necessarily come from H^+ ions, but rather from inorganic acids such as sulphuric acid or hydrochloric acid (Dold & Fontbote, 2002). Four different acidity pools may attribute to low pH conditions in tailings (not just active and reserve acidity) (Hudson-Edwards *et al*., 1995; Kossoff *et al*., 2011; Weisener *et al*., 2011). The buffering capacity of most tailings is very low, due to lack of ion exchange, with the exception of kimberlite. Tailings rarely have any expansive properties due to the lack of 2:1 expansive clays (exception of kimberlite tailings that may contain 2:1 expansive clays in mineralogical composition) (Huang *et al*., 2012). Tailings materials are not easily mechanically compacted, as a result of their uniform particle size. Tailings are usually well graded whereas most natural soils are well sorted. In natural soils, the wide variation in particle size allows the smaller particle fraction to fill the pore spaces resulting in interlocking (Huang *et al*., 2011; Wehr *et al*., 2006).

Compared to agricultural and natural soils, mine tailings contain virtually no %C, attributable to the chemistry of ore and metallurgical extraction process. Many macro- and micronutrients are limited in tailings as nutrients are fixated or still in the primary mineral form, caused by the high redox potential (Akala & Lal, 2001; Asensio *et al*., 2013; Barrutia *et al.,* 2011; Krzaklewski & Pietrzykowski, 2002). The high redox potential causes drastic variability in pH and Eh. Phosphorus (P) and potassium (K) are consequently fixated. K is usually fixated into a new mineral, most common in gold tailings as K-Jarosites ($KFe^{3+}{}_3(OH)_6(SO_4)_2$) (van Deventer & Hattingh, 2004).

4.1.2.3 Microbial activity in natural soils and mine tailings

Microbiologists consider normal physiological conditions for microorganisms to typically be an aerobic atmosphere with no overpressure, with temperatures at around ± 37˚C, neutral pH, ± 1% salinity and glucose as main energy and C source (Sylvia *et al*., 1997).

The mine waste environment, however, does not fit into these “normal” conditions. In terms of microbiota, mine waste environments are considered an extreme environment based on the fact that there are certainly physical and chemical limitations to microbial cellular processes. These

physical and chemical limitations are typically at the end of gradients, for example, low and high-temperature, low and high pH and Eh, high salinity, high concentrations of potentially toxic trace metal element compounds, and extremely low nutrient concentrations (Mendez *et al*., 2007; Torsvik & Øvreås, 2008). The microbial diversity is more often lower in extreme soils than in natural soils. The microbial communities of extreme soil environments tend to reach unique equilibria, corresponding to either under- or overrepresentation of certain microbial community components (Torsvik & Øvreås, 2008).

Metallomorphic environments, such as mine TSFs sites, provide a unique habitat for microbial life (Wakelin *et al*., 2012). Soil enzymes are considered a group of enzymes that typically resides in the soil and are constantly playing an important role in supporting soil ecology, physical and chemical properties, fertility, and soil quality (Das & Varma, 2011). Soil enzymatic activity within the soil environment reacts fairly quickly to changes in the soil condition (Zhang *et al*., 2010) such as natural and anthropogenic disturbances (Karlen *et al*., 2003; McCarthy, 1994). Soil enzyme activities can be considered an important index of soil fertility and can reflect any changes in the soil quality (Bandick & Dick, 1999). It can serve as an early and sensitive indicator of soil ecological disturbances or rehabilitation performance in different ecosystems (Dick, 1996; Dick *et al*., 1994; Pascual *et al*., 2000). An early and sensitive indicator cannot be acquired with standard soil physical/chemical measurements and/or higher organism diversity analyses.

DHA fall within the Oxidoreductase enzyme class (Gu *et al*., 2009). Soil DHA is considered one of the most important among all the enzymes in the soil environment. DHA is considered a good indicator of the overall microbial activity in soil and relates to the total viable microorganisms within the soil environment (Gu *et al*., 2009; Salazar *et al*., 2011; Taylor *et al*., 2002; Quilchano & Marañon, 2002; Wolińska & Stępniewska, 2012). DHA take place intercellular in the soil in all living microbial cells (Moeskops *et al*., 2010; Wolińska & Stępniewska, 2012; Zhao *et al.,* 2010). Furthermore, DHA is strongly linked with microbial oxidoreduction processes (Moeskops *et al.,* 2010; Wolińska & Stępniewska, 2012).

4.2 Materials, methods and site description

To familiarise oneself with the difference between naturals soils and mine tailings, the materials, methods and site description of the different substrates are provided. As this study's focus is the rehabilitation of gold tailings more detail is given on gold mines. In order to obtain a baseline enzymatic activity of one mining site (New Machavie) detailed sampling was done, consequently, a more comprehensive description of New Machavie is given (refer to **4.2.1** site sampling and description).

4.2.1 Site sampling and description

Samples were obtained from nine TSFs of different lithological units and mineralogical composition (**Table 4-1**). Different mine tailings materials were used to compare the microbial activities of these tailings.

1. The tailings materials used for this project originate from three different gold mining operations. This includes:
    - Dominionville situated ± 24km from Klerksdorp in the North-West.
    - Gold tailings from a gold mining operation near Stilfontein in the North-West province.
    - New Machavie gold mining operation located ± 21km West of Potchefstroom in the North-West province.

These three gold mines are located on different lithological units in auriferous palaeoplacer deposits, i.e., Dominion Group, Witwatersrand - and Transvaal Supergroup. The Dominionville mine forms part of the Dominion Group. Gold ore of the Stilfontein area was mined on the Vaal Reef in the Klerksdorp Goldfield. This forms part of the Witwatersrand Supergroup, i.e., the Central Rand Group, which has provided the bulk of gold ore mined in the area. New Machavie is situated on the Black Reef Formation of the Transvaal Supergroup. The geological map with the location of the gold TSFs are presented in **Figure 4-1**.

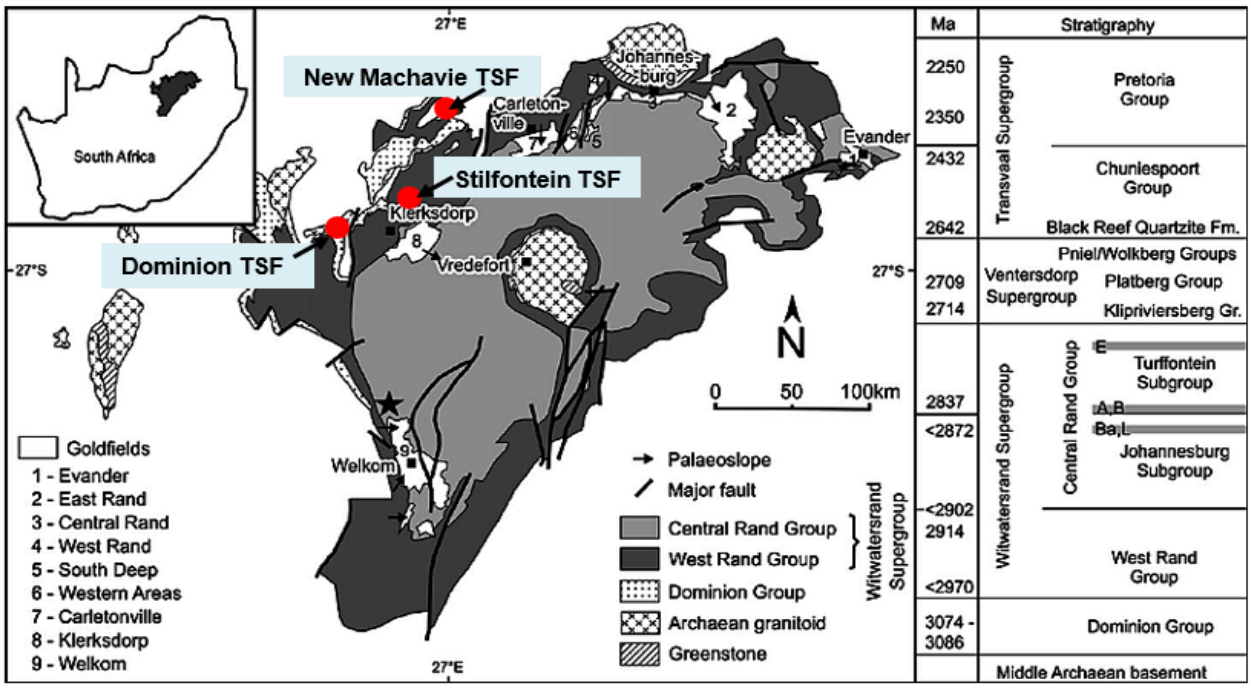


Figure 4-1: Geological map and stratigraphic column of the auriferous Witwatersrand Basin (after Koglin *et al*., 2010; adapted from Frimmel *et al*., 2009). Red markers indicate the position of the different gold mine TSFs mentioned in the text.

In addition to the gold TSFs, the following tailings materials were also sampled for comparison purposes:

1. Platinum tailings are from the Merensky and Upper Group No. 2 Reefs, which forms part of the Bushveld Igneous Complex.
2. Kimberlite tailings are from the Cullinan kimberlite pipe, which occurs within the stable, Kaapvaal Craton and intrudes rocks of the Transvaal Supergroup, Bushveld Igneous Complex and the younger Waterberg Group.
3. Coal fines and discard originate from the Witbank coal seam, which is situated in the northern sector of the Karoo Supergroup (Johnson *et al*., 2006).

These TSFs are situated in the climate region of the typical South African Highveld. In this region, the rainfall is typically between 300-700mm, annually. The Highveld region of South Africa typically gets their rainfall in the summer and during the dry winter months (May to August) frost occurs. The average maximum temperature for the summer months' ranges from 22°C to 34°C, while the average winter temperature is 16°C but can range from an average of 2 to 20°C in a single day. Summer temperatures exceeding 30°C and winter temperature below -2°C are common in the Potchefstroom and surrounding regions (Climate-Data, 2016).

Table 4-1: List of tailings used and tailings numbering system.

Tailings	Origin	Number	Geological environment
		Anthropogenic mine tailings	
Dominion Gold	Klerksdorp	Domreef	Dominion Reef placer conglomerates
Stilfontein Gold	Stilfontein	Stilfontein	Witwatersrand placer conglomerates
New Machavie TSF 1	Potchefstroom	NM-1	
New Machavie TSF 2	Potchefstroom	NM-2	Black Reef placer conglomerates, shales and quartzite
New Machavie TSF 3	Potchefstroom	NM-3	
Platinum	Rustenburg	Platinum	Bushveld Igneous Complex
Coal fines	Witbank	Coal fines	Karoo sediment coal deposits
Coal discard	Witbank	Coal discard	
Kimberlite	Cullinan	Kimberlite	Post Karoo kimberlite intrusive
		Natural soils	
NM-Soil Ochric A	New Machavie	NM-soil	
Ochric A horizon	Potchefstroom	Control ochric	Natural unpolluted soil
Exposed argic B	Potchefstroom	Nitisol argic B	

4.2.1.1 A detailed description of New Machavie gold mine

In order to demonstrate the difference in soil enzymatic activities on one mining sites TSFs, a detailed sampling was done on the New Machavie gold mine (**Figure 4-2**). Gold was mined at New Machavie from 1930 to mid-1940 which resulted in 5200kg gold and 5 TSFs (Aucamp, 2000; Botha, 2015; Koch, 2014; Schimmer *et al.,* 2015).

New Machavie TSFs was subjected to both wind and water erosion since its abandonment and the surrounding land is covered in tailings materials varying from 0.2m to up to 1.5m in certain areas. These TSFs were unable to "naturally" rehabilitate itself during the period of abandonment due to a combination of chemical and physical factors. New Machavie TSFs is situated on the Transvaal Basin that is made up of sedimentary rocks. These TSFs are found on the dolomite of the Monte Christo and Oaktree Formation. The Black Reef Formation outcrops near the tailings and is known for its low-grade sporadic palaeoplacer gold. Situated within the Malmani Subgroup, this quartz-auriferous deposit is associated with pyritic carbonaceous shales and cross-cutting veins (Eriksson *et al*., 2006). The Black Reef Formation consists of a succession of interbedded quartzites and carbonaceous shales with weak basal conglomerates (Coetzee, 1996). The weak basal conglomerate is followed by thicker quartzite and thinner shales, forming an upward-fining structure (Johnson *et al*., 2006).

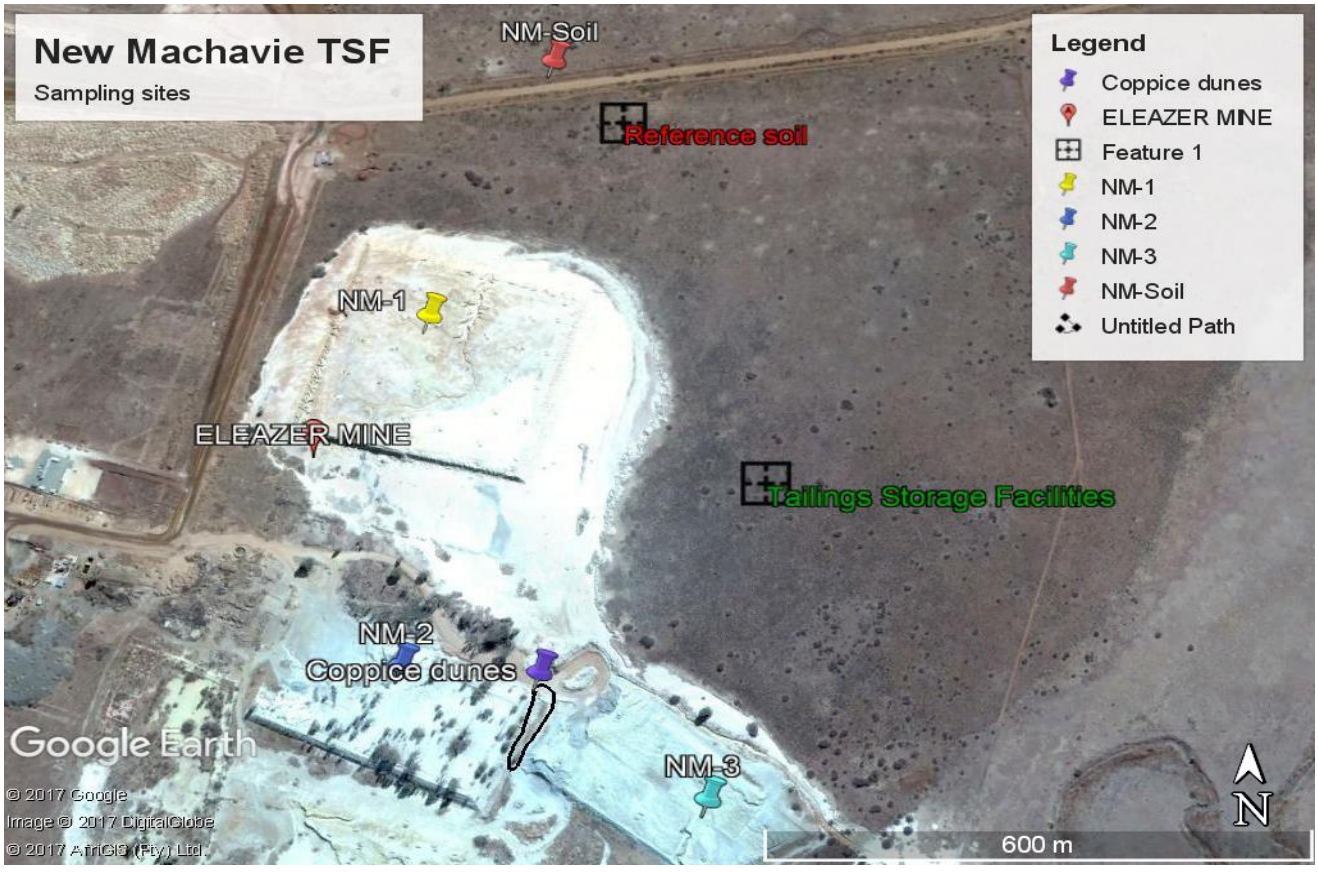


Figure 4-2: New Machavie TSFs and sampling areas (Google Earth, 2017).

4.2.1.2 Physical description of the different New Machavie TSFs.

After decades of neglect and abandonment, no vegetation has naturally established on New Machavie, apart from NM-2.

Iron-rich weathering products of pyrite (jarosite and goethite) indicate that the sulphide minerals in the gold tailings are actively oxidising (**Figure 4-3** and **Figure 4-4**).

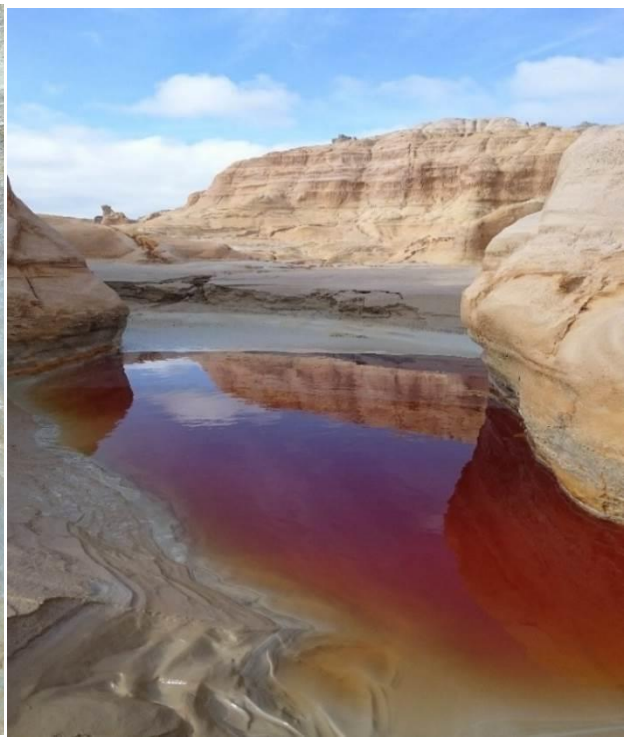
Figure 4-3: Shows the iron-rich weathering product of pyrite (A and B) yellowish colour indicates jarosite.

Figure 4-4: Formation of goethite. (A) The reddish colour shows the formation of goethite within the cracks in the tailings material. (B) Show the goethite layer developed as a crust on the weathered tailings surface. Photo credit to Angelique Daniels (B).

Goethite (**Figure 4-4**) is the most widespread mineral associated with AMD. AMD arises when sulphide-bearing material primarily pyrite, is exposed to oxygenated water. A common weathering product of sulphide oxidation is the formation of iron hydroxide [$Fe(OH)_3$], a red/orange coloured

precipitate and leachate found in streams and drainage systems. Clear signs of AMD can be seen at New Machavie (Aucamp, 2000; Botha, 2015; Koch, 2014; Schimmer *et al.,* 2015) (**Figure 4-5**).

A - NM-1 dry gully structure with AMD water



B - AMD seepage water NM-1

C - AMD seepage water on NM-1

D - NM-3 AMD seepage water stream

Figure 4-5: AMD seepage water. Characteristic red/orange colour AMD water can be seen at the different New Machavie sites. (A, B and C)-NM-1 dry gully-erosion structure with AMD, (D)-NM-3 seepage water and streams.

Particle size distribution, grading as well as illuviation and traffic, compaction and crusting are common and can be seen (**Figure 4-6**). Because of the compaction and crusting, the site has a low infiltration rate and a high runoff rate. In some areas where water runoff is present, stagnation and seepage are common phenomena. During dry seasons, the area is also susceptible to wind erosion due to the TSF's small PSD. As a result of abandonment and total lack of maintenance

practices huge dry gully-like erosion structures have formed all over the site (**Figure 4-7**). Above mentioned characteristics can be seen on all of the New Machavie TSFs.

A - Compaction and layering on NM-1 **B - Crust formation on NM-3**

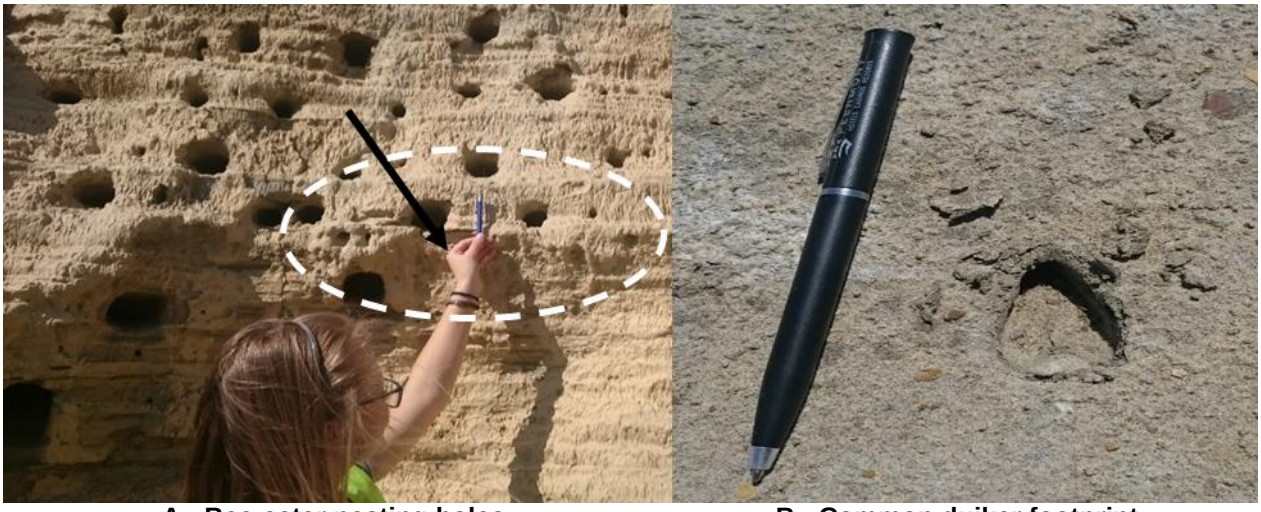
Figure 4-6: Signs of compaction and crust formation (A) NM-3 and (B) NM-1.

A - Dry gully-like erosion structures formed on NM-1 **B - Dry gully-like erosion structures formed on NM-3**

Figure 4-7: Severe erosion forming dry gully-like structures formed on (A) NM-1 and (B) NM-3.

4.2.1.3 Biota

New Machavie mining environment is considered as an extreme environment, potentially environmentally toxic, physically and chemically unbalanced, and ecologically unstable. However, even under these conditions animals inhabits the area. This includes a number of animals such as the bee-eater birds (**Figure 4-8**), Guinea fowl, and some small antelope species (prominently the common duiker). Most of these animals either inhabit the TSFs such as barn owls and bee-eater birds or graze/feed in the surrounding areas (barn owl and duiker).



A - Bee-eater nesting holes **B - Common duiker footprint**

Figure 4-8: Nesting-holes of bee-eater birds located on NM-2 (A) and duiker footprint (B) close to NM-3.

4.2.2 Sampling procedure

Two composite samples were indiscriminately collected from each of the TSF sampling sites. These composite samples consisted of four subsamples taken from the top 15cm of soil (Dick *et al*., 1996; Taylor *et al*., 2002). Sampling was carried out in spring (October 2016) as soil microbial activity is higher in this season. The samples were brought to the laboratory the same day, homogenised and kept refrigerated at 4°C to keep them field-moist and preserve biological characteristics pending analyses.

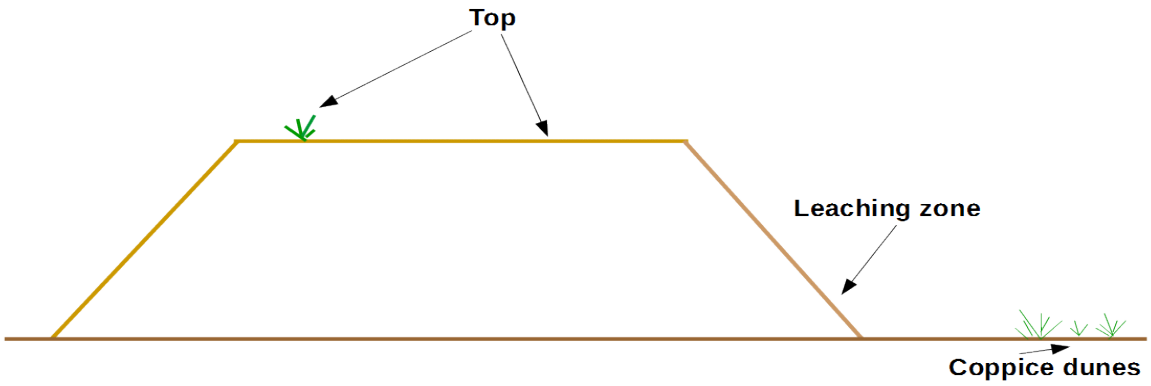


Figure 4-9: Shows the sampling design of each of the different TSFs.

Sampling was done at different site locations on and in the surrounding area of the New Machavie TSF. This includes sampling on top of each of the different TSFs, the coppice dunes and on the leaching zone (**Figure 4-9**). On NM-2 and NM-3 some self-established vegetation occurred, rhizospheric soil samples were taken from these plant species rhizospheres. On NM-3, a grass rehabilitation trial was conducted with the addition of a mixture of amendments. To compare natural vegetation establishment to assisted establishment, rhizospheric soil samples were taken from the established *Eragrostis curvula.* New Machavie has no irrigation and relies on dryland conditions for vegetation establishment. As New Machavie has no suitable control sites, a random site from the immediate area comprising - of fairly natural veldt, was chosen as a reference site and referred to as NM-soil. For the other mine tailings locations excluding New Machavie, homogenised composite site samples were taken. Each sample was characterised by analyses of the chemical and physical characteristics of the different tailings materials; and soil enzymatic activities.

4.2.3 Tailings and soil analyses

Physical and chemical substrate analyses were conducted by Eco-Analytica®, an independent laboratory, using standard methods (Non-Affiliated Soil Analysis Work Committee, 1990; Soil and Plant Analysis Council, 1999).

For each of the chemical and physical analysis, appropriate pre-treatment requirements were met. Substrate samples were dried, sieved <2mm and analysed according to standard methods (Non-Affiliated Soil Analysis Work Committee, 1990).

The chemical and physical analyses include the following:

- Extractable P was analysed using the P-Bray 1 extraction method, using UV-Vis.
- Exchangeable cations (Ca^{2+}, Mg^{2+}, K^{+} and Na^{2+}) were determined by leaching method using ammonium acetate (1 mol/dm^3 and pH buffered at 7.0), using AAS.
- pH(KCl) and pH(H_2O) were determined using a calibrated pH/EC multi-meter. The pH was determined using a ratio of 1:2.5 substrate samples to de-ionised water/KCl suspension on a mass basis. Electrical conductivity (EC) and pH values were measured simultaneously by means of a substrate saturated paste with a calibrated pH/EC multi-meter.
- Soluble anions (SO_4-S, NO_3-N, Cl, F, and PO_4-P) were determined from saturated substrate paste, using an ion chromatograph.
- CEC was analysed using the sodium acetate (1 mol/dm^3 and pH buffered at 7.0) method.
- Extractable and exchangeable micro-nutrient elements were quantified by means of a 0.02 mol/dm^3 $(NH_4)_2$ EDTA.H_2O solution using AAS.

- %N, %C and %S was measured using the LECO approach.
- Total trace element concentrations were extracted by the HNO_3/H_2O_2 method (USEPA 3050b acid digestion) and measured via inductively coupled plasma mass spectrometry (ICP-MS).
- Quantification of particle size distribution of the substrate samples was conducted based on a method advocated by the Non-Affiliated Soil Analysis Work Committee (1990).

4.2.4 Enzymatic activity assays

Soil enzymatic assays were done by Eco-Analytica®, using appropriate standard methods. Acid phosphatase (orthophosphoric monoester phosphohydrolase, buffered to pH 6.5) and alkaline phosphatase (orthophosphoric monoester phosphohydrolase, buffered to pH 11.0), β-glucosidase (β-*D*-glucoside glucohydrolase), urease (urea amidohydrolase) and dehydrogenase were assayed (Alef & Nannipieri, 1995; Dick *et al*., 1996; Claassens, 2003; Claassens *et al*., 2008; Tabatabai, 2000).

- DHA with the substrate INT is based on the method described by von Mersi and Schinner (1991). The DHA was expressed as µg INF/g dry weight/2h.
- Urease hydrolysing activity was assayed by the method of Kandeler and Gerber (1988), as described by Alef and Nannipieri (1995). The method is based on the release of ammonia after the soil samples were incubated at 37˚C with a urea solution for 2h, and colourimetric determined. Urease activity was expressed as µg NH_4-N/g dry weight/2h
- β-glucosidase, acid phosphatase and alkaline phosphatase activity were determined based on *p*-nitrophenol release and spectrophotometric detection (Tabatabai, 1982; Tabatabai, 2000; Tabatabai & Bremner, 1969). This method is based on the release of p-nitrophenol after cleaving to an artificial substrate (and *p*-nitrophenyl glucoside and *p*-nitrophenyl phosphate, respectively) (Alef & Nannipieri, 1995; Dick *et al*., 1996). Acid phosphatase, alkaline phosphatase activity and β-glucosidase, are expressed as µg p-nitrophenol/g dry weight/h.
- Soil moisture content was measured after air drying substrate samples (Alef & Nannipieri, 1995; Claassens *et al*., 2008).

4.2.5 Statistical analyses

The difference in enzyme activity, chemical and physical properties of each of the various tailings materials were evaluated by means of ANOVA.

- The quantitative data for each compound were analysed by both ANOVA and PCA using XLSTAT 2017 (Addinsoft, Paris, France).
- Tukey's HSD test for mean separation was done, statistical significance ($p = 0.05$) and variability between groups were indicated.
- Standard deviation (SD) values were calculated using Microsoft Excel and depicted in graphs as error bars.
- The Pearson's correlation coefficient (Pearson's r) was calculated. Positive correlation coefficients indicate that pairs of variables increase together, whereas negative values indicate an inverse relationship. For pairs with $p < 0.05$, there is a significant association between the variables.

4.3 Results and discussion

Results obtained from physical, chemical and enzyme activities of the various tailings materials are presented in the results and discussion section.

4.3.1 Physical characteristics

Particle size distribution was used as initial baseline parameter for physical properties. Results obtained for the physical characterisation of the different tailings materials and natural soils are summarised in **Table 4-2**.

Table 4-2: Particle size distribution for the various substrates.

Substrates	> 2mm	Sand	Silt	Clay	Texture Class
	(%)	(%)	(%)	(%)	(USDA, 2016)
		Gold, platinum, kimberlite and coal tailings			
Stilfontein Gold	0.2[a]	70.4[g]	24.7[g]	4.8[e]	Sandy loam
Dominion Gold	0.0[a]	68.8[f]	27.6[h]	3.7[c]	Sandy loam
Platinum	0.1[a]	92.6[k]	8.9[d]	2.1[a]	Sand
Kimberlite	13.6[c]	90.0[j]	7.5[c]	2.4[a]	Sand
Coal discard	0.2[a]	92.6[k]	5.2[b]	2.1[a]	Sand
Coal fines	0.0[a]	59.4[d]	20.9[f]	19.7[i]	Sandy loam
		Natural soils			
Exposed argic B	1.3[b]	48.8[b]	28.5[hi]	22.7[j]	Sandy clay loam
Arenosol ochric A	0.0[a]	92.6[k]	2.9[a]	4.3[d]	Sand
Arenosols NM-soil	0.9[b]	78.2[h]	9.7[d]	12.1[g]	Loamy sand
		New Machavie			
NM-1	0.0[a]	64.1[e]	29.3[i]	6.7[f]	Sandy loam
NM-2	0.0[a]	57.9[c]	35.3[j]	6.8[f]	Sandy loam
NM-3	0.0[a]	83.4[i]	13.2[e]	3.3[b]	Loamy sand
NM-leaching zone	1.3[b]	28.8[a]	55.5[k]	15.7[h]	Silty loam
Pr > F	<0.001	<0.001	<0.001	<0.001	

† Data in column with similar superscript alphabetic letters indicate no statistical significance p>0.05, in contrast, those with different letters show significant variance at Tukey's HSD p<0.05.

Sand/sandy loam is clearly the dominant soil textural class in most of the tailings materials with a few exceptions that include NM-3 and NM-leaching zone. In terms of physical constraints, the small particle fraction of the tailings materials, are prone to compaction and crusting. Subsequently, it leads to low infiltration rate and a high runoff rate. In terms of microbial activity and soil texture, Carney and Matson (2005) postulated that fine-textured soils support more microbial biomass compared to coarse-textured soils. The distribution of microorganisms in different soil textural classes might be related to soil moisture and nutrient status. According to Heritage *et al*. (2003), sandy soils tend to retain moisture poorly and are known to drain rapidly. Comparatively, clay loam has a high moisture-holding capacity and retains nutrient for longer (Heritage *et al*., 2003).

4.3.2 Chemical characteristics

Various chemical properties of the materials are used for the evaluation of DHA. It is common knowledge that soil pH (acidity and alkalinity) has influenced many soil reactions and soil quality indicators.

Therefore, it is necessary to neutralise the growth substrates to a pH that enables plant growth. The data in **Table 4-3** represent the analytical data of the "lime requirement" analysis of the materials.

Table 4-3: Lime requirements for the different substrates.

Substrates	pH(KCl)	pH(H_2O)	Neutral. pot. (t/ha)	Titr. acid (t/ha)	Acid pot (t/ha)	Lime req. (t/ha)	Compost required (t/ha)
Gold, platinum, kimberlite and coal tailings							
Stilfontein Gold	5.4^{e}	5.7^{e}	0^{a}	1^{b}	115^{i}	116^{i}	55^{d}
Dominion Gold	3.5^{c}	4.2^{d}	0^{a}	18^{f}	105^{h}	122^{j}	40^{c}
Platinum	8.0^{g}	6.6^{f}	34^{b}	0^{a}	2^{b}	-32^{b}	35^{b}
Kimberlite	7.6^{g}	9.7^{h}	121^{c}	0^{a}	0^{a}	-121^{a}	35^{b}
Coal discard	2.3^{a}	2.4^{a}	0^{a}	76^{h}	92^{g}	168^{k}	65^{e}
Coal fines	3.8^{cd}	2.5^{a}	0^{a}	103^{i}	152^{j}	255^{l}	55^{d}
Natural soils							
Ochric A	4.1^{d}	4.1^{cd}	0^{a}	6^{d}	0^{a}	6^{d}	30^{a}
Exposed argic B	6.6^{f}	7.4^{g}	0^{a}	0^{a}	0^{a}	0^{c}	30^{a}
NM-soil	5.7^{e}	6.5^{f}	0^{a}	0^{a}	0^{a}	0^{c}	30^{a}
New Machavie							
NM-1 gold	2.5^{a}	2.8^{ab}	0^{a}	22^{g}	41^{e}	63^{g}	40^{c}
NM-2 gold	3.6^{cd}	4.0^{cd}	0^{a}	16^{e}	25^{d}	41^{f}	40^{c}
NM-3 gold	2.8^{ab}	3.5^{bc}	0^{a}	21^{g}	54^{f}	75^{h}	40^{c}
NM-leaching zone	2.4^{a}	2.9^{ab}	0^{a}	141^{j}	294^{k}	435^{m}	40^{c}
NM-dunes	3.3^{bc}	3.8^{cd}	0^{a}	3^{c}	6^{c}	9^{e}	30^{a}
Pr > F	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

† Data in column with similar superscript alphabetic letters indicate no statistical significance p>0.05, in contrast, those with different letters show significant variance at Tukey's HSD p<0.05.

