Investigating soil algae and cyanoprokaryotes on gold tailings material in South Africa

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Wisdom begins in Wonder

-Socrates-

Abstract

Gold mine tailings material facilities are characterized by sparse vegetation and an abundance of dust. Mine tailings facilities are examples of extreme geotechnical and geochemical conditions which make it almost impossible for higher plants to establish and grow without rehabilitation intervention. In most cases higher plants such as grasses and trees are the focus areas for rehabilitation, but, having a look at something a little smaller such as biological crusts, it is seen that these micro-organisms play very important roles in any ecosystem.

Various studies have shown that biological crusts, consisting of micro-organisms such as lichens, algae and cyanoprokaryotes enhance the soil quality by binding soil particles together, forming aggregates which counteract the erosive forces of wind and water. They play a part in nitrogen and carbon fixation, increase the soil surface temperature and increase the water retention of the soil. Thus, these organisms improve the overall health of the soil, which will in time encourage the successful establishment of higher plants.

The aim of this study was to investigate the presence of cyanoprokaryotes and soil algae on mine tailings storage facilities that have been rehabilitated for different periods of time as well as to correlate the presence of these species with the physical and chemical characteristics of the mine tailings material. Chemical, physical and biological analyses of soil samples were done. Some of the ecologically important and dominant species were isolated and protocols were developed in order to identify the most successful manner in which to re-inoculate the organisms to a chosen substrate and how to measure biomass.

Due to the immense cost of standard rehabilitation practices there is a need for a more cost effective, sustainable manner in which to protect the tailings material against the erosive forces of wind and water with as little input as possible. The influence of an organism cultured in normal Bold's Basal medium (BBM) growth medium, BBM growth medium with half the phosphate concentration and BBM growth medium with half the nitrate concentration on the establishment of a biological soil crust (BSC) was tested. To test the influence of the inoculums already present in the tailings material and in the air, trials with mulch, water and nutrients without the addition of an organism was also investigated. This was done in the controlled environment of a glasshouse, as well as in field conditions. The biomass of the cyanoprokaryotes and algae, as well as the soil surface strength was also tested.

The results show that the time of rehabilitation did not have an influence on the cyanoprokaryotes as well as algal species that occurred on the tailings material. *Chlorella* sp., *Chlorococcum* sp. and *Klebsormidium* sp. were present on all six sites, except on the

fresh material and 15 year old material where no rehabilitation has been done. As for dominance; *Chlamydomonas* sp., *Chlorococcum* sp., *Klebsormidium* sp. and *Phormidium* sp. were dominant on all six sites except for the fresh material, where nothing grew.

An array of methods exists for measuring algal biomass as a measure of growth. During the development of protocols for further use in investigating the growth of algae, the extraction solvent ethanol, for use in chlorophyll *a* extraction, was identified as the most sufficient. The re-inoculation of cyanoprokaryotes and soil algae onto a chosen substrate is most successful when pouring the organisms, cultured in growth medium and 0.1% agar, over the substrate.

During the glasshouse trials the influence of the growth medium and growth medium with half the nitrate and half the phosphate concentrations showed that *Chlamydomonas* sp. produced the highest biomass when cultured in BBM. With *Nostoc* sp. the highest biomass occurred with culturing in BBM and BBM with half the phosphate concentration. *Microcoleus vaginatus* showed no significant difference when cultured in the three different growth mediums (BBM, BBM with half the nitrate concentration and BBM with half the phosphate concentration). Overall *Nostoc* sp. produced the highest biomass (34.33 μg/g), followed by *Microcoleus vaginatus* (17.05 μg/g) and *Chlamydomonas* sp. (6.12 μg/g).

Soil surface strength, measured with a hand held penetrometer showed that *Chlamydomonas* sp. cultured in BBM growth medium produced the most stable crust (2.58 kg/cm^2), although it had the lowest biomass measurements (6.12 μg/g). *Nostoc* sp. produced the highest biomass (34.44 μg/g), but had the lowest soil surface strength results (1.75 kg/cm^2). *Microcoleus vaginatus* proved to be the species with high biomass production (17.05 μg/g), as well as high soil surface strength (2.08 kg/cm^2). *M. vaginatus* is also a pioneer species and is therefore a good choice as primary inoculum on bare tailings material.

It was decided to use *Nostoc* sp. in the field trials due to its high biomass and *Microcoleus vaginatus* due to the high soil surface strength produced. Despite the occurrence of a severe thunder storm on the afternoon of application and poor water management during the field trials the significance of water on the establishment of soil algae and cyanoprokaryotes on tailings material was determined.

Key words: Gold mine tailings facilities, biological soil crusts (BSC), soil algae, cyanoprokaryotes, chlorophyll-*a* extraction

Uittreksel

Goudmynslikdamme word gekenmerk deur yl plantegroei en oorvloed stof. Mynslikdamme is voorbeelde van uiterste geotegniese en geochemiese toestande wat veroorsaak dat dit byna onmoontlik is vir plante om te vestig en te groei sonder rehabilitasie. In die meeste gevalle is hoër plante soos grasse en bome die focus areas vir rehabilitasie, maar wanneer daar gekyk word na iets kleiner, soos `n biologiese grondkors, word daar gesien dat hierdie mikro-organismes `n baie belangrike rol in die ekosisteem waarin hul teenwoordig is, speel.

Verskeie studies toon dat biologiese korse, wat uit mikro-organismes soos ligene, alge en sianoprokariote bestaan, die kwaliteit van die grond verhoog deur gronddeeltjies saam te bind en sodoende aggregate te vorm wat beskerming teen wind- en watererosie bied. Die korse speel `n rol in stikstof- en stikstoffiksering, verhoog die temperatuur van die grondoppervlak asook die waterhoudingskapasiteit van die grond die algehele gesondheid van die grond word dus verbeter wat die suksesvolle vestiging van plante kan verseker.

Die doel van hierdie studie was om die teenwoordigheid van grondalge en sianoprokariote op mynslikdamme, wat vir verskillende periodes gerehabiliteer is, te ondersoek. Daar is ook vasgestel of die teenwoordigheid van die spesies met die fisiese en chemiese eienskappe van die mynslikmateriaal gekoppel kan word. Chemiese, fisiese en biologiese ontleding van grondmonsters is gedoen. Sommige van die ekologies belangrik en dominante spesies is vir verder eksperimente geïsoleer. Protokolle is ontwikkel om die mees suksesvolle wyse waarop die spesies weer aan `n gekose substraat toegedien kan word te bepaal. Daar is ook protokolle ontwikkel vir die mees effektiewe wyse waarop biomassa gemeet kan word.

As gevolg van die geweldige kostes wat gepaard gaan met standaard rehabilitasie praktyke is daar `n behoefte aan meer koste-effektiewe, volhoubare metodes waarop die slikmateriaal beskerm kan word teen die vernietigende effek van wind- en water erosie met so min as moontlik insette. Die vestiging van `n organisme gekweek in normale Bold's Basal groeimedium, Bold's Basal groeimedium met die helfte van die fosfaatkonsentrasie en Bold's Basal groeimedium met die helfte van die nitraatkonsentrasie op goudslik is getoets. Die teenwoordigheid van die inokulum wat reeds in die lug en slik teenwoordig is, is bepaal deur behandelings waar net water, voedingstowwe en `n beskermende deklaag toegedien is. Hierdie eksperimente is in `n gekontroleerde omgewing van `n glashuis, sowel as *in situ* op `n uitskothoop getoets. Die biomassa van die alge en sianoprokariote, sowel as die stabiliteit van die grondoppervlak is getoets.

Die resultate toon dat die tyd van rehabilitasie nie `n invloed op die sianoprokariote en grondalgspesies wat op die slikmateriaal voorkom, het nie. *Chlorella* sp., *Chlorococcum* sp.

en *Klebsormidium* sp. was op al ses persele teenwoordig, behalwe op die varsmateriaal en 15 jaar oue materiaal waar geen rehabilitasie gedoen is nie. *Chlamydomonas* sp., *Chlorococcum* sp., *Klebsormidium* sp. en *Phormidium* sp. was op al ses die persele dominant, behalwe vir die varsmateriaal, waar niks gegroei het nie.

Daar bestaan `n reeks metodes om die biomassa van alge te meet, as `n maatstaaf vir groei. Die verkose metode vir hierdie studie was chlorofil-*a* ekstraksie. Chlorofil *a* ekstraksie sluit die gebruik van `n oplosmiddel in. Tydens die ontwikkeling van die protokolle is etanol as die mees effektiewe oplosmiddel geïdentifiseer. Die mees effektiewe inokulering van organismes op `n verkose substraat geskied deur die organisme, gekweek in ‘n groeimedium met 0.1 % agar, oor die substraat te giet.