The lime requirement of the different substrates can be summarised as follows:

- The two alkaline tailings (kimberlite and platinum) both have a negative lime requirement as anticipated from the > pH 6.5 values.
- Natural soils, i.e., Arenosols, Nitisols and ochric A have a very low lime requirement due to low acidity reactions present and due to equilibrium in pedo-chemical conditions.
- Gold tailings and coal fines all have high net acid potential and lime requirement. Except for the coppice dune sand in the toe-paddock (NM-dunes) of the New Machavie TSFs. This is a secondary aeolian deposit derived from the TSFs. The vegetation cover is also an indication that the acidification is not as extreme.

Table 4-4: Chemical properties of the different tailings and soils.

Substrates	pH (H_2O)	EC	P	K	Ca	Mg	Na	Cl^-	CEC	SO_4	NO_3	Tot S	ESP
		(mS/m)	(mg/kg)	(mg/kg)	(mg/kg$^-$	(mg/kg$^-$	(mg/kg$^-$	(mg/l)	(cmol/kg)	(mg/l)	(mg/l)	(%)	(%)
Gold, platinum, kimberlite and coal tailings													
Stilfontein gold	5.7^{e}	286^{g}	4.9^{de}	19^{d}	1226.1^{f}	96^{d}	32.0^{c}	100.7^{g}	6.5^{f}	3808.2^{e}	168.6^{k}	0.3^{ab}	2.2^{d}
Dominion gold	4.2^{d}	820^{k}	1.3^{abc}	13^{b}	6142.5^{m}	252^{h}	2.5^{a}	5.9^{b}	9.2^{g}	24571.3^{h}	92.1^{i}	1.4^{e}	0.3^{a}
Platinum	6.6^{f}	218^{e}	6.0^{e}	26^{e}	325.4^{c}	33^{b}	64.0^{d}	549.4^{h}	2.2^{c}	1109.9^{d}	48.5^{g}	0.1^{a}	12.9^{e}
Kimberlite	9.7^{h}	108^{d}	4.0^{cde}	880^{i}	1695.6^{i}	226^{g}	1843.0^{e}	10.6^{c}	20.5^{j}	448.3^{c}	7.4^{a}	1.0^{cd}	39.1^{g}
Coal discard	2.4^{a}	1090^{l}	0.0^{a}	25^{e}	1324.0^{g}	156^{e}	22.0^{b}	7.0^{b}	0.6^{a}	114585.0^{k}	12.4^{b}	1.5^{e}	15.8^{f}
Coal fines	2.5^{a}	390^{h}	1.0^{ab}	25^{e}	2237.0^{k}	211^{f}	3.0^{a}	3.0^{a}	0.8^{a}	30340.0^{i}	13.3^{c}	1.4^{de}	1.5^{c}
New Machavie gold tailings													
NM-1 gold	2.8^{a}	1228^{m}	2.1^{abcd}	5^{a}	761.5^{d}	483^{j}	0.5^{a}	12.7^{d}	3.9^{d}	35155.6^{j}	15.6^{d}	0.7^{bc}	0.0^{a}
NM-2 gold	4.0^{bcd}	776^{j}	5.0^{e}	2^{a}	1946.0^{j}	376^{i}	4.0^{a}	16.5^{e}	5.1^{e}	14290.3^{f}	16.9^{e}	0.5^{ab}	0.4^{a}
NM-3 gold	3.5^{b}	734^{i}	12.6^{f}	14^{bc}	88.5^{a}	541^{k}	0.5^{a}	2.6^{a}	6.6^{f}	16184.4^{g}	115.8^{j}	0.7^{bc}	0.0^{a}
NM-leaching zone	2.9^{a}	34^{b}	1.0^{ab}	17^{cd}	2329.0^{l}	478^{j}	0.0^{a}	10.5^{c}	5.0^{e}	184207.0^{l}	56.0^{h}	3.3^{f}	0.1^{a}
NM- dunes	3.8^{bc}	259^{f}	2.0^{abcd}	4^{a}	123.0^{b}	20^{a}	1.0^{a}	-	3.58^{d}	-	-	0.1^{a}	0.1^{a}
Natural soils													
Arenosol NM soil	5.7^{e}	23^{ab}	17.4^{g}	267^{h}	971.1^{e}	215^{f}	29.5^{c}	4.1^{a}	11.1^{h}	20.8^{a}	42.3^{f}	0.1^{a}	1.2^{bc}
Ochric A horizon	4.1^{cd}	13^{a}	4.0^{cde}	62^{f}	88.0^{a}	49^{c}	1.0^{a}	3.4^{a}	1.4^{b}	31.1^{a}	48.8^{g}	0.0^{a}	0.3^{a}
Nitisol argic B horizon	7.4^{g}	77^{c}	3.5^{bcde}	149^{g}	1457.6^{h}	721^{l}	62.0^{d}	59.9^{f}	13.5^{i}	106.3^{b}	330.6^{l}	0.1^{a}	1.0^{b}
Pr > F	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

† Exchangeable cations: 1M NH_4-acetate pH=7; EC: Saturated Extraction; CEC: 1 M Na-acetate pH=7; pH (H_2O/KCl): 1:2.5 Extraction; Phosphorus: P-Bray 1 Extraction.
†† Data in column with similar superscript alphabetic letters indicate no statistical significance p>0.05, in contrast, those with different letters show significant variance at Tukey's HSD p<0.05.

Chemical characterisation results obtained for the TSFs sites and natural soils from various localities are summarised in **Table 4-4**. Based on the chemical characteristics, the following observations can be made.

- The gold TSF's possesses acidic pH(H_2O) and pH(KCl) whilst the opposite is noted for kimberlite tailings (**Table 4-3**). Plant growth and soil processes, including nutrient availability and microbial activity, are favoured by a soil pH range of 5.5-8. Low pH affects microbial activity observed in all the low pH TSF's DHA (**Table 4-5**; **Table 4-6** and **Table 4-7**).
- High sulphates dominate the tailings with the exception of the platinum and kimberlite.
- Natural soils, i.e., Arenosols, Nitisols and the ochric A-horizon have a marginal to low salinity (EC) < 50mS/m. The EC for the gold tailings ranges between 34 to 1228mS/m. The minimum EC value is present in the NM-leaching zone indicative of leached free salts.
- In the case of the acidic tailings materials, Al is soluble. In this form, Al retards plant root growth, restricting access to moisture and nutrients. In very acidic substrates (e.g., gold tailings and coal fines), all the major plant nutrients (N, P, K, S, Ca, Mn and trace element Mo) may be unavailable, or only available in insufficient quantities. The exchangeable macro elements vary greatly in the different materials.

4.3.3 Dehydrogenase activity of different New Machavie's TSFs.

DHA was obtained from different types of gold tailings as well as in a natural, undisturbed and polluted soil in the same vicinity. New Machavie, NM-3 rhizospheric substrate sample was taken from a research study done in 2014 by Taute (2014). The results are shown in **Table 4-5.**

Table 4-5: DHA (INF µg/g/2h) of New Machavie's TSFs.

TSFs sites	Bare/Rhizospheric substrates of grass	Horizon	DHA (INF µg/g/2h)
NM- 1	Bare zone	A	1.47^{a}
NM-2	Rhizospheric *Hyparrhenia hirta*	A	2.99^{ab}
		B	2.03^{ab}
	Rhizospheric *Cynodon dactylon*	A	34.67^{bc}
		B	1.01^{a}
	Bare zone	A	2.19^{ab}
	Rhizospheric *Eragrostis curvula*	A	75.73^{d}
NM-3	Bare zone	A	58.57^{cd}
	Leaching zone	A	75.52^{d}
Coppice dunes	Rhizospheric *Cynodon dactylon*	A	4.51^{ab}
		B	85.36^{d}
NM-soil	Rhizospheric *Hyparrhenia hirta*	A	124.73^{e}
P> F			< 0.001

† Data in column with similar superscript alphabetic letters indicate no statistical significance p>0.05, in contrast, those with different letters show significant variance at Tukey's HSD p<0.05.

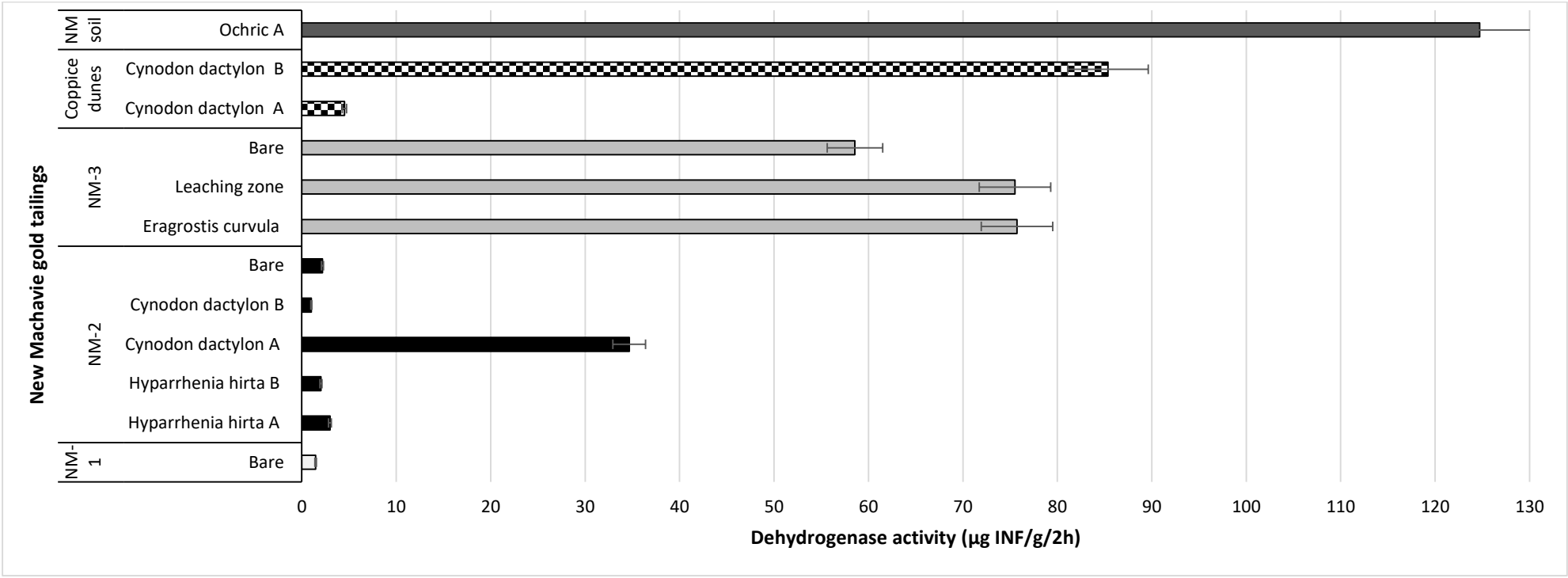


Figure 4-10: DHA of the different New Machavie gold tailings materials. This graph shows the DHA (µg INF/g/2h) for the different sampling sites on New Machavie, this includes the barren and vegetated areas.

When comparing different New Machavies TSF's a variation in DHA can be seen. Compared to the reference sites all the TSF's DHA is lower, indicating the anthropogenic disturbances/degradation effects of tailings materials on DHA. Unvegetated barren areas have the lowest microbial activity, with the exception of NM-3. When looking at the DHA of a single mine, such as New Machavie, one can see that the microbial activity can be highly variable. This variability in DHA can be attributed to the difference in chemical (**Table 4-3** and **Table 4-4**), physical (**Table 4-2**) and biological composition of each TSFs, which constraints both the microbial activity and successful vegetation establishment. Comparatively, NM-1 and NM-2 have the lowest DHA, these two TSFs are also the most problematic to rehabilitate.

Individual TSF's DHA is discussed separately below (refer to **Figure 4-10 to Figure 4-18**).

Figure 4-11: Inspection of coppice dunes. Representation of site with voluntary grass establishment with voluntary grass establishment and (B) Aeolian sand accumulation with coppice dunes in the background.

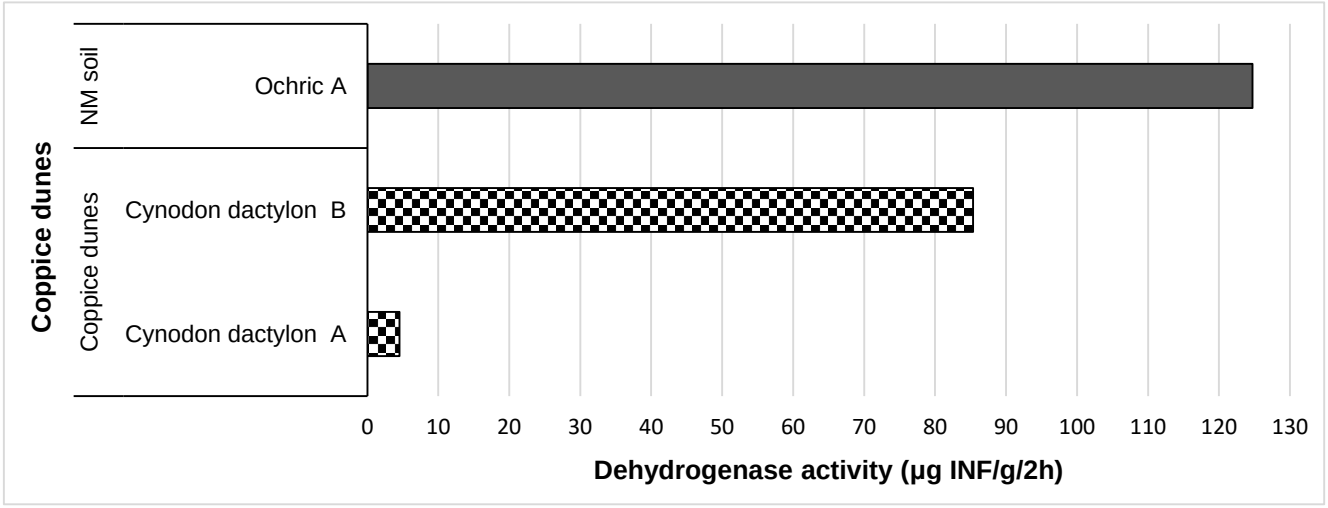


Figure 4-12: A comparison of the dehydrogenase activity (INF µg/g/2h) of the coppice dunes compared to those of the reference soil (NM soil).

4.1.1.1 Coppice dunes

Figure 4-11(A): Two dominant grass species, voluntary and well established, on the coppice dunes.

Figure 4-11(B): Aeolian sands (shown by arrow) compared to A-horizons (TSF deposition) at coppice dune.

Figure 4-12: Microbial activity and microbial richness declines with depth (Taylor *et al.*, 2002). This however is not the case with the coppice dunes; the DHA on the B-horizon is much higher compared to A-horizon. This is due to the B-horizon comprising of more Aeolian sands and the A-horizons of more tailings materials.

Note: Coppice dunes seem to be ecosystem functionally stable with sustainable vegetation establishment.

Figure 4-13: Inspection of NM-1. Representation of NM-1 site: barren of vegetation since abandonment with severe erosions.

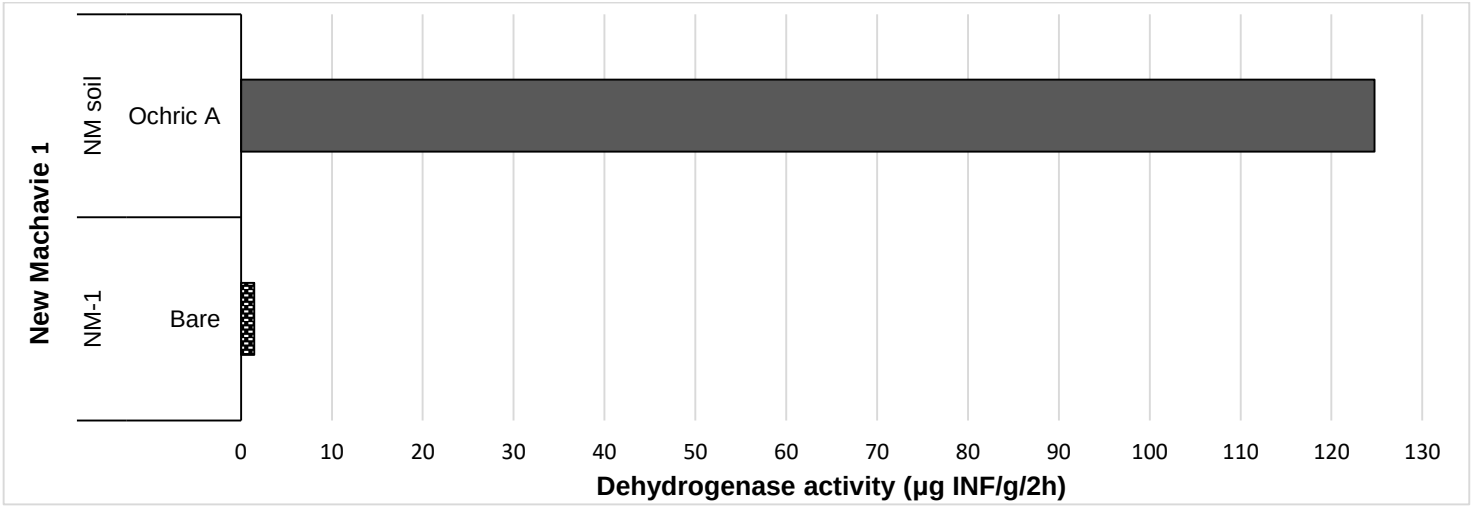


Figure 4-14: A comparison of the dehydrogenase activity (INF µg/g/2h) of the NM-1 compared to those of the reference soil (NM soil).

4.1.1.2 NM-1

Figure 4-13: NM-1 is the most environmentally unstable TSF. Having no plant growth, severe erosion, the worst physically (**Table 4-2**) and chemically properties (**Table 4-4**) compared to the rest of New Machavie TSF.

Figure 4-14: NM-1 has a very low DHA compared to NM-soil, resulting in a poor substrate quality/degradation.

Note: Characteristics of NM-1 indicates that it can be considered as a hostile environment and functionally unstable.

Figure 4-15: Physical inspection of NM-2. Sparse growth of two grass species on top of NM-2 (top left A) and barren areas with invasive Blue Gum tree (Eucalyptus) (top right B).

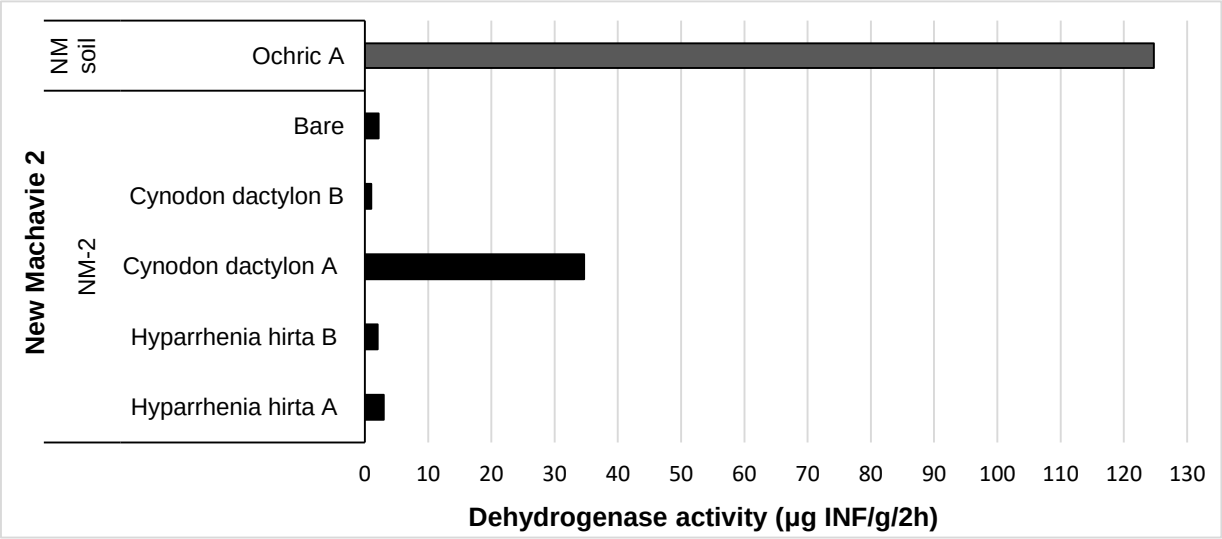


Figure 4-16: A comparison of the DHA (INF µg/g/2h) of NM-2 compared to those of the reference soil (NM soil).

4.1.1.3 NM-2

Figure 4-15(A): NM-2 TSF has two grass species voluntary established on this site**,** i.e. common thatching grass (*Hypharrenia hirta)* and couch grass (*Cynodon dactylon).*

Figure 4-15(B): NM-2 TSF has barren areas and some areas with invasive eucalyptus trees**.** NM-2 TSF produced the highest voluntary plant establishment and specie richness.

Figure 4-16: Assessed to other TSF, NM-2 is also chemically (**Table 4-4**) and physically (**Table 4-2**) less hostile. The two different grass species differ in DHA, with couch grass having the higher DHA. Both grass species on the A horizon is higher in DHA compared to the B-horizon.

Note: In terms of ecosystem stability and functionality NM-2 TSF is less environmental hostile and sustains vegetation establishment.

Figure 4-17: Representation of the NM-3 site. NM-3 is barren (A) and severe donga erosion (B).

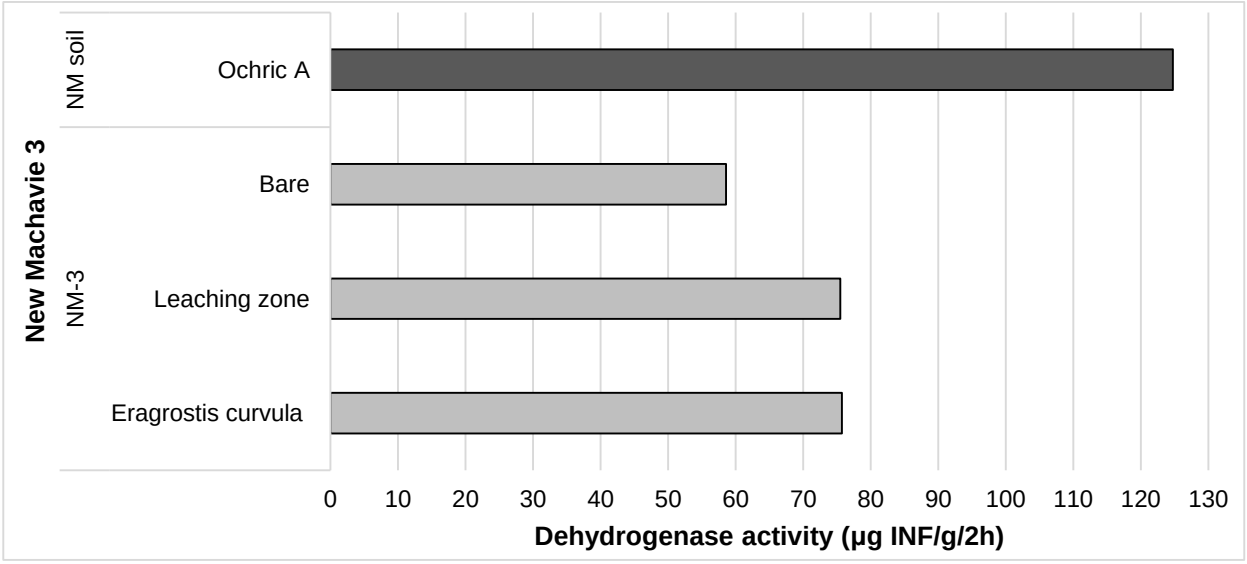


Figure 4-18: A comparison of the DHA (INF µg/g/2h) of the NM-3 compared to those of the reference soil (NM soil).

4.1.1.4 NM-3

Figure 4-17: At NM-3 TSF a combination of chemical (**Table 4-4**) and physical (**Table 4-2**) properties have constraint the proper rehabilitation of this site. No voluntary grass establishment has taken place. Although a pilot study done a year ago still remains with stunted grass growth.

Figure 4-18: When comparing the DHA, NM-3 has the highest activity of all the other TSF. This can be attributed to a high amount of inorganic carbon and pyrite concentration within this TSF. Consequential a greater number of sulphate oxidisers and carbon users is present. The leaching zone has a larger surface that is exposed to oxidizing conditions resulting in higher DHA compared to barren sites.

The average DHA assayed are presented in **Table 4-5**. By comparing the different TSF's DHA one can see a difference. The highest DHA is present at the reference site (NM-soil=125 µg/g/2h) (**Table 4-5**). This reference site represents an agricultural, natural soil adjoining the New Machavie mine. In terms of the soil DHA of the TSFs, the highest DHA can be seen on the coppice dunes B-horizon (**Figure 4-12**=85 µg/g/2h).

The bare areas of the different TSF's represent sites without any humic materials and plant organic waste, subsequently, the %C content of these sites could be used as a proxy for microbial biomass that represents an oligotrophic environment. In general, these barren sites have very low microbial activity (**Table 4-5**). These sites have the lowest %C content (**Table 4-4**), as well as the lowest percentage ground and crown vegetation coverage.

The microbial activity of the sparsely vegetated zones is higher compared to those of the bare areas and is probably related to the higher %C content of these sites. Aon and Colaneri (2001) and Aon *et al.* (2001), affirmed that plants influence microbial activity and diversity, particularly in the rhizospheric zone. This implies that the high microbial activity may be a consequence of plant C input (SOM), indicating a plant-driven organisation of soil microbiology and biochemistry (Aon *et al.,* 2001; Claassens, 2003; Claassens *et al*., 2008). Consequently, it can be assumed that the soil quality and fertility of the barren sites of the different New Machavie TSFs are lower because of decreased microbial activity due to insufficient biodegradable SOM and inadequate nutrient cycling. The opposite applies to the vegetated zones of the different TSFs sites with higher DHA.

A Principe Component Analysis (PCA) ordination diagram demonstrating the association among the chemical, physical and DHA of the different New Machavie TSFs is presented in **Figure 4-19**. The eigenvalue for the first two ordination axes of the PCA were 0.533 and 0.235, respectively. These two axes accounted for 76.8% of the total observed variance.

Based on these results, it can be concluded that the physical, chemical and DHA characteristics of a single mining site, such as New Machavie, can vary remarkably.

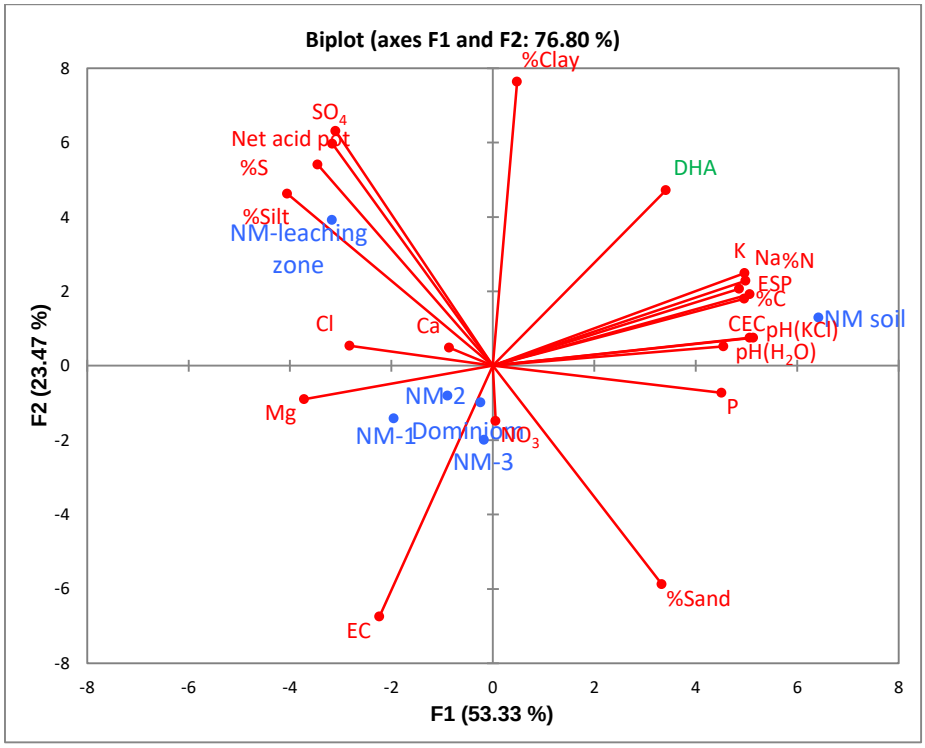


Figure 4-19: Principe Component Analysis (PCA) ordination diagram of the microbial, physical and chemical characteristics of the different New Machavie gold tailings samples.

A positive association exists (significance <0.05) between pH(H_2O) (r0.994), pH(KCl) (r0.994), potassium (r0.908), CEC (r0.885), ESP (r0.972), %N (r0.913) and %C (r0.903) which emphasises the necessity of C and N, even at low concentrations that can serve as an energy source for various microbial groups.

A negative association was also apparent between salinity (EC) and DHA (r-0,868; p<0.05), assayed during the study. These results are in accordance with the results obtained by García and Hernández (1996); Frankenberger and Bingham (1982); Rietz and Haynes (2003) and Tripathi *et al*. (2007), that an increase in soil salinity inhibits DHA. Salination is an abiotic soil factor, which is considered as a major constriction to soil fertility and affect vegetation establishment. Soil EC influences plant responses by altering the diversity and composition of the microbial community present and consequently their enzymatic activity (Singh, 2016; Yan *et al*., 2015; Yuan & Yue, 2012). The altered and/or reduced microbial activities are likely due to either the direct toxic effects of high saline concentrations on microbial communities (Rietz & Haynes, 2003) or a negative consequence of salinity on vegetation (Wong *et al*., 2010). The toxic effects of salinity on plants lead to diminished SOM input (residue, litter and roots), and as a result drastically reduces microbial activities (Singh, 2015; 2016).

4.3.4 Soil enzymatic activity of different tailings and natural soil.

Table 4-6: DHA of other tailings materials and soils (Ferreira, 2015; Zanella *et al*., 2018).

Substrate	DHA (µg INF/g/2h)	Ferreira (2015) DHA (µg INF/g/2h)	Average DHA (µg INF/g/2h)
Platinum	6	2	4.0[d]
Stilfontein gold	5	1	3.0[d]
Dominion gold	4	n/a	2.7[d]
Rooikraal gold	2	-	2.3[d]
Cullinan Kimberlite	34	9	21.5[d]
Coal fines	101	n/a	110.5[b]
Coal discard	68	n/a	71.5[c]
	Natural soils		
Ochric NM A-horizon	120	115	117.5[b]
Rooikraal soil	351	-	351[a]
Exposed argic B-horizon	10	14	12.0[d]
Granite saprolite	n/a	4	4.0[d]
Aeolian Kalahari sand	n/a	4	4.0[d]
Ottosdal kaolinite clay	n/a	7	7.0[d]
Pr > F			< 0.001

† INF: iodonitrotetrazolium chloride-formazan; DHA: dehydrogenase activity.
†† Data in column with similar superscript alphabetic letters indicate no statistical significance p>0.05, in contrast, those with different letters show significant variance at Tukey's HSD p<0.05.

Table 4-6 and **Figure 4-20,** represents data collected from Zanella *et al*. (2018). A full description of the DHA of natural soils and other types of tailing materials is available in Humusica 2, Article 16. This table is a comparison between the DHA of different acid and alkaline tailings materials to those of natural soils. When comparing the DHA of natural soils to tailings materials, natural soils tend to have a higher microbial activity. Low microbial activity is not unique to gold tailings materials, rather most other tailings materials, such as platinum, kimberlite, etc. display low DHA when compared to natural soils. However, exceptions do exist e.g., coal fines and coal discard (refer to **Table 4-6).** The low DHA within the different tailings materials demonstrates the degree of soil ecological stress associated with anthropogenic soils.

In terms of natural soils, sub-horizons have lower microbial activity compared to A-horizons. Ferreira (2015), compared the DHA and C/N ratio of certain substrates. Some of the substrates used for this study comes from the same localities as Ferreira (2015) and are included for comparisons. The degree of microbial activity variation within the natural soils and tailings

materials provide insight into the soil quality and health (i.e., the degree of degradation). Like most natural soils, the reference ochric A-horizon (**Table 4-6**=115µg INF/g/2h) has a much higher microbial activity compared to that of tailings materials. Rooikraal-soil's DHA supports these findings, i.e., naturals soils possess higher microbial activity. The high DHA of the Rooikraal soil may be attributed to the high amount of %C present in the reference area surrounding the Rooikraal TSF. For a detailed description of Rooikraal TSF refer to Kruger (2017). The B-horizon soils include the granite saprolite, Kalahari aeolian sand (ochric A-horizon) and Ottosdal kaolinite clay. These B-horizon soils have a lower DHA compared to those of the A-horizon. These findings correspond with previous results by Blume *et al*. (2002), Fierer *et al*. (2003) and Šantrůčková *et al*. (2010), concerning soil depth and microbial activity. The exposed argic B-horizons lower microbial activity relates to the argic B-horizon being exposed and subsequently indicates a degree of degradation that the horizon underwent.

In general, factors such as pH, nutrient status, SOM tend to change with depth (Aon & Colaneri, 2001; Claassens *et al.,* 2008; Eilers *et al*., 2012; Rumple & Kögel-Knabner, 2011; Taylor *et al*., 2002). Along this gradient microbial biomass also tend to decline (Blume *et al*., 2002; Fierer *et al*., 2003; Šantrůčková *et al*., 2010), additionally a shift in the microbial community composition occurs (Eilers *et al*., 2012; Ekelund *et al*., 2001; Hartmann *et al*., 2009).

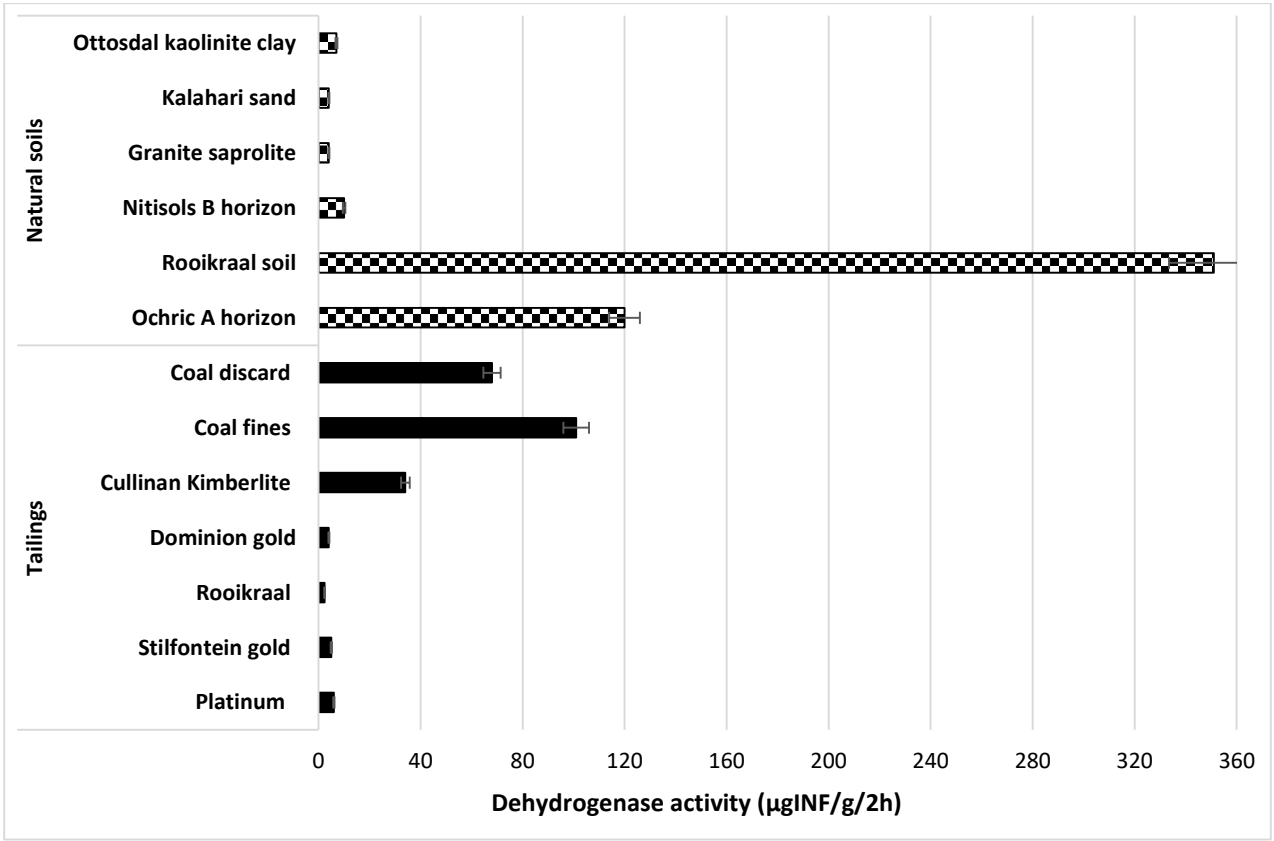


Figure 4-20: Comparison of the DHA of different tailings materials with natural soils.

Table 4-7 Soil enzymatic activities of different gold tailings materials.

Substrates	Dehydrogenase	β-glucosidase	Alkaline phosphatase	Acid phosphatase	Urease
	(µg INF/g/2h)	(µg PNP/g/h)	(µg PNP/g/h)	(µg PNP/g/h)	(NH_4-N µg/g/2h)
NM-1	74.42[a]	0.00[b]	1.40[b]	3.84[a]	6.50[c]
NM-2	1.76[b]	1.02[a]	8.16[a]	0.42[b]	54.15[b]
Dominion	1.31[b]	1.15[a]	2.79[b]	0.49[b]	138.61[a]
Ochric A	120.00[b]	1.29[a]	1.19[b]	0.14[b]	136.45[a]
Pr > F	< 0.0001	0.003	0.001	< 0.0001	< 0.0001

† INF: iodonitrotetrazolium chloride-formazan; PNP: para-nitrophenol.
†† Data in column with similar superscript alphabetic letters indicate no statistical significance p>0.05, in contrast, those with different letters show significant variance at Tukey's HSD p<0.05.

The average activities of enzyme assayed are presented in **Table 4-7**. Enzymatic activities assayed showed an overall statistically significant difference (ANOVA p<0.05) between the different gold tailings materials. DHA was measured as an estimation of overall microbial activity. Acid-, alkaline phosphatases, β-Glucosidase and urease, and are enzymes that carry out specific hydrolyses and catalyse reactions involved in the biogeochemical transformations of C, N, and P, respectively. β-Glucosidase activity is very useful for the monitoring of soil ecosystems changes as it plays a key role in SOM cycling, it is seldom substrate limited and it is the most abundant of the three enzymes involved in cellulose degradation (Claassens, 2003; Claassens *et al*., 2008; Taylor *et al*., 2002). Urease and phosphatase activities were assessed because of their significance in the N and P biogeochemical cycles. Soil enzymatic activities respond relatively quickly to small changes in soil conditions (Zhang *et al.,* 2010), thus reflecting changes in soil quality before detection by physical and chemical soil assessments (Gómez -Sagasti *et al*., 2012; Izquierdo *et al*., 2005).

The low DHA, β-glucosidase, urease, acid phosphatase and alkaline phosphatase activity observed at all the gold mining sites indicates an overall low microbial activity for these sites. Consequently, it can be assumed that the soil quality and fertility of these sites are low as a consequence of low microbial activity, due to the deficit biodegradable SOM and limited nutrient cycling. The low soil enzymatic activity of these gold tailings brings current rehabilitation approaches into question. Firstly, the necessity of microbiological properties as monitoring assessment criteria for rehabilitation performance/quality. Secondly, the integration of biotic factors into rehabilitation specifications.

Natural soils, in general, have a higher soil enzymatic activity compared to tailings materials. This was not the case with the ochric A-horizon, with the exception of DHA. The β-glucosidase, acid and alkaline phosphatase activities are either equal to or below the gold tailings materials activity. The lower microbial activity of the natural soil may be attributed to the physical and chemical characteristics of the ochric soil, i.e., low moisture holding capacity and unfavourable C/N ratio (Xiang *et al*., 2008). Previous research has also indicated that a relationship exists between barren/decrease vegetation establishments and reduced soil microbial activity (Claassens *et al*., 2008; García *et al*., 2002; Lange *et al*., 2014) as is the case with the gold TSFs presented in this research.

A Principal Component Analysis (PCA) ordination diagram demonstrating the association between the dominant environmental variable and the soil enzymatic activities for different gold mining sites can be seen in **Figure 4-21**. The eigenvalue for the first two ordination axes of the PCA were 0.520 and 0.264, respectively. These two axes accounted for 78.4% of the total observed variance. Based on these results, it can be concluded that the physical, chemical and soil enzymatic characteristics of gold mines can vary remarkably.

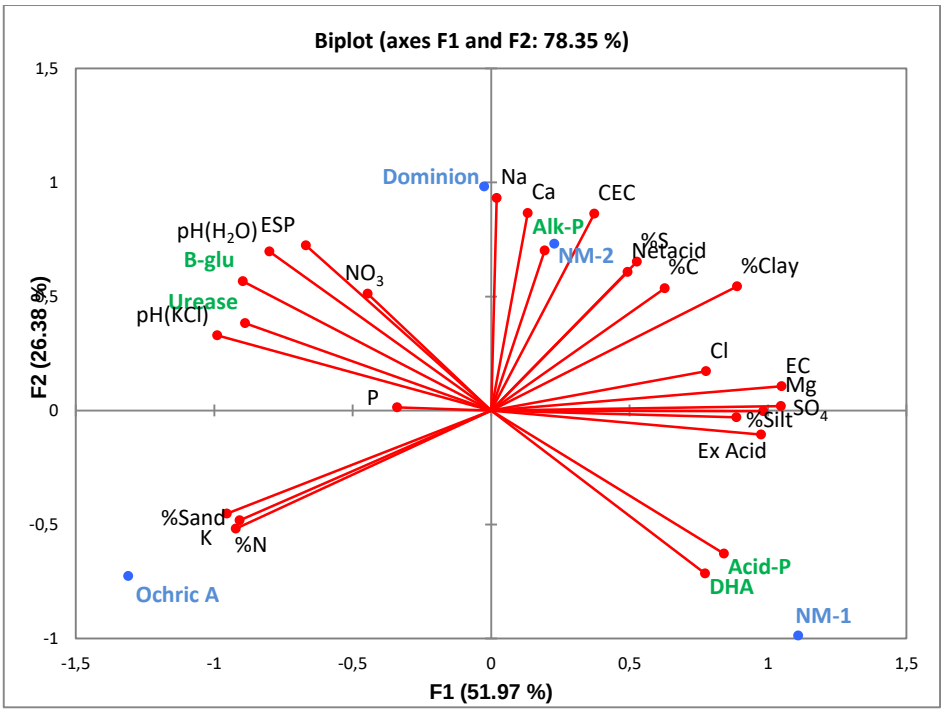


Figure 4-21: Principe Component Analysis (PCA) ordination diagram of the microbial, physical and chemical characteristics of different gold tailings samples.

Based on the results obtained, it is evident that the dominant chemical, physical and microbial characteristics of gold mining sites varied significantly between sites. When soil enzyme activities were correlated to each other, significant correlations were found between the DHA and acid phosphatase (r0.994; $p<0.05$). A negative correlation existed between DHA and β-glucosidase

activity (r-0.979; $p<0.05$), as well as between acid phosphatase and β-glucosidase activity. Furthermore, β-glucosidase activity strongly associated with both pH(H_2O) (r0.988; $p<0.05$) and pH(KCl) (r0.961; $p<0.05$).

The DHA and acid phosphatase activity were negatively associated with pH and ESP (ranged from r-0.960 to -0.994), with a significance of $p<0.05$. A relationship exists between pH and phosphatase activities, as the alkaline and acid phosphatase displays a characteristic pH-dependent activity profile, possessing an optimum pH for its highest activity and specific stability, which relates to the soil pH. The negative association with ESP supports previous findings that saline-affected tailings materials reduced soil enzyme activities. These findings are also in accordance with the results obtained by Siddikee *et al*. (2011). One of the important parameters affecting biological activity within the soil is pH. Soil pH alters the solid phase equilibrium, nutrient availability/toxicity, microbial community structure and diversity present in the soil and subsequently their soil enzymatic activity. Investigations done by Wolińska and Stępniewska (2012), demonstrate that the optimal pH range for DHA is between 5.5 - 5.73. Acidic soils possess lower DHA values, in the case of acidic tailings materials; the DHA is extremely low/absent (**Table 4-5** and **Table 4-7**). One of the contributing factors is likely to be the acid pH value of gold tailings. In natural soils, several studies have indicated that phosphatase activity is affected by soil pH even when the phosphatase assay is carried out in a buffered medium (Acosta-Martínez & Tabatabai, 2000; Gianfreda & Bollag, 1996; Tabatabai, 2000).

4.4 Conclusion

The findings for this chapter can be summarised as follows:

- The DHA of different gold TSF, although slightly variable, are all low compared to reference soils.
- Low DHA is not exclusive to gold TSF but most other mining TSF possesses low DHA.
- When comparing DHA of the different grass species' rhizosphere substrates, the DHA of the gold TSF self-established grass species is low, with the exception of the coppice dunes. DHA of the self-established grass is, however, higher than the barren areas.
- The low microbial activities, in gold TSF, are not only exclusive to DHA, the majority of microbial enzymatic activities assayed during this phase were low (β-glucosidase, urease, acid and alkaline phosphatase).

- A strong negative correlation exists between New Machavie's EC and DHA. Consequently, indicating a relationship between the chemical and microbial properties of gold tailings.

- The poor soil enzymatic activities of the tailings are a consequence of detrimental physical and chemical characteristics, resulting in poor soil quality.

Mine TSF's substrate samples have highly selective physicochemical properties and all the tailings materials used for this research have low microbial activity relative to natural soils. Indicating that most tailings are degraded to an extent that the minimum number of microbial species necessary to support the ecosystem is absent. Under steady conditions, a minimum number of soil microbial species are necessary for ecosystem functioning, however, a far greater amount of soil microbial species is required to maintain and balance activities in fluctuating environments (Subhani *et al*., 2001) such as those present in the mine waste environment. This research shows that a full understanding of the mine waste environments cannot be achieved unless the microbiological processes are taken into consideration. In other words, microbial processes, microbial richness and microbial diversity must be included in models, theories, analyses and rehabilitation mine closure specifications. Soil enzymatic activity results obtained from the different TSF highlight the importance of taking microbiological constraints into consideration when rehabilitating mine waste environments. Through the incorporation of soil microbial activity and microbial functional diversity, one can get a better understanding of the linkage between nutrient availability, microbial community structure/function and ecosystem processes that can influence rehabilitation success. The current practice monitoring criteria used to determine rehabilitation performance/quality often include a dependence on a few assessments, i.e., using soil fertility, SOM and plant species selection with emphases on the physiochemical aspects of mine wastes. In contrast, the microbiological characteristics have received relatively little attention. In general, very few biological indicators are used to determine soil functioning. The integration of biological properties is of vital importance to accurately evaluate soil quality. According to García *et al*. (2002), the dynamics of soil ecosystem are so complex that one cannot successfully use a single microbial characteristic for determining microbial activity.

van Straalen and Krivolutsky (1995) emphasised the importance of identifying an integrated set of indicators to form an indicator system that can be used as a benchmark in environmental remediation. As a result of the soil system's complexity, single soil indicators cannot be used to determine soil functioning (degree of rehabilitation/degradation). Rather, it requires a minimum data set of attributes that include physical, chemical and biological properties of soil (Karlen *et al*., 2003). A detailed conclusion and recommendation refer to **Chapter 6** and **Chapter 7**.

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CHAPTER 5

PHASE 2

BIOLOGICAL AMENDMENTS AND BIO-STIMULANTS EFFECT ON VARIOUS MOTHER CROPS

"Wonder is the seed of knowledge"

–**Francis Bacon**

Abstract

Abandoned gold tailings storage facilities pose numerous threats to the environment and need to be rehabilitated according to legislation. Identifying methods for rehabilitation of a given site is a challenging procedure and requires consideration of several factors and sometimes alternative and more innovative methods are necessary. This chapter will focus on an alternative multi-phase rehabilitation approach on various abandoned gold TSFs that has no signs of any voluntary establishment of vegetation. A combination of chemical, physical and microbiological properties were identified as the major rehabilitation constraints; pH of 1.7, a net acid lime requirement of 300t/ha; low DHA (1.4 INF µg/g/2h) and compost requirement of 65 t/ha are amongst the worst. The focus of this study was the synergistic use of soil microbes and plants to facilitate the rehabilitation of a number of abandoned TSFs. Research was conducted to determine the effects of various bio-stimulants on different mother crop species' survivability and growth in deleterious environments, anticipating that, the synergistic use of bio-stimulants and mother crop species would improve vegetation recovery and subsequently revegetation efficiency. It was decided to follow a multi-phase approach with proper time built into it to address each of the rehabilitation attributes which have a negative effect on the physical, chemical and microbial characteristics. It must be emphasised that the focus of this research is to confirm the vegetation performance improvement and microbial activity. This phase included inter alia annual ryegrass (*Lolium multiflorum*) and certain Brassicaceae species together with various bio-stimulants. Results showed that both bio-stimulants and plant root activity stimulated soil rhizosphere dehydrogenase activity. Tailings from NM-geel with canola/carbohydrates treatment possessing the highest DHA increase (857 INF µg/g/2h), and all un-treated tailings the lowest. Various bio-stimulants significantly increased the mother crop species germination and survival rate (ANOVA $p<0.005$). Results also indicate that rehabilitation is substrate specific, i.e., certain bio-stimulants and different mother crop species performed better on different gold tailings. As such, the contribution microbes make to the ecosystems functioning in extreme environments, such as mine waste disposal environments are vastly underrated. Consequently, microbial processes need to be

included into mine rehabilitation practices, hypotheses, models, and interpretation of rehabilitation findings.

Keywords: *mine waste environment, multi-phase approach, soil dehydrogenase activity, bio-stimulants; mother crop species; ecosystem functioning, rehabilitation assistance.*

In general, soil amendments are a key necessity for successful vegetation establishment in the mine waste environment. Biological-ameliorative vegetation establishment approaches will assist with the transformation, land efficiency and fertility of degraded land ecosystems and new ecosystems such as mine tailings. Inorganic and organic amendments are beneficial to the stabilisation of mine waste prior to revegetation (Alvarenga *et al.*, 2008). To overcome physiochemical constraints present within the mine waste materials, fertilisers and organic amendments (e.g., compost, sewage sludge) are added, which is usually too limited to sustain plant growth (Bradshaw, 1997; Mains *et al*., 2006; Munshower, 1994; Tordoff *et al*., 2000; Wong, 2003). The positive effects obtained from amendments are for a limited period, before reverting to its original degraded state. The heterotrophic bacteria in tailings are typically extremely low ($<10^3$ CFU/g) (de-Bashan *et al*., 2012; Mendez, 2007; Mendez *et al*., 2007), whilst extremely acidic tailings possess even lower heterotrophic bacterial count (10-75 CFU/g) (Mendez, 2007). In most rehabilitation techniques, microbial deficiencies contribute towards the difficulties in vegetation establishment. In order to improve mine rehabilitation success, the recovery of the belowground microbial community should be incorporated into rehabilitation approaches (Li *et al*., 2014; Mukhopadhyay *et al*., 2013).

5.1 Background

The use of bio-stimulants (i.e., plant growth promoting rhizobacteria (PGPR) inoculation, humic acid, amino acid etc.) has been proposed to support the successful cultivation of plants on tailings (de-Bashan *et al*., 2012; Mendez & Maier, 2008; Solís-Domínguez *et al*., 2011; Zhuang *et al*., 2007). This approach assumes that the bio-stimulants will improve the microbial community structure and microbial activity, thus providing an improved plant survival and growth support (de-Bashan *et al*., 2012). The mechanisms through which these bio-stimulant enhances plant growth and development according to various researchers (de-Bashan *et al*., 2012; Glick, 2003; Grandlic *et al*., 2009; Huang *et al*., 2012; Li *et al*., 2007; Rajkumar & Freitas, 2008; Reed & Glick, 2005; Wu *et al*., 2006) includes:

- Increasing nutrient availability.
- Improving resistance to trace metal toxicity.
- Reducing bioavailability of toxic trace metal elements in the rhizosphere.

- Eliciting plant protection and exudation of metabolites such as hormones (e.g., indole acetic acid (IAA) and metabolites (e.g., siderophore).

Rehabilitation of heterotrophic microbial communities in tailings can be achieved via the application of SOM and bio-stimulants (de-Bashan *et al*., 2012; Grandlic *et al*., 2008; Huang *et al*., 2012). These bio-stimulants may include local or commercial isolates of PGPM and other bio-substances that may improve tailings native heterotrophic microbial communities (Huang *et al*., 2012). The use of bio-fertilisers and bio-stimulants for reclamation and rehabilitation purposes can lead to the improved fertility of barren TSFs in a shorter period, improving the tailings materials thus, creating a better environment to sustain long-term vegetation establishment. The end goal of revegetation is not only to establish a vegetative cover to stabilise TSF's surface through plant cover and root zone cultivation but to transform the soil (paedogenesis) in such a way as to augment plant succession. If possible, such an ecological transition would include a more diverse native microbial community, capable of sustaining plant growth and development (de-Bashan *et al*., 2012).

The positive effects of bio-stimulants and bio-fertilisers (rhizobacteria) on plant growth and health in sustainable agriculture and horticulture has been thoroughly documented by various researchers (e.g., Barea *et al*., 2002; Barea *et al*., 2005; Cabello *et al*., 2005; Caravaca *et al*., 2004; Compant *et al*., 2010; Grandlic, 2008; Ryan *et al*., 2009; Toro *et al*., 1998; Vestberg *et al*., 2002). In contrast, the synergistic effects of mother crop species and bio-stimulants microbial communities and microbial activities in the mine waste environment are poorly understood. This has led to an intense pursuit for microorganisms that are site-adapted and able to promote plant growth in disturbed soils and mine-waste environments. In order to understand the impact of microorganisms on plant development in disturbed mining environments, knowledge of plant physiological properties and their associated microorganisms is necessary. The presence of a positive plant-microbial interaction in the mine waste environment will facilitate plant growth and development.

Microorganisms contribute towards plant protection against unfavourable soil conditions by elevating abiotic and biotic stresses. Plant growth is influenced by the physical condition (e.g., soil aggregation), chemical composition (e.g., nutrient cycling) and microbial qualities of soils. However, in many cases, unfavourable conditions existing in mine waste environments causing a lack of vegetation or a diminished vegetation development. During a plant's entire lifespan, plants are subjected to various environmental stresses that influence their plant growth and productivity. Abiotic and biotic stresses influence plants characteristics at a molecular, biochemical, physiological and morphological level (Chakraborty *et al*., 2015). Rhizosphere microorganisms are especially critical for plant colonisation of unfavourable mine waste

environments since they can alleviate the biotic and abiotic stress of plants (Hrynkiewicz & Baum, 2011).

Suitable plant species utilised primarily for improving soil conditions for the benefits of the subsequent plant establishment phase is known as mother crops. Mother crops can be used for various purposes including improving soil structure, fixing N, nurturing soil biological life, and controlling soil moisture.

For this research purposes, the mother crop species consisted of *Brassica* species and grass species with and/or without additional bio-stimulants. Bio-stimulants potential for microbial activity improvement and plant growth promotion (revegetation potential) will be researched in this chapter. The species include three *Brassica* species, i.e., canola (*Brassica napus*), fodder rape (*B. napus*), radish (*Raphanus sativus*) and one grass species, ryegrass (*Lolium multiflorum*) for control and comparison purposes. The mother crop species selected for this research germinates very quickly, consequently ensuring quick erosion protection and stabilisation of TSFs. These mother crop species are gradually replaced by more sustainable perennial species that guarantees long-term stabilisation. Several Brassicaceae species have the ability to extract trace metal elements and are able to tolerate saline conditions. Most hyperaccumulators are characterised as slow-growing with low plant biomass (van Ginneken *et al.*, 2007), however, certain species within the *Brassica* genus (*Brassica napus, Brassica juncea*, and *Brassica rapa*) are hyperaccumulators that are fast growing with high biomass (Ebbs & Kochian, 1997). *Brassica* or grass, or a grass mixture with *Brassica* can scavenge around 18kg residual N from the soil and even more when SOM or pre-plant fertilisers have been applied. Additionally, this causes a rapid increase in total biomass and a higher total nutrient availability for subsequent plant establishment (Natural Resources Conservation Service (NRCS), 2017). The deep tap root system of Brassicas, penetrate and open compacted soils, improving infiltration and reducing compaction. Consequently, as a mother crop, Brassicaceae species can prevent erosion, suppress weeds and soil-borne pests, alleviate soil compaction, scavenge nutrients and extract certain trace metal elements, thus improving soil conditions (Sarrantonio, 2012).

The aims of this study were to determine the effects of the application of different bio-stimulants and/or a mother crop species (i) soil dehydrogenase activity and (ii) plant performance. It was hypothesised that the rehabilitation measures (bio-stimulant application and mother crop selection) would enhance soil microbial activity and improves plant performance. To prove these hypotheses, (i) determined and compared the effects of the different treatments on chemical variables (pH and EC), (ii) soil dehydrogenase activity; (iii) plant performance was monitored by measuring plant growth and recording survivability.

In this chapter, the research focuses primarily on the synergistic effects of a selection of mother crop species and bio-stimulants to improve plant growth and establishment. The applicability of these bio-stimulants and mother crops to improve microbial activity was assessed by means of DHA. It must be emphasised that the focus of this research is to confirm the vegetation performance improvement and not on DHA or other enzymes in the growth substrates.

5.2 Amelioration and bio-stimulants

By definition, soil amendments include all inorganic and organic substances added to the soil to achieve a better soil condition concerning plant performance. Soil amendments provide a better environment for roots and plant growth, by improving the moisture-holding capacity and soil structure, nutrient availability, and the microbiome environment. These characteristics are important to plant growth and establishment. Within the amendment group, a broad class of formulations exists, e.g., bio-stimulants, biocontrol and soil conditioners.

Currently, no universal, legal or regulatory definition of bio-stimulants exists. Bio-stimulants can be described as a diverse formulation that contains one or more substances and/or microorganisms whose function, when applied to either plants or rhizosphere is to stimulate natural processes to improve/assist nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and crop quality (Calvo *et al*., 2014; de Vasconcelos *et al*., 2009; European Bio-stimulant Industry Council (EBIC), 2012). These bio-stimulant effects are usually more evident under stressful conditions (abiotic and biotic), boosting the stress tolerance of plants and repairing the damage caused by these unfavourable conditions. EBIC, 2012 considers bio-stimulants substances that complement other plant protection products and fertilisers rather than replacements (EBIC, 2012). Bio-stimulants are said to boost rhizosphere microbial activity and soil enzymes, produce soil and plant hormones and/or growth regulators, and photosynthetic process (Giannattasio *et al*., 2013; Nardi *et al*., 2009).

According to EBIC (2012), bio-stimulants promote plant growth and development during the entire plant's lifecycle, i.e., from seed germination to plant maturity. The plant's growth and development promoting capabilities include:

- Improving plant metabolism efficiency that stimulates a yield increase,
- Enhancing plant quality by augmenting plant tolerance and plant recovery from abiotic stresses.
- Facilitating nutrient assimilation, translocation and consumption.
- Improving plant produce quality characteristics i.e., sugar content, colour, fruit seeding.
- Enhancing certain physicochemical properties of the soil.
- Fostering the development of complementary soil microorganisms (EBIC, 2012).

5.2.1 Main categories of plant bio-stimulants

Bio-stimulants can be classified into seven categories; humic substances; protein hydrolysates and other N-containing compounds; seaweed extracts; chitosan and other biopolymers; inorganic compounds; beneficial fungi and bacteria (Bulgari *et al.,* 2015; Calvo *et al.,* 2014; du Jardin, 2015; La Torre *et al*., 2016; Yakhin *et al.,* 2017). These categories include both non-microbial substances and microbial inoculants.

5.2.1.1 Humic substances

Humic substances (HS) are the largest natural component of SOM (up to 75%) responsible for many complex chemical reactions in the soil environment (Stevenson, 1994). HS arise from the decomposition and transformation of the plant, animal and microbial residue (MacCarthy, 2001). HS are assemblages of heterogeneous compounds, categorised according to their molecular weights and solubility into humin (not soluble in aqueous solutions), humic acids (HA, soluble at basic pH) and fulvic acids (FA, soluble at any pH range) (Hayes & Malcom, 2001; Stevenson, 1994). Humic material and SOM performs a key role in supporting soil functions and plant yields (Lal, 2004; Sparling *et al*., 2006) as such the loss of humic material, accompanied by an overall reduction in SOM is a major concern.

The low SOM is one of the contributing constraints in the mine waste environment. Consequently, attention has been drawn to HS-based amendment applied to organically low agricultural and other degraded systems to reverse these trends (Piccolo & Mbagwu, 1997; Quilty & Cattle, 2011). HS can be extracted from various sources (Halpern *et al*., 2015), including soils (Nardi *et al*., 2000; Varanini *et al*., 1993; Zandonadi *et al*., 2007), municipal waste (Ayuso *et al*., 1996), peat (Ayuso *et al*., 1996; Schmidt *et al*., 2007), several coal deposits (Kulikova & Perminova, 2002), vermicompost and earthworm casts (Arancon *et al*., 2006; Canellas *et al*., 2002; Russels *et al*., 2006) and from mineral deposits such as leonardite (du Jardin, 2012; Nikbakht *et al*., 2008). In soil, humic acid derivatives play key roles in several soil and plant functions (Berbara & García, 2014; Calvo *et al*., 2014). HS has been acknowledged as an important contributor to soil fertility, acting on both the physicochemical and biological properties of the soil. HS has the ability to interact with metal ions, oxides, hydroxides, and organic compounds (Albers *et al.*, 2008) to form water-soluble and - insoluble complexes (Piccolo & Spiteller, 2003; Trevisan *et al*., 2010). The accumulation of these complexes reduces trace metal toxicity (Chen *et al*., 2004; Elkins & Nelson, 2002; Imbufe *et al*., 2005; Peiris *et al*., 2002; Piccolo & Mbagwu, 1989; Piccolo *et al*., 1997). In the mine waste environment, these complexes (Elkins & Nelson, 2002) will facilitate with the revegetation of TSFs. Other documented HS soil amendments benefits include increased pH buffering and cation exchange capacity, moisture retention capacity and bioavailability of nutrients

(such as N, P, K, S, Zn and Fe), specifically at very low concentrations (Arslan & Pehlivan, 2008), and improved soil aggregation and structure. Depending on the type of HS, it can also increase the soil microfauna and microflora. Microorganisms interact with HS in the soil and rhizosphere environment in various ways (Puglisi *et al*., 2013). Following application of soil-derived HS to two soil types, Visser (1985b), found that HS has a stimulating influence on amylolytic, proteolytic and denitrifying microorganisms. Furthermore, in a related study, Visser (1985a), found that HS stimulated the growth of starch decomposers, aerobic cellulose decomposers and nitrifiers. HS is also known to influence the microbial community composition, abundance and microbial activity (Anderson *et al*., 2011; Dong *et al*., 2009; Paranychianakis *et al*., 2013; Puglisi *et al*., 2009; Valdrighi *et al*., 1996; Visser, 1985a).

Microorganisms that can degrade HS to some extent include the bacteria, such as *Streptomyces* and *Pseudomonas* (Challis & Hopwood, 2003; Steffen, 2003). In addition to indirectly influencing plant productivity through soil properties alteration, HS can also directly impact physical and metabolic plant processes (Rose *et al*., 2014). Direct effects are said to be a consequence of the HS flow into the apoplast, and this may include the induction of H^+-ATPase synthesis and transport proteins, hormone-like effects and effects on glycolysis and other enzymes involved in the Krebs cycle (Canellas *et al*., 2002; Nardi *et al*., 2002; Pinton *et al*., 1997; Pinton *et al*., 1999; Pinton *et al*., 2009). Furthermore, HS is recognised to have various morphological, physiological and biochemical effects on higher plants (Nardi *et al*., 2002; Trevisan *et al*., 2010; Vaughan & Malcom, 1985).

Several studies have discovered that HS from various sources stimulate root, leaf and shoot growth, improves the yield of a variety of crops, including grains and vegetables (e.g., Arancon *et al*., 2006; Eyheraguibel *et al*., 2008; Lee & Bartlett, 1976; Nardi *et al*., 2002; Piccolo *et al*., 1993; Puglisi *et al*., 2009) as well as the germination rate of some plants (Canellas *et al*., 2015; Piccolo *et al*., 1993). Plant growth benefits are dependent on several factors, including the origin of the HS, application rate, crop type and soil type (Rose *et al*., 2014). However, the mechanisms for improved plant growth in response to the HS application have not been completely verified.

5.2.1.2 Seaweed extracts

Seaweed has been used as soil conditioners for centuries as seaweed is a rich source of SOM and fertiliser nutrients, however, their biological stimulatory effects have only recently been recorded. Seaweeds have beneficial functions on both soil and plants. In terms of plant growth and health, seaweed extracts showed improvement in root growth and development, enhanced plant chlorophyll content and improved crop yields (Craigie, 2011; Craigie *et al*., 2008; Khan *et al*., 2009). Chemical constituents of commercial seaweed extract and purified seaweed compounds that affect plant growth include polymers such as agars, laminarin, alginates, fucan,

phlorotannin and carrageenan and their breakdown products (Connan *et al*., 2006; Khan *et al*., 2009). Other plant growth promotion constituents include macro- and micro-element nutrients, sterols, N-containing compounds like betaines, amino acids, vitamins, and phytohormones such as cytokines, auxins, and abscisic acid (ABA)-like growth substances (Jameson, 1993; Tarakhovskaya *et al*., 2007; Taylor & Wilkinson, 1977; Yokoya *et al*., 2010; Zhang & Ervin, 2004; Zhang *et al*., 1991). Several of these compounds mentioned are unique to their algal source. Most of the algal species belong to the brown algae phylum with main genera being *Ascophyllum*, *Fucus*, and *Laminaria*. Conversely, seaweed extracts with carrageenans originate from red seaweeds (Rhodophyta). Khan *et al*. (2009) identified more than twenty commercial seaweed products that are used as plant growth stimulants.

In addition to stimulating plant growth-promoting effect, seaweeds also affect the physical, chemical, and biological properties of soil which in turn influence plant growth. Furthermore, the application of seaweeds and seaweed extracts causes the growth of soil microorganisms (Khan *et al*., 2009; Yakhin *et al*., 2017). Several reviews and published research papers exist on seaweed extractants as a bio-stimulant. This includes reviews (e.g., Battacharyya, 2015; Calvo *et al*., 2014; Craigie, 2011; Crouch & van Staden, 1993; Khan *et al*., 2009; Sharma *et al*., 2014; Tuhy *et al*., 2013), and published research papers (e.g., Billard *et al*., 2014, Elansary *et al*., 2016; Godlewska & Ciepiela, 2016; Jannin *et al*., 2013; Sangha *et al*., 2014).

5.2.1.3 Protein hydrolysates and amino acids

Bio-stimulants may also be based on protein hydrolysates and amino acids from either animal origin, including wastes and by-products e.g., collagen, epithelial tissues; or from plant sources, e.g., crop residues (Calvo *et al*., 2014; Cavani *et al*., 2006; Colla *et al*., 2014; du Jardin, 2015; Halpern *et al*., 2015; Mladenova *et al*., 1998; Rodríguez-Morgado *et al*., 2014; Yakhin *et al*., 2017). Formulations containing amino acids, peptides, polyamines, betaines and other protein hydrolysates have been proven to take part in various roles as bio-stimulants of plant growth (Calvo *et al*., 2014; Cavani *et al*., 2006; Ertani *et al*., 2013; Halpern *et al*., 2015; Subbarao *et al*., 2015). Direct plant effects include the modulation of N uptake and assimilation. Some amino acids, such as proline have shown to have chelating effects (du Jardin, 2015). Colla *et al*. (2014) reported that certain complex protein and tissue hydrolysates showed phytohormonal activities. Furthermore, some of the nitrogenous compounds, including glycine, betaine and proline showed antioxidant activity that helps with the mitigation of environmental stress. Indirectly, protein hydrolysates are also known to improve microbial activity and biomass, soil respiration and improve the overall soil fertility (du Jardin, 2015).

Various studies have reported the advantageous effects of protein hydrolysates applications on growth, yield and fruit quality of agricultural crops (Colla *et al*., 2014; Đurić *et al.,* 2014; Ertani *et al*., 2009; Gurav & Jadhav, 2013; Morales-Payan & Stall, 2003; Parrado *et al*., 2007; Subbarao *et al*., 2015; Yunsheng *et al*., 2015).

5.2.1.4 Chitosan and other biopolymers

Chitosan is a deacetylated form of the biopolymer chitin, that is produced naturally (a component of fungal cell walls, marine diatoms and algae, arthropod exoskeletons and crustacean shells) or industrially (Sharp, 2013). Chitosan stimulates various plant responses, including increased disease and abiotic (drought, salinity, cold) stress resistance, plant growth and yield improvement, and enhanced flowers and fruit shelf life, improved quality traits related to primary and secondary metabolites such as polyphenolics, lignin, flavonoids, and phytoalexins (El Hadrami *et al*., 2010; Hadwiger, 2013; Sharp, 2013). The physiological effects of chitosan in plants may be attributed to the ability of the polycationic compound to bind an extensive variety of cellular components, including plasma membrane and cell wall constituents as well as DNA. Furthermore, chitosan also has the ability to bind specific receptors participating in defence gene activation, in a similar way as plant defence elicitors (El Hadrami *et al*., 2010; Hadwiger, 2013; Katiyar *et al.*, 2015; Yin *et al*., 2010) inducing pathogen-related protein production, for example, chitinases and other hydrolytic enzymes. These enzymes hydrolyse chitin and chitosan in fungal cell walls, causing growth inhibition and/or mortality (Doares *et al*., 1995; Mason & Davis, 1996).

Chitosan has been extensively studied as a means to inhibit microbial growth and decrease microbial membrane integrity (Hadwiger, 2013; Iriti & Varoni, 2015; Kulikov *et al*., 2006; Palma-Guerrero *et al*., 2008; Rabea *et al*., 2003; Xu *et al*., 2007). The stomatal closure prompted by chitosan via abscisic acid (ABA) dependent mechanism (Iriti *et al*., 2009) contributes towards the environmental stress protection.