Tydens die glashuisproewe is die invloed van groeimedium en groeimedium met helfte van die nitraat- en die helfte van die fosfaatkonsentrasies getoets. Die resultate het getoon dat *Chlamydomonas* sp. die hoogste biomassa produseer wanneer dit in Bold’s Basal groeimedium gekweek word. *Nostoc* sp. groei die beste in Bold’s Basal groeimedium en Bold’s Basal groeimedium met helfte van die fosfaatkonsentrasie. *Microcoleus vaginatus* het geen betekenisvolle verskil getoon wanneer dit in die drie verskillende groeimediums (Bold’s Basal groeimedium, Bold’s Basal groeimedium met helfte fosfaatkonsentasie en helftenitraat konsentrasie) gekweek word nie. Algeheel het *Nostoc* sp. die hoogste biomassa (34.44μg/g) geproduseer, gevolg deur *Microcoleus vaginatus* (17.05 μg/g) en *Chlamydomonas* sp. (6.12 μg/g).

Grondoppervlaksterkte is met ‘n drukmeter gemeet en daar is getoon dat *Chlamydomonas* sp., gekweek in Bold’s Basal groeimedium, die mees stabiele kors produseer (2.58 kg/cm^2), maar *Chlamydomonas* sp. het ook die laagste biomassametings gehad (6.12 μg/g). *Nostoc* sp. het die hoogste biomassa (34.44 μg/g), maar die laagste grondoppervlaksterkte resultate (1.75 kg/cm^2). *Microcoleus vaginatus* blyk die voordeligste spesie te wees met 'n hoë biomasssaproduksie (17.05 μg/g), sowel as 'n hoë grondoppervlaksterkte (2.08 kg/cm^2). *M. vaginatus* is ook 'n pionierspesie en is dus 'n goeie keuse om as primêre inokulum op slikmateriaal te gebruik.

Vir die veldproewe daar is besluit om *Nostoc* sp. te gebruik as gevolg van sy hoë biomassa en *M. vaginatus* wat hoë grondoppervlaksterkte geproduseer het. Ten spyte van 'n donderstorm tydens die middag van inokulering en die swak bestuur van water tydens die eksperiment het die veldproewe aangetoon dat die toediening van water ‘n belangrike rol speel in die vestiging van grondalge en sianoprokariote op slik.

Sleutelterme: Goudmynslikdamme, biologiese grondkorse, grondalge, sianoprokariote, chlorofil-*a* ekstraksie

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Disclaimer

"Any opinion, findings and conclusions or recommendations expressed in this material are those of the author(s) and therefore the NRF/THRIP, Geology Department of the North-West University and Agreenco Environmental Projects do not accept any liability in regard thereto."

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Chapter 1

1. Introduction

1.1 Problem statement

Tailings storage facilities (TSF) are mine residue storage facilities for potential harmful waste products such as waste rock, cyanide sand and slime, surplus mine water and discarded solutions (Reichardt, 2012), known as tailings material or mill tailings (Haagner, 2008). The tailings material investigated in this study is produced through the process of gold mining.

Sparse vegetation, which is a common sight on tailings material, are not aesthetically very pleasing, exposes a surface to the erosive forces of wind and water, decreases infiltration of water and thereby increases the possibility of runoff, erosion, sedimentation and air pollution through the generation of dust from the tailings material (Hattingh and van Deventer, 2004). Dust emission from the tailings has many health effects on the communities closely associated with the TSF's (Reichardt, 2012 and Smallhorne, 2012). The low pH, presence of acids and high salt content of tailings material causes a harsh, infertile environment for vegetation to establish and grow (Hattingh and van Deventer, 2004 and Martin *et al*., 2008). Due to the chemical nature of the tailings material (possible toxicity of heavy metals, and immobilization of some essential nutrients) the establishment of vegetation for rehabilitation purposes is a very costly process (Haagner, 2008). There is therefore a serious need for a cost effective alternative to rehabilitate tailings material and provide effective protection against the erosive effects of wind and water.

Most of the gold tailings material dumped on TSF's contain up to 3.5% pyrite, which oxidizes in the presence of oxygen, thereby producing an acidic solution (Haagner, 2008, and Martin *et al*., 2008) that can be explained through the following reaction by Stumm and Morgan: $FeS_2 + 3.75O_2 \rightarrow Fe(OH)_3 + 2H_2SO_4$ (Martin *et al*., 2008). The acidic solution produced then infiltrates the soil, lowering the pH of the soil to as low as 2.0 (Haagner, 2008, Martin *et al*., 2008), which may lead to heavy metal liberation, such as aluminium (Al) which becomes available for plant uptake (Winegardner, 1995). This acidic infiltrate also leads to morphological, compositional and mineralogical changes within the soil profile that include decline in cation exchange capacity (CEC), texture variation, greater electrical conductivity (EC) and the appearance of horizons with colour variations (Martin *et al*., 2008). CEC is a relative measure of nutrient holding capacity of the soil (Winegarder, 1995) and should be between 5 and 20 $cmolkg^{-1}$ (van Wyk, 2002). Low CEC causes nutrients to leach from the soil, causing nutrient deficiencies, leading to limited plant growth on these substrates. EC is

an indication of the electrical conductivity of the soil (Aucamp, 2003) and should ideally be between 60 – 100 mSm^{-1}(van Wyk, 2002). High EC values are usually an indication of dispersion potential (van Deventer, 2013). It is thus clear that tailings material poses quite some challenges for rehabilitation purposes.

The presence and significance of biological soil crusts (BSC) have been researched extensively on coal mines, agricultural lands and especially natural soils (Tsujimura *et al*., 2000; Frouz *et al*., 2001; Issa *et al*., 2007). The organisms associated with BSCs are known to establish in extreme environments (Shields and Durrell, 1964; Fogg *et al*., 1973; Eldridge *et al*., 2000; Zancan *et al*., 2005.) and contribute significantly to the stabilization of soils through soil aggregate formation (Lange, 2001; Hu *et al*., 2003; Flechtner, 2007 and Bowker 2007). Biological soil crusts could therefore potentially provide protection against the erosive forces of wind and water, especially on tailings material.

To the best of our knowledge, there have not been any similar research studies done on gold mine tailings facilities in South Africa.

1.2 Research aims and objectives

Revegetation of tailings facilities has been considered the most effective means of reducing water and wind-borne erosion but these methods have proven ecological and economically unsustainable (Straker *et al*., 2007). The use of BSC in restoration of mine tailings may be a cheaper alternative and it is therefore important to investigate this possibility. Specific objectives to achieve this were:

1. To investigate the occurrence of soil algae and cyanoprokaryotes on both rehabilitated and un-rehabilitated gold mine tailings. This was done by studying the inoculums present in the air around the sampling sites as well as identifying and isolating the algal and cyanoprokaryote species in the tailings material.
2. To determine protocols to do growth studies with soil algae and cyanoprokaryotes.
    a. This includes the determination of the best growth medium to work with;
    b. the best extraction method of chlorophyll-*a* from soil
    c. as well as the best method to reintroduce inoculums on the tailings material.
3. To compare the growth of a few chosen algae and cyanoprokaryotes on tailings material in the controlled environment of a glass house.

4. To investigate the establishment of chosen algae and cyanoprokaryotes on tailings storage facilities *in situ*.

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Chapter 2

2. Literature Review

2.1 Challenges associated with gold tailings material

Gold mining in South Africa dates as far back as 1806, when the Secretary Governor of the Cape announced the discovery of gold in an area between the Witwatersrand and Magaliesberg (Stanley, 1987). Since then gold mining has gone from strength to strength in South Africa and the country was even one of the leaders in gold production worldwide (Vermeulen, 2001). Vermeulen (2001) describes the gold extraction process under four main stages namely: winning mineral ores, ore dressing, metallurgical extraction and refining. The metallurgical extraction phase describes the chemical extraction of the ore-mineral through one of two processes: amalgamation or cyanidation (Vermeulen, 2001). For the purpose of the literature study emphasis will be placed on the cyanidation technique, as this is the technique used on the tailings material investigated in this study. Gold is leached from a pulp with the use of sodium cyanide or calcium cyanide (Vermeulen, 2001). To stabilize the cyanide radical and maintain a pH of 10 to 11 oxygen and calcium hydroxide are used. Cyanidation produces a solution with anionic metal cyanide complexes from which the gold complexes must be separated (Vermeulen, 2001). To recover the gold complexes, zinc precipitation is used. Lead nitrate, together with zinc dust or shavings, are added to the pulp where the zinc with a high electro-negative charge precipitates the gold due to its high electro positive charge (Vermeulen, 2001). The gold and zinc salts slime is then filtrated and treated with dilute sulphuric acid to remove the zinc and other impurities. The precipitate is then dried and impurities such as lead and zinc is oxidised (Vermeulen, 2001). The products are then smelted in a flux of borax and silica, where the base metals combine with the silica to form a slag, which separates form the gold. The bullion is poured into bars and sent for refinery, whereas the slag is transported for dumping on a TSF (Vermeulen, 2001). The slag dumped on the TSF still contains the residue of active chemicals, such as cyanide, sulphuric acid, metals and salts, and may have negative impacts on the environment (Rossouw, 2010).

In 1977 the Chamber of Mines commissioned the formulation of a guideline for the design, operation and closure of TSF (Blight, 2010). TSF have to be managed very closely as many environmental and social impact concerns arise, including air pollution, water pollution, erosion and tailings stability (Hattingh and van Deventer, 2004). Exposure to dust, radon

emission, acids, salts, heavy metals, radio nucleotides, cyanides and sediment load can cause health problems, physical discomfort to people and crop damage (Petavratzi *et al*., 2005). Health problems associated with excessive dust exposure includes respiratory problems as well as skin diseases, not only in humans but in animals as well (Petavratzi *et al*., 2005). Therefore mine waste storages should be constructed and protected in such a way that their adverse effects on human health and the natural environment are minimized on a long term and continual basis.