5.2.1.5 Inorganic compounds

Inorganic compounds are chemical elements comprising of negatively charged anions and positively charged cations (Deliopoulos *et al*., 2010). Inorganic compounds are beneficial elements that may be fundamental to particular plant taxa, but not necessarily essential to all plant types (du Jardin, 2015; Pilon-Smits *et al*., 2009). This includes phosphites, phosphonates, bicarbonates, chlorides, silicates, sulphates and nitrates (du Jardin, 2012; 2015). Silicon (Si) has quite a few effects on plant performance and growth. It is proven to increase fungi resistance, amend nutrient imbalances, alleviate trace metal toxicity, and enhance abiotic stress tolerance (Deliopoulos *et al*., 2010; Savvas & Ntatsi, 2015). At present, Si is used in a few commercial horticultural crops (Hwang *et al*., 2005; Pilon *et al*., 2013; Pilon-Smits *et al*., 2009; Wang & Galleta,

1998) to stimulate abiotic stress, diseases, and pest resistance, however, the use of Si is still limited (Savvas & Ntatsi, 2015). Phosphate salts as a beneficial element have a direct effect on plant metabolism, plant defence responses and stomatal function (Deliopoulos *et al*., 2010; du Jardin, 2012; Gómez-Merino & Trejo-Téllez, 2015).

Pilon-Smits *et al*. (2009) reported that the beneficial effects of inorganic salts include enhanced resistance to biotic stresses such as pathogens and herbivory, improved abiotic resistance to stresses such as drought, salinity, and increased resistance to nutrient toxicity or deficiency.

5.2.1.6 Beneficial microorganisms

Rhizosphere microorganisms that are beneficial to plants by stimulating plant growth and controlling diseases are referred to as plant growth promoting microbes (PGPM) (Kloepper *et al*., 1980). In terms of plant life, PGPMs are multifunctional and affect all plant life aspects such as morphogenesis and development, nutrition and growth, response to biotic and abiotic stress, interactions with other organisms in the agroecosystems (Ahemad *et al*., 2009; Berendsen *et al*., 2012; Berg *et al*., 2014; Chandler *et al*., 2008; de-Bashan *et al*., 2012). Most of these functions are generally completed by the same microorganisms, however, some functions are strain-specific, and others are dependent on synergisms within a bacterial consortium.

PGPM possess regular plant growth promoting traits such as, phytohormone that includes auxins, cytokinins, gibberellins, ethylene, abscisic acid, brassinosteroids, and jasmonates (Krouk *et al*., 2011; Patten & Glick, 2002; Tank & Saraf, 2010; Vallad & Goodman, 2004). These phytohormones are involved in several aspects of plant growth and development, from stem cell niches during embryogenesis (Wolters & Jürgens, 2009) to organogenesis and growth during postembryonic development (Stamm & Kumar, 2010).

Another way that beneficial microbes promote plant growth is by increasing nutrients availability via N fixation, soil P, K, Zn and Fe solubilisation, by means of microbial mechanisms such as:

- Siderophore formation (Gamalero & Glick, 2011; Siebner-Freibach *et al*., 2003; Verma *et al*., 2011).
- Enzyme activity, i.e., nitrogenase activity (Glick, 2012; Khan, 2005).
- Facilitating nutrient acquisition (Barea *et al*., 2002; Rodríguez & Fraga, 2004).
- Alleviate plant stress by secreting ACC (1-aminocyclopropane-1-carboxylate) deaminase enzyme (Glick, 2014; Penrose & Glick, 2001).

Certain bacteria have the ability to mediate direct plant protection, known as induced systemic resistance (ISR). This type of protection is induced in plants by certain bacteria, generally

Pseudomonas and *Bacillus* (Kloepper *et al.*, 2004). Induced systemic resistance is considered a latent defence that only activates when the plant is under herbivore/pathogen or insect attack (Conrath *et al.*, 2006).

In addition to the regular plant growth promoting traits, a wide range of new microbial traits has been identified that includes:

- Trace metal element detoxifying potentials (Ahemad, 2012; Ahemad & Malik, 2011; Braud *et al*., 2009; Gamalero *et al*., 2009; Hayat *et al*., 2010; Kumar *et al*., 2015; Ma *et al*., 2011a; 2011b; 2011c; Rajkumar *et al*., 2010; Wani & Khan, 2008; 2010).
- Salinity tolerance (Bashan *et al*., 2014; Egamberdieva & Lugtenberg, 2014; Kang *et al*., 2014; Khan *et al*., 2011; Khan *et al*., 2017; Mayak *et al*., 2004; Tank & Saraf, 2010).
- Phytopathogen biocontrol potentials (Copping, 2004; Hynes *et al*., 2008; Joo *et al*., 2005; Kabaluk & Gazdik, 2005; Murphy *et al*., 2000; Partida-Martinez & Heil, 2011; Pineda *et al*., 2010; Russo *et al*., 2008; Sanchez *et al*., 2005; van Wees *et al*., 2008).
- Pesticide degradation/tolerance (Ahemad & Khan, 2009a; 2009b; Ahemad & Khan, 2012a; 2012b).

5.2.1.6.1 Beneficial bacteria

Rhizobacteria with PGP-activity occur in several bacterial phyla (Actinobacteria, Proteobacteria and Firmicutes), including strains belonging to genera *Bacillus*, *Pseudomonas*, *Azospirillum, Azotobacter, Alcaligens, Arthobacter, Agrobacterium, Burkholderia, Comamonas, Pantoea, Rhizobium, Serratia, and Variovorax* (Hurek & Reinhold-Hurek, 2003; Kloepper *et al*., 1989; Kloepper *et al*., 1991; Steenhoudt & Vanderleyden, 2000). Symbiotic (*Rhizobium, Bradyrhizobium, Mesorhizobium*) and non-symbiotic (*Pseudomonas, Bacillus, Klebsiella, Azotobacter, Azospirillum, Azomonas*), rhizobacteria are used worldwide as bio-inoculants to promote plant growth and development under various environmental stress conditions. Given that spore-forming bacteria are very persistent, the inoculation of *Bacillus*-related species to enhance plant growth has a great advantage over non-spore forming bacteria. For research purpose, the *Bacillus* spp. was selected to represent the beneficial bacteria, subsequently, a more specific reference will be made for *Bacillus*.

Bacillus spp. are gram-positive aerobic endospore-forming bacteria (AEFB). Bacteria belonging to the *Bacillus* spp. are fundamentally ubiquitous present in the soil and in the phylloplane (leaves), adapt to living as endophytes and occur in abundance in most rhizosphere soils. Various *Bacillus* strains have been identified that possess the ability to affect plant growth and health through three different ecological mechanisms, namely, antagonism against fungal, bacterial,

nematode pathogens and insect pests, promotion of host plant nutrition and growth, and stimulation of host defence mechanisms (Govindasamy *et al*., 2010).

In terms of the nutrient acquisition, various strains of the *Bacillus* genera have been identified to facilitate nutrient acquisition. Ectorhizospheric strains of *Pseudomonas* and *Bacillus* have been described as being able to effectively solubilise soil phosphate (Whitelaw, 2000). With *Bacillus megaterium, B. circulans, B. subtilis, B. polymyxa, B. sircalmous, Pseudomonas striata*, and *Enterobacter* as the most influential bacterial strains (Harvey *et al*., 2009; Khan *et al*., 2007).

Another fundamental characteristic of *Bacillus*-related species is their N-fixing ability. Diazotrophy or atmospheric N-fixation ability is an essential trait that seems to be irregularly spread out among the *Bacillus* and *Paenibacillus* genus strains (Selvakumar *et al*., 2016). With *Paenibacillus azotofixans* being identified as the most efficient N-fixer prevalent in the rhizosphere of forage grass, sorghum, wheat and maize (Rosado *et al*., 1998). In terms of plant protection, several Bacillus spp. have been reported to be effective against plant disease most commonly *B. subtilis, B. cereus* and *B. amyloliquefaciens*. One of the major advantages as a biocontrol agent is that *Bacillus* strains have the ability to form spores that are resilient against chemicals and mechanical damage. *Bacillus* produces many antibiotic compounds, for example, fengycin, Iturin and zwittermycin (Raaijmakers *et al*., 2002; Romero *et al*., 2007). Members of *Bacillus* were reported to inhibit various phytopathogens including *Fusarium oxysporum f.* sp*. ciceri* (Kumar, 1999) and *Rhizoctonia solani* (Asaka & Shoda, 1996). Members of *Bacillus* spp. are also considered to be phytostimulators attributable to their plant hormone production (Halpern *et al*., 2015) that includes cytokinin (Ortíz-Castro *et al*., 2008), IAA (Idris *et al*., 2007), and gibberellins (Gutiérrez Mañero *et al*., 2001).

5.2.1.6.2 Beneficial fungi

A wide range of beneficial fungi interacts with different plant species' roots, these fungal-root interactions facilitate plant nutrient uptake, substituting them for carbohydrates and other organic metabolites (Zuccaro *et al*., 2014). On account of their filamentous organisation, fungi are able to utilise diverse substrates depending on their nutritional strategy. For example, Saprobes are the group of fungi that act as decomposers, thriving on the feeding of dead and decaying wood, leaves, litter, and SOM. Many fungi are involved in symbiotic relationships, including parasitism and mutualism (Carlile *et al*., 2001). *Trichoderma* species are ubiquitous soil-borne Ascomycetes noted for their positive impacts on cultivated plants.

Trichoderma spp., have been extensively studied and are commercially used for their mycoparasitic and biocontrol capacities (Verma *et al*., 2007). The *Trichoderma* species more

commonly used in biocontrol are T*. asperellum, T. atroviride, T. harzianum, T. virens,* and *T. viride* (López-Bucio *et al*., 2015). Most of these *Trichoderma* species also display great horticultural crop bio-stimulant action. Several reports noted the beneficial effects of *Trichoderma* species on horticultural crops such as cucumber, tomatoes, chrysanthemum and other high-value crops (Mastouri *et al*., 2010; Prasanna *et al*., 2016; Zhao & Zhang, 2015). Bio-stimulant characteristics of *Trichoderma* are determined by fungal-root communication via plant hormones and growth regulators. Several root development characteristics such as lateral root formation are controlled by *Trichoderma* metabolites, which include standard phytohormones such as IAA, cytokinins and ethylene, and volatile blends (López-Bucio *et al*., 2015). For example, *Trichoderma harzianum* promotes plant growth through changes in phytohormone levels, specifically auxins and cytokinins (Sofo *et al*., 2011). Research done by Bareen *et al*. (2012) showed that inoculation with *Trichoderma pseudokoningii* induced the activities of stress tolerance-related, antioxidative enzymes catalase (CAT) and superoxide dismutase (SOD) with increasing levels of metal uptake in plants as part of abiotic stress mechanism. *Trichoderma* spp. also has the ability to synthesise peptaibols, a family of peptides with antibiotic function (Whitmore & Wallace, 2004), that facilitate plant protection. The antibiotic function results from membrane-insertion and pore-forming abilities. Shi *et al*. (2012) showed that the antibiotic effects of peptaibols produced by *Trichoderma pseudokoningii* can induce programmed cell death in plant fungal pathogens.

Several research publications have supported the use of *Trichoderma* spp. in remediation and vegetation establishment in trace metal elements contaminated soils and TSFs (Babu *et al*., 2014a; Babu *et al*., 2014b; Babu *et al*., 2014c; Bareen *et al*., 2012). *Trichoderma* fungi colonise roots of various plant species with several isolates having been reported to enhance plant growth and increase trace metal elements availability in contaminated soils (Adams *et al*., 2007; Babu *et al*., 2014a; Babu *et al*., 2014b; Babu *et al*., 2014c; Bareen *et al*., 2012; Cao *et al*., 2008).

5.3 Mother crop definition and benefits

Mother crops have multiple benefits depending on the species. These species are known to improve the soil in various ways, these include reducing soil erosion, providing SOM, reducing compaction, and providing either a habitat/food source for certain soil organisms. Indirectly, these mother crops also contribute towards soil quality by scavenging nutrients, or in the case of legumes, adding N to the soil (Clark, 2007; Thomas *et al*., 2017). They also convert unavailable nutrients to more readily available forms. Mother crops also facilitate nutrient uptake from deep soil layers to upper soil layers. For example, Ca and K are two macro-nutrients that tend to travel downwards into the soil with water, i.e., leaching effect (Clark, 2007). Certain mother crop species can suppress or limit pests and weeds (Clark, 2007; Kruidhof *et al*., 2008; Spies *et al*., 2011).

5.3.1 *Brassica* characteristics

Brassica is among the most widely grown and important crops worldwide. The Brassicaceae family comprises of several essential field crops such as oilseed rape (*Brassica napus*), cauliflower, broccoli, and kale (*Brassica oleracea* group*),* rape (*Brassica rapa)* and radish (*Raphanus sativus*) (Rakow, 2004). As a mother crop, *Brassica* members have several beneficial attributes including high biomass production, a well-developed taproot, rapid growth, nutrient-scavenging ability, high receptiveness to N; compete with other plants and pest-resistance capabilities.

Pest suppression capabilities of *Brassica* may be attributed to the degradation of glucosinolates (Gardiner *et al*., 1999; Petersen *et al*., 2001). Upon cellular injury to the *Brassica* plant, glucosinolates are enzymatically degraded by myrosinase (a thioglucosidase), leading to the formation of a variety of breakdown products. These breakdown products include sulphate, glucose, and depending on the specific Brassicas chemical structure, isothiocyanates, nitriles, epithionitriles, oxazolidinethions, indolyl alcohols, thiocyanate, and amines (Aghajanzadeh *et al*., 2014; Ahuja *et al*., 2010; Bones & Rossiter, 2006; Kissen *et al*., 2009; Petersen *et al*., 2001). These compounds are known to be toxic to certain microorganisms, nematodes, fungi and insects (Ahuja *et al*., 2010; Bones & Rossiter, 1996).

In terms of fertiliser requirements, Brassicas need sufficient amounts of N and S fertiliser, as Brassicas S nutrition requirements and S-uptake capacity exceed those of most other plant species. This high S requirement can be attributed by the high S necessary for the production of oil and glucosinolate (Aghajanzadeh *et al*., 2014). The Brassicaceae members are also particularly efficient at acquiring P from the soil (Greenwood *et al*., 2005; Greenwood *et al*., 2006). Their ability to acquire P from soil seems to be related to their ability to mobilise P via organic acid exudation (Hoffland, 1992), additionally, their extensive tap root system also facilitates P-scavenging from the soil (Dechassa *et al*., 2003; Hammond *et al*., 2009). Under orthophosphate stress, specific plant species and cultivars developed an arrangement of physiological, morphological, biochemical and molecular modifications that allow them to scavenge P from inadequately soluble P-soil fractions (Akhtar *et al*., 2007).

Brassicaceae is one of the natural metal hyperaccumulator families that include 34 different plant families, including Asteraceae, Brassicaceae, Caryophyllaceae, Poaceae, Violaceae and Fabaceae (Prasad & Freitas, 2003). These families developed the ability to take up, tolerate and accumulate extremely high concentrations of trace metal elements and metalloids present in the soil and in their aboveground biomass devoid of observable toxicity symptoms.

The Brassicaceae family is considered the top representative amongst the metal hyperaccumulator families, with 87 species identified and classified as metal hyperaccumulators (Anjum *et al.*, 2012; Broadley *et al*., 2001; Cappa & Pilon-Smits, 2014; Cecchi *et al*., 2010; Koch & German, 2013; Krämer, 2010; Roosens *et al*., 2008; Verbruggen *et al*., 2009). Several representatives of the family Brassicaceae are either known for or have the potential to remediate a variety of environmental contaminants (Cecchi *et al*., 2010; Krämer, 2010; Milner & Kochian, 2008). A substantial amount of research has been done, concerning the tolerance, uptake and defence mechanism of several species, particularly *Brassica juncea* and *B. napus*, against stress induced by trace metal elements. For example, Hernández-Allica *et al*. (2008) did an extensive study relating to the trace metal elements tolerance of different species (including several cultivars of *B. campestris, B. rapa, B. napus, B. oleracea* and *B. carinata*). Hernández-Allica *et al*. (2008), findings concluded that a high level of metal tolerance exists within the *Brassica* species, primarily to Zn, and to a lesser extend Pb and Cd (Hernández-Allica *et al*., 2008).

Various microorganisms are indigenous to the rhizosphere of *Brassica* species; this includes *Phyllobacterium brassicacearum, Serratia plymuthica, Comamonas terrigena, Stenotrophomonas maltophilia, Agromyces cerinus, Acinetobacter rhizospaerae, Microbacterium oxydans, Paenibacillus lautus, Arthrobacter globiformis, Pseudomonas fluorescens, Variovorax paradoxus,* and *Trichoderma* spp. etc. (Croes *et al*., 2013). These soil microorganism species have been reported to influence the growth and phytoremediation potential of *Brassica* plant species (Adams *et al*., 2007; Ahmed *et al*., 2012; Larcher *et al*., 2003; Larcher *et al.*, 2008; Liang *et al*., 2014). Research done by Wang *et al*. (2011) demonstrates that the application of beneficial microorganisms colonising the rhizosphere of plants considerably reduces trace metal toxicity to plants and enhances the accumulation of trace metal elements in the plants. **Table 5-1** shows examples of research that has been done on PGPR and various *Brassica* crop species growth and their sequential results.

Table 5-1: Examples of PGPR used for improved *Brassica* phytostabilisation (adapted from Ahemad, 2014; Hansda *et al*., 2014).

PGPR	Plant	Experiment settings	Results of bacteria addition	Reference
Pseudomonas sp.	*Alyssum serpyllifolium, Brassica juncea* (Indian mustard)	Pot trial	Increased biomass (*B. juncea*) and Ni content (*Alyssum*) grown in Ni - stressed soil	Ma *et al*. (2011a)
Pseudomonas sp., *Psychrobacter* sp., *Bacillus* sp.	*Brassica juncea, Brassica oxyrrhina* (Smoothstem turnip)	Pot trial	Improved biomass of test plants and enhanced Ni accumulation in plant tissues	Ma *et al.* (2009a)
Psychrobacter sp., *Bacillus cereus*	*Brassica juncea, Brassica oxyrrhina*	Pot trial	Improved metal build-up in plant tissues by aiding Ni release from insoluble phases	Ma *et al*. (2009b)
Achromobacter xylosoxidans strain	*Brassica juncea*	Pot trial	Enhanced Cu plant uptake and increased root and shoot length, and plant biomass	Ma *et al*. (2009c)
Bacillus edaphicus	*Brassica juncea*	Pot trial	Promoted plant growth, reduced Cd uptake	Sinha and Mukherjee (2008)
Pseudomonas aeruginosa	*Brassica juncea*	Pot trial	Stimulated plant growth, aided soil Pb mobilisation & build-up	Sheng *et al.* (2008)
Pseudomonas tolaasii, *Pseudomonas fluorescens*, *Alcaligenes*, *Mycobacterium*	*Brassica napus* (Canola)	Pot trial	Protected canola against inhibitory effects of cadmium (Cd)	Dell'Amico *et al.* (2008)
Sinorhizobium sp.	*Brassica juncea*	Microcosms	Improved efficiency of Pb phytoextraction	DiGregorio *et al.* (2006)
Xanthomonas, *Azomonas*, *Pseudomonas*, *Bacillus*	*Brassica napus*	Pot trial	Stimulated plant growth, improved Cd accumulation	Sheng and Xia (2006)
Pantoea agglomerans	*Brassica oleracea var. capitata*	Pots, greenhouse conditions	Improved growth, nutrient, and hormone content	Turan *et al.* (2014)
Brevibacillus reuszeria *Rhizobium rubib*	*Brassica oleracea var. italica*	Field	Increased yield, plant height, head diameter, chlorophyll content, macro-nutrient and micronutrient uptake	Yildirim *et al.* (2011)
Pseudomonas fluorescens MTCC103	*Brassica oleracea var. italica*	Pot trials, greenhouse conditions	Enhanced plant growth, nutrient uptake and yield	Tanwar *et al.* (2014)

5.3.1.1 Selected species characterisation

Characterisation of the different species used for this research phase is discussed below. The individual species characteristics as a mother crop compared to other well-known mother crop species can be seen in **Table 5-2.** This table compares the soil tolerance and requirements, soil ecology, soil performance and impact of the mother crop species used in this research phase to those of wheat (*Triticum aestivum*) and oats (*Avena sativa*).

1. ***B. napus var. napus* (*L.*)** - **canola, oilseed rape, rapeseed, rape**

Canola is considered a winter crop with a growth optimum temperature of 21°C. Typically, temperatures below 10°C result in progressively weaker germination and plant development. Depending on the cultivar and growth conditions, mature plants can reach ± 1.7m in height. Canola possesses a taproot system and can grow rapidly after establishment, with 85% of the root dry matter in the top 25cm of soil. Days to flowering can differ between 70 days for late planted (middle to end June) and 120 days for early (May) planted crops. Seeds are round, black, brown or yellow and comparatively small. Mature pods contain ±23 seeds (DAFF, 2010).

2. ***Brassica napus var. oleifera* (*L.*) - fodder rape**

Fodder rape is a short-seasoned, leafy *Brassica* of which the leaves and stems are used for grazing purpose. Two types of fodder rape exist, i.e., a leafy upright giant type and a short-branched dwarf type. As forage rape is used for grazing it possesses a high leaf-to-stem ratio and is winter hardy. Spitfire cultivar is a modern rape produced by crossing rape with kale. Fodder rape is regarded as a suitable companion to other fast-establishing *Brassica* species. Spitfire is a medium variety with high dry matter yield, excellent aphid tolerance, good stock palatability and rapid maturity. Maturity is indicated by a transformation of leaf colour to a purplish colour (Ayres, 2002).

3. ***Lolium multiflorum var. italicum (L.)*- Annual ryegrass or Italian ryegrass**

Annual ryegrass is a very competitive winter annual, with good seedling vigour, fast germination (with adequate moisture) and rapid establishment. Germination usually occurs within a week to ten days under good conditions Annual ryegrass is a fast-growing bunch grass, yellowish-green at the base, with long glossy green leaves. Annual ryegrass root system comprises of extensive shallow roots that can tolerate compaction. Develops an extensive fibrous root system and can be quite competitive with other plants (Truter *et al*., 2016). Annual ryegrass produces several

upright tillers from each plant. Italian ryegrass is diploids (2n = 14) (Anon, 1998). The ryegrass served as a control plant, as ryegrass growth in tailings is known.

4. *Raphanus raphanistrum var. sativus* (L.) - Cherry Bell, Belle Cerise radish

Cherry belle radish is small, bright, red-skinned, fast-growing heirloom variety with creamy, white flesh. Radish reached maturity quite fast (± 4 weeks) while plants remain small (± 15cm height). This variety tends to bloom in late summer (blooms ± 0.4cm in diameter). Radish prefers cool and moist weather conditions (10°C to 18°C). In mild climate regions, radish can be planted throughout the year. The maintenance requirements for cherry belle radish are rather low; they are easy to grow (Folia, 2017).

5. *Brassica oleracea (L.) var. viridis (L.)* – fodder kale, leaf cabbage

Kale is characterised as a tall, bulky *Brassica* that is grown primarily for winter feed for cattle. Sovereign kale is a high yielding intermediate height kale with a high leaf to stem ratio. Kale is considered to be winter-hardy (survival tolerance to -12˚C) and possesses a greater cold tolerance compared to other *Brassica* species. Generally, more tolerant to dry rot and club root and has a deep root system. Kale has a slower maturation rate than traditional grazing *Brassica* species and is more suited to cool, summer moist climates (de Ruiter, 2009).

For detailed characteristics of various mother crops see **Table 5-2.** This table describes the specific plant's performance and role, tolerance to certain stress factors, their soil ecology and soil impact, and other necessary plant characteristics.

Table 5-2: Table of plant characteristics of some mother crops (adapted from Agricol, 2017; Clark, 2007).

Species	Performance and Roles								Tolerances					Soil ecology				Soil impact							Planting	
	Total N(kg/ha)[1]	Dry matter (t/ha/yr.)	N Scavenger[2]	Soil Builder[3]	Erosion Fighter[4]	Weed Fighter[5]	Good Grazing[6]	Quick Growth	Heat	Drought	Shade	Flood	Low fertiliser	Nematodes	Allelopathic	Disease	Attracts beneficial	Subsoiler	Free P & K	Loosen topsoil	Type	pH (preferred)	Best Established[7]	Min. Germin. Temp. (°C)	Seeding rate (kg/ha)	Reseeds
Annual rye grass	-	2.2-10	V	V	V	V	V	V	F	F	V	V	F	G	G	G	F	G	G	E	Winter annual	6,0-7,0	Esp, Lsu, EF, F	4	18 to 25	Reliably
Radish	55-220	4.5-7.9	E	V	V	E	G	V	G	F	G	F	F	V	V	G	F	E	V	G	Cool annual	6,0-7,5	Sp, Lsu, EF	7	2 to 4	Sometimes
Rapeseed (Canola & fodder rape)	45-180	2.2-5.6	V	G	V	V	G	V	F	G	G	F	F	V	V	G	G	G	F	G	Winter annual	5,5-8,0	F, Sp	5	3 to 4	Sometimes
Spring oats	-	2,2-11,2	V	G	V	E	G	E	F	F	F	G	G	P	V	G	F	P	E	V	Cool annual	4,5-7,5	LSu, Esp, W	3,3	50-100	Sometimes
Wheat	-	3,3-8,9	V	V	V	V	V	V	G	G	G	P	G	F	F	F	F	G	V	V	Winter annual	6,0-7,5	LSu, F	3,3	100-180	Sometimes

[1]Total N —Total N from all plant. Grasses not considered N source.
[2]N Scavenger—Ability to take up/store excess nitrogen.
[3]Soil Builder—Organic matter yield and soil structure improvement.
[4]Erosion Fighter—Soil-holding ability.
[5]Weed Fighter—suppress weeds and reduce damage by diseases, insects and nematodes
[6]Good Grazing—Production, nutritional quality and palatability.
[7] E=Early; M=Mid; L=Late; F=Fall; Sp=Spring; Su=Summer; W=Winter

See **Appendix B** for a full explanation of plant characteristic parameters.

Symbols: P=Poor; F=Fair; G= Good; V= Very Good; and E= Excellent

5.4 Materials and methods

Phase 2 of this study was conducted at the nursery for Soil and Plant research for Mine Rehabilitation on the premises of the North-West University (NWU) at Potchefstroom. For this phase, different types of bio-stimulants were applied to three gold tailings materials and a control soil. The difference in DHA within these substrates was determined after three weeks. Selected members of the Brassicaceae family and *L. multiflorum* was established with different bio-stimulant treatments. The mother crop species used for this research, with the exception of radish, were provided by Agricol.

5.4.1 Substrate selection

The different gold tailings materials were chosen based on their physical and chemical constraints, with a wide range of lime requirements selected to illustrate the effects of different acidic pH on microbial activity and plant performance. The different gold tailings materials include samples from Dominionville Gold Mine (Dominion Reef) and New Machavie Gold Mining (Transvaal Supergroup) (**Figure 4-1** and **Figure 4-2**). Two additional samples of New Machavie gold tailings (i.e., NM-700 and NM-C2) and coal discard were selected for the supplementary research study on biological amendments and bio-stimulants (refer to **Table 5-7** and **Figure 5-4**). For a detailed description of the geology of these materials refer to **Table 5-3**.

Table 5-3: List of gold tailings used and tailings numbering system.

Tailings	Origin	Number
Dominion gold	Dominion Reef	Dominion
New Machavie Sanddam	Transvaal Supergroup Black Reef Formation	NM-C1
New Machavie geel	Black Reef Formation	NM-geel
Red sandy loam	Control	Control
New Machavie 700	Black Reef Formation	NM-700
New Machavie Black Reef	Black Reef Formation	NM-C2
Coal discard	Witbank coal seam in the Karoo Supergroup	Coal

5.4.2 Amendments and bio-stimulant selection and application

In order to stimulate microbial activity and improve microbial communities, five different amendments were selected. These amendment materials were selected for their differences in biochemical quality and their potential abilities as bio-stimulants. This section will give a discussion of the bio-stimulants utilised. These bio-stimulants target specific microbial functional groups and plant traits. **Table 5-4** summarises the type of amendments used. The bio-stimulants

application per pot was done based on the recommended amount from the manufacturer. The amendments were applied as soil treatments two weeks before establishing phase 2 mother crop species.

Table 5-4: List of amendments.

Amendment	Type of amendment
Carbohydrate derivatives	Bio-stimulant for fungi and bacteria
Protein hydrolysates, amino acids and metabolites	Fungal bio-stimulant
Beneficial microbes and microbial activator	Bio-fertiliser and bio-stimulant for microbes
K-humate	Organic humic acid bio stimulant source of organic carbon
Mix	Combination of all of the above
No treatment	Serves as control

5.4.2.1 Amendments and bio-stimulant characterisation

This section gives a description of the different amendments and bio-stimulants used during phase 2. This includes both inorganic and organic amendments. du Jardin (2015), proposed that bio-fertilisers be placed in one of the sub-categories of bio-stimulants. For this purpose of this research, the bio-fertilisers used are considered to be bio-stimulants.

K-humate

K-humate was incorporated into gold tailings rehabilitation trials as a soil ameliorant (bio-stimulant) due to claims that it is beneficial for plant growth and soil microorganisms. Humates are mineral salts of humic (HA) or fulvic acids (FA). It is dark brown potassium (K)-humate-containing liquid. Humic substances such as this product have been proven to improve soil structure, CEC, and microbial activity, partly because of their complex, organic chemistry (Hamza & Suggars, 2001).

Protein hydrolysates, amino acids and metabolites:

Serves as a bio-stimulant that specifically targets fungal growth by supplying nutrients necessary for fungal growth. It is formulated to retain the biological diversity in the soil and primarily, to stimulate fungi growth. This product contains beneficial elements to enhance fungal growth. Additionally, it contains naturally chelated minerals, chlorophyll, 25 vitamins, proteins, carbohydrates, auxins, cytokinins, amino acids, humate and fulvates. This product contains no active microbe inoculants.

Beneficial microbe with microbial activator

This product is a high strength microbial inoculant specifically formulated to address all aspects of bio-fertilisation. The bio-fertiliser contains four *Bacillus* species with a high microbial strength ranging from 5 x 10^8 to 1.5 x 10^{10} colony-forming unit/ml (15,000,000,000 bacteria/ml). These bacteria are able to solubilise P, nitrify the soil, and as a bio-fertiliser increase plant nutrition through ATP production and improved mineral uptake. PGPR attributes include the production of plant growth hormones such as auxins, gibberellins and cytokinins. The presence of this bacilli bacterial complex in the soil shows that there is a high degree of suppression of various common root diseases. This increases the ability of plants to experience healthier root growth. The bacterial inoculant was applied with a microbial growth stimulant; these stimulants provide C energy to the bacteria to sustain their activity. This product contains active ingredients such as naturally occurring plant sugars, proteins and fulvic acid.

Carbohydrate derivatives

Carbohydrate derivatives were chosen as a model compound for organic materials with large amounts of readily available C. Carbohydrate derivatives are a great carbon source for crops. Boosting plant and soil energy as well as being a very effective bio-stimulant. One of the primary functions as a bio-stimulant, carbohydrate derivatives serves as a food source supplying carbon, sugars and carbohydrates for soil microorganisms, specifically bacteria. They also function as a chelating agent, which converts nutrients tied up in the soil to more plant available forms (Soil Guy, 2017). In terms of carbohydrate derivatives as a soil amendment, several studies have indicated their benefits, such as enhancing the structural stability of sodic soils (Suriadi *et al*., 2002) or facilitating the degradation of organic contaminants as a co-substrate in soil (Boopathy, 2001).

5.4.3 Plant species selection and establishment

Plant species were established after the gold tailings materials were pretreated with fertiliser and lime. The crop species selected for the experiment were based on their tolerance and ability to grow on gold tailings materials. The pot trials were irrigated regularly after seeding. For this study, the *Brassica* plant species selected included canola, fodder rape, kale, and radish. In addition to the Brassicaceae species, *Lolium multiflorum* (annual ryegrass) was used as a model plant. Due to *Brassica oleracea L. var. viridis* (kale) low mean germination rate and high mortality rate in all the growth substrates, the kale was removed at week 3 to plant an alternative species, *Raphanus sativa* (cherry belle radish). The kale's growth in the four growth substrates can be seen in **Appendix D**. Examples of the different Brassicaceae species used for this research phase can

be seen in **Figure 5-1.** These species are known to be a good indicator plant and was subsequently chosen for these characteristics. The scientific and common names of the selected species are listed in **Table 5-5.**

Table 5-5: List of established mother crop species.

Common name	Cultivar name	Scientific name
Canola	Garnet	*Brassica napus*
Fodder rape	Spitfire	*Brassica napus*
Kale	Sovereign	*Brassica oleracea var. sabellica*
Radish	Cherry Belle	*Raphanus sativa*
Italian ryegrass	Agriboost	*Lolium multiflorum*

A- Fodder rape **B- Canola** **C- Radish**

Figure 5-1: Examples of the different mother crop species.

5.4.4 Effect of bio-stimulants on plant performance

In order to determine the effect of the bio-stimulants on plant performance, nursery germination trials were conducted. This was accomplished by placing 10 seeds per species in 616cm^2 pots with four replicates per treatment in four different growth substrates (n=560). The seeds were sown in a circular arrangement as seen in **Figure 5-2.** For each substrate and each bio-stimulant, three treatments were used: (i) tailing/soil with bio-stimulants, (ii) tailing/soil with bio-stimulant and mother crop species (iii) tailing/soil with mother crop species, without bio-stimulants. Untreated tailings/soil was used as control treatments. Germination was considered to have occurred when the radicles were half of the seed length. Growth of the different mother crop species was initially measured weekly for a period of five weeks to determine the germination rate and

mortality/survivability of each mother crops/bio-stimulant treatments (five measurements in total for each plant species). The list of each mother crop species and bio-stimulant combinations with abbreviations are summarised in **Appendix C: Table C-1.**

Germination and survival rate are expressed as a percentage (%) and calculated as follows:

Germination rate

$$\textbf{\textit{Germination rate}}\ (\%) = \frac{\textbf{\textit{number of seeds germinated}}}{\textbf{\textit{total number of seeds}}} \times \mathbf{100}$$

Survival rate

$$\textbf{\textit{Finite survival rate}}\ (\%) = \frac{\textbf{\textit{individuals alive at end of time period}}}{\textbf{\textit{individuals alive at start of time period}}} \times \mathbf{100}$$

Figure 5-2: Weekly plant growth monitoring of the different mother crop species.

5.4.5 Effect of plant species and bio-stimulants on DHA

As a result of economic constraints, only untreated treatments and bio-stimulants/mother crop treatments showing the best growth improvement based on germination rate were sampled for DHA. For this reason, other enzymes were not analysed. In order to determine the overall microbial activity of the different treated growth substrates, DHA was measured. DHA of the six selected treatments and untreated control from four different substrates with four replicates each were sampled. Rhizosphere soil samples were collected from the pot trial at the end of the mother crop species growth season. Composite samples were taken for each bio-stimulants/plant species combination (n=4). The hand-held auger was centred over the rhizosphere to maximise the retrieval of rhizosphere-influenced soil. All substrate samples were stored on cooldown (on ice) after collection and brought to the laboratory.

DHA activity was assayed, three weeks after the application of the various bio-stimulants and again at the end of the mother crops growth season. The DHA was determined with the substrate with iodonitrotetrazolium violet (INT) according to the method by von Mersi and Schinner (1991), explained by Alef and Nannipieri (1995).

- Moist soil was collected after harvesting the mother crops.
- For microbial enzyme assay purposes, three bio-stimulant/substrate treatment composite samples were taken.
- Each of these composite samples consisted of four subsamples taken from the top 15cm of soil (rhizosphere) (Dick *et al*., 1996; Taylor *et al*., 2002).
- A soil auger was used to obtain substrate samples and sampling was carried out in autumn (April 2016) three weeks before the phase 3 winter crop establishment.
- In order to ensure unbiased microbial activity determination, the sampling of the amended substrates took place three weeks after bio-stimulant application. This ensures that the DHA represents a more stabilised microbial growth, rather than exponential phase growth.
- The samples were brought to the laboratory the same day, homogenised and kept in a refrigerator at 4°C to keep them field moist and preserve biological characteristics until analyses.
- The DHA of the mother crop/bio-stimulant treatments was measured by Eco-Analytica. DHA assays for the bio-stimulants (before mother crop establishment) were conducted in collaboration with the Agricultural Research Council (ARC). For detailed method see **Chapter 3**.
- Refer to **Appendix E** for the seasonal difference in DHA during summer and winter months.