A number of methods to protect tailing's slopes against erosion by geotechnical means have been developed and experimented with. These methods include: gravel mulching, covering the slopes with a thin gravel layer or 'gravel mulch', rock cladding, covering the entire surface with a layer of waste rock, and rock armouring which involves the discontinuous layering of gravel and rock on the surface (Blight, 2011). Vegetation on TSF also has many advantages as these areas are aesthetically more pleasing, it reduces the potential for erosion, increases infiltration, thereby ensuring sustainability. Vegetation can create a suitable habitat for the establishment of biodiversity (Hattingh and van Deventer, 2004). It is well known that gold TSF are not considered an inhabitable area for sustainable growth by vegetation, unless species that are tolerant to these environments are able to establish (Rossouw, 2010). Characteristics associated with these areas include compaction, acidity, low macronutrient availability, high availability of heavy metals such as aluminium, high salt content and acid generating potential (van Deventer and Hattingh, 2008, Rossouw, 2010 as well as Haagner, 2008).It has to be pointed out, however, that continued irrigation, fertilization and overall maintenance are required to prevent degradation, as in the case of good farm soils (Rossouw, 2010).

2.2 What are biological soil crusts?

Biological soil crusts (BSC), also known as microbiotic, microphytic, cryptogamic or cryprobiotic crusts (Belnap *et al*., 2001, Li *et al*., 2005 and Rosentreter *et al.*, 2007) comprise of diverse communities, including soil algae, cyanoprokaryotes, fungi and mosses (Belnap and Lange, 2001, Bowker, 2007, Issa *et al*., 2007, Flechtner, 2007 and Langhans *et al*., 2009). Research on BSC date as far back as 1941 when Booth investigated the relationship between soil algae and soil aggregate stability. Although algae and cyanoprokaryotes are commonly known as freshwater and marine organisms, they are now generally accepted as main constitutes in BSC, occupying a variety of terrestrial habitats (Hoffman, 1989, Frouz *et al.*, 2001 and Bowker, 2007).

Soil algae and cyanoprokaryotes are present in soils and occur as spores in the air (Shields and Durrell, 1964, Zancan *et al*., 2005) and initiate the formation of BSC with episodic events of available moisture (Fogg *et al*., 1973 and Hu *et al*., 2003).

According to Bowker (2007) the formation of BSC occurs in stages as organisms colonize the soils in successional stages (Belnap *et al*., 2001). Initially filamentous cyanoprokaryotes such as *Scytonema* sp. and *Microcoleus* sp. penetrate and stabilize the soil for the single celled green algae such as *Chlamydomonas* sp. and *Chlorococcum* sp., followed by fungi, lichens and eventually mosses (Belnap *et al*., 2001). In Figure 2.1 an illustration of a BSC with all components can be seen.

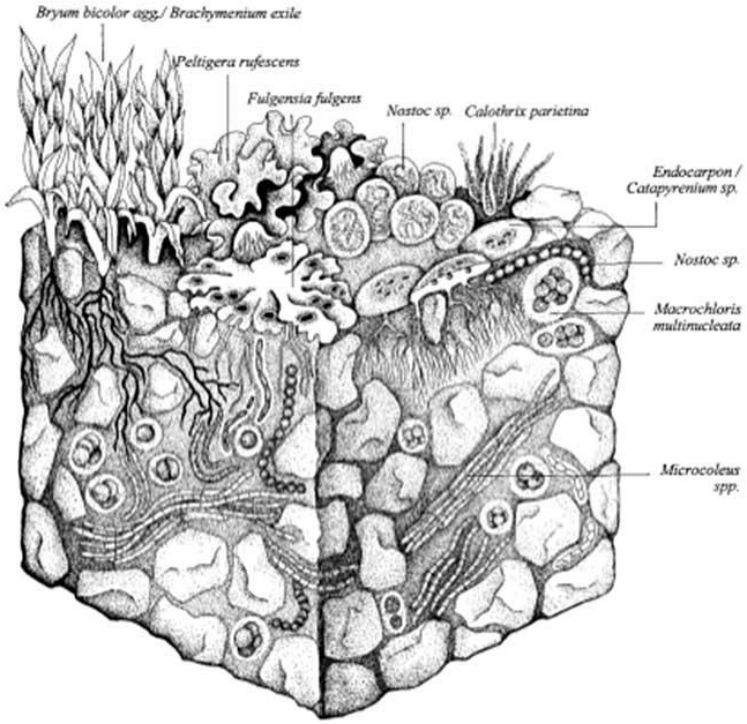

Figure 2.1: An illustration of a BSC, with typical colonizers (Belnap *et al*., 2001).

2.3 Biological, Chemical and Physical Soil Crusts

Much confusion about the difference between biological, chemical and physical crusts exists as many of these crusts may seem the same to the untrained eye. Biological crusts, seen in Figure 2.2 (a), are living crusts containing many organisms such as algae, fungi and lichens, as explained previously (Natural Resources Conservation Services, 2001, Bowker, 2007, Flechtner, 2007 and Langhans *et al*., 2009).

Physical crusts, as seen in Figure 2.2 (b), indicate low organic content, low aggregate stability and susceptibility to erosion (Natural Resources Conservation Services, 2001), all characteristics that have to be dealt with on tailings material. Chemical crusts form mainly in

the presence of high salt content and can be seen in Figure 2.2 (c) (Natural Resources Conservation Services, 2001).

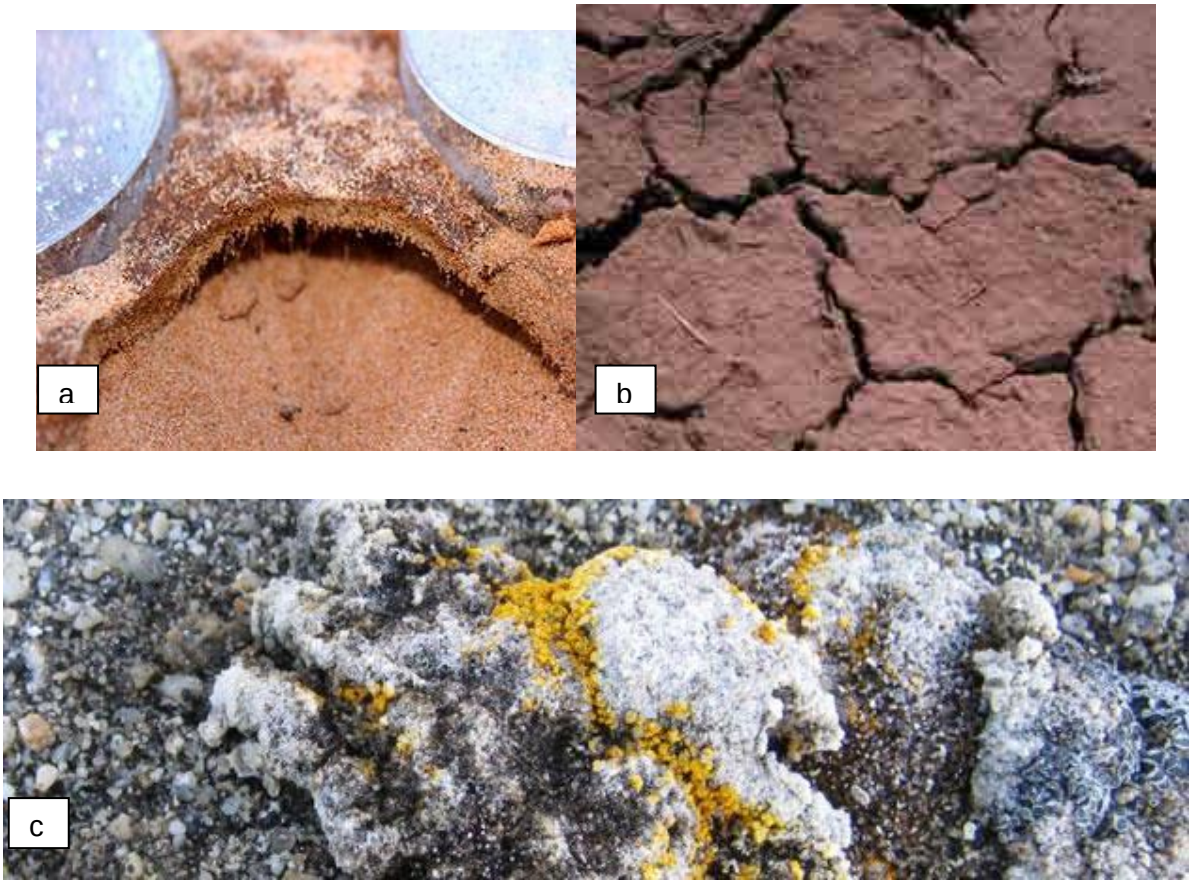

Figure 2.2: Example of a (a) well established biological soil crust (http://www.terradaily.com), b) a physical crust (http://ecomerge.blogspot.com) and c) a chemical crust (http://www.uni-kl.de).