5.4.6 Statistical analyses

Statistical analyses were performed and graphs generated. The differences between the bio-stimulant treatments from the four different substrates were evaluated by means of ANOVA. Tukey's Honest Significant Difference test (HSD) was used to identify significant differences between sites, with error rate set at p 0.05. PCA was constructed in order to determine sample relatedness using XLSTAT 2017(Addinsoft, Paris, France). SD values were calculated using Microsoft Excel and depicted in graphs as error bars. The Pearson's correlation coefficient (Pearson's r) was calculated. Positive correlation coefficients indicate that pairs of variables increase together, whereas negative values indicate an inverse relationship. For pairs with $p < 0.05$, a significant association exists between the variables.

5.5 Results and discussion

Results obtained from the bio-stimulants application and mother crops plant performance results and DHA are presented and discussed in this part of the chapter.

5.5.1 Bio-stimulants effect on DHA (before mother crop establishment)

The DHA was determined after the application of different bio-stimulants without mother crop establishment, to determine whether these bio-stimulants had any significant effect on the microbial activity within the different growth substrates. A study on biological amendments and bio-stimulants were done on other types of tailings materials shown in **Table 5-7** and **Figure 5-4,** in order to get additional research data. It was hypothesised that the bio-stimulants would increase the DHA to various degrees depending on the type of bio-stimulant and type of tailings. The DHA results presented in **Table 5-6** and **Table 5-7** supports this hypothesis with statistical significance ($p<0.05$) for selected growth substrates/bio-stimulant treatments. In the additional study, some amendments were included that cannot be defined as bio-stimulants, as they contain either fertilisers (e.g., microfine langfos) or amendments that are used to improve soil conditions (e.g., bentonite). Consequently, hereafter these amendments are known as biological amendments.

Table 5-6: Bio-stimulants DHA after 3 weeks.

Bio-stimulants	NM-geel	Dominion	Ochric A	NM-C1
	Dehydrogenase (µg INF/g/2h)			
K-humate	291.0 ± 49.6^{ab}	198.4 ± 37.9^{a}	192.7 ± 52.6^{a}	156.0 ± 83.2^{a}
Amino acids	374.3 ± 106.2^{a}	295.4 ± 62.4^{a}	139.3 ± 54.1^{bc}	161.2 ± 86.5^{a}
Carbohydrate	235.3 ± 33.73^{b}	284.3 ± 41.85^{a}	155.2 ± 26.21^{b}	174.0 ± 21.14^{a}
Beneficial bacteria	270.1 ± 54.2^{ab}	277.1 ± 38.8^{a}	126.5 ± 27.2^{c}	187.4 ± 46.3^{a}
Mix	212.8 ± 38.7^{b}	276.1 ± 50.6^{a}	156.6 ± 91.0^{b}	166.1 ± 55.4^{a}
Native untreated	1.5 ± 4.6^{c}	3.8 ± 3.9^{b}	12.7 ± 4.1^{d}	0.2 ± 0.4^{b}
Pr > F	0.003	0.015	< 0.001	0.004

[1]Values given are mean ± standard error values.
[2] Data in column with similar superscript alphabetic letters indicate no statistical significance $p>0.05$, in contrast, those with different letters show significant variance at Tukey's HSD $p< 0.05$.
[3] INF: iodonitrotetrazolium chloride-formazan.

ANOVA and Tukey's HSD shows a significant variation ($p<0.05$) in DHA amongst the different bio-stimulants and substrates. Statistical significance and variability between bio-stimulant groups and untreated control can be seen for all the growth substrates, i.e., NM-geel (p±0.003), Dominion (p±0.015), ochric A ($p<0.001$), and NM-C1. The different bio-stimulant treatments improved the DHA of all the different growth substrates to various degrees when compared to the untreated (control) tailings and soil. The significance and variability between bio-stimulant treatments are

shown in **Table 5-6.** A variation can be seen in the general DHA, i.e., NM-C1> ochric A > NM-geel >Dominion. This variation can be attributed to the growth substrates chemical and physical properties (**Table 4-2** to **Table 4-4**).

The difference in DHA for the four growth substrates can be seen in **Figure 5-3.** This graph shows the variability in DHA between the various biostimulants and growth substrates. In NM-geel tailings, DHA data indicated a significant increase in the microbial activity due to the use of amino acids and protein hydrolysates. The DHA in the NM-geel/amino acid treatment (374µg INF/g/2h) exhibited a 250-fold higher activity than in the untreated tailings (2µg INF/g/2h). In the Dominion and NM-C1 tailings, DHA data indicated a significant increase in the microbial metabolic activity compared to the untreated tailings, however, there exists no variation between the bio-stimulants. Consequently, the bio-stimulants equally increase the DHA. In the ochric A, the K-humate indicated a significant increase in DHA when compared to the untreated tailings. The DHA in the ochric A/K-humate treatment (193µg INF/g/2h) exhibited a 15-fold higher activity than in the untreated tailings (13µg INF/g/2h). K-humate has a greater resistance to further decomposition. As a result, the K-humate serves as a more long-term microbial SOM (Asli & Neumann, 2010; Pizzeghello *et al*., 2002; Schiavon *et al*., 2010). The lowest improvement in NM-geel DHA was the bio-stimulants mixture, in Dominion and NM-C1 tailings it was K-humate and in the ochric A-horizon, it was the beneficial bacteria.

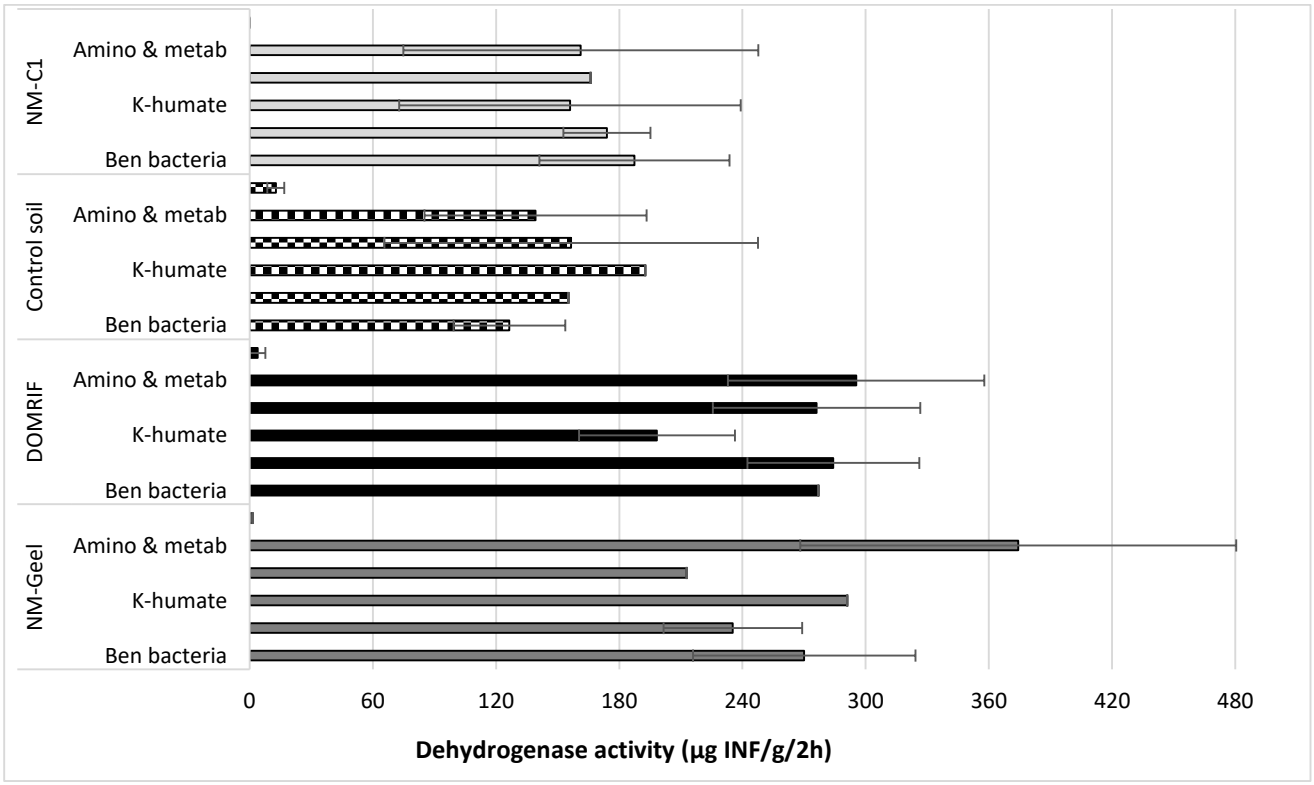

Figure 5-3: DHA associated with the various bio-stimulants of the four growth substrates.

Table 5-7: DHA of additional biological amendments and bio-stimulants.

Bio-stimulants	NM-700	Control soil	Coal discard	NM-C2
	Dehydrogenase (µg INF/g/2h)			
Leonardite	200.58^{abcd}	176.81^{abc}	229.79^{abc}	95.40^{a}
Bacterial food source	76.05^{d}	116.43^{abcd}	192.29^{abc}	77.22^{a}
Beneficial bacteria	264.17^{abcd}	106.95^{abcd}	258.74^{abc}	65.87^{a}
Amino acid	161.12^{bcd}	84.79^{abcd}	167.73^{abc}	176.73^{a}
K-humate	95.43^{cd}	159.00^{ab}	126.74^{bc}	147.36^{a}
Ca -Seaweed extract	164.51^{abcd}	121.02^{ab}	271.13^{abc}	114.55^{a}
Microfine langfos	380.61^{a}	79.63^{bcd}	307.33^{ab}	163.30^{a}
Humic/fluvic acid	307.94^{abc}	49.79^{cd}	297.03^{ab}	140.28^{a}
Bentonite	174.92^{abcd}	141.10^{abc}	358.81^{a}	209.88^{a}
Carbohydrate x1	365.82^{ab}	188.05^{a}	312.28^{ab}	81.10^{a}
Carbohydrate x2[1]	202.57^{abcd}	124.10^{abc}	175.57^{abc}	105.21^{a}
Carbohydrate x3[1]	188.06^{abcd}	139.81^{abc}	277.13^{abc}	119.64^{a}
Carbohydrate x4[1]	216.99^{abcd}	104.66^{abcd}	200.61^{abc}	76.03^{a}
Lime	144.99^{cd}	76.17^{bcd}	191.58^{abc}	62.11^{a}
Native untreated	75.52^{d}	27.03^{d}	101.45^{c}	161.45^{a}
Pr > F	**0.034**	**0.033**	**0.041**	0.159

[1] Carbohydrate concentration increased two, three and four times the standard application rate.
† Data in column with similar superscript alphabetic letters indicate no statistical significance p>0.05, in contrast, those with different letters show significant variance at Tukey's HSD p<0.05.
‡INF: iodonitrotetrazolium chloride-formazan.

ANOVA and Tukey's HSD shows a significant difference in DHA between the different bio-stimulants and growth substrates. These differences were statistically significant and variable for NM-700 (p±0.034), control soil (p±0.033) and coal discards (p±0.041). No significant statistical difference was observed in the NM-C2 gold tailings (p±0.159). All the biological amendments applied to the different growth substrates increase the DHA to various degrees compared to the untreated DHA. A clear difference in the general DHA of the different growth substrates can be seen. For example, DHA is coal discard < NM-700 < NM-C2 < control soil. The control soil possesses a noticeable low DHA. The low DHA in the control soil can be attributed to the soils natural characteristics, i.e., poor moisture-holding capacity, high water infiltration and crust formation. This results in increased drying of the soil, wilting of plants and leaching of bio-stimulants. It should be noted that the control soil used in this additional study is not the same as the ochric A used in this research phase.

As presented in **Figure 5-4** and **Table 5-7**, in the NM-700 tailings, the microfine langfos (381 µg INF/g/2h) and the carbohydrate (366µg INF/g/2h) biological amendments had the highest

significant increase in DHA. The DHA in the NM-700 microfine langfos exhibited a 5-fold higher activity than in the untreated NM-700 tailings (control). Langfos is registered as a sedimentary rock phosphate. Langfos is different from normal P-fertiliser, in the fact that langfos not only contains phosphorous and gypsum, but also micronutrients (i.e., S, Fe, Mn, B, Mg, Zn, Mo, Cu and Co), and calcitic lime. The increase in DHA in the NM-700 tailings microfine langfos treatment can probably be contributed to nutrient increases P. Fertiliser can directly stimulate the microbial population growth and activity by supplying nutrients to the microbial population (Khonje *et al*., 1989). Results obtained by Chu *et al*. (2007), revealed that DHA increased significantly with the addition of P comparative to other macronutrients (N and K), consequently emphasising the importance of P in soil microbial metabolism. In the control soil, the carbohydrates (188µg INF/g/2h) had the most significant increase in DHA. The DHA in the control soil carbohydrates exhibited a 7-fold higher activity than in the untreated control soil. In the coal discard, the bentonite and carbohydrates (Bent=359µg INF/g/2h; Carb=312µg INF/g/2h) had the most significant increase in DHA. The DHA in the coal discards bentonite exhibited a 4-fold higher activity than in the untreated coal discards (control). Although not significant, in the NM-C2, the bentonite (210µg INF/g/2h) had the highest increased in DHA. In NM-C2 no variability exists between bio-stimulants and control.

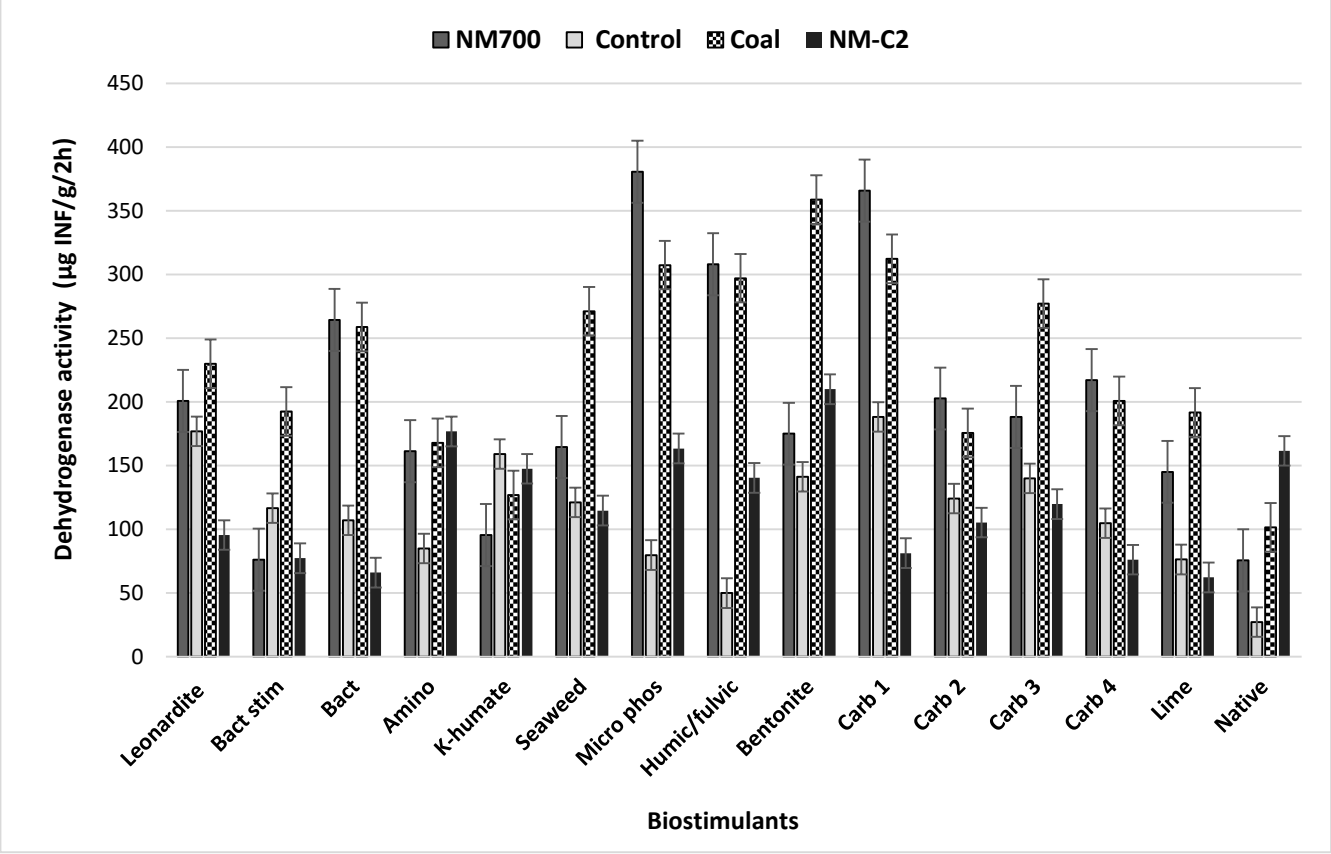

Figure 5-4: Graph illustrating the relative DHA response of the additional biological amendments trial on four growth substrates.

The high increase in DHA relating to the carbohydrates and bentonite amendments seems to redundantly improve the DHA throughout all the growth substrates. Carbohydrates' contribution towards increased DHA can be attributed to the energy source that the carbohydrates provide to microorganisms. These carbohydrate derivatives usually contain macronutrient and trace elements which can be utilised by soil microbes (Ward, 2016). Tate (1995) found that C resources most commonly controls soil microbial development. Consequently, the application of carbohydrate bio-stimulant with energy-rich C has a substantial microbial activity enhancing capability for short periods of time. The problem, however, arises in the fact that carbohydrates provide a fast nutrient source to microorganisms, it is also highly leachable through the soil and is therefore quickly depleted. Bentonite is a clay soil that usually contains at least 70% of the three-layered (2:1) clay mineral montmorillonite. Limited research has been reported for bentonite as a soil amendment. Bentonite has the ability to immobilise trace metal elements and metalloids present in the tailings materials. A decrease in trace metal toxicity in these growth substrates could possibly explain the increase in DHA in the tailings. This result concurs with Kiikilä *et al*. (2001), who reported that immobilisation of trace metals and metalloids caused an increase in basal respiration, litter decomposition, and microbial activity. In addition, bentonite possesses a high moisture-absorption and swelling potential that improves soil conditions by improving moisture-holding capacity and buffering capacity.

In terms of lowest/smallest increase in DHA, in the NM-700 tailings, the bacterial food source had the lowest DHA increase (76µg INF/g/2h), which is similar to the untreated control DHA. This indicates that the bacterial food source had no effect on the microbial activity. In the control soil, the lime application had the lowest increase in DHA (76µg INF/g/2h) which is still a 3-fold increase in DHA. In the coal discard, the K-humate had the lowest increase in DHA (127µg INF/g/2h), which is only around ± 25g INF/g/2h higher than untreated tailings DHA. In the NM-C2 tailings, most of the biological amendments had a lower DHA when compared to the untreated tailings DHA, apart from microfine langfos and bentonite. The lack of significant effects found on NM-C2 DHA with biological amendments in this study was possibly as a result of large variation observed between measurements, on different samples of the same treatment (microbial activity varies on spatial scale) (van Schoor, 2009). Another plausible reason for this contrasting DHA levels in NM-C2 tailings was due to the leaching effects of biological amendments in trail pots during irrigation. The biological amendments DHA was subjected to nursery trials external environmental factors consequently influencing the microbial activity (Guwahati, 2014).

5.5.2 Plant establishment and influence of bio-stimulants on plant growth

For this part of the research, it was hypothesised that the bio-stimulants would improve mother crop germination (emergence) and survival rate. Additionally, the different mother crops species' ability to successfully establish the different gold tailings was investigated. The bio-stimulants exhibited varying degrees of stimulatory effect on plant growth. Germination results revealed a positive influence of the bio-stimulants on the cultivated plants. Data presented in **Figure 5-5** and **Table 5-8** show the effect of applying bio-stimulants on vegetative growth characters of different Brassicas mother crop species during the winter growing season. Radar graphs presented in **Figure 5-6** illustrates the relative survival (%) rate of different mother crop species to several bio-stimulants grown in the different growth substrates. In most cases, plants in the experimental bio-stimulant/mother crop treatments performance were higher than those in the control treatments (no bio-stimulants). The various bio-stimulants significantly increased the mother crop species germination and survival rate ($p<0.005$). ANOVA and Tukey's HSD shows that a difference in germination and survival rate (%) can be seen between the different bio-stimulant/mother crop species treatments (**Table 5-8**). The differences were statistically significant and variable for NM-geel germination rate and survival ($p=0.001$) and Dominion germination rate ($p=0.024$) and survival ($p=0.022$). Tukey's HSD showed significant variability between the germination and survival rate between different mother crop/bio-stimulant treatments. Results obtained from control soil was significant for the germination rate and survival ($p=0.003$) but not variable. No significance was observed in the NM-C1 gold tailings.

Plant performance of the different mother crop species is also highly variable for the different bio-stimulants within the different gold tailings and control soils. The ryegrass served as a control pioneer grass species for this phase. The best plant performance in terms of survival versus germination for the gold tailings was ryegrass, with best results varying in bio-stimulants treatments. For example, the best result for ryegrass was NM-geel and Dominion – ryegrass with K-humate (57.5% for NM-geel and 97.5% for Dominion), NM-C1- ryegrass with carbohydrates (95%) (reoccurring positive results present in all substrates for bio-stimulants, i.e., Rahum, Racarb, Chum, Ccarb and Ryhum, refer to **Appendix C**). The control soil had no difficulties in terms of plant germination or establishment, having 100% plant survivability for several fodder rape treatments. In terms of control soil bio-stimulants, the amino acid, mixture of bio-stimulants and beneficial bacteria performed well with the mother-crops. The mother crop species with no additional bio-stimulants had the lowest establishment in the gold tailings materials. The lowest mother crop species establishment for the gold tailings include NM-geel canola (7.5%), Dominion fodder rape (42.5%) and NM-C1 ryegrass (67.5%).

5.5.2.1 Germination rate and survival rate

Table 5-8: Different plant species and bio-stimulant combinations mean germination and survival rate (%).

Plant/bio-stimulants	NM-geel		Dominion		NM-C1		Control soil	
	Germination (%)	Survival (%)	Germination (%)	Survival (%)	Germination (%)	Survival (%)	Germination (%)	Survival (%)
C-	7.5 ± 7.5^{c}	7.5 ± 7.5^{c}	60.0 ± 25.5^{ab}	55.0 ± 29.6^{ab}	85.0 ± 7.5^{a}	85.0 ± 7.5^{a}	97.5 ± 4.3^{a}	97.5 ± 4.3^{a}
Caa	30.0 ± 20.0^{abc}	30.0 ± 20.0^{abc}	60.0 ± 21.2^{ab}	60.0 ± 21.2^{ab}	92.5 ± 11.3^{a}	92.5 ± 11.3^{a}	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}
Chum	47.50 ± 17.5^{abc}	42.5 ± 13.8^{abc}	80.0 ± 12.2^{ab}	70.0 ± 7.1^{ab}	87.5 ± 12.5^{a}	87.5 ± 12.5^{a}	95.0 ± 5.0^{a}	95.0 ± 5.0^{a}
Cbac	22.5 ± 13.8^{abc}	20.0 ± 10.0^{abc}	57.5 ± 8.3^{ab}	57.5 ± 8.3^{ab}	90.0 ± 10.0^{a}	90.0 ± 10.0^{a}	97.5 ± 4.3^{a}	97.5 ± 4.3^{a}
Cmix	30.0 ± 25.0^{abc}	27.5 ± 22.5^{abc}	77.5 ± 22.8^{ab}	75.0 ± 20.6^{ab}	92.5 ± 8.3^{a}	92.5 ± 8.3^{a}	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}
Ccarb	47.5 ± 32.5^{ab}	45.0 ± 30.0^{ab}	72.5 ± 17.9^{ab}	72.5 ± 17.8^{ab}	90.0 ± 5.0^{a}	90.0 ± 5.0^{a}	92.5 ± 8.3^{a}	92.5 ± 8.3^{a}
Ra-	32.5 ± 8.8^{abc}	32.5 ± 8.8^{abc}	45.0 ± 15.0^{ab}	42.5 ± 10.9^{ab}	75.0 ± 5.0^{a}	72.5 ± 7.5^{a}	85.0 ± 16.6^{a}	85.0 ± 16.6^{a}
Raaa	17.5 ± 3.8^{abc}	10.0 ± 5.0^{abc}	75.0 ± 5.1^{ab}	72.5 ± 8.3^{ab}	90.0 ± 10.0^{a}	90.0 ± 10.0^{a}	97.5 ± 4.3^{a}	97.5 ± 4.3^{a}
Rahum	42.5 ± 11.3^{abc}	42.5 ± 11.3^{abc}	70.0 ± 12.2^{ab}	67.5 ± 13.0^{ab}	87.5 ± 12.5^{a}	87.5 ± 12.5^{a}	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}
Rabac	20.0 ± 20.0^{abc}	20.0 ± 20.0^{abc}	72.5 ± 16.4^{ab}	72.5 ± 16.4^{ab}	85.0 ± 15.0^{a}	85.0 ± 15.0a	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}
Ramix	12.5 ± 12.5^{bc}	10.0 ± 10.0^{bc}	60.0 ± 25.5^{ab}	60.0 ± 25.5^{ab}	85.0 ± 7.5^{a}	85.0 ± 7.5^{a}	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}
Racarb	40.0 ± 20.0^{abc}	40.0 ± 20.0^{abc}	45.0 ± 32.0^{b}	45.0 ± 32.0^{b}	80.0 ± 15.0^{a}	80.0 ± 15.0^{a}	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}
Rad-	25.0 ± 12.5^{abc}	15.0 ± 7.5^{abc}	60.0 ± 14.1^{ab}	57.5 ± 10.9^{ab}	70.0 ± 5.0^{a}	70.0 ± 5.0^{a}	90.0 ± 7.1^{a}	90.0 ± 7.1^{a}
Radaa	32.5 ± 12.5^{abc}	22.5 ± 7.5^{abc}	74.0 ± 17.4^{ab}	68.0 ± 19.4^{ab}	82.5 ± 12.5^{a}	82.5 ± 12.5^{a}	95.0 ± 5.0^{a}	95.0 ± 5.0^{a}
Radhum	25.0 ± 17.5^{abc}	20.0 ± 10.0^{abc}	62.5 ± 16.4^{ab}	60.0 ± 18.7^{ab}	85.0 ± 5.0^{a}	85.0 ± 5.0^{a}	85.0 ± 8.7^{a}	85.0 ± 8.7^{a}
Radbac	30.0 ± 10.0^{abc}	25.0 ± 12.5^{abc}	60.0 ± 7.1^{ab}	60.0 ± 7.1^{ab}	85.0 ± 10.0^{a}	77.5 ± 11.3^{a}	90.0 ± 7.1^{a}	90.0 ± 7.1^{a}
Radmix	25.0 ± 7.5^{abc}	25.0 ± 7.5^{abc}	77.5 ± 27.7^{ab}	77.5 ± 27.7^{ab}	80.0 ± 10.0^{a}	77.5 ± 8.8^{a}	87.5 ± 4.3^{a}	87.5 ± 4.3^{a}
Radcarb	27.5 ± 17.5^{abc}	22.5 ± 18.8^{abc}	82.5 ± 17.9^{ab}	75.0 ± 11.2^{ab}	75.0 ± 5.0^{a}	72.5 ± 3.8^{a}	92.5 ± 4.3^{a}	92.5 ± 4.3^{a}
Ry-	30.0 ± 10.0^{abc}	27.5 ± 8.8^{abc}	85.0 ± 15.0^{ab}	85.0 ± 15.0^{ab}	72.5 ± 3.8^{a}	67.5 ± 7.5^{a}	97.5 ± 4.3^{a}	97.5 ± 4.3^{a}
Ryaa	50.0 ± 15.0^{ab}	50.0 ± 15.0^{ab}	75.0 ± 15.0^{ab}	75.0 ± 15.0^{ab}	82.5 ± 8.8^{a}	82.5 ± 8.8^{a}	80.0 ± 10.0^{a}	80.0 ± 10.0^{a}
Ryhum	62.5 ± 8.8^{a}	57.5 ± 12.5^{a}	97.5 ± 4.3^{a}	97.5 ± 4.3^{a}	85.0 ± 5.0^{a}	82.5 ± 3.8^{a}	80.0 ± 15.8^{a}	80.0 ± 15.8^{a}
Rybac	35.0 ± 15.0^{abc}	32.5 ± 13.8^{abc}	85.0 ± 15.0^{ab}	85.0 ± 15.0^{ab}	95.0 ± 5.0^{a}	92.5 ± 7.5^{a}	90.0 ± 7.1^{a}	90.0 ± 7.1^{a}
Rymix	45.0 ± 25.0^{abc}	42.5 ± 27.5^{abc}	82.5 ± 14.8^{ab}	82.5 ± 14.8^{ab}	85.0 ± 10.0^{a}	85.0 ± 10.0^{a}	90.0 ± 7.1^{a}	90.0 ± 7.1^{a}
Rycarb	40.0 ± 30.0^{abc}	37.5 ± 27.5^{abc}	92.5 ± 8.3^{a}	90.0 ± 10.0^{a}	95.0 ± 7.5^{a}	95.0 ± 7.5^{a}	82.5 ± 4.3^{a}	82.5 ± 4.3^{a}
Pr > F	**0.001**	**0.001**	**0.024**	**0.022**	0.360	0.336	**0.003**	**0.003**

† Values given are mean ± standard error values. Represent the mean of 4 replicates (n=4) ± standard error.

‡Treatments labelled with the same letters within a column were not statistical significance p>0.05, in contrast, those with different letters show significant variance at Tukey's HSD p<0.05. See **Appendix C-1** for a full list of abbreviation used in the result.

Figure 5-5 illustrates the relative germination rate of the four different *Brassica* species and ryegrass used in this phase. The different plant species established performed differently with the different amendments. Overall, these nursery trials displayed variable responses to applied bio-stimulant treatments. By comparing the overall germination and survival rate of the different growth substrates, NM-geel had the lowest germination and survivability and the control soil the highest. In addition to low germination and establishment, the NM-geel plant growth is visually poor with reduced plant health (see **Figure 5-7** and **Figure 5-10**).

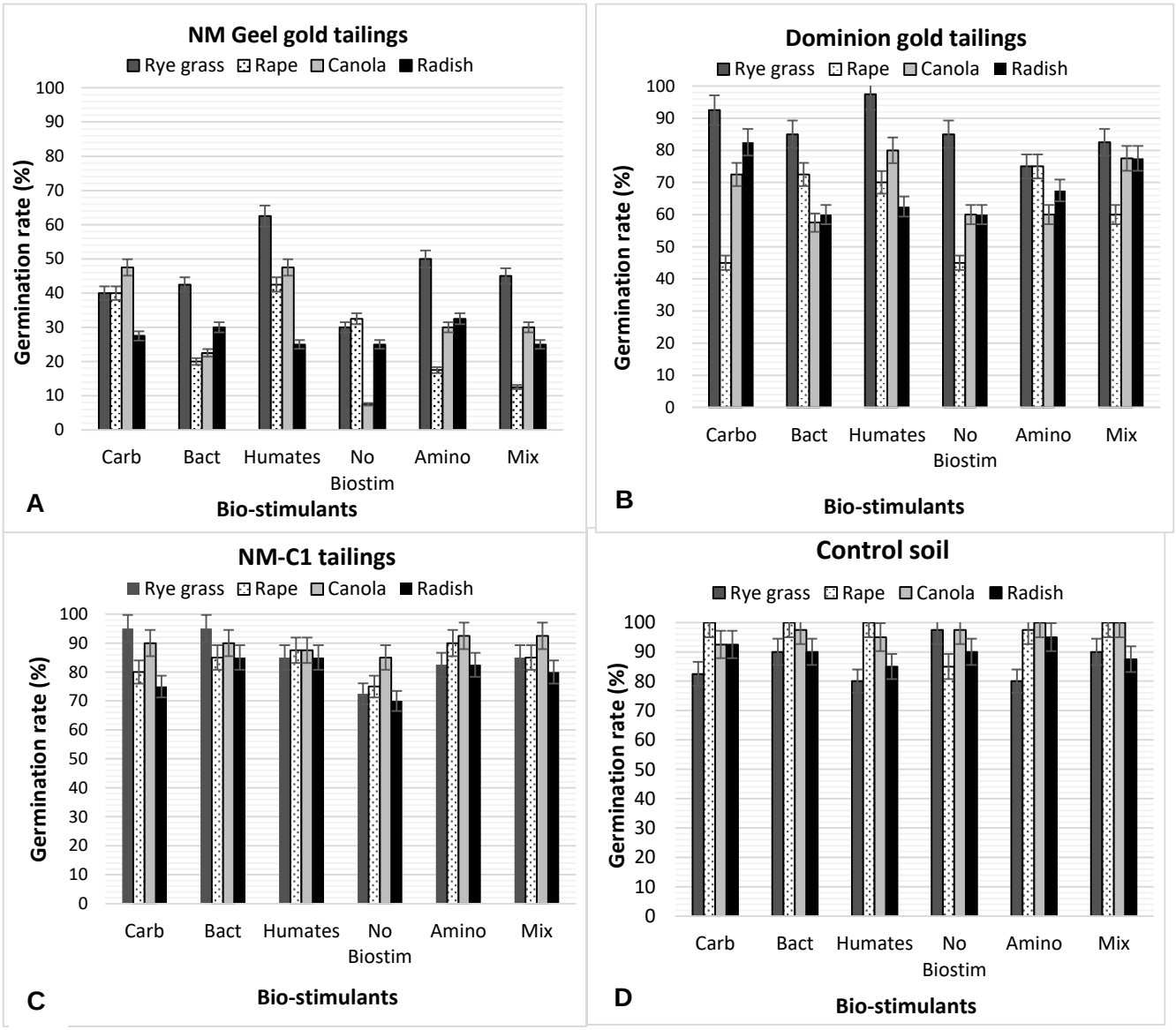

Figure 5-5: Germination rate (%) of different mother crop species and bio-stimulant treatments grown in four growth substrates. From above left (A) NM-geel; above right (B) Dominion; below left (C) NM-C1 and below right (D) Control soil. Carb- Carbohydrates; Bact- Beneficial bacteria; Humate-K-humate; No biost- no bio-stimulants; Amino- amino acids, Mix- a mixture of amendments.

When comparing the mother crop species performance of gold tailings to the control soil, contrasting results are observed, i.e., ryegrass with K-humate performing well in tailings whilst having the worst performance in the control soil. It is evident from the results that most of the applied bio-stimulant treatments greatly improved the mother crop species growth, compared with those of controls (no bio-stimulants). The application of K-humate, carbohydrates and beneficial bacteria had the highest growth-enhancing effects on the mother crop plant growth. The survival rate for the individual mother crop species established in the different gold tailings and control soil are presented in **Figure 5-6**. Results indicate that the mother crop and bio-stimulants establishment is substrate specific, i.e., certain bio-stimulants and mother crop species combinations performed better on different gold tailings.

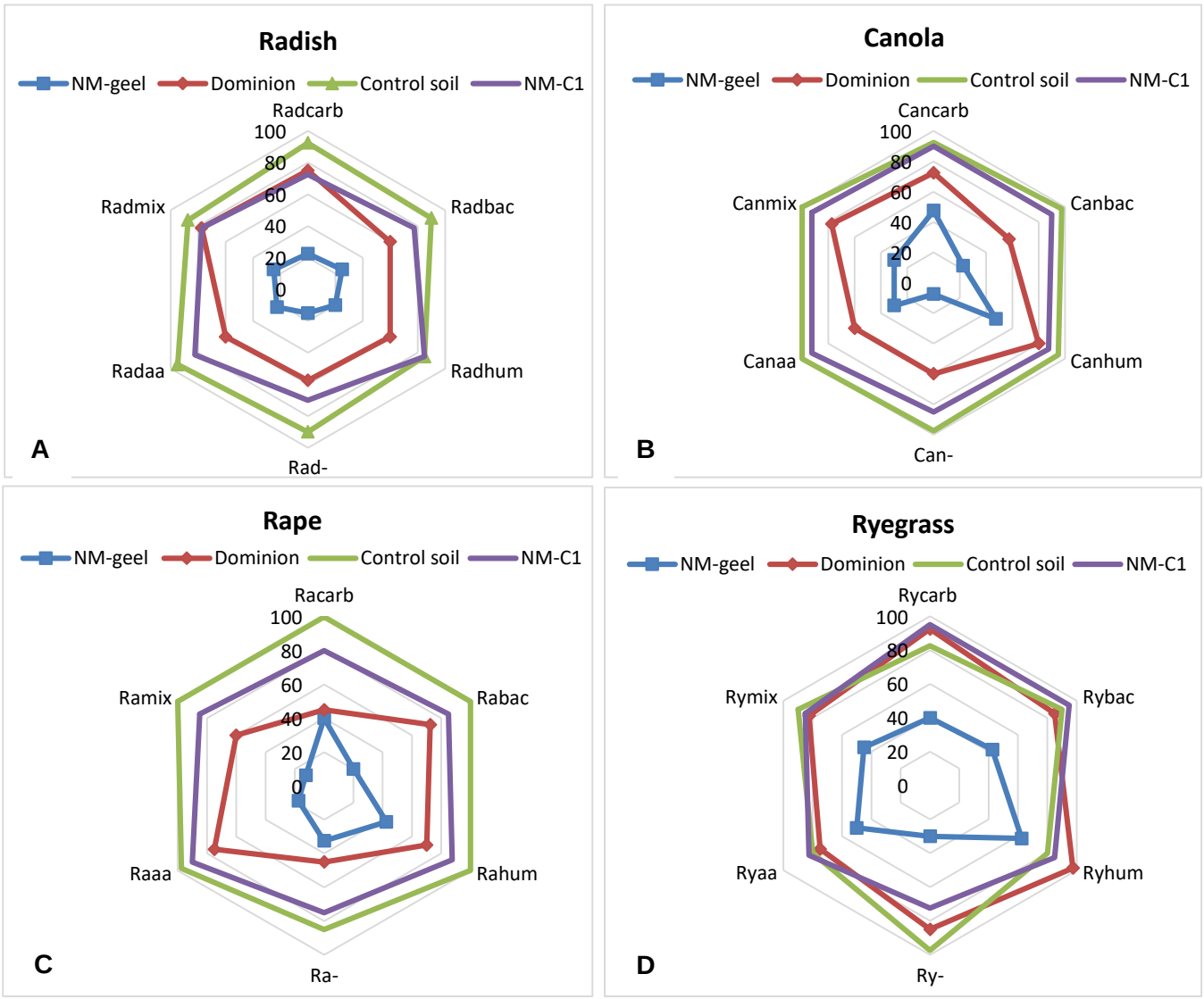

Figure 5-6: Radar graphs illustrating the relative survival (%) response of different mother crop species to several bio-stimulants grown in four substrates. Bio-stimulants abbreviations are listed in Appendix C. Mother crops are (A) radish (B) canola (C) fodder rape and (D) ryegrass.

Bio-stimulant treated canola germination rate over a period of 5 weeks in the four growth substrates.

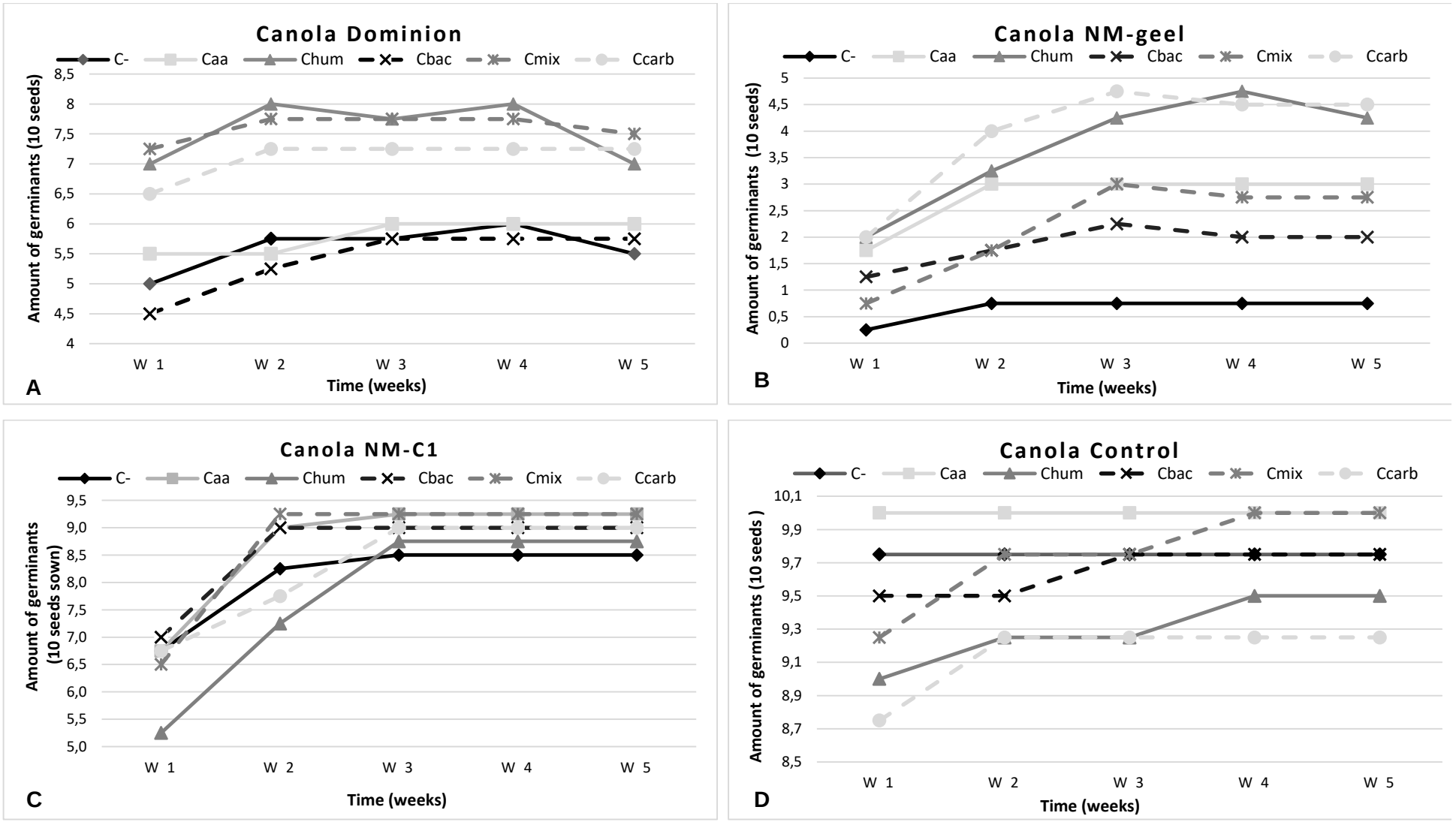

Figure 5-7: Germination rate of canola/bio-stimulant treatment combinations established in different gold tailings and control soil. From above left (A) Dominion, above right (B) NM-geel, below left (C) NM-C1, and below right (D) control soil.

Bio-stimulant treated fodder rape germination rate over a period of 5 weeks in the four growth substrates.

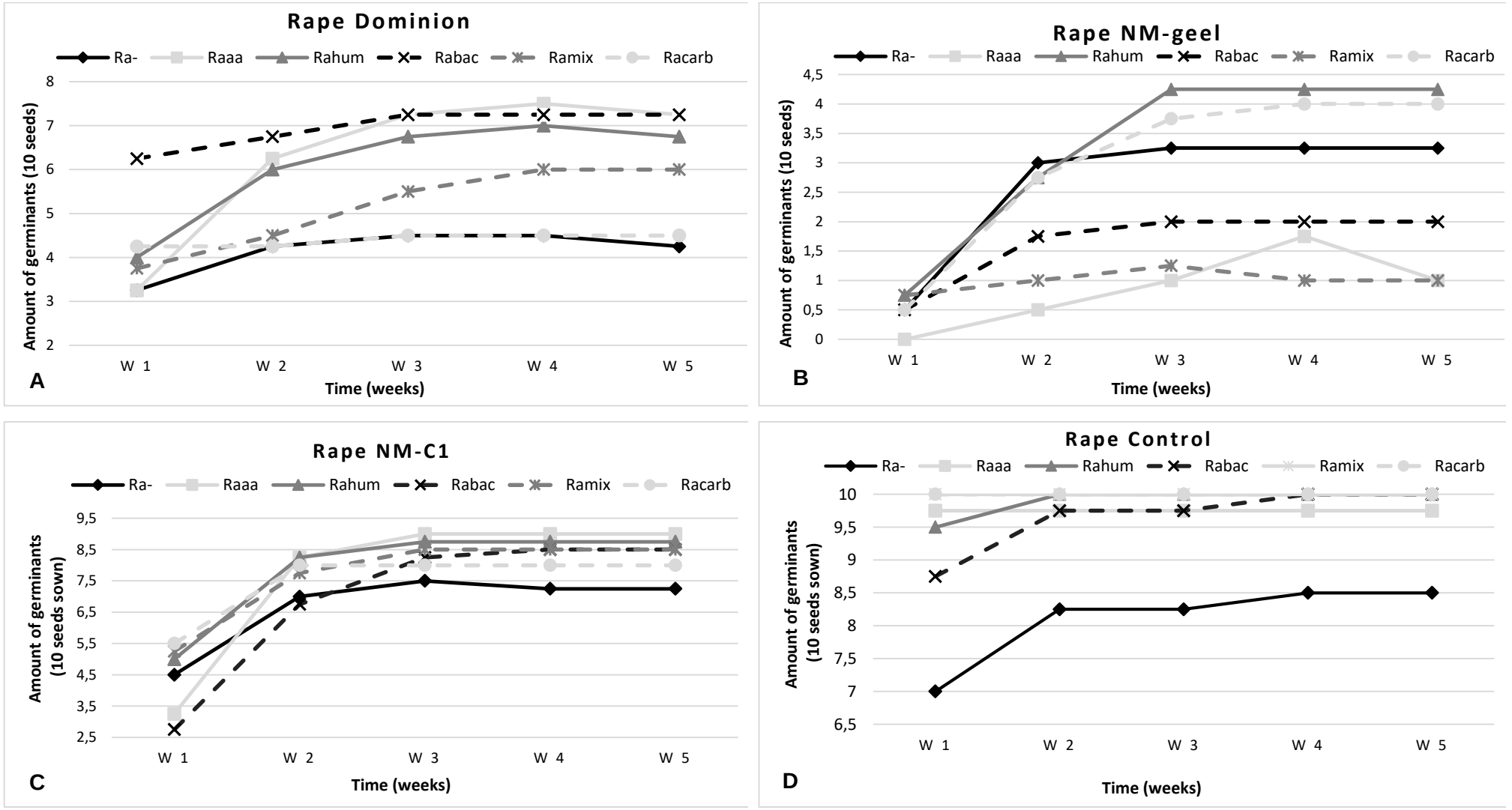

Figure 5-8: Germination rate of fodder rape/bio-stimulant treatment combinations established in different gold tailings and control soil. From above left (A) Dominion, above right (B) NM-geel, below left (C) NM-C1, and below right (D) control soil.

Bio-stimulant treated radish germination rate over a period of 5 weeks in the four growth substrates.

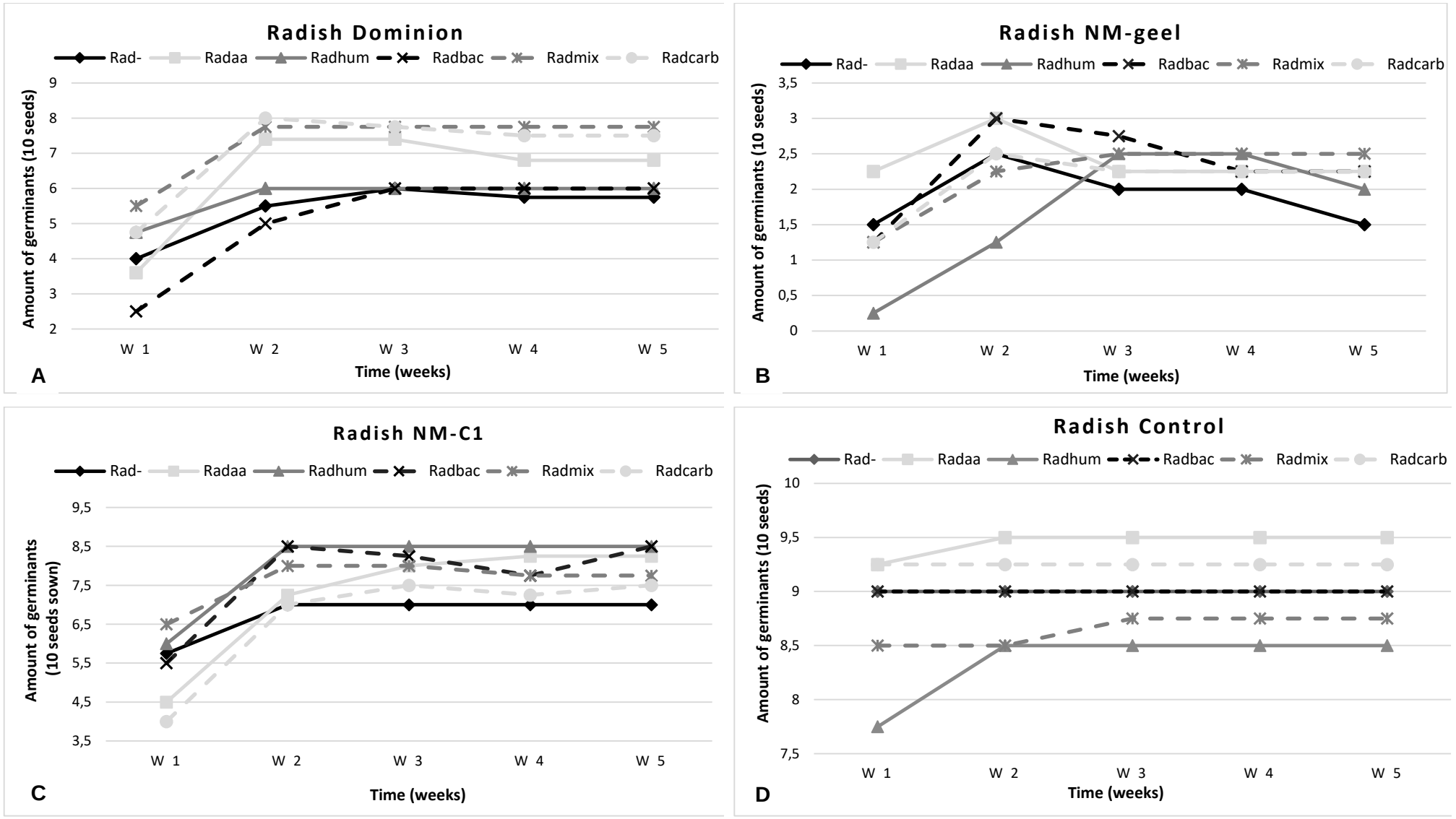

Figure 5-9: Germination rate of forage radish/bio-stimulant treatment combinations established in different gold tailings and control soil. From above left (A) Dominion, above right (B) NM-geel, below left (C) NM-C1, and below right (D) control soil.

Bio-stimulant treated ryegrass germination rate over a period of 5 weeks in the four growth substrates.

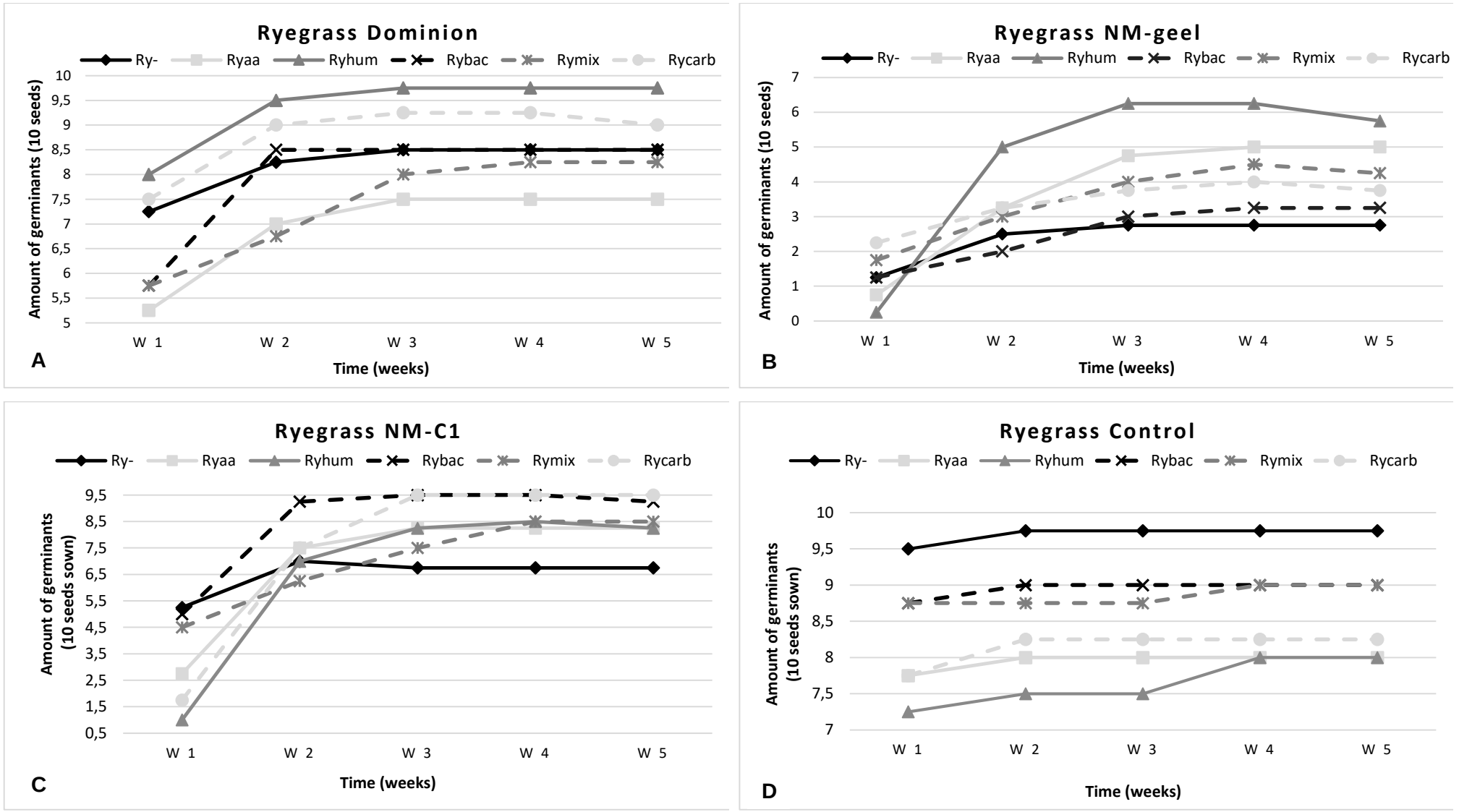

Figure 5-10: Germination rate of ryegrass/bio-stimulant treatment combinations established in different gold tailings and control soil. From above left (A) Dominion, above right (B) NM-geel, below left (C) NM-C1, and below right (D) control soil.

Initially, sovereign kale was also established with the other selected Brassicaceae family members. The kale showed slow germination and high seedling death in the various growth substrates, including the control soil. Consequently, sovereign kale was regarded as an unsuitable cultivar and was subsequently eliminated from treatment trials (refer to **Appendix D**). and replaced with radish. Different Brassicaceae species planted within the various growth substrates elicited different germination rate responses to the bio-stimulants used. This study showed that in most cases the bio-stimulants accelerated the germination of seeds within the different growth substrates.

Table 5-9: Different plant species and bio-stimulant combinations mean germination and survival rate.

Mother crop	Growth substrate	Early seedling development	Best survival rate	Slowest seedling development	Lowest survival rate
Canola	NM-geel	Carbohydrates	Carbohydrates	No biostimulants	No biostimulants
	Dominion	Mixture	Mixture K-humate	Bacteria	No biostimulants
	NM-C1	Bacteria	Mixture	K-humate	No biostimulants
	Control soil	Amino acid	Amino acid Mixture	Carbohydrates	Carbohydrates
Fodder rape	NM-geel	Bacteria	Bacteria Amino acid	No biostimulants Amino acid	No biostimulants
	Dominion	K-humate	K-humate Carbohydrates	Amino acid	Amino acid
	NM-C1	Carbohydrates	Amino acid	Bacteria	No biostimulants
	Control soil	Mixture Carbohydrates	Mixture Carbohydrates Bacteria	No biostimulants	No biostimulants
Radish	NM-geel	Mixture	Mixture	Bacteria	No biostimulants
	Dominion	Amino acid	Mixture	K-humate	No biostimulants
	NM-C1	Mixture	K-humate Bacteria	Carbohydrates	No biostimulants
	Control soil	Amino acid Carbohydrates	Amino acid	K-humate	K-humate
Rye grass	NM-geel	K-humate	K-humate	Amino acid	Amino acid
	Dominion	Carbohydrates	K-humate	K-humate	No biostimulants
	NM-C1	No biostimulants Bacteria	Bacteria Carbohydrates	K-humate	No biostimulants
	Control soil	No biostimulants	No biostimulants	K-humate	K-humate Amino acid

* Amino acids are an abbreviation for amino acids, protein hydrolysates and metabolites.

Table 5-9 summarises the results obtained from **Figure 5-7** to **Figure 5-10.** This table indicates which bio-stimulants had the highest/lowest seedling growth, and highest/lowest survivability for each mother crop species seeded in the four growth substrates. Overall, these nursery trials displayed variable responses to applied bio-stimulant treatments.

Some of the bio-stimulants used did show some similarities between the different growth substrates, for example, amino acids elicited early seedling development and possessed the highest survivability in both the radish and canola control soil treatments. Similarly, the K-humate and mixture of bio-stimulants treated seedlings elicited the highest survivability for all of the mother crop species used (canola, forage rape, radish and ryegrass) in all four growth substrates. The improvement of these mother crop species establishment may be due to the role of K (from K-humate) in regulating plant nutrition, as well as potentially mitigating and osmoregulating the adverse effects of high saline concentrations in the tailings materials (Aguilar *et al*., 2003; Marschner, 2012; Munns, 2002; Taiz & Zeiger, 2006; Wang *et al*., 2013), or HS positive effects on plant growth and productivity (Billingham, 2012; Chen *et al*., 2004; Quilty & Cattle, 2011; Rose *et al*., 2014). Reports have also postulated the existence of a complex relationship between K and specific metabolic functions that modifies the host-parasite-environment compatibility, which decreases the susceptibility of diseases, provided that K persists in the tissues and facilitates cellular wall thickening (Cakmak, 2005; Perrenoud, 1977; Römheld & Kirkby, 2010; Wang *et al*., 2013). Xue *et al*. (1994) also observed better performance when HS was applied together with chemical fertiliser in rapeseed, sesame, maize, wheat, and cotton production.

The beneficial bacteria inoculant showed contradictory results, that is, in some treatment combinations it elicited early seed development (e.g., NM-C1 canola; NM-geel fodder rape) and in other treatments, it possessed the slowest seedling growth (e.g., Dominion canola; NM-C1 fodder rape; and NM-geel radish). These results further demonstrate the variability between plant species and soil type responses to bio-stimulants. During the developmental phase of microbial-assisted revegetation, several aspects should be considered. For instance, the plant species and cultivar used for revegetation can be an influential factor.

In terms of slowest seedling growth, no bio-stimulants and amino acid treatments showed similarities in NM-geel canola, forage rape and ryegrass. No bio-stimulants control treatment elicited the least beneficial responses in regards to increasing seedling growth and survivability of most of the mother crop species established in all the growth substrates. The complex multi-component nature of many bio-stimulants used in this research, obscure clear findings pertaining to their role in microbial methods of action, assemblage, and activity.

5.5.2.2 Visual difference in plant growth

During the plant monitoring of the different mother crop species, NM-geel had a low germination rate and a high mortality rate (**Table 5-8**). NM-geel tailings also produced a salt crust (**Figure 5-11**). The EC of NM-geel was initially before treatment 1228mS/m (**Table 4-4**), the NM-geel salt crust EC measured at ± 4596mS/m (46dS/m), and after treatments 575mS/m (6dS/m). NM-geel tailings are considered moderately saline (4-8dS/m), but the salt crust formation is considered strongly saline (>16dS/m) (Wentz, 2001). NM-geel EC was the highest compared to the other gold tailings materials. Although the tailings' EC were reduced during the treatment, a high EC salt crust formed that constraint plant growth. The mother crop species that manage to establish in the NM-geel tailings seem to possess poor plant health (**Figure 5-12** and **Figure 5-13**). This negative association with EC observed in this research chapter correlates with similar findings in **Chapter 4**. A negative correlation exists between DHA and EC (**Figure 4-19**, r-0.868; $p<0.05$) for the New Machavie gold tailings material characteristics. These findings support García and Hernández (1996) observations, that salinity negatively influences the microbial activity by means of high osmotic strength, which can be ascribed to the toxic effect on microbial growth, except for tolerant halophytic bacteria.

Figure 5-11: Salt crust formation in NM-geel.

Plant establishment on mine tailings in South Africa is hindered by several physicochemical factors owing to the semi-arid to arid climatic conditions, i.e., extreme temperatures particularly on the tailings surface, low precipitation, and strong winds. Resulting in high evapotranspiration, low moisture infiltration and increased surface salinity due to capillary uptake (Munshower, 1994).

As a consequence, on the tailings surface a saline crust develops (Grandlic, 2008; Mendez, 2007; Newson & Fahey, 2003). In such cases, the formation of salt crusts on the tailings surface causes intense physiological stress on plants. Saline stress adversely influences germination, plant growth, and reproduction by disturbing the plant physiological processes (Akbarimoghaddam *et al*., 2011; Barkla & Pantoja, 2011; Kang *et al*., 2014; Nawaz *et al*., 2010; Neocleous *et al*., 2014; Paul, 2012; Shibli *et al*., 2007; Singh & Chatrath, 2001; Singh *et al*., 2011).

Figure 5-12: Plant health of ryegrass/amino acid treatment grown in two different gold tailings materials with restricted/stunted root growth (A) NM-geel compared to the same-aged ryegrass root of (B) Dominion.

A difference in plant species growth could be visually detected within the different growth substrates. In **Figure 5-12** the growth of the ryegrass can be seen in the **(A)** NM-geel and **(B)** Dominion tailings. NM-geel ryegrass **Figure 5-12 (A)** possessed virtually no roots and upon root tissue examination, the roots once extended further but were burned off (salt crust) in the NM-geel tailings. Furthermore, during the germination of the different mother crops, yellow discolouration on the shoots was observed in NM-geel, indicating stress-related to not ideal growth conditions. The Dominion tailings ryegrass **Figure 5-12 (B)** and NM-C1 (not shown) had healthier root systems, with only some stunted shoot growth observed. **Figure 5-12 (B**) shows an ideal example of hampered shoot growth. The stunted stem growth may be attributed to the fact that the gold tailings are known to form crusts. In the case of the Dominion tailings, the crust resistance was too great, consequently, the stem continued to grow beneath the crust, twisting the plant stems and shoots, only to emerge where a crack occurred in the crust or where a thinner, weaker crust was encountered. This supports research done by Timm *et al*. (1971) and Velde (1995).

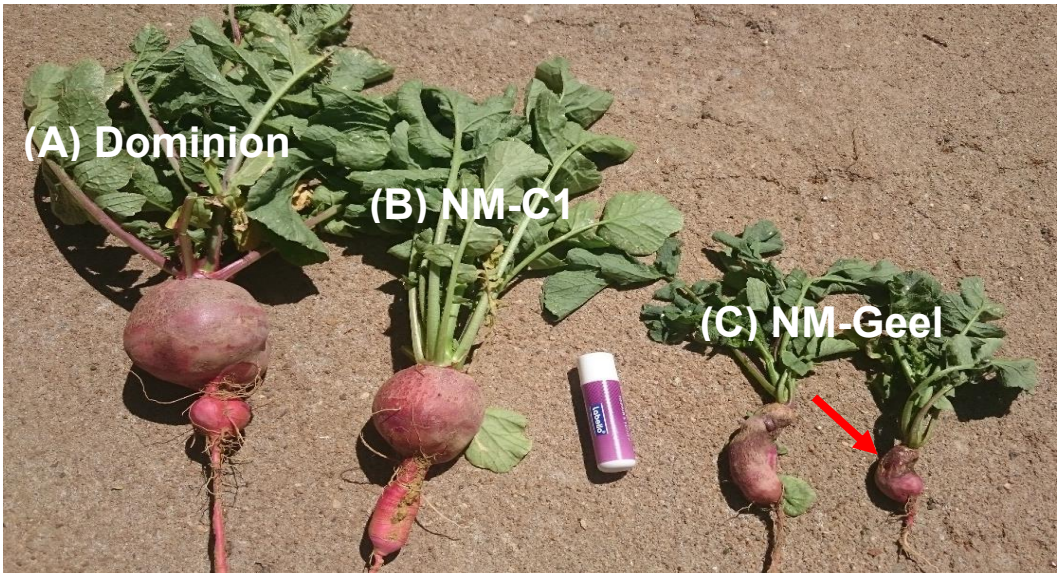

Figure 5-13: Difference in size and health of radish/K-humate treatments grown in different gold tailings materials. (A) Dominion tailings, (B) NM-C1 tailings and (C) NM-geel gold tailings.

The same observations in **Figure 5-12** can also be seen in **Figure 5-13**. When comparing the radish fusiform taproot growth in the different gold tailings, clear size differences can be distinguished. With Dominion tailings possess the largest radish, then by NM-C1, and NM-geel possessing the smallest taproot. Based on the depth uniformity of the point of necrosis/burn on the radish's taproot of NM-geel, the obvious physiochemical characteristic contributing to poor growth performance is due to the extreme salt crust. A possible explanation for the high death rate of the mother crops in NM-geel tailings could be the build-up of salts and consequently the formation of salt crusts. Annual ryegrass (*Lolium multiflorum*) is ranked as a more salt-sensitive plant, tolerating soil EC from 3 to 6dS/m. Most of the *Brassica* species have been classified as moderately salt tolerant, with canola threshold EC of 11dS/m (FAO, 2017). Absolute salinity tolerances vary, depending on climate conditions, soil conditions, and plant cultivar/ecotype.

5.5.3 Bio-stimulants/mother crops establishments effect on DHA

After the subsequent mother crop winter-growth season, the DHA was measured in mid-September 2016. It was hypothesised that the DHA of the mother crop/bio-stimulant treatments would be higher compared to mother crop established without bio-stimulants. It was also hypothesised that the mother crop species would also positively influence DHA, as plant establishment creates plant-microbe interactions. Additionally, it was hypothesised that the DHA of the different mother crop/bio-stimulant combinations would vary. The results of the mother crop/bio-stimulant treatments DHA are shown in **Table 5-10**.

Table 5-10: DHA (µg INF/g/2h) of different bio-stimulant/mother crop species combinations.

Plant species	Bio-stimulant	NM-geel	Dominion	Control	NM-C1
		Dehydrogenase activity (µg INF/g/2h)			
Ryegrass	Bacteria	224.05^{bc}	93.46^{def}	61.42^{cde}	164.64^{cde}
	Carbohydrates	107.90^{c}	140.75^{def}	109.26^{cde}	125.86^{defg}
	K-Humate	252.36^{bc}	131.98^{def}	117.09^{cde}	119.59^{defg}
	Amino acid	192.20^{bc}	105.30^{def}	246.86^{ab}	142.18^{def}
	None	159.73^{c}	93.65^{def}	124.47^{bcde}	75.14^{hij}
Canola	Bacteria	262.32^{bc}	170.52^{bcd}	37.07^{de}	81.58^{ghij}
	Carbohydrates	856.82^{a}	202.02^{abcd}	141.68^{abcd}	150.53^{cdef}
	K-Humate	264.22^{bc}	328.12^{a}	95.61^{cde}	356.86^{a}
	Amino acid	202.60^{bc}	104.31^{def}	171.42^{abc}	190.01^{bc}
	None	156.46^{c}	89.69^{def}	119.24^{cde}	121.09^{efg}
Rape	Bacteria	237.84^{bc}	207.62^{abcd}	33.72^{de}	224.93^{b}
	Carbohydrates	212.26^{bc}	230.00^{abc}	100.35^{cde}	68.98^{hijk}
	K-Humate	417.79^{b}	207.49^{abcd}	51.45^{cde}	25.04^{k}
	Amino acid	236.84^{bc}	307.84^{a}	101.36^{de}	166.65^{cd}
	None	198.27^{bc}	97.27^{def}	55.51^{cde}	107.81^{fgh}
Radish	Bacteria	205.81^{bc}	187.34^{bcd}	254.96^{a}	139.16^{def}
	Carbohydrates	214.65^{bc}	167.67^{bcde}	129.30^{bcde}	90.44^{ghi}
	K-Humate	285.73^{bc}	187.79^{bcd}	82.01^{cde}	190.81^{bc}
	Amino acid	250.72^{bc}	59.24^{ef}	73.10^{cde}	49.90^{ijk}
	None	242.90^{bc}	65.37^{ef}	106.78^{cde}	124.85^{defg}
Bare	None	104.94^{c}	28.97^{f}	9.44^{e}	43.23^{jk}
Pr>F		0.002	0.002	0.003	<0.0001

* Significance at 0.05 probability level between columns and rows.
‡ Treatments labelled with the similiar letters within a column were not statistical significance p>0.05, in contrast, those with different letters show significant variance at Tukey's HSD p< 0.05.

DHA results obtained from the bio-stimulant/mother crop treatments were statistically significant for all four growth substrates, NM-geel (p±0.002), Dominion (p±0.002), control (p±0.003) and NM-C1 (p<0.001). As previously mentioned in both discussion for **Table 5-6/Table 5-7** and **Table 5-8**, significant variability in terms of performance exist between growth substrates, i.e., bio-stimulant specific and plant specific. For each of the different species established a control mother crop was established for that specific species without bio-stimulants (i.e., rape without bio-stimulant). **Figure 5-14** illustrates the radar diagrams for the relative DHA response of different mother crop/bio-stimulants treatments grown in four growth substrates, whilst **Figure 5-15** to **Figure 5-18**, represents the individual growth substrates bio-stimulant/mother crop treatments.