2.4 Biological Soil Crusts and their adaptations

Soil algae and cyanoprokaryotes are known to colonize substrates that may seem uninhabitable to other organisms and even higher plants. There have been accounts of these organisms being the initial stage in succession (Shields and Durrell, 1964), able to withstand conditions such as extreme drought, high temperature, low pH and high UV intensity (Flechtner, 2007, Issa *et al*., 2007 and Zhang *et al*., 2009). BSC samples are in most cases very diverse and that may be in part the reason for their success (Büdel *et al.*, 2009). Many algae such as *Chlorococcum* sp. produce UV-absorbing pigments (see Figure 2.3) that reflect and/ or absorb excessive radiation, thereby protecting their cell structure, (Belnap and Lange, 2001, Belnap, 2001, Flechtner, 2007 and Issa *et al*., 2007). Organisms, such as *Microcoleus* sp., do not produce UV protecting pigments and occur beneath the other organisms, deeper in the soil, producing thick polysaccharide sheaths that aid in water

retention as well as protection against excessive light concentrations (Flechtner, 2007 and Issa *et al*., 2007). In addition to providing protection against desiccation, these polysaccharide sheaths glue soil particles together, assisting in the production of soil aggregates (Issa et al., 2007, Flechtner, 2007 and Belnap, 2001).

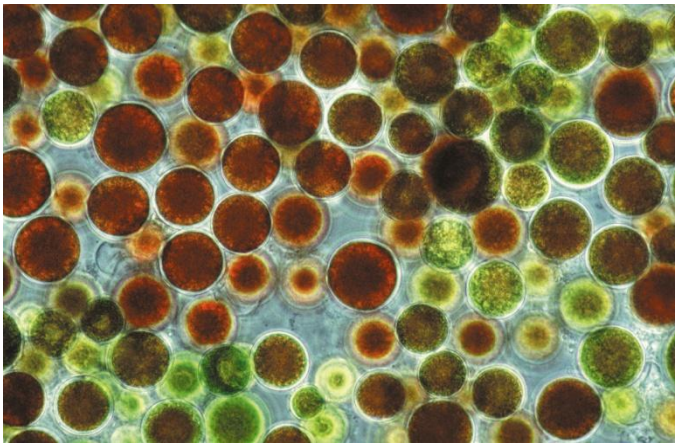

Figure 2.3: *Chlorococcum* sp. producing UV protecting pigments that can keep up to 50 – 93% of radiation from reaching the cell interior (Belnap, 2001).

2.5 The importance of biological soil crusts

Most soil algae and cyanoprokaryotes are cosmopolitan organisms and are known to play a significant role in the system where they inhabit. Soil algae can function as a bio-indicator for soil quality (Zancan *et al*., 2005) as they play a role in an array of ecological benefits which include assistance in nitrogen and carbon fixation, increasing soil nutrient status, decreasing water runoff, increasing water infiltration and assistance in stabilizing soil surfaces (Belnap and Lange, 2001, Hu *et al*., 2003, Sun *et al*., 2004, Flechtner 2007, Bowker, 2007 and Issa *et al*., 2007).

Nitrogen (N) occurs in an unusable form for plants (N_2 gas) in the atmosphere. In order for plants to absorb nitrogen, it has to be reduced to NH_4^+ or NO_3^- (Belnap, 2001a) and Nabors, 2004). This reduction occurs through prokaryotic organisms such as eubacteria and cyanoprokaryotes (Nabors, 2004). Nitrogen-fixating bacteria occur as free-living organisms in the soil, or in some cases complexes are formed with certain plant species (Nabors, 2004). Nitrogen gas is converted to ammonia (NH_3), after which the ammonia adds an H^+ ion from the soil to form ammonium (NH_4+) (Nabors, 2004). Common nitrogen fixating cyanoprokaryotes that occur in soils are *Anabaena* sp., *Nostoc* sp., *Scytonema* sp. and

Calothrix sp. As nitrogen fixation is an anaerobic process, these cyanoprokaryote species form thick-walled cells called heterocysts where fixation takes place (Belnap, 2001a).

The ability of BSC to stabilize soils lay mainly in their production of mucus or polysaccharide sheaths (Flechtner, 2007 and Isssa *et al*., 2007).

Belnap (2001b) explains the soil aggregation properties of specifically *Microcoleus* sp. as follows: Bundles of *Microcoleus* filaments are surrounded by polysaccharide sheaths. As the filaments move through the soil particles to the upper soil layers they are bound together upon soil wetting. When it becomes dryer the filaments retreat within the soil and new polysaccharide sheaths are produced. Even the dry mucus sheaths contribute significantly to soil aggregate stability.

Another soil stabilizing strategy, especially in well-developed crusts, is 'cyanoprokaryote layering' (Belnap, 2001b). Organisms which are not mobile (such as *Nostoc* and *Scytonema* species) produce UV-protecting pigments and occur on the soil surface, whereas species such as *Microcoleus* with no UV protecting pigments, are mobile and occur deeper within the soil (Belnap, 2001b).

The presence of Biological Soil Crusts has been observed on many growth mediums that may seem unfavourable to other organisms (Shields and Durrell, 1964). Their presence on coal discard have been investigated considerably (Lukesova, 2001; Frouz *et al*., 2001). There is however no accounts of studies for the presence of biological soil crusts on gold tailings storage facilities.

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Chapter 3

3.1 Introduction

Here follows a paper published in *Nova Hedwigia*, 2013, volume 97 issues 3-4, pages 281-194 written on the presence of soil algae and cyanoprokaryotes on gold mine tailings facilities in the Stilfontein area in the North West province. As previously mentioned, soil algae and cyanoprokaryotes, as constituents of biological soil crusts play ecological significant roles in the system they occur in. The aim of this study was to compare the species present in, and dominant on, gold tailings material that has been rehabilitated over a chrono-sequence. The algal inoculum present in the air was also investigated. Attached after the article is a photo page of some of the identified soil algal and cyanoprokaryote species present in the tailings material (see Figure 3.4, page 29). In the article the term cyanobacteria is used instead of cyanoprokaryotes as in the rest of the thesis.

Article as PDF file on CD

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Cyanobacteria and algae of gold mine tailings in the Northwest Province of South Africa

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With 3 figures and 6 tables

Abstract: Cryptogamic crusts are important components in arid and semiarid lands and could play a vital role in the rehabilitation of mine tailings. The aim of this study was therefore to investigate the occurrence of cyanobacteria and algae on gold mine tailings storage facilities in the Northwest province in South Africa that are characterized by extreme geotechnical and geochemical conditions. Samples for chemical, physical as well as biological analysis were collected from un-rehabilitated tailings as well as a chrono-sequence of biologically rehabilitated tailings materials. Results show that *Chlorella* (Trebouxiophyceae), *Chlorococcum* (Chlorophyceae) and *Klebsormidium* (Klebsormidiophyceae) species were present at all the rehabilitated sampling sites. The dominant genera on these sites were *Chlamydomonas* (Chlorophyceae), *Chlorococcum*, *Klebsormidium* and *Phormidium* (Phormidiaceae). No cyanobacterial or algal growth was found on freshly deposited tailings material and only a few species such as *Chlamydomonas* sp., *Chlorella ellipsoidea* Gerneck, *Chlorococcum* sp., *Microcoleus vaginatus* (Phormidiaceae) and a *Phormidium* species were found on un-rehabilitated material. It was evident that the time of rehabilitation did not have an influence on the algal as well as cyanobacterial species present in the tailings material and that the presence of a grass cover on the rehabilitated sites may have provided a microclimate enhancing the growth of these organisms.

Key words: cyanobacteria, terrestrial algae, biological crusts, algal and cyanobacterial diversity.

Introduction

Gold mine tailings storage facilities are characterized by extreme geotechnical and geochemical conditions which severely inhibits the growth and establishment of higher plants. The absence of higher plants such as grasses and trees leaves the soil surface exposed to extreme temperatures and solar radiation levels and poses a significant challenge for rehabilitation practitioners. Revegetation of tailings facilities has been considered the most effective means of reducing water and wind-borne erosion as well as protection for the surrounding environment. Most of these methods have proven

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ecologically and economically unsustainable (Straker et al. 2007) as a result of the inhospitable landscape and soil environment. Although the use of microbial organisms has been largely ignored in restoration practices (Bowker 2007) these communities can be linked to plant establishment and plant-microbe interactions which are important for promoting nutrient cycling, soil aggregation and plant nutrient uptake (Mendez & Maier 2008, Mummey et al. 2002). As far back as 1941 Booth investigated the use of algae for erosion control and concluded that algal resistance to erosion is the result of binding surface particles of soil into a non-erodible layer which also is very effective in breaking the force of falling water. Authors such as Langhans et al. (2009) and Harper & Belnap (2001) reported that certain organisms such as cyanobacteria, eukaryotic algae and mosses are able to survive harsh conditions, such as low soil nutrient and soil moisture levels. These organisms form a dense matrix that glues soil particles together adding to the stability of the soil and counteracting the erosive forces of wind and water (Belnap et al. 2001, Rosentreter et al. 2007, Langhans et al. 2009, Bowker 2007, Campbell et al. 1989); play a vital role in the fixation of atmospheric nitrogen and carbon sequestration; retain soil moisture and contribute to soil organic matter (Metting 1981, Issa et al. 2007, Thomas et al. 2006, Belnap et al. 2001, Harper & Belnap 2001, Rosentreter et al. 2007, Bowker 2007, Campbell et al. 1989). The most common species found in these biological crusts include cyanobacterial genera such as *Microcoleus*, *Nostoc*, *Scytonema* and *Calothrix* but also green algae such as *Chlorella*, *Chlorococcum*, *Coccomyxa* and *Klebsormidium* (Langhans et al. 2009).