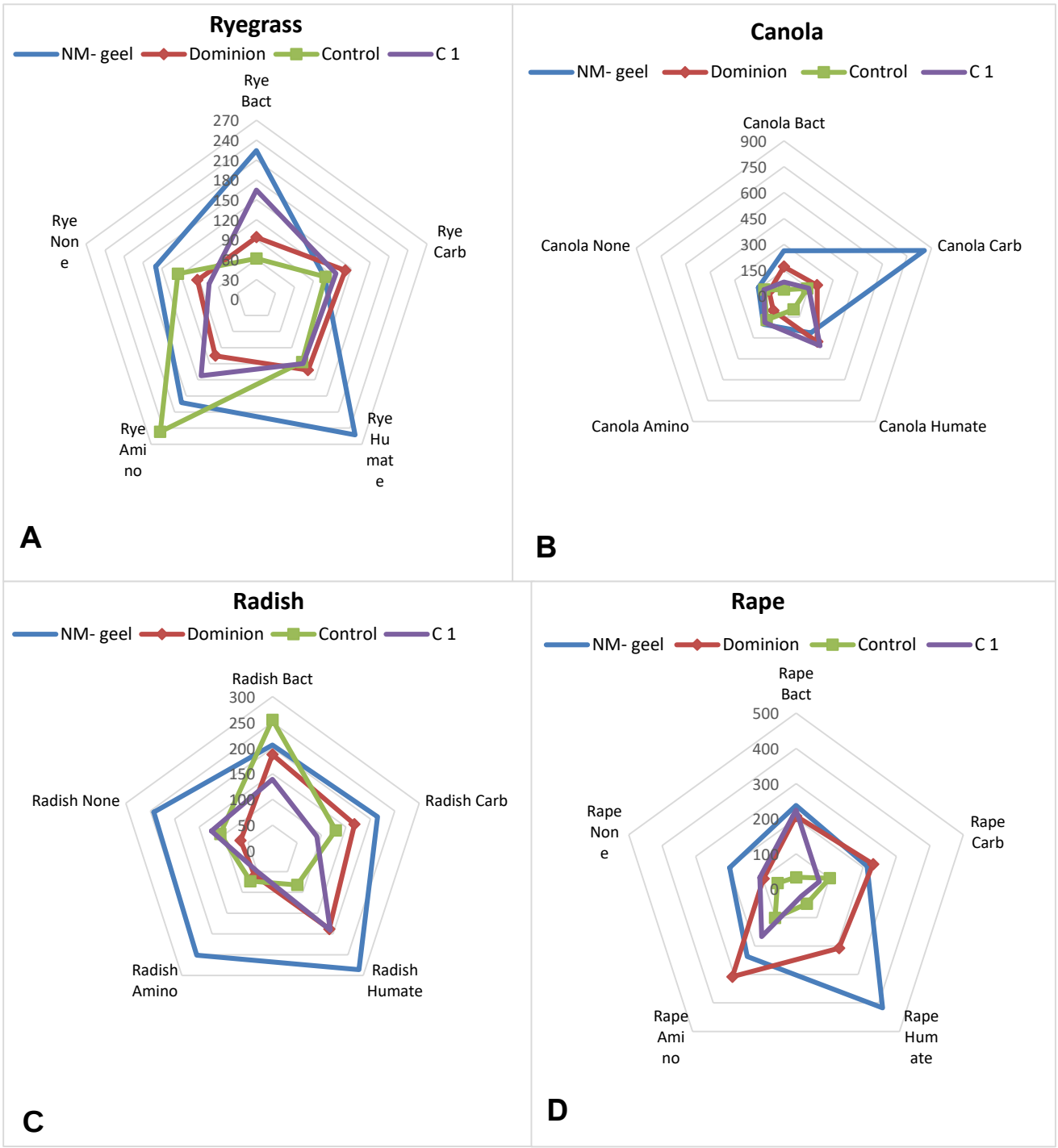

Figure 5-14: Radar graphs demonstrating the relative DHA response of different mother crop/bio-stimulants treatments grown in four growth substrates (A) ryegrass, (B) canola, (C) radish and (D) fodder rape. Bio-stimulants abbreviations: Amino- amino acids; Carb-carbohydrates; Bact-bacteria; None- no bio-stimulants; and Humate- K-humate.

1. New Machavie geel

The DHA of all the bio-stimulant/mother crop treatments, except for ryegrass/carbohydrates, improved compared to the initial untreated NM-geel DHA (105µg INF/g/2h) (**Figure 5-15**). The lowest performing treatment was ryegrass/carbohydrates (108µg INF/g/2h) treatment, with a DHA

close to untreated. As previously stated, the carbohydrates provide a nutritional source to microorganisms; consequently, the carbohydrate can easily be depleted in a short time by means of leaching out to sub-soil. This can possibly provide a reason why DHA of some mother crop/carbohydrates is lower when compared to DHA of mother crop/no bio-stimulants. In addition, carbohydrates do not differentiate between beneficial and pathogenic microbes, consequently stimulating both groups. Only a small Tukey's HSD variability exists for the ryegrass/bio-stimulant treatments. All the ryegrass/bio-stimulant treatments improved DHA, with exception of ryegrass/carbohydrates (105µg INF/g/2h) compared to ryegrass control treatment (160µg INF/g/2h).

The ryegrass/carbohydrates had a lower DHA compared to control treatment (ryegrass/no bio-stimulant). In the canola treatments, all canola/bio-stimulant treatments significantly improved the DHA compared to control (156µg INF/g/2h). Only a small Tukey's HSD variability exists between rape/bio-stimulant treatments. All the rape/bio-stimulant treatments improved DHA (i.e., >198µg INF/g/2h) compared to control treatment (198µg INF/g/2h). However, rape/carbohydrates treatments (212µg INF/g/2h) DHA is close to the control treatments DHA. In terms of Tukey's HSD variability between groups, only rape/K-humate (418µg INF/g/2h) showed significant improvement. Finally, in the radish/bio-stimulant treatments, no variability between treatments existed, with radish/K-humate (286µg INF/g/2h) possessing the highest improvement in DHA compared to control treatment (243µg INF/g/2h). Consequently, in terms of DHA improvement, bio-stimulant addition to mother crop seems to improve the overall DHA.

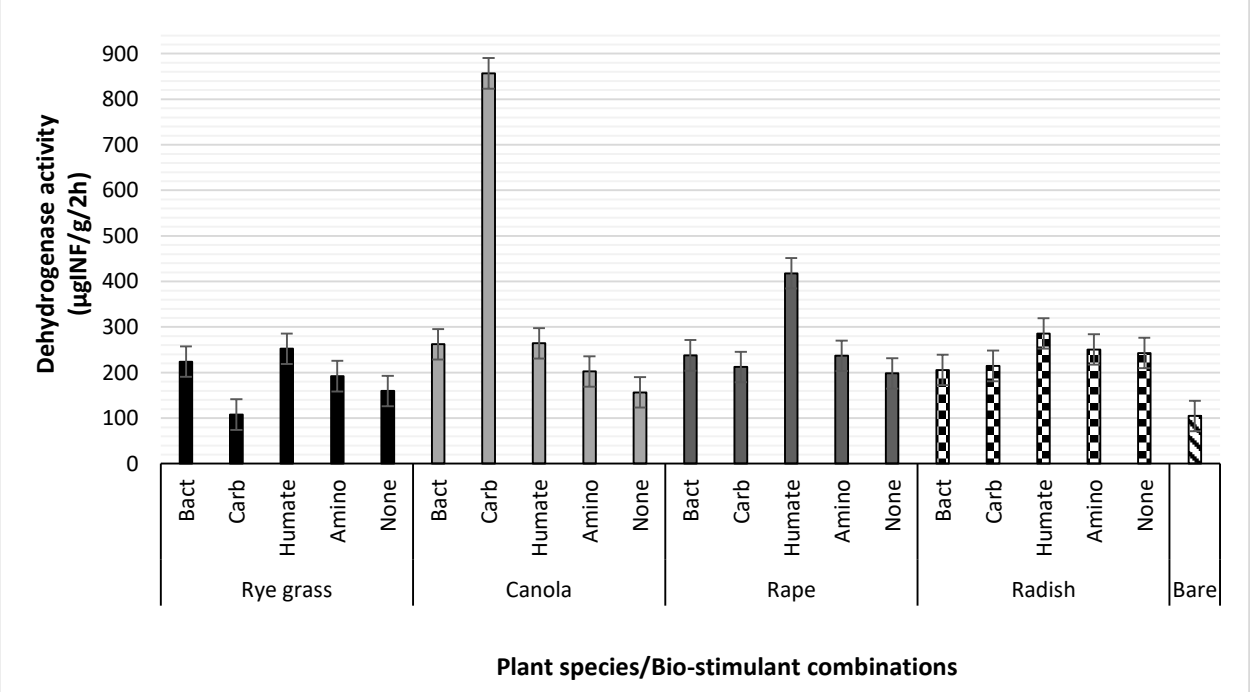

Figure 5-15: DHA associated with the various bio-stimulants/mother crop treatments for NM-geel gold tailings.

2. Dominion

Results for Dominion are presented in **Figure 5-14** and **Figure 5-16**. The Dominion's DHA for the entire bio-stimulant/mother crop treatments improved, compared to the untreated tailings DHA (29µg INF/g/2h). The highest increase in DHA was observed in the rape/amino acid treatment (308µg INF/g/2h) and the canola/K-humate treatment (318µg INF/g/2h). The smallest increase in DHA was in the radish/amino acid (59µg INF/g/2h) and radish/no bio-stimulant treatment (65µg INF/g/2h). Although these two treatments had the lowest increase in DHA, it was still higher (2-fold greater) than the untreated DHA (29µg INF/g/2h).

Tukey's HSD variability did not exist between the ryegrass/bio-stimulant treatments. All the ryegrass/bio-stimulant treatments improved the DHA to a small degree, relative to the ryegrass/no bio-stimulants (94µg INF/g/2h). With ryegrass/carbohydrates (141µg INF/g/2h) having the highest DHA of all the treatments. All the canola/bio-stimulant treatments significantly improved the DHA (i.e.,>89µg INF/g/2h) compared to canola/no bio-stimulants (89µg INF/g/2h). With canola/K-humate (328µg INF/g/2h) and canola/carbohydrates (202µg INF/g/2h) having the highest increase in DHA compared to control treatment. Whereas, the canola/amino acid treatment had the smallest DHA improvement (104µg INF/g/2h). The rape/bio-stimulant treatments significantly and variably improved DHA compared to control treatments (97µg INF/g/2h). The rape/amino acid treatment (230µg INF/g/2h) possesses the highest increase in DHA. In the radish/bio-stimulant treatments, only a small variability exists between treatments (Tukey's HSD), with radish/K-humate (188µg INF/g/2h) and radish/bacteria (187µg INF/g/2h) possessing the highest DHA improvement. Consequently, in terms of DHA the bio-stimulant addition to mother crop improve the overall DHA.

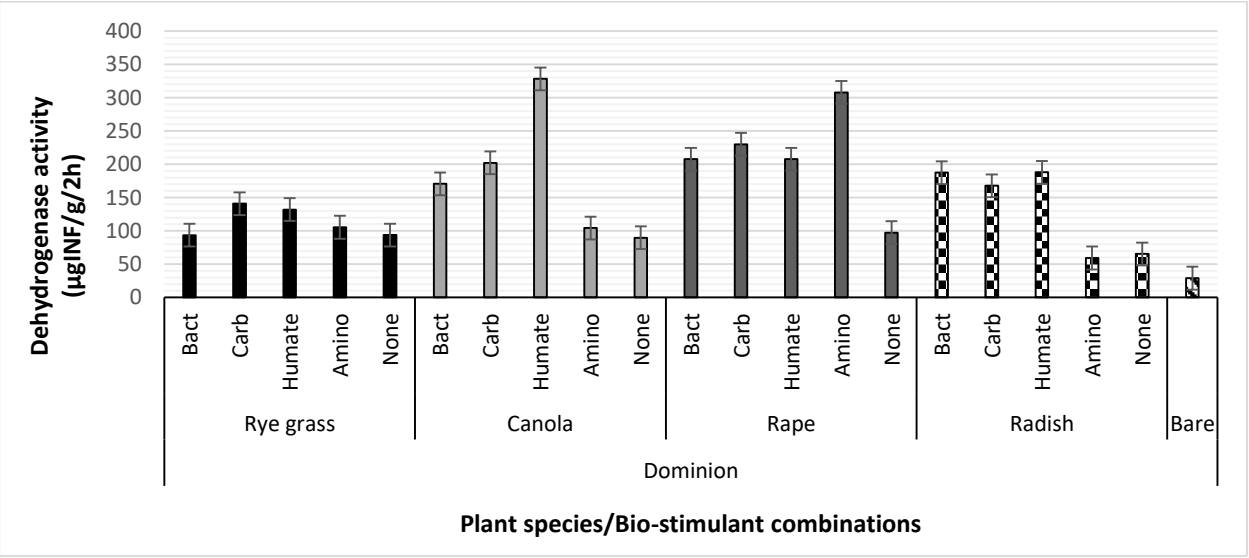

Figure 5-16: **DHA associated with the various bio-stimulants/mother crop treatments for Dominion gold tailings.**

3. Control soil

Results for the control soil is presented in **Figure 5-14** and **Figure 5-17.** Relative to the gold tailings, the control soil seems to behave differently. In the control soil, quite a few mother crop/bio-stimulant combinations exhibited lower DHA values compared to the control mother crop/no bio-stimulant treatments. A possible explanation for this phenomenon is that due to the soil textural class and higher water regime necessary to sustain plant growth, most of the bio-stimulants were leached out. The DHA of the bio-stimulant/mother crop treatments improved compared to the initial untreated control soil DHA (9µg INF/g/2h).

The highest increase in DHA was observed in the radish/bacteria treatment (255µg INF/g/2h). The least improvement in DHA was observed in canola/bacteria (37µg INF/g/2h) treatment and the rape/bacteria treatment (33µg INF/g/2h). Although these two treatment combinations had the lowest increase in DHA, it was still higher (4-fold greater) than the untreated DHA (9µg INF/g/2h). Radish/bacteria had the highest DHA increase, whereas the rape/bacteria and canola/bacteria had the lowest, perhaps exemplify the importance of plant species/bio-stimulant selection and their subsequent influence on microbial populations. For example, the plant species and cultivar selected for revegetation purposes can be an influential factor in acquiring benefits from bacterial inoculants (Calvo *et al*., 2014; Dalmastri *et al*., 1999; Khalid *et al.,* 2004; Remans *et al*., 2008). The use of PGPR in the phytostabilisation of mine tailings was proposed by various researchers (i.e., de-Bashan *et al.*, 2010; Mendez & Maier, 2008; Zhuang *et al*., 2007).

Tukey's HSD variability exists between ryegrass treatments/bio-stimulant treatments. Apart from ryegrass/amino acid (247µg INF/g/2h), all other bio-stimulant treatments had a lower DHA compared to control treatments (124µg INF/g/2h). Tukey's HSD variability exists between canola/bio-stimulant treatments. Apart from canola/carbohydrates (142µg INF/g/2h) and canola/amino acids (171µg INF/g/2h), all other bio-stimulant treatments had a lower DHA than the control treatment (119µg INF/g/2h). Tukey's HSD variability exists between rape/bio-stimulant treatments. Within the rape treatments, only rape/carbohydrates (100µg INF/g/2h) and rape/amino acids (101µg INF/g/2h) had improved DHA compared to the control treatment (56µg INF/g/2h). Tukey's HSD variability exists between the radish/bio-stimulant treatments. In the radish bio-stimulant treatments only radish/carbohydrates (129µg INF/g/2h) and radish/bacteria (255µg INF/g/2h) had improved DHA compared to the control treatment (107µg INF/g/2h). Consequently, it appears that only the carbohydrates and amino acids treatments (with some exceptions) improved DHA better than mother crop/no bio-stimulant treatments. Indicating that mother crop selection played a more dominant role in DHA improvement compared to bio-stimulant addition.

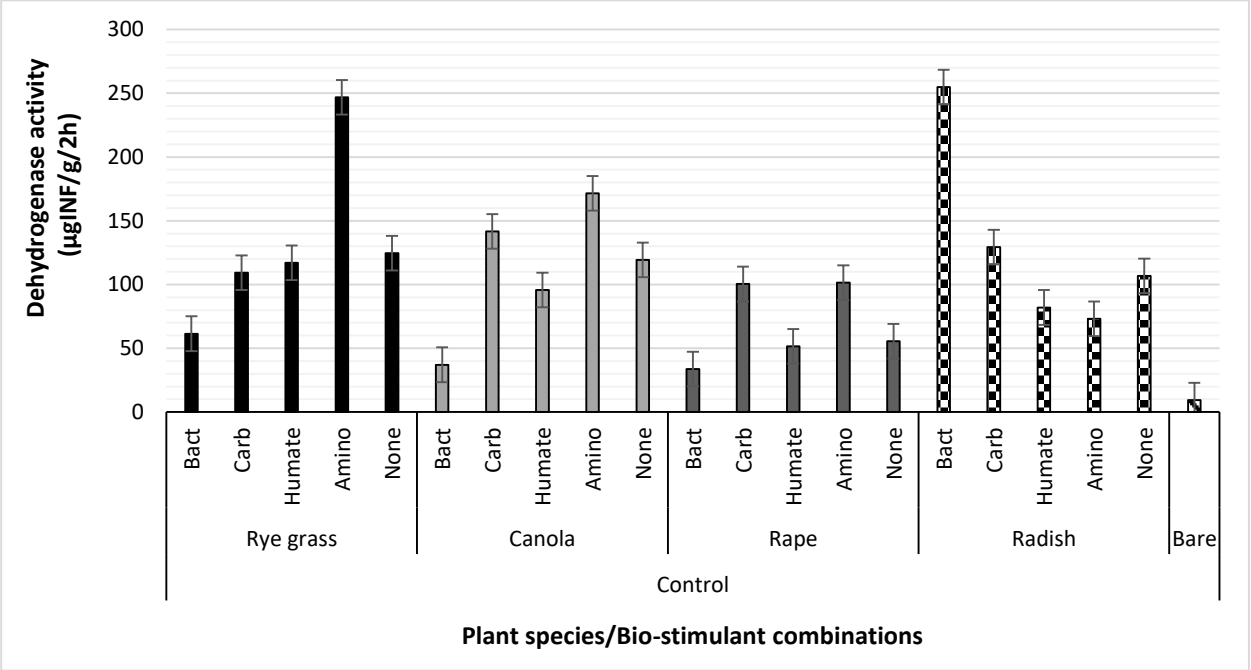

Figure 5-17: DHA associated with the various bio-stimulants/mother crop treatments for control soil.

4. New Machavie C1

Results for the NM-C1 gold tailings are presented in **Figure 5-14** and **Figure 5-18.** In terms of variability in bio-stimulant addition and mother crop species in tailings, NM-C1 showed the most inconsistencies. For example, not many of the other gold tailings, mother crop/bio-stimulant treatments' DHA were lower than the control treatment (mother crop/no bio-stimulant). In NM-C1 however, a few mother crop/bio-stimulant treatments had lower DHA values compared to the control, i.e., rape/K-humate (25µg INF/g/2h) and radish/amino acid treatments (50µg INF/g/2h) possessing either lower/close to untreated DHA (43µg INF/g/2h). All other NM-C1 treatments performed better compared to the untreated treatments DHA (43µg INF/g/2h). The highest increase in DHA for NM-C1 was observed in the canola/amino acid treatment (357µg INF/g/2h), whilst the least improved DHA was observed in the rape/K-humate (25µg INF/g/2h) treatment.

Tukey's HSD variability exists between ryegrass/bio-stimulant treatments. The ryegrass/bio-stimulant treatments improved the DHA, relative to the ryegrass/no bio-stimulants (75µg INF/g/2h). In comparison, ryegrass/bacteria (165µg INF/g/2h) possessed the highest DHA of all the ryegrass/bio-stimulant treatments. All canola/bio-stimulant treatments significantly improved the DHA (i.e., >121µg INF/g/2h), except for canola/bacteria (82µg INF/g/2h). The canola/K-humate treatment (357µg INF/g/2h) had the largest increase in DHA. Apart from the rape/bacteria (225µg INF/g/2h) and rape/amino acid (167µg INF/g/2h) treatments, all rape treatments possessed lower DHA than the control treatments (108µg INF/g/2h). Within the radish treatments,

only radish/bacteria (139µg INF/g/2h) and radish/K-humate (191µg INF/g/2h) improved DHA compared to control treatments (125µg INF/g/2h). Bacterial community adaptation and evolution in pioneer plants rhizosphere (such as mother crops) seems to play a key role in plant colonisation and survival on mine tailings (Carrasco *et al*., 2010). Similarly, Marschner and Timonen (2005) stated that different plant species rhizosphere microbial communities established in identical soil are often distinct.

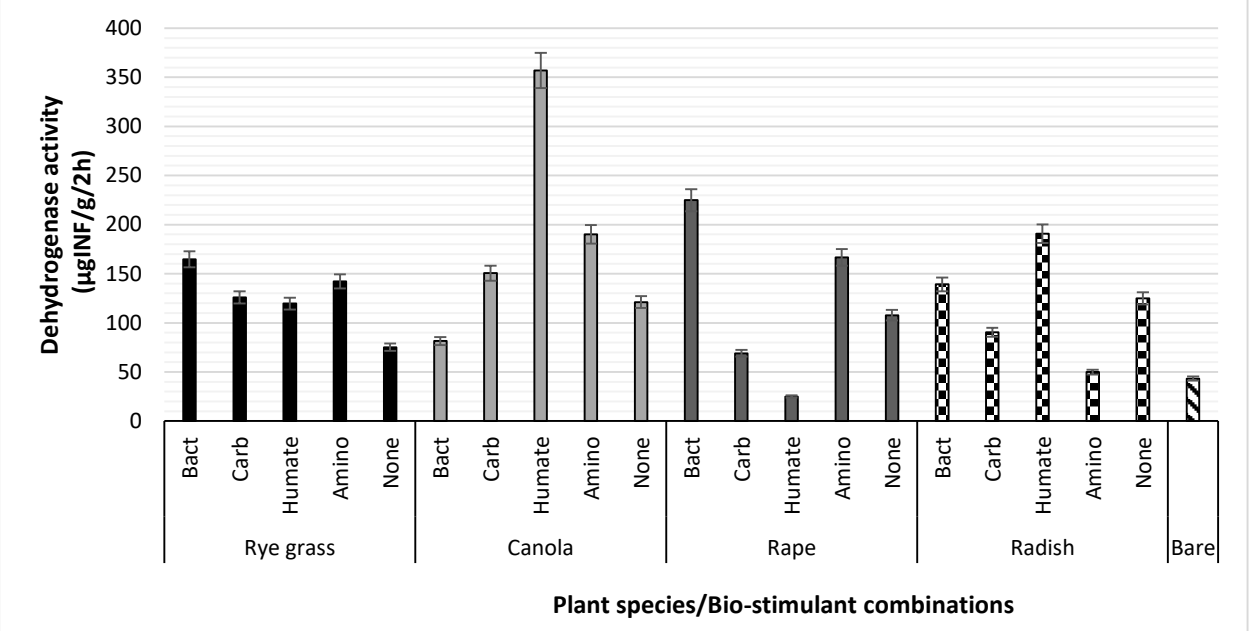

Figure 5-18: DHA associated with the various bio-stimulants/mother crop treatments for NM-C1 gold tailings.

The benefit of the added bio-stimulants can be observed in initial higher DHA after six months of mother crop plant growth. When determining whether the bio-stimulants had an effect on DHA in all the gold tailings, in most cases all the bio-stimulants improved DHA. With bacterial inoculant, K-humate and carbohydrates having the most profound effects persistently. In control soil, the amino acids, bacteria and carbohydrates were the only bio-stimulants that seemed to improve DHA compared to the no bio-stimulant treatments. The difference in DHA for the different mother crop/no bio-stimulant treatments may possibly be due to different root exudates constituents and plant litter, which differs from plant species to plant species. In some cases, certain mother crop species without bio-stimulants had a higher DHA compared to the combined treatments. Previous work has revealed that amended tailings with plant growth during mine wastes revegetation has a major influence on soil microbial communities' abundance and stability (Li *et al*., 2014; Mummey *et al*., 2002a; Mummey *et al*., 2002b; Pérez-de-Mora *et al*., 2006; Rosario *et al*., 2007; Valentín-Vargas *et al*., 2014). Both Li *et al*. (2014) and Pérez-de-Mora *et al*. (2006) revealed that for the revegetation of mine wastes, the plant species selection had a greater influence on microbial

community structure and activity than the soil amendment and bio-stimulant used (Valentín-Vargas *et al*., 2014).

A Principal Component Analysis (PCA) ordination diagram demonstrating the association between the plant growth characteristics and the microbial enzymatic activities for different gold mining sites are presented in **Figure 5-19**. The eigenvalue for the first two ordination axes of the PCA were 0.314 and 0.222, respectively. These two axes accounted for 53.6% of the total observed variance.

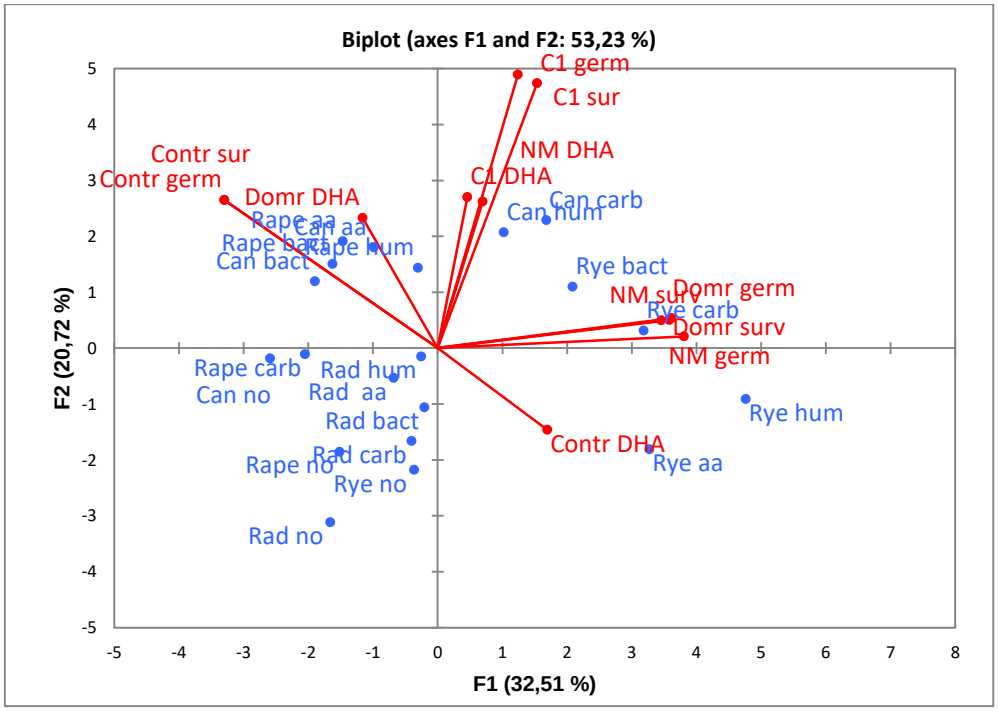

Figure 5-19: Principal component analysis (PCA) diagram demonstrating plant growth characteristics (plant germination and plant survival) in relation to various soil bio-stimulant/mother crop treatments for the different growth substrates.

Based on these results in **Figure 5-19**, it can be concluded that the plant growth characteristics and DHA of the gold tailings can vary remarkably. The germination rate was strongly associated with the survival rate with a statistical significance of <0.05 for NM-geel (r0.971), Dominion (r0.968); control soil (r0.989) and NM-C1 (r0.978). With NM-C1 closely associated with canola/carbohydrates and canola/K-humate, NM-geel with rye/bacteria and rye/carbohydrates. These results further indicate that no clear relationship exists between plant growth characteristics and DHA. A weak association exists between the growth substrates survival and DHA. It is recommended that additional soil enzymatic activities are included into future research studies. Soil enzymatic activity (i.e., β-glucosidase, urease, alkaline and acid phosphatase) play a key role in SOM decomposition and nutrient cycling specifically in the rhizosphere of plants. Considering that rhizosphere fitness is one of the most essential conditions for succesful PGPR stabilisation (Bashan *et al.*, 2014; Egamberdieva & Lugtenberg, 2014). NM-geel DHA was only weakly

positively associated with germination (r0.305) and survival rate (r0.299). For NM-C1, the DHA was positively associated with germination with a poor correlation (r0.303), and survival rate (r0.316). Both Dominion and control soil seem to have a negative association with DHA. As seen in **Figure 5-19**, the control soil is negatively associated with the plant characteristics (germination and survival), although the correlation between plant characteristics are poor r-0.313 (germination) and r-0.248 (survival), with a statistical significance of $p<0.005$. Overall, in the control soil, the bio-stimulants did not significantly improve either DHA or plant growth. A possible reason is that the control soil is a natural soil with few properties that might limit plant growth. Consequently, the difference in plant growth would not be of the same magnitude as for gold tailings. Therefore, the effects of the bio-stimulants will show a higher magnitude of improvement in the gold tailings (i.e., high levels of chemical, physical and microbiological constraints). Association between plant growth characteristics and DHA of each mother crop/bio-stimulant treatment established in the four growth substrates see **Table 5-11.**

Table 5-11: Summary of best plant survival associated with bio-stimulants versus DHA.

Growth substrate	Mother crop species	Highest plant survival (10 seed)		Bio-stimulant DHA (µg INF/g/2h)	
NM-geel	Ryegrass	K-humate	5.8	K-humate	252
	Canola	Carbohydrates	4.5	Carbohydrates	857
	Rape	K-humate	4.3	K-humate	418
	Radish	Carbohydrates	2.3	Carbohydrates	214
		Bacteria	2.3	Bacteria	206
Dominion	Ryegrass	Carbohydrates	9.0	Carbohydrates	141
		K-humate	9.8	K-humate	132
	Canola	Carbohydrates	7.3	Carbohydrates	202
		K-humate	7.0	K-humate	328
	Rape	Amino acid	7.3	Amino acid	309
		Bacteria	7.3	Bacteria	230
	*Radish	Carbohydrates	7.5	Bacteria	187
				K-humate	187
Control	*Ryegrass	No bio-stimulant	9.8	Amino acid	247
	Canola	Amino acid	10	Amino acid	171
	Rape	Carbohydrates	10	Carbohydrates	100
	Radish	Bacteria	9.0	Bacteria	255
NM-C1	Ryegrass	Bacteria	9.3	Bacteria	165
	Canola	K-humate	8.8	K-humate	357
		Amino acid	10	Amino acid	190
	Rape	Bacteria	8.5	Bacteria	224
	Radish	K-humate	8.5	K-humate	191
		Bacteria	10	Bacteria	139

* indicates no association between best performing mother crop/bio-stimulant treatment and DHA. Includes either the highest/second highest treatments.

As mentioned in **Chapter 4**, the control soil's chemical and physical characteristics negatively contribute to the poor DHA (i.e., virtually no cohesion between particles, poor moisture retention capacity, and crusting). Even though a poor association exists between DHA and plant growth characteristics some comparative association exists. For example, in all the growth substrates the best performing mother crop/bio-stimulants in terms of survival is also positively associated with the highest improvement in DHA. In NM-geel/ryegrass treatments, the highest survival was ryegrass/K-humate treatment which also had the highest DHA, some exceptions do exist (e.g., cases were no variability exists between treatments).

5.6 Conclusion

The findings for this research phase can be summarised as follows:

1. The various bio-stimulants applied to the different growth substrates (before mother crop establishment) improved DHA to various degrees. Bio-stimulants showed variability in improving DHA, i.e., in general bio-stimulant showed to be substrate specific.

- Additional studies using amendments and bio-stimulants supports the above-mentioned findings. For example, microfine langfos showed the highest improvement in DHA for NM-700.

2. The mother crops established in the different growth substrates showed variability in revegetation potential, i.e., indicating plant species are substrate specific.

- Plant performance of the different bio-stimulant/mother crop combinations is highly variable. Visual differences in plant health were also noted, the same mother crop/bio-stimulant combination grown in the tailings showed variability in plant health.

- Plant performance of bio-stimulant/mother crop combination indicates substrate specific results, i.e., certain bio-stimulants and mother crop species performed better on different mine tailings.

- Bio-stimulant/mother crop combinations showed improved germination and survival rate compared to untreated mother crops establishment. Some exceptions to this finding were also noted see **Table 5-9**.

3. Bio-stimulant/mother crop combinations improved DHA to various degrees compared to untreated tailings.

- DHA results from the bio-stimulant/mother crop treatments were statistically different for all four growth substrates.

- As with the case with plant performance, the DHA improvement seemed to show variability in bio-stimulant/mother crop treatments, i.e., substrate specific and plant species specific.

- PCA results show no clear association between plant growth characteristics and DHA. Only a weak association exists between the different growth substrates survival and DHA. Some correlations do exist between best performing bio-stimulant/mother crop treatments survival and highest DHA.

4. Although the different mother crop species and bio-stimulants showed substrate specific preferences some similarities existed, i.e., amino acids elicited early seedling development and highest survivability in both the radish and canola treatments.

5. Chemical-physical and microbial characteristics of the different growth substrates played an important role in mother crop species revegetation potential.

- NM-geel (known as NM-1 in Chapter 4) showed the lowest plant germination and survival. One of the possible explanations is the extremely high EC salt crust formation of these tailings. These findings correlate with the strongly negative association with EC, identified in **Chapter 4**.

In conclusion, the study gains novel insights into the possibility of using mother crops and bio-stimulants to improve revegetation stability and DHA of the tailings materials. Both the germination, survival rate and enzyme activity in this study support the hypothesis that permanent vegetative cover in combination with bio-stimulants application provides favourable conditions reflected by higher DHA, and improved mother crop establishment compared to untreated tailings. The application of several bio-stimulants promoted early seedling development and improved mother crop survivability. The combination of bio-stimulants and mother crop species improved the DHA more effectively than the sole establishment of the mother crops (no bio-stimulants). Consequently, the addition of bio-stimulants to the different mother crop/gold tailings not only improved plant growth characteristics but also the DHA in the rhizosphere. Although not researched, it should be mentioned that micro-environmental between soil-plant-microbe continuum plays a key role in both microbial activity and plant growth (Hatfield & Prueger, 2015; Okabe *et al*., 2001; Schupp, 1995).

Due to economic constraints, only DHA was evaluated, however in order to get a more comprehensive understanding of bio-stimulants/plant interactions and their successive role in improving soil biological characteristics, additional biochemical assays such as microbial biomass-C and enzymatic activities (β-glucosidase, urease, alkaline and acid phosphatase) should be assayed in future studies. Glucosidase, urease, and acid and alkaline phosphatases are enzymes that carry out specific hydrolyses and catalyse reactions encompassed in the biogeochemical transformations of C, N, and P, respectively. Consequently, these microbial enzymatic activities provided important soil fertility indicators. In addition, research studies should be carried out in other climatic regions to observe differences in soil biological characteristics and plant performance in different weather conditions.

The complex multi-component nature of the bio-stimulants and mother crop species used in this research obscures clear findings, as enzymatic activity is limited in this study only to DHA, enzymes such as urease, β-glucosidase etc. should also be integrated. The soil itself is a product of mutual actions between plants and microorganisms. Comprehending the complex soil nature requires that soil should not be regarded as an inert substrate but rather as one of the dynamic members of the soil-rhizosphere-plant continuum. For that reason, soil should be seen as a component of a larger plant-microbe interactome (Rosselli & Squartini, 2015). For a detailed conclusion and recommendation refer to **Chapter 6** and **Chapter 7**.