According to Hoffmann (1989) the most important environmental factors that control algal populations in soil seem to be light intensity, humidity, temperature, availability of nutrients and pH. Shubert & Stark (1979) found a positive correlation between the abundance of algae with levels of nitrogen, phosphate, silicon, manganese, aluminum and lead, while sodium, copper, lithium, molybdenum and strontium had negative correlations. However, algal growth varies from species to species in their requirements or tolerance of most elements (Stark & Shubert 1982). Lukesova (2001) found that green algae is typical for acid to slightly alkaline post mining areas while diatoms and cyanobacteria prefer neutral to alkaline as well as saline and calcium rich habitats. Cyanobacteria do not occur in soils with pH below 4.0 (Lukesova 2001).

Several researchers claimed that cryptogamic soil crusts are critically important components of arid and semiarid lands in which they occur (Johansen et al. 1993). Some evidence indicates the crusts may increase vascular seedling establishment (St. Clair et al. 1984) and reduce soil erosion (Bowker 2007), factors that can be helpful when rehabilitating mine tailings facilities. The aim of this study therefore was to investigate the occurrence of cyanobacteria and other algae on both un-rehabilitated and rehabilitated gold mine tailings by studying the inoculum present in the air around the sampling sites as well as identifying the algal and cyanobacterial species in the tailings material.

Materials and Methods

STUDY AREA AND RESEARCH SITES: The study area is located near Stilfontein (26.85°S, 26.78°E) in the Northwest Province of South Africa. This area is characterized by an average rainfall of 610 mm per

year and midday temperatures as low as 18°C in June and as warm as 29.1°C in January (http://www.saexplorer.co.za/southafrica/climate/stilfontein_climate.asp, Low & Rebelo 1996). The year 2010 was exceptionally wet, with approximately 900 mm of rain. The study sites are mostly redundant gold tailings storage facilities (TSF) in the Stilfontein area, namely Buffelsfontein, Stilfontein and Hartebeesfontein TSF and are all within a 15 km radius. The tailings material of the different sampling localities was mined from the same geological unit, the Witwatersrand basin quartzite. The rehabilitated tailings materials are all of the same age although it has been rehabilitated at different time intervals over the past 15 years. The same rehabilitation methods that is liming, leaching and the application of equal amounts of ameliorants were applied to all the rehabilitated sites by the same contractor.

The Buffelsfontein TSF was rehabilitated 1 year ago and grass species such as *Chloris gayana* Kunth, *Cynodon dactylon* (L.) Pers., *Dactyloctenium aegyptum* (L.) Willd, *Eragrostis tef* (Zucc.) Trotter and *Eragrostis curvula* (Schard.) Nees were established. The Stilfontein TSF and Hartebeesfontein TSF were rehabilitated 10 and 15 years ago respectively and grass species such as *Chloris gayana* Kunth, *Cynodon dactylon* (L.) Pers., *Hyparrhenia hirta* (L.) Stapf and *Eragrostis curvula* (Schard.) Nees dominated. Three other sites at Buffelsfontein TSF were also sampled: freshly dumped tailings material; a 15 years old site where no rehabilitation has been done and an undisturbed site near the TSF.

Sampling procedures: Six main representative sites on the western slopes at each of the sampling localities were selected. At each of these six sites, composite soil samples were taken which consisted of between 10 and 15 random samples. These samples were taken using a teaspoon, gently removing soil approximately 1 cm deep from the most representative areas of about 10–50 cm^2 in area. Samples were stored in sterilized envelopes. Three composite soil samples were taken 10 cm deep at each of the study sites.

Cultivation and identification of algae: Standard plastic Petri dishes with 1.5% agar, enriched with Bold Basal Medium (BBM) (Stein 1973), were used for collection of the algal spores to determine the inoculum present in the air around the study sites. Six Petri dishes were randomly placed at each of the sampling localities and left open for exactly one hour, after which the Petri dishes were sealed off and incubated at 20°C and a light intensity of 35 $\mu mol \cdot m^{-2}s^{-1}$ until visible colonies were formed. Seven days after incubation algal colonies were counted and identified microscopically. The sampled tailings material (10–20 mg) was incubated on 1.5% agar plates enriched with BBM at 20°C with continuous light at a light intensity of 35 $\mu mol \cdot m^{-2}s^{-1}$. After approximately one week colonies of the different algal species started to grow and were identified microscopically. To identify the dominant species sampled tailings material was put into a Petri dish and wetted with distilled water. After 24 hours, three sterile cover glasses per Petri dish were placed on the tailings material and incubated at 20°C with light intensity of 35 $\mu mol \cdot m^{-2}s^{-1}$. After approximately two weeks the cover glasses were studied microscopically to identify the species present.

Soil analyses: The soil analysis was done, by Eco Analytica, in accordance with the standards, as set out by the Agricultural Laboratory Association of Southern Africa and the International Soil Analytical Exchange (ISE), Wageningen, The Netherlands, control schemes (http://www.agrilasa.co.za/AgriLASACertifiedLaboratories). The heavy metals in the soil were determined with Environmental Protection Agency (EPA) method 3050b (http://www.epa.gov/osw/hazard/testmethods).

Data analyses: The different species on the sites, the nutrient status of each site, the exchangeable ions as well as the heavy metals on each site was compared by using the Kruskal-Wallis ANOVA (non-parametric data) for comparing multiple independent samples to determine differences between the sampling sites. The p-values were given in Tables 2–6. Similarities between the species composition of the different sites were analyzed by using the Bray-Curtis dissimilarity index (Hahs & McDonnell 2006). This was done with the software program Primer 5 (Clarke & Gorley 2001). Canonical Correspondence Analysis (CCA) was done with Canoco 4.51. In the CCA the algal and cyanobacterial families instead of the species were used to decrease the amount of variables. However, due to an extensive range of variables measured, unequal replications and many similarities between species diversity of the sites, the Eigenvalues were very low.

Results

Diaspores from 35 cyanobacterial and algal species (see Table 1) were found in the air surrounding the tailing storage facilities. Three genera and 5 species belong to Cyanophyta (Cyanobacteria), 16 genera and 27 species belong to Chlorophyta (green algae), 3 genera and 3 species to Xanthophyceae (yellow-green algae) and 1 species to Eustigmatophyceae. The most common species, which occurred on all the sampling localities, were *Klebsormidium dissectum* (F.Gay) Ettl et. G.Gärter (Chlorophyceae), *Chlorococcum* sp. and *Interfilum* sp. Many of the species present are typical soil algal species, such as *Chlorosarcinopsis minor* (Gerneck) Herndon (Chlorophyceae), *Tetracystis aggregata* R.M.Brown et H.Bold (Chlorophyceae), and *Bracteacoccus minor* (Chod.) Petrova (Chlorophyceae).

The highest density of diaspores were identified on the sampling locality that was rehabilitated 10 years ago, with an average of 39 383 diaspores per 1 m^{-2} per hour. The sampling locality that had been rehabilitated one year ago had an average density of 33 979 diaspores per 1 m^2; the sampling locality that had been rehabilitated 15 years ago an average of 2296 diaspores per 1 m^2 and the sampling locality with fresh material that had not undergone any rehabilitation intervention had an average of 471 diaspores per 1 m^2 per hour.

A total of 40 algal and cyanobacterial species were identified in the tailings material using the agar plate and cover glass methods (see Table 2). Five species were identified at the 15 year old un-rehabilitated site; 25 species at the site that had been rehabilitated the year before; 30 species at the site that had been rehabilitated 10 years ago; 28 species at the site that had been rehabilitated 15 years ago and 34 species at the undisturbed site. No algal or cyanobacterial growth had been found on the freshly dumped tailings material. The information in Fig. 1 shows the different algal classes on the different sites.

The results from the statistical analysis showed no significant difference between the species present at the different rehabilitated sites (p-values are included in Table 2; $p>0.05$). The Bray-Curtis dissimilarity index (Hahs & McDonell 2006) grouped the sites that were rehabilitated 10 and 15 years ago together with the natural undisturbed site; with the site that was rehabilitated the previous year close by (Fig. 2). The further the distance between the sites in the ordination space the higher the degree of dissimilarity between the sites (Kent & Coker 1992). It can therefore be concluded that the time laps after rehabilitation did not influence algal or cyanobacteria diversity as there is no significant difference between the rehabilitated sites. According to Clark (1993) all ordination methods are a compromise because high-dimensional data are being viewed in a two dimensional plot. The stress value can be used to assess the success of an ordination. A stress value smaller than 0.05 corresponds to a good ordination with no prospect of misinterpretation (Clark 1993).

Tables 3, 4, 5 and 6 present the results of the soil analysis of the different sampling sites. The p-values, included in the tables, show that there is no significant difference between the tailings materials from the different rehabilitated sites except for the calcium content of the tailings material ($p = 0.02$).

Table 1: Species present in the air surrounding the storage facilities. Legend: 0=not present; 1=present

Species	Fresh	1y rehab	10y rehab	15y rehab
Anabaena sp.	1	1	0	0
Botrydiopsis arhiza Borzi	0	1	0	0
Botrydium granulatum Greville	0	1	1	0
Bracteacoccus minor (Chodat) Petrová	1	0	0	0
Chlamydomonas sp.	1	1	0	0
Chlorella ellipsoidea Gerneck	0	1	0	0
Chlorella minitissima Fott et Nováková	1	1	1	0
Chlorella sp.	0	1	1	1
Chlorococcum sp.	1	1	1	1
Chlorococcum vacuolatum Starr	0	1	0	0
Chlorosarcinopsis minor Herndon	1	1	1	0
Desmococcus olivaceus (Pers. ex Ach.) Laundon	0	0	1	1
Ellipsoidion perminimum Pascher	0	0	1	1
Eustigmatos magnus (B.Petersen) Hibberd	0	0	1	0
Interfilum sp.	1	1	1	1
Klebsormidium crenulatum (Kützing) Ettl et Gärtner	0	1	1	1
Klebsormidium dissectum (Gay) Ettl et Gärtner	1	1	1	1
Klebsormidium flaccidum (Kützing) Silva et al.	0	1	1	1
Klebsormidium pseudostichococcus (Heering) Péterfi L. et al.	0	0	1	0
Klebsormidium sp.	0	1	0	0
Leptosira erumpens (Deason et Bold) Lukešová	0	0	1	0
Leptosira sp.	0	0	1	0
Leptosira terrestris (Fritsch et John) Printz	1	1	0	0
Macrochloris sp.	0	0	1	0
Myrmecia biatorellae (Tschermak-Woess et Plesl) B. Petersen	0	0	0	1
Nostoc linckia (Roth) Bornet et Flahault	0	1	0	0
Nostoc punctiforme (Kützing) Hariot	0	1	0	0
Nostoc sp.	0	1	0	0
Palmellopsis gelatinosa Korschikov	1	1	1	0
Phormidium sp.	1	1	0	0
Radiosphaera minuta Herndon	0	1	0	0
Stichococcus chodatii (Bialosuknia) Heering	0	0	0	1
Stichococcus minor Nägeli	0	0	0	1
Tetracystis aggregata Brown et Bold	0	1	0	0

Table 2: Species identified at each site using agar plates and the cover glass method. Legend: 0=not present; 1=present; 2=dominant

Species	p-values	Undis-turbed site	15 y no rehab	Fresh	1 y rehab	10 y rehab	15 y rehab
Apodochloris polymorpha (Bischoff et Bold) Komárek		1	0	0	0	0	1
Botrydium granulatum Greville	0.23	0	0	0	1	1	0
Bracteacoccus minor (Chodat) Petrová	0.71	1	0	0	1	1	1
Characium sp.	0.24	1	0	0	2	0	0
Chlamydomonas sp.	0.17	2	2	0	2	2	2
Chlorella ellipsoidea Gerneck		1	1	0	0	0	1
Chlorella minutissima Fott et Nováková	0.71	1	0	0	1	1	1
Chlorella sp.	0.74	1	0	0	2	1	1
Chlorella vulgaris Beijerinck	0.74	1	0	0	2	1	1
Chlorococcum sp.	0.96	1	1	0	2	2	2
Chlorosarcina sp.		0	0	0	0	0	2
Chlorosarcinopsis minor Herndon		0	0	0	1	1	1
Chlorosarcinopsis sp.	0.48	1	0	0	1	1	1
Cylindrocystis brebissonii Meneghini	0.39	1	0	0	1	0	0
Desmococcus sp.	0.48	0	0	0	1	1	0
Ellipsoidion perminimum Pascher		1	0	0	0	1	0
Eustigmatos magnus (B.Petersen) Hibberd	0.71	1	0	0	1	1	1
Hantzschia amphyoxis (Ehrenberg) Grunow in Cleve et Grunow	0.28	2	0	0	0	2	1
Interfilum sp.		1	0	0	0	1	1
Klebsormidium crenulatum (Kützing) Ettl et Gärtner		1	0	0	1	0	1
Klebsormidium dissectum (Gay) Ettl et Gärtner		1	0	0	1	1	1
Klebsormidium flaccidum (Kützing) Silva et al.	0.79	1	0	0	2	2	2
Klebsormidium sp.	0.86	0	0	0	1	1	2
Microcoleus vaginatus (Vaucher) Gomont	0.17	2	1	0	0	1	0
Navicula pellioulosa (Brebisson) Hilse		1	0	0	0	1	1
Nostoc linckia (Roth) Bornet et Flahault	0.71	2	0	0	2	2	2
Nostoc sp.	0.71	2	0	0	2	2	2

Palmellopsis gelatinosa Korschikov		1	0	0	1	0	1
Phormidium autumnale (Agardh) Gomont	0.48	2	0	0	0	2	0
Phormidium foveolarum Rabenhorst ex Gomont	0.39	2	0	0	1	1	1
Phormidium sp.	0.39	2	1	0	2	2	2
Pinnularia borealis Ehrenberg	0.22	1	0	0	0	1	2
Pleurastrum terristris (Bristol) John		1	0	0	1		
Scotiellopsis terrestris (Reisigl) Punčochářová et Kalina	0.96	1	0	0	0	1	0
Scytonema sp.	0.48	2	0	0	0	2	1
Stichococcos minor Nägeli		1	0	0	1	0	0
Synechocystis sp.	0.96	0	0	0	0	2	0
Tetracystis aggregata Brown et Bold	0.24	1	0	0	2	1	1

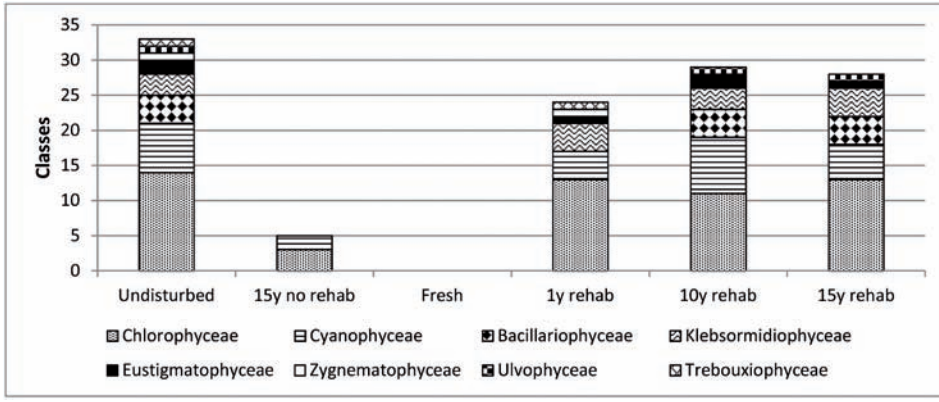

Fig. 1: Representation of the different algal and cyanobacterial classes at the different sampling sites.

The Canonical Correspondence Analysis (CCA) revealed a strong correlation between Merismopediaceae and the site that was rehabilitated 10 years ago (site 4; Fig. 3). Pleurastraceae, Palmellopsidaceae, Mesotaeniaceae and Prasiolaceae correlate with sodium and Characiaceae with magnesium. The site that was rehabilitated 1 year ago (site 2; Fig. 3) correlates strongly with potassium (K) and cation exchange capacity (CEC). Base saturation (Base sat) is the proportion of the cation exchange sites in the soil that are occupied by the various cations and the S-value is the total amount of exchangeable cations present. Fig. 3 shows that the calcium, pH (determined in potassium chloride and water), Base sat and S-value correlate with Pseudocharaciopsidaceae, Ulotrichaceae, Bacillariophyceae, Naviculaceae, Scytonemataceae, Oocystaceae and Pinnulariaceae.

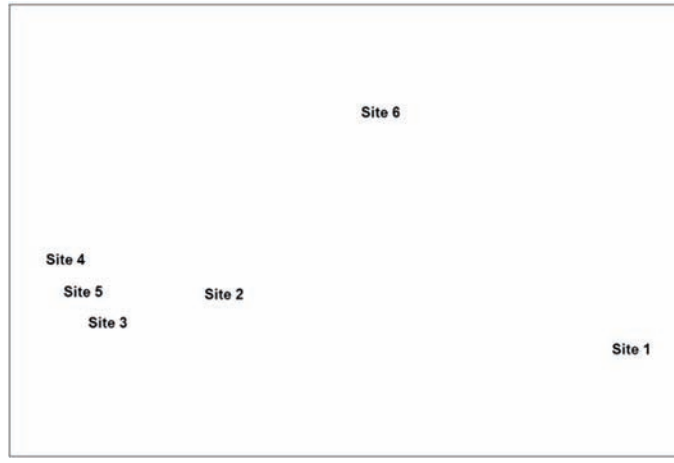

Fig. 2: The non-metric multidimensional scaling ordination diagram of sites similarities according to the Bray-Curtis dissimilarity index. Site 1 = freshly dumped material; 2 = rehabilitated 1 year ago; site 3 = undisturbed; site 4 = rehabilitated 10 years ago; site 5 = rehabilitated 15 years ago; site 6 = un-rehabilitated site 15 years old.