References

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CHAPTER 6

CONCLUSION AND CONCLUDING REMARKS

"We know very little, and yet it is astonishing that we know so much, and still more astonishing that so little knowledge can give us so much power."

–**Bertrand Russell**

6.1 Integration of results obtained

Conventional rehabilitation of mine tailings focuses primarily on soil fertility, SOM and plant species selection. Whilst the chemical and physical characteristics of mine tailings are important for plant establishment and growth monitoring, the biotic factors are also essential for soil fertility maintenance and stable soil/plant system evolution (Ehrenfeld *et al*., 2005). Soil biological characteristics receive minor attention prior to and after rehabilitation of mine waste. While most conventional rehabilitation procedures make use of pioneer species, SOM (decomposition), and nutrient cycling (fertiliser) as part of the main components for ecosystem stability. The problem, however, arises due to the fact that sustainable vegetation growth and ecosystem stability is highly dependent on the biological characteristics of the mining environment. That is, essential nutrient release rate, SOM accumulation and decomposition are reliant on the soil-plant-microbe system. This research emphasised the importance of integrating the biological characteristics as part of rehabilitation design criteria and rehabilitation performance monitoring.

For a detailed discussion of results obtained for each phase, refer to **Chapter 4** and **Chapter 5**.

Phase 1: Enzymatic activity of different mine tailings and natural soils – a baseline study.

Results from **Chapter 4** can be summarised and concluded as follows:

1. Un-rehabilitated mine tailings from various mines' TSFs, including gold tailings, kimberlite, platinum etc. all possess low enzymatic activity.

   a. The various gold tailings samples collected from different geological stratigraphic formation units, mining locations and/or mining techniques showed low enzymatic activity (β-glucosidase, urease, DHA, acid and alkaline phosphatase; although slight variability occurred). Some exceptions, however, did exist i.e., Dominion gold tailings had a higher urease activity (139 NH_4-N µg/g/2h) compared to the reference soil (136 NH_4-N µg/g/2h). A possible explanation for this might be due to the mining history of the Dominion mine. During the mining period, gold production was the primary

objective but sporadically uranium extracting also took place. The extraction method for uranium uses ammonium nitrate/nitric acid or ammonium sulphate and ammonia which reacts and precipitates with the other elements in the ore. Refer to **Figure 6-1** for uranium extraction methods used in the past in South Africa.

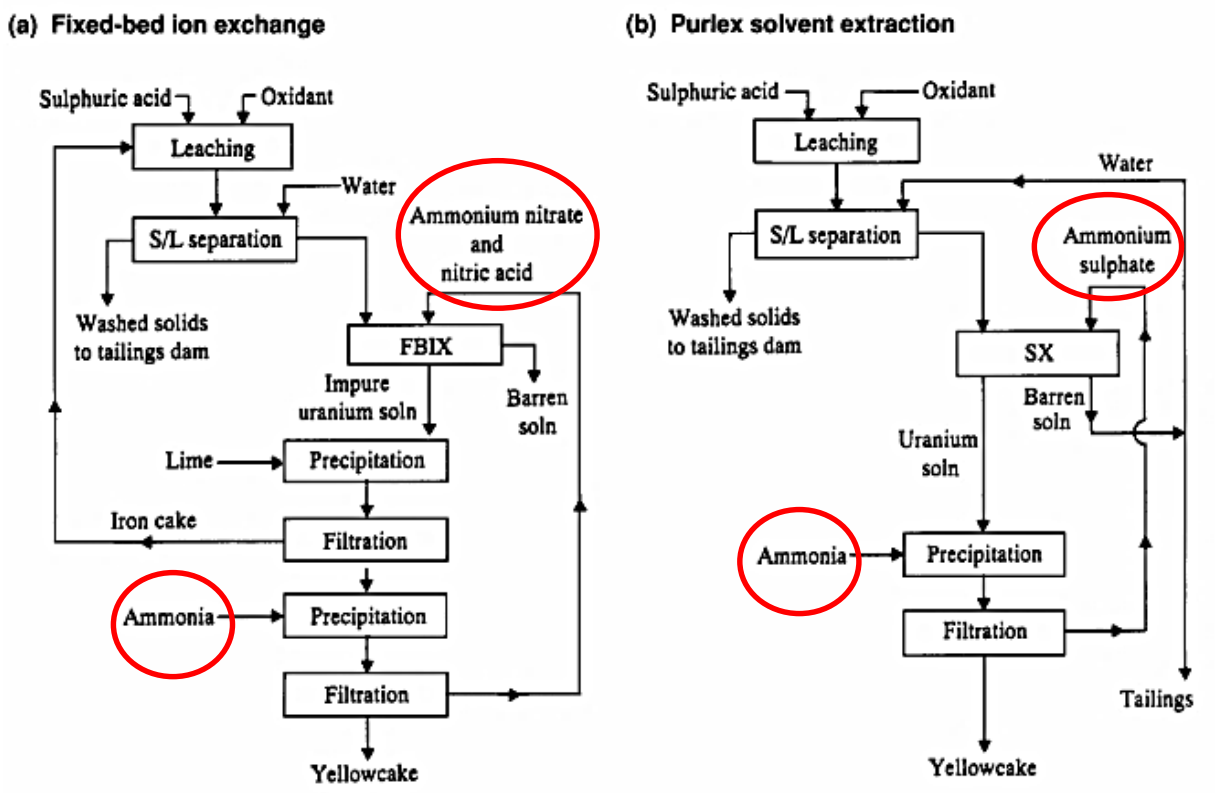

Figure 6-1: **Uranium extraction methods commonly used in the past in the Dominion, Witwatersrand and New Machavie gold mines (summary from Fordt, 1993).**

b. When comparing the DHA of tailings materials to reference soils. Most tailings possessed a lower DHA compared to the reference soils. However, some exceptions were also present. For example, coal fines possessed a DHA (111µg INF/g/2h) close to some natural soils (i.e., ochric A=118 INF/g/2h). In addition, soil depth played an important role in the microbial activity, i.e., all B-horizon soils assayed during this research had lower microbial activity compared to A-horizon.

c. The enzymatic activity of a single gold mining site also varies greatly, i.e. due to different gold-bearing geological and lithological units mined. A good example can be seen between NM-1 and NM-2 (e.g., DHA of NM-1=74 INF/g/2h and NM-2=2 INF/g/2h). Refer to **Table 4-7**.

2. In terms of natural plant establishment on gold TSFs, the self-established grasses' rhizosphere DHA is low, apart from the coppice dunes (for explanations refer to **Chapter 4**). The DHA of the self-established grasses, however, is higher compared to the barren non-vegetated areas, indicating the importance of microbial-plant interactions for a stable ecosystem, which seems to be insufficient in the self-established grasses' rhizosphere (i.e., low microbial activity).

3. A strong negative correlation exists between New Machavie's EC and DHA (r-0.868; $p<0.05$). ANOVA showed an overall statistically significant difference among the different gold mining sites' enzymatic activities. When integrating all the gold mining tailings researched during phase 1, a strong negative association exists between pH(H_2O), DHA and acid phosphatase (r-0.960; $p<0.05$). Exemplifying, the integrated relationship between the physical, chemical and microbial characteristics of tailings materials.

4. In comparison to the natural soils, the tailings possessed physical and chemical characteristics that constraints both plant - and microbial growth (e.g., extreme pH, high EC, low %C and %N; poor physical structure and aggregation, compaction and crusting).

5. In conclusion, it can be assumed that the soil quality and fertility of the different TSFs' research during phase 1 are non-existent (only slightly developed). This is due to poor physical characteristics, detrimental chemical characteristics and decreased soil enzymatic activity due to the biodegradable SOM deficit and limited nutrient cycling. It is therefore concluded that TSF's possesses poor soil quality.

A combination of chemical and physical characteristics inhibits microbial activity in the tailings. To summarise, compared to the undisturbed natural soils, the tailings were characterised by low microbial activity, low %C and %N, low pH, high EC, and low levels of plant essential nutrients such as P. Gold mining areas vary depending on the different lithological units, mining and metallurgical extraction methods, consequently the physical and chemical properties of these gold tailings may vary greatly (e.g., low pH, high trace metal content, poor aggregate stability, EC etc.). As a result, the soil enzymatic activity may vary depending on the physical and chemical properties (Ehrenfeld *et al*., 2005). Identifying methods for rehabilitation of a given site is a challenging procedure and requires consideration of several factors. Physical-chemical characteristics generally change at a decelerated temporal scale and are unsuited for short-term rehabilitation performance/quality monitoring (Melero *et al*., 2007; Mijangos *et al*., 2006; Sastre-Conde *et al*., 2007). Conversely, biochemical indicators such as microbial biomass-C and soil enzymatic activities respond more rapidly and with a higher degree of sensitivity to soil disturbance, consequently, determining soil condition and functionality more accurately (García-

Gil *et al*., 2000). The enzyme activities in this study support the hypothesis that in addition to chemical and physical characteristics, the microbial activity also constraints proper TSFs' rehabilitation and plant establishment. Additionally, low microbial activity is dominant at all tailings when compared to reference soils indicating poor soil quality, limited nutrient cycling and SOM decomposition (supported by chemical results). Therefore, it is conclusive that, under extreme environmental conditions created by mining activities the microbial activity decreases.

Phase 2: Biological amendments and bio-stimulants effect on different mother crop species.

Results from **Chapter 5** can be summarised and concluded as follows:

1. The various bio-stimulants applied to the different growth substrates (before mother crop establishment) improved DHA to various degrees. Bio-stimulants showed variability in improving DHA, i.e., in general bio-stimulants showed to improve tailings but are substrate specific.

   a. Additional studies were done and using amendments and bio-stimulants supports the above-mentioned findings. For example, microfine langfos showed the highest improvement in DHA for NM-700. In addition, the DHA improvement showed variability between bio-stimulant and growth substrates.

2. The mother crops established in the different growth substrates showed variability in revegetation potential, i.e., indicating plant species are also substrate specific.

   a. Plant performance of the different bio-stimulant/mother crop combinations is highly variable. Visual differences in plant health were also noted in the same mother crop/bio-stimulant combination.

   b. Plant performance of bio-stimulant/mother crop combination indicates substrate specific results, i.e., certain bio-stimulants and mother crop species performed better/worse on different mine tailings.

   c. Bio-stimulant/mother crop combinations showed improved germination and survival rate compared to untreated mother crops establishment. Some exceptions to these results were observed. Refer to **Table 5-9**.

3. Bio-stimulant/mother crop combinations improved DHA to various degrees compared to untreated tailings.

a. DHA results obtained from the bio-stimulant/mother crop treatments were statistically different for the different growth substrates.

b. The same pattern for plant performance seemed to be true for DHA, i.e., substrate specific and plant species specific.

c. PCA results show no clear relationship between plant growth characteristics and DHA. Only a weak association exists between the different growth substrates survival and DHA. Some correlations do however exist between bio-stimulant/mother crop treatments best survival and highest DHA.

4. Although the different mother crop species and bio-stimulants performed substrate specific, some similarities existed, i.e., amino acids elicited early seedling development and possessed the highest survivability in both the radish and canola treatments.

5. Chemical, physical and microbial characteristics of the different growth substrates played an important role in mother crop species revegetation potential.

a. NM-geel (aka NM-1) showed the lowest plant germination and survival. One of the possible explanations is the extremely high EC and crust formation of these tailings. These findings correlate with the strongly negative association with EC, identified in **Chapter 4**.

b. Consequently, both positive and negative correlation between the chemical, physical and microbial properties (i.e., microbial activity) exist. For example, a strong negative association exists between DHA and salinity (EC).

Results indicated that the mother crop and bio-stimulants establishment is substrate specific, i.e., certain bio-stimulants and mother crop species combinations performed better on different gold tailings. Several researchers (Bashan, 1998; de-Bashan *et al*., 2012; Glick, 2003; 2012; 2014; Grandlic, 2008; Mendez *et al*., 2007; Mendez & Maier, 2008a; 2008b; Reed & Glick, 2005; Zhuang *et al*., 2007) have verified the capability of bio-stimulants to improve plant germination, improve seedling emergence and plant establishment. This research study validates these researchers' findings to a certain degree, as plant species selection and type of tailings (substrate site-specific) also played a role in the microbial-assisted revegetation. For example, Bachmann and Kinzel (1992) investigated the soil-plant interactions of six different plant species in four agricultural soils and discovered that the soil was frequently the dominating factor in the combination. Bachmann and Kinzel (1992) findings are supported by da Silva *et al*. (2003) and Salles *et al*. (2004). Whereas, research done by Bashan (1998), Lucy *et al*. (2004) and Schweitzer *et al*. (2008),

postulated that bacterial growth-promoting ability may be highly specific to certain plant species, cultivar and genotype. Examples of bacterial host plant preferences in agricultural crops were identified by several researchers (Berg & Smalla, 2009; Haichar *et al*., 2008; Kowalchuk *et al*., 2002; Wieland *et al*., 2001). Findings from Marschner *et al*. (2001) and Marschner *et al*. (2004) concluded that the complex interaction between soil type, plant species and rhizosphere micro-environment determines the rhizospheric microbial community structure and microbial activity (Berg & Smalla, 2009).

Plant performance- and DHA results in this study support the hypothesis that the synergistic use of soil microbes (bio-stimulants) and plants (mother crops) improves revegetation efficiency, therefore facilitating gold tailings rehabilitation. To summarise, vegetative cover (mother crops) in combination with bio-stimulants provides favourable conditions for more effective revegetation, improving both plant performance and DHA activity.

6.2 Overall conclusion

In conclusion, the study gains novel insights into the use of microbial enzymatic activity as a rehabilitation soil quality indicator. Additionally, the study highlights the importance of integrating microbial characteristics into rehabilitation designs and monitoring criteria. Consequently, due to microbial enzymatic activities sensitivity, rapid response, and their integrated response to various environmental factors, biological parameters are useful tools in determining the soil quality (degree of initial degradation), and rehabilitation performance monitoring (improvement/change in ecosystem stability). The results of this study (synergistic use of soil microbes and plants) show potential for future revegetation designs considering that germination and early growth represent crucial phases of the plant life cycle. Considering that bio-stimulant/mother crop treatments improved plant performance. Nonetheless, additional research is necessary to determine the sustainability of the design as the period of plant succession and ecosystem stability is a complex and lengthy process.

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CHAPTER 7

RECOMMENDATION AND FUTURE RESEARCH

"Science, my lad, is made up of mistakes, but they are mistakes which it is useful to make because they lead little by little to the truth."

–**Jules Verne**, **A Journey to the Centre of the Earth**

7.1 Recommendations

While global knowledge of soil microbial diversity and functioning is rising at a fast rate, knowledge of microbes is limited in the South African mine waste rehabilitation industry.

- Firstly, one of the future research goals would be identifying and understanding the microfauna within the different types of mine waste. Restricted knowledge exists on how soil microbial communities' structure and function vary within the different mining waste materials.

- In order to re-establish a dynamic, healthy and supportable ecosystem suitable for post-mining land use, an alternative mind-set is required in order to rehabilitate critical ecosystems.

- Mining waste environment should be investigated with a microbiological perspective, identifying available sources of microbial energy, electron acceptors, trace metal element availability and abiotic conditions that might have an effect on the microbial population.

Future research and knowledge gaps that need to be addressed includes

- DNA sequencing each of the different mine waste environments (i.e., coal, platinum, gold, kimberlite etc.).

- DNA sequencing on different localities of mine waste environments of the same type of mine waste; most probable numbers of the important groups found in the mine waste environment (e.g., Fe-oxidisers, Fe-reducers and S-reducers), and methods to measure the microbial enzymatic activity.

One of the key challenges in mine rehabilitation is the successful establishment of a self-sustaining vegetative cover on the TSFs and disturbed areas. Further research goals include the identification, isolation and cultivation of existing microbes within the rhizosphere of voluntarily established plant species on the different TSFs. Applying these adaptive microbes to selected

plant species and studying the synergistic effects. Integrating the abiotic and biotic environmental factors affecting successful rehabilitation, one can improve the likelihood of sustainable revegetation and ecological stability.

This thesis' research and results need to be confirmed across multiple field sites in order to draw general conclusions about whether this is just a local trend or a general pattern across plant species and gold tailings types. Considering that nursery-trials are under more ideal conditions compared to dryland field conditions.

In addition, verification of this multiphase approach needs to be tested on the standard rehabilitation climax grass establishment. It is hypothesised that the multiphase approach will have a positive effect on climax grass species establishment, soil physical properties (e.g., soil aggregate stability and structure), chemical properties (e.g., %C and %N) and microbial properties (e.g., soil enzymatic activity).

Comprehension about microbial communities and microbial activity's long-term dynamics during assisted-revegetation of mine tailings is lacking, specifically, how the microbial communities and microbial activity (Valentín-Vargas *et al*., 2014) might

- Respond to bio-stimulant treatments.
- Influence of microbial-assisted vegetation establishment on soil quality.
- Influence on long-term sustainability/success of plant establishment.

An alternative question arising from this research is whether bio-stimulants can be used on poorly established grasses on TSFs (soil application vs. foliar). It is therefore recommended that the thesis approach should be researched under more long-term, dryland/field trials. The microbial-assisted approach should be tested across multiple TSFs types, ranging from mildly to extremely constraint in terms of rehabilitation, across different locations and climates. In order to determine whether these results are just local trends or spatially correlated.

Identifiable limitations within research

Soil enzyme activities only indicate microbial activity potential rather than the actual rates of enzymatically catalysed reactions under natural *in situ* conditions. The fact is that laboratory conditions, whilst assaying enzymatic activity, are different to the relative field conditions. Consequently, the chemical reactions and the different sources of the enzymes influence the microbial activity measurements (Nannipieri *et al*., 2002). For example, DHA is regarded as an

intracellular enzyme, however the enzyme can be found extracellular in soil due to cell lysis and SOM and colloid associations (Månsson *et al.*, 2014; Marumoto *et al.*, 1982; Nannipieri *et al*., 2002; Udawatta *et al*., 2009).

Most bio-stimulants, with the exception of microbial inoculants, do not differentiate between beneficial and pathogenic microbes when stimulating microbial growth, but rather creates competition (du Jardin, 2015). Resulting in the more resilient microorganisms to outcompete the more sensitive species, which may create detrimental consequences. Additionally, the bio-stimulant were applied according to standard agricultural norms, which might not be applicable to tailings as naturals soils are not considered a harsh environment for plant growth. Consequently, the difference in bio-stimulant application concentrations should be investigated in future studies.

Due to economic (bursary budget) constraints, only DHA was measured. Refer to **Chapter 5**. In order to get a more comprehensive understanding of bio-stimulants/plant interactions and their successive role in improving soil biological characteristics, additional biochemical analyses, such as microbial biomass-C and enzymatic activities (β-glucosidase, urease, alkaline and acid phosphatase) should be assayed in future studies.

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ANNEXURE A

Table A-1 Neutralisation period for the different tailings materials.

Growth substrate	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5
NM-geel	2.7 ± 0.05	4.88 ± 0.18	5.50 ± 0.20	4.65 ± 0.05	5.075 ± 0.08	6.20 ± 0.05
NM-C1	3.68 ± 0.08	5.50 ± 0.10	6.68 ± 0.08	7.45 ± 0.08	5.95 ± 0.05	6.65 ± 0.05
DOMRIF	3.43 ± 0.04	5.08 ± 0.09	6.28 ± 0.13	6.65 ± 0.15	5.78 ± 0.13	6.33 ± 0.08

‡ Value given is mean ± standard error values.

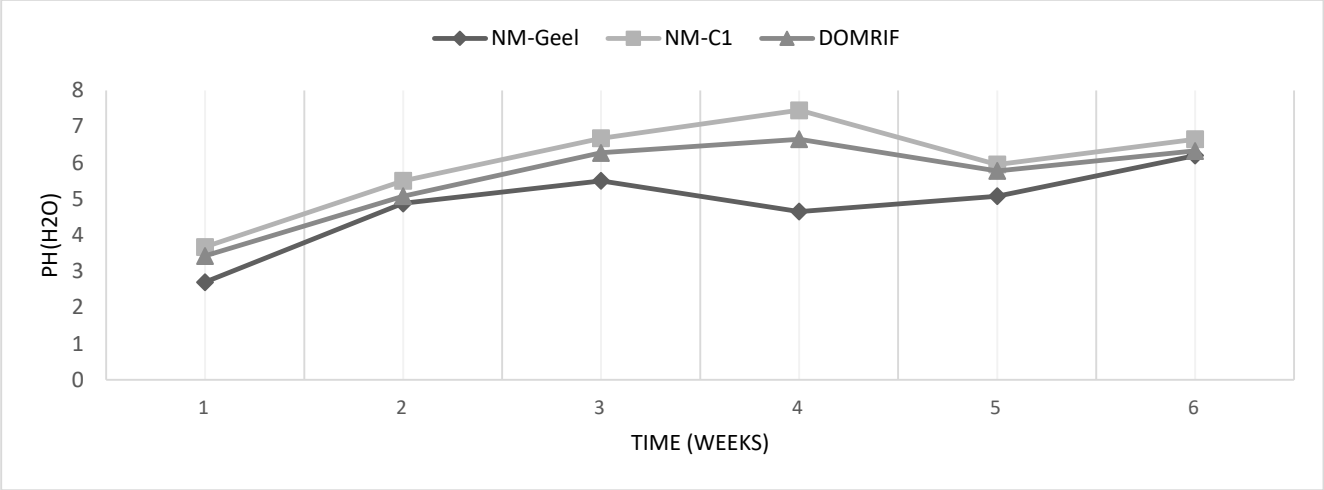

Figure A-1: Timeframe for neutralisation to take place for the gold tailings.

Table A-2 New Machavie TSFs substrate names for different chapters.

Chapter 4 New Machavie TSFs substrate name	Chapter 5 New Machavie TSFs substrate name
NM-1	NM-geel
NM-2	NM-C1

Table A-3 Fertiliser requirement for the mother crop species established in the different growth substrates.

Mother crop	NPK 5:3:4(33)	LAN(28)
Ryegrass	370kg/ha	300kg/ha
Brassica spp.	1220kg/ha	-

ANNEXURE B

Table B-1 Plant characteristics of some mother crops parameters explained:

N Scavenger-.	Evaluates mother crop's ability to take up and store excess nitrogen.
Soil builder-	Evaluates mother crop's ability to produce SOM and improve soil structure.
Erosion fighter-	Evaluates how extensive and quickly the root system develops, and its toleration and prevention of erosion.
Weed fighter-	Evaluates the ability of mother crops to out-compete weeds via its lifecycle.
Good grazing-	Forage capabilities including relative production, nutritional quality and palatability
Quick growth	Evaluates the establishment speed and growth.
pH preferred	pH range of which the mother crop species are expected to perform relatively well.
Soil impact.	Evaluates mother crops ability to loosen subsoil, make soil P and K more freely available, or amend topsoil.
Soil ecology.	Evaluates mother crops ability to oppose pests by repressing/reducing damage from nematodes, fungal/bacterial infection, or weeds via natural herbicidal (allelopathic) or competition/smothering action.
Minimum germination temperature.	The minimum soil temperature (˚C) required for successful germination and establishment.

ANNEXURE C

Table C-1: List of bio-stimulant and mother crop species combinations abbreviations:

Plant bio-stimulant abbreviation	Plant	Bio-stimulant
C-	Canola	None
Caa	Canola	Amino acids
Chum	Canola	K-humate
Cbac	Canola	Beneficial bacteria
Cmix	Canola	Mixture
Ccarb	Canola	Carbohydrates
Ra-	Rape	None
Raaa	Rape	Amino acids
Rahum	Rape	K-humate
Rabac	Rape	Beneficial bacteria
Ramix	Rape	Mixture
Racarb	Rape	Carbohydrates
Rad-	Radish	None
Radaa	Radish	Amino acids
Radhum	Radish	K-humate
Radbac	Radish	Beneficial bacteria
Radmix	Radish	Mixture
Radcarb	Radish	Carbohydrates
Ry-	Ryegrass	None
Ryaa	Ryegrass	Amino acids
Ryhum	Ryegrass	K-humate
Rybac	Ryegrass	Beneficial bacteria
Rymix	Ryegrass	Mixture
Rycarb	Ryegrass	Carbohydrates

* These abbreviations are used in the results.

Table C-2: Comparison of various mother crop/bio-stimulant treatments germination and survival rate (%) in the four growth substrates. Comparing canola and radish treatment combinations (A).

Growth substrates		Canola					
		C-	Caa	Chum	Cbac	Cmix	Ccarb
		(%)					
NM-Geel	Germ	7.5±7.5^{c}	30.0±20.0^{b}	47.50±17.5^{b}	22.5±13.8^{c}	30.0±25.0^{b}	47.5±32.5^{a}
	Survival	7.5±7.5^{c}	30.0±20.0^{b}	42.5±13.8^{b}	20.0±10.0^{c}	27.5±22.5^{b}	45.0±30.0^{a}
Dominion	Germ	60.0±25.5^{ab}	60.0±21.2^{ab}	80.0±12.2^{a}	57.5±8.3^{b}	77.5±22.8^{a}	72.5±17.9^{a}
	Survival	55.0±29.6^{b}	60.0±21.2^{ab}	70.0±7.1^{ab}	57.5±8.3^{b}	75.0±20.6^{ab}	72.5±17.8^{a}
NM-C1	Germ	85.0±7.5^{ab}	92.5±11.3^{a}	87.5±12.5^{a}	90.0±10.0^{a}	92.5±8.3^{a}	90.0±5.0^{a}
	Survival	85.0±7.5^{ab}	92.5±11.3^{a}	87.5±12.5^{a}	90.0±10.0^{a}	92.5±8.3^{a}	90.0±5.0^{a}
Control soil	Germ	97.5±4.3^{a}	100.0±0.0^{a}	95.0±5.0^{a}	97.5±4.3^{a}	100.0±0.0^{a}	92.5±8.3^{a}
	Survival	97.5±4.3^{a}	100.0±0.0^{a}	95.0±5.0^{a}	97.5±4.3^{a}	100.0±0.0^{a}	92.5±8.3^{a}
Pr > F		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.012
Growth substrates		**Radish**					
		Rad-	**Radaa**	**Radhum**	**Radbac**	**Radmix**	**Radcarb**
NM-Geel	Germ	25.0±12.5^{c}	32.5±12.5^{b}	25.0±17.5^{b}	30.0±10.0^{c}	25.0±7.5^{b}	27.5±17.5^{b}
	Survival	15.0±7.5^{c}	22.5±7.5^{b}	20.0±10.0^{b}	25.0±12.5^{c}	25.0±7.5^{b}	22.5±18.8^{b}
Dominion	Germ	60.0±14.1^{b}	74.0±17.4^{a}	62.5±16.4^{a}	60.0±7.1^{b}	77.5±27.7^{a}	82.5±17.9^{a}
	Survival	57.5±10.9^{b}	68.0±19.4^{a}	60.0±18.7^{a}	60.0±7.1^{b}	77.5±27.7^{a}	75.0±11.2^{a}
NM-C1	Germ	70.0±5.0^{ab}	82.5±12.5^{a}	85.0±5.0^{a}	85.0±10.0^{ab}	80.0±10.0^{a}	75.0±5.0^{a}
	Survival	70.0±5.0^{ab}	82.5±12.5^{a}	85.0±5.0^{a}	77.5±11.3^{ab}	77.5±8.8^{a}	72.5±3.8^{a}
Control soil	Germ	90.0±7.1^{a}	95.0±5.0^{a}	85.0±8.7^{a}	90.0±7.1^{a}	87.5±4.3^{a}	92.5±4.3^{a}
	Survival	90.0±7.1^{a}	95.0±5.0^{a}	85.0±8.7^{a}	90.0±7.1^{a}	87.5±4.3^{a}	92.5±4.3^{a}
Pr > F	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

† Values given are mean ± standard error values. Represent the mean of 4 replicates (n=4) ± standard error.
* Significance at 0.05 probability level between columns and rows.
‡Treatments labelled with the same letters within a column were not significantly different from each other (p>0.05). While those with different letters show significant variance at Tukey's HSD p< 0.05.

Table C-3: Comparison of various mother crop/bio-stimulant treatments germination and survival rate (%) in the four growth substrates. Comparing rape and ryegrass treatment combinations (B) (continued).

Growth substrates		Rape					
		Ra-	Raaa	Rahum	Rabac	Ramix	Racarb
		(%)					
NM-Geel	Germ	32.5±8.8c	17.5±3.8c	42.5±11.3c	20.0±20.0b	12.5±12.5c	40.0±20.0b
	Survival	32.5±8.8c	10.0±5.0c	42.5±11.3c	20.0±20.0b	10.0±10.0c	40.0±20.0b
Dominion	Germ	45.0±15.0bc	75.0±5.1b	70.0±12.2abc	72.5±16.4a	60.0±25.5b	45.0±32.0ab
	Survival	42.5±10.9bc	72.5±8.3b	67.5±13.0bc	72.5±16.4a	60.0±25.5b	45.0±32.0ab
NM-C1	Germ	75.0±5.0ab	90.0±10.0ab	87.5±12.5ab	85.0±15.0a	85.0±7.5ab	80.0±15.0ab
	Survival	72.5±7.5ab	90.0±10.0ab	87.5±12.5ab	85.0±15.0a	85.0±7.5ab	80.0±15.0ab
Control soil	Germ	85.0±16.6a	97.5±4.3a	100.0±0.0a	100.0±0.0a	100.0±0.0a	100.0±0.0a
	Survival	85.0±16.6a	97.5±4.3a	100.0±0.0a	100.0±0.0a	100.0±0.0a	100.0±0.0a
Pr > F		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.002
Growth substrates		**Annual ryegrass**					
		Ry-	**Ryaa**	**Ryhum**	**Rybac**	**Rymix**	**Rycarb**
NM-Geel	Germ	30.0±10.0c	50.0±15.0a	62.5±8.8b	35.0±15.0b	45.0±25.0b	40.0±30.0b
	Survival	27.5±8.8c	50.0±15.0a	57.5±12.5b	32.5±13.8b	42.5±27.5b	37.5±27.5b
Dominion	Germ	85.0±15.0ab	75.0±15.0a	97.5±4.3a	85.0±15.0a	82.5±14.8ab	92.5±8.3a
	Survival	85.0±15.0ab	75.0±15,0a	97.5±4.3a	85.0±15.0a	82.5±14.8ab	90.0±10.0a
NM-C1	Germ	72.5±3.8ab	82.5±8.8a	85.0±5.0ab	95.0±5.0a	85.0±10.0ab	95.0±7.5a
	Survival	67.5±7.5b	82.5±8.8a	82.5±3.8ab	92.5±7.5a	85.0±10.0ab	95.0±7.5a
Control soil	Germ	97.5±4.3a	80.0±10.0a	80.0±15.8ab	90.0±7.1a	90.0±7.1a	82.5±4.3a
	Survival	97.5±4.3a	80.0±10.0a	80.0±15.8ab	90.0±7.1a	90.0±7.1a	82.5±4.3a
Pr > F		< 0.0001	0.025	0.001	< 0.0001	0.004	< 0.0001

† Values given are mean ± standard error values. Represent the mean of 4replicates (n=4) ± standard error.
* Significance at 0.05 probability level between columns and rows.
‡Treatments labelled with the same letters within a column were not significantly different from each other (p>0.05), while those with different letters show significant variance at Tukey's HSD p< 0.05.

ANNEXURE D

Kale seedling health in the different growth substrates.

NM-geel gold tailings | Dominion gold tailings

NM-C1 gold tailings | Control soil

Figure D-1: Seedlings of sovereign kale in the different growth substrates.

ANNEXURE E

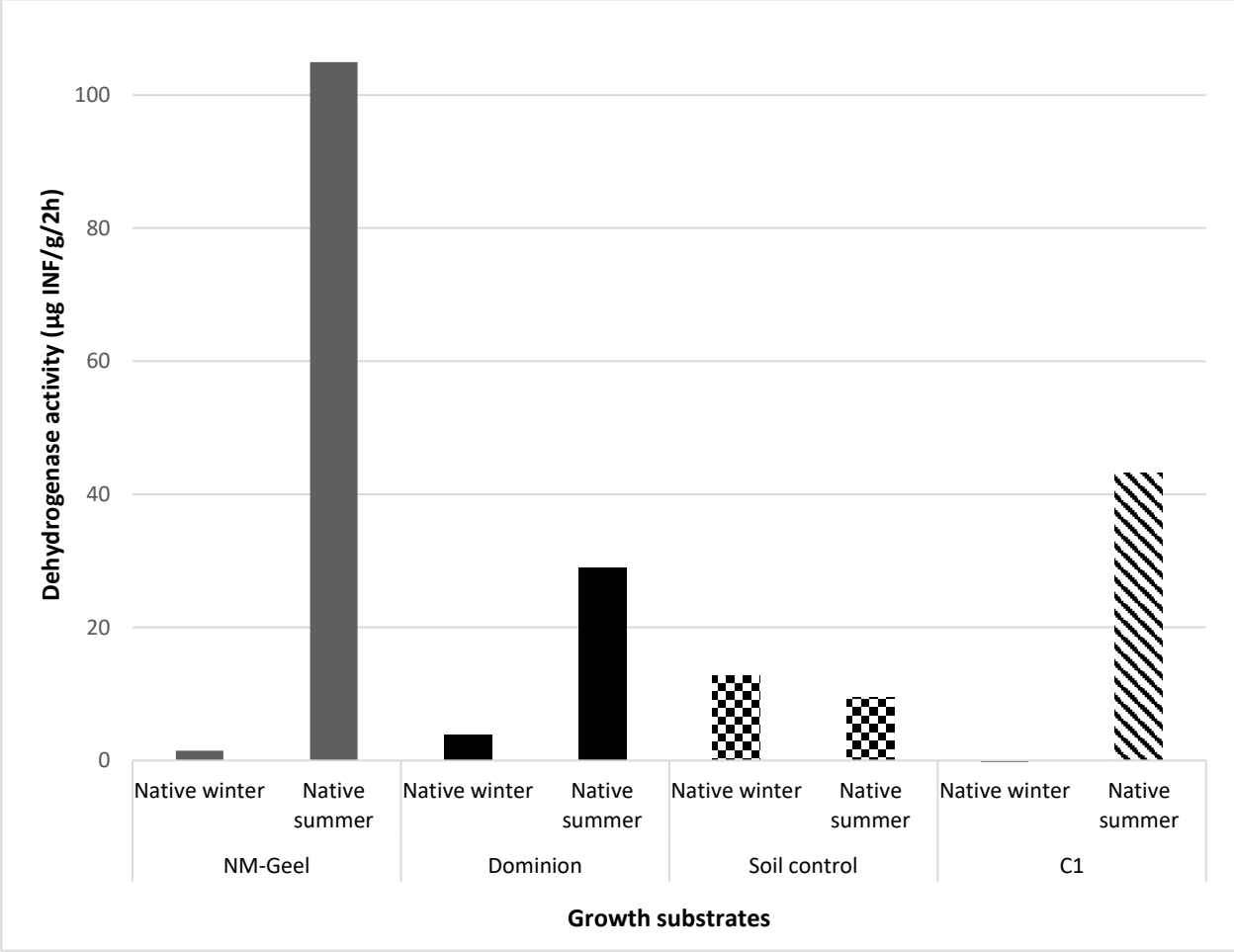

Figure E-1. Winter/Summer differences in DHA (µg INF/g/2h) of the four growth substrates used in Chapter 5.

ANNEXURE F

Humusica 2, article 16: Techno humus systems and recycling of waste.

Published in Applied Soil Ecology INPRESS (2018).

ANNEXURE G

Humusica 3, article 12: Baseline status of microbial activity on gold tailings facilities in South Africa.

Published in Applied Soil Ecology INPRESS (2018).