Table 3: Average nutrient status of the different sampling sites.

Sampling sites	Ca (mg/kg)	Mg (mg/kg)	K (mg/kg)	Na (mg/kg)	P (mg/kg)	pH (H_2O)	pH (KCl)	EC (mS/m)	LOI %C
p-values	0.02	0.66	0.73	0.15	0.29	0.27	0.17	0.71	0.72
Fresh material	3764.83	136.67	212.83	667.33	3.57	8.15	8.43	1177.33	0.01
1 Year rehabilitation	3895.83	137.67	48.50	80.67	197.30	5.73	5.55	296.33	1.68
Undisturbed area	487.17	94.67	225.67	57.50	9.94	5.97	4.82	44.00	0.88
10 Years rehabilitation	676.50	99.67	30.83	48.33	20.97	6.64	6.41	105.67	0.65
15 Years rehabilitation	674.67	67.50	55.50	30.17	10.70	6.51	6.38	125.00	0.64
15 Years, no rehabilitation	1969.00	455.67	6.00	65.00	27.10	3.37	3.29	1432.00	0.18

Discussion

The presence of high diaspore counts in the air above the rehabilitated sites (1 and 10 years) were expected as measurements were done at the end of a good rainy season. Near the 15 year old rehabilitated site was an unplanted agricultural field which could explain the significant lower diaspore count at the site. The diaspore count in the air surrounding the un-rehabilitated site was significantly lower than the rehabilitated

Table 4: Average exchangeable ions of the different sampling sites.

Sampling sites	Ca (cmol (+)kg)	Mg (cmol (+)kg)	K (cmol (+)kg)	Na (cmol (+)kg)	Cation Exchange Capacity	S-Value	Base saturation %	ESP
p-values	0.02	0.66	0.72	0.15	0.77	0.04	0.1	0.24
Fresh material	18.79	1.12	0.54	2.90	3.69	23.36	628.92	1.35
1 Year rehabilitation	19.44	1.13	0.13	0.35	7.14	21.05	333.99	20.24
Undisturbed site	2.43	0.78	0.58	0.25	7.45	4.04	54.60	
10 Years rehabilitation	3.38	0.82	0.08	0.21	5.38	4.49	81.74	26.99
15 Years rehabilitation	3.37	0.56	0.14	0.13	5.68	4.20	74.27	46.15
15 Years, no rehabilitation	9.38	3.75	0.02	0.28	4.14	13.87	336.87	16.44

Table 5: Some heavy metal concentrations (ppm) of the different sampling localities.

Sampling sites	Cr 53	Co 59	Ni 60	Cu 63	Zn 66	As 75	Pd 105	Hg 202	Pb 208	U 238	Mn 55
p-values	0.46	0.47	0.77	0.06	0.72	0.76	0.72	0.34	0.27	0.09	0.93
Fresh material	1.9	1.1	5	1.6	2.6	2.6	0.018	0.21	4.1	3.5	8.8
1 Year rehab	1.1	0.61	2.1	1.3	4.1	4.6	0.019	0.1	1.9	2.6	7.1
Undisturbed soil	2.8	0.89	4.9	1.4	3.5	0.89	0.0054	0.01	1.1	0.87	170
10 Years rehab	0.48	0.44	1.6	1.2	2	1.6	0.012	0.11	1.4	0.63	4.6
15 Years rehab	0.69	0.92	4.9	1	7	3.1	0.028	0.14	2.7	1.3	24
15 Years, no rehab	1.3	0.74	3.1	1.1	3.4	9.3	0.0079	0.014	1.6	2.3	3.4

sites emphasizing the importance of rehabilitation. However it was unexpected that there was no statistical difference between the tailings material from the different rehabilitated sites even though there was a time difference.

According to Haagner (2008) tailings storage facilities are often stacked at their natural angle of repose (ca. 35°) and with long slope lengths, decreasing their infiltration capacity and increasing run-off rate and hence susceptibility to erosion. The tailings material from all the sampling sites lacks a wide particle size distribution (Table 6) as well as organic material (% LOI in Table 3). The tailings materials are characterized by elevated metal concentrations such as arsenic, lead, mercury and uranium (Table 5) and high exchangeable sodium percentage (Table 4; ESP) values. ESP in the soil solution is of importance, as Na^+ has a high hydration potential and thus soils that are rich in sodium retain greater volumes of water, causing dispersion, making these soils more prone to erosion (MacVicar & De Villiers 1991). According to soil classification

Table 6: Particle size distribution (µm), of the different sampling sites.

Sampling sites	> 2%	Very coarse sand	Coarse sand	Medium-sand	Fine sand	Very fine sand	Silt	Clay
p-values		0.89	0.86	0.78	0.72	0.96	0.98	0.77
Fresh material	0.00	0.00	0.10	2.20	37.60	38.10	18.00	4.00
1 Year rehab	0.6	1.5	1.6	2.6	23.6	35.3	27.5	7.9
Undisturbed soil	4.4	8.3	9.8	15.7	21.3	20.7	20.0	4.1
10 Years rehab	0	0.5	1.2	6.8	41.9	31.1	13.6	4.9
15 Years rehab	1.6	2.7	2.9	4	24.8	30.7	29.6	5.4
15 Years, no rehab	0	0	0	0.3	13	48.7	26.3	9.6

(MacVicar & De Villiers 1991) the desired ESP value for South African top soils are below 10. Values above 10 (sodic soils) can have a toxic effect on plants, and can also create mineral nutrition problems, such as Ca^{2+} deficiencies (Sparks 2003). Fig. 3 shows that ESP plays a role in the site that was rehabilitated 15 years ago and can have an influence on the growth of Phormidaceae.

The fresh tailings material has a very high salt content as reflected in the electrical conductivity (EC; Table 3). Winegardner (1995) describes electrical conductivity as the total amount of salts that are present in the soil solution, influenced by the soil's water content, texture and proportion of soluble salts. The EC of the rest of the sampling localities is below the maximum of 400 $mS \cdot m^{-1}$ proposed for South African top soils (MacVicar & De Villiers 1991).

The cation exchange capacity (Table 4; CEC) as described by Winegardner (1995) and Sylvia et al. (2005) is the sum total of exchangeable cations that a soil can adsorb or hold. It determines the capacity of soil to retain ions, so that it is available for uptake by plants and not easily leached out from the soil profile (Sparks 2003). The CEC of the tailings material from all the sampling localities were below the minimum 8 $cmol \cdot kg^{-1}$ proposed for South African top soils (MacVicar & De Villiers 1991). Fig. 3 show that it plays a role in the rehabilitated site where there was a time lapse of 1 year.

Other physico-chemical factors such as extreme temperatures, low precipitation and high winds can impede the growth and establishment of organisms and higher plants (Mendez & Maier 2008). Soil pH also plays a vital role in the availability of nutrients, solubility of the elements, absorption capacity by plants as well as the occurrence of micro-organisms. The freshly dumped material was found to be non acidic (Table 3) but the oxidation of pyrite and other sulphideswhich frequents gold tailings, can lower the pH increasing the solubility of aluminum; manganese and iron and reduce the availability of most essential plant nutrients such as phosphorous and potassium (Haagner 2008). The pH of the rehabilitated sites and the undisturbed area ranged from 5.5 to 6.6, while the un-rehabilitated site had a pH of 3.3 (Table 3). This change is due to targeted rehabilitation methods, such as leaching through frequent irrigation, and liming applied to neutralize the acidic tailings material.

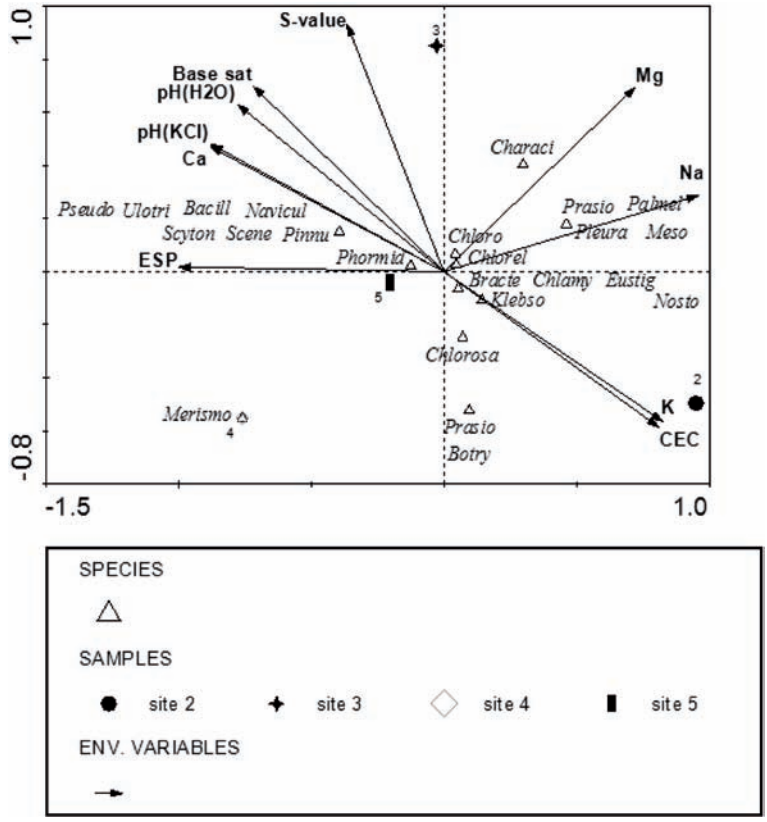

Fig. 3: CCA of the average exchangeable ions as well as algal and cyanobacterial families of the different sampling sites. Site 2 = rehabilitated 1 year ago; site 3 = undisturbed; site 4 = rehabilitated 10 years ago; site 5 = rehabilitated 15 years ago. Axis 1: eigenvalue 0.139, variance explained 64.6%; axis 2: eigenvalue 0.076, variance explained 35.4%. The environmental variables are explained in the text. Bacillariaceae (Bacill), Botriaceae (Botry), Bracteacoccaceae (Bracte), Characiaceae (Characi), Chlamydomonaceae (Chlamy), Chlorococcaceae (Chloro), Chlorosarcinaceae (Chlorosa), Eustigmataceae (Eustig), Klebsormidiaceae (Klebso), Merismopediaceae (Merismo), Mesotaeniaceae (Meso), Naviculaceae (Navicul), Nostocaceae (Nosto), Palmellopsidaceae (Palmel), Phormidiaceae (Phormid), Pinnulariaceae (Pinnu), Pleurastraceae (Pleura), Prasiolaceae (Prasio), Pseudocharaciopsidaceae (Pseudo), Scenedesmaceae (Scene), Scytonemataceae (Scyton), Ulotrichaceae (Ulotri).

The physical and chemical analysis of the tailings material confirmed that the sampled tailings material presents a harsh environment for organisms and higher plants to grow and spontaneously establish. If compared to the un-rehabilitated sites, it seems that the addition of lime, fertilizers and irrigation as well as the presence of higher plants did improve conditions for algal growth. Most algal crust formations in arid areas are initiated by the growth of cyanobacteria (Belnap & Gardner 1993) and are key factors in crust cohesion (Hu et al. 2002). It was therefore surprising to find that the Chlorophyceae was dominant and limited cyanobacterial species were present even in

young rehabilitated material (Fig. 1). According to Lukesova (2001) the more acidic nature of the tailings material (Table 3) could have favored green algae. This was however not confirmed by the CCA (Fig. 3). Another factor can be the use of Bold's Basal growth medium that did not encourage luxurious growth of fungi and bacteria (Brown et al. 1964) and could have contributed to the low cyanobacterial numbers.

Several studies (Hu et al. 2002 and Belnap & Gardner 1993) found that cyanobacteria such as *Microcoleus*, *Phormidium* and *Nostoc* found on the tailings material have the potential to lessen problems such as erosion and airborne dust on TSFs. Hu et al. (2002) found that a year old crust consisting of only algae and cyanobacteria such a *Microcoleus vaginatus* were strong enough to withstand a 25 $m \cdot s^{-1}$ sand storm for more than 8 h. This study also found that filamentous cyanobacteria such as *Microcoleus*, *Phormidium* and *Nostoc* species have a greater capacity to stabilize sand than single celled species (Hu et al. 2002). Belnap & Gardner (1993) found that even dead algae adhere and bind soil particles together, contributing to increased moisture and nutrient retention and gradually changing soil physico-chemical properties.

It can be concluded that algae as well as cyanobacteria are able to grow and colonize the tailings material despite the harsh conditions and can be vital in rehabilitating the TSF. Although there are high concentrations of inoculum present in the atmosphere surrounding the TSF the introduction of inoculum into the sites may speed up the formation of biological crusts. According to Bowker (2007) unassisted recovery of biological crusts can take from 6 years to centuries, but assisted recovery may reduce this time period to a scale more manageable in the context of a rehabilitation project.

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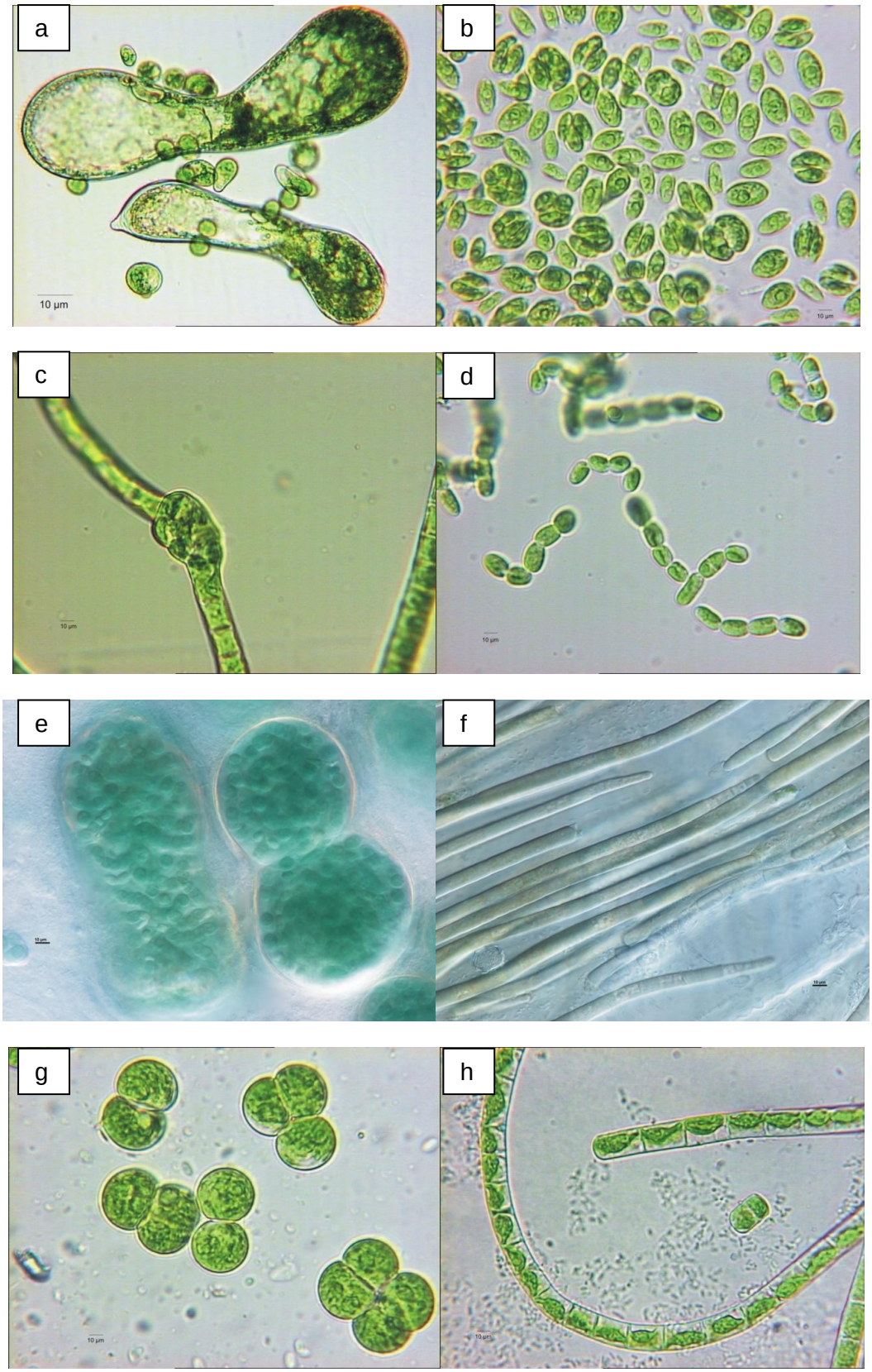


Figure 3.4: Illustration of some of the soil algal and cyanoprokaryote species identified in the gold tailings material. a) *Botrydium granulatum*, b) *Chlamydomonas* sp., c) *Klebsormidium crenulatum*, d) *Klebsormidium dissectum*, e) *Nostoc* sp., f) *Microcoleus vaginatus*, g) *Tetracystis aggregata,* h) *Klebsormidium flaccidum*.

Chapter 4

4.1 Introduction

The experimental work of this study started with the identification of soil algal and cyanoprokaryote species that occurred in the mine tailings material. The next step was to isolate some of the dominant algae and cyanoprokaryotes and then establish the best way to determine the biomass of the organisms and how to inoculate them onto the selected substrate.

4.2 Methods

4.2.1 Isolation of prevalent algae and cyanoprokaryote species

In Chapter 3 the identification of biologically important and dominant algal and cyanoprokaryote species are discussed. These included *Chlamydomonas*, *Chlorococcum*, *Klebsormidium*, *Nostoc* and *Phormidium* species.

Chlamydomonas and *Chlorococcum* species are unicellular, single celled organisms which form part of the Chlorophyta, more commonly known as the green algae group (Pickett-Heaps, 1975). *Chlorococcum* sp. is a common soil alga (Prescott, 1978, John *et al*., 2002). *Klebsormidium* species form part of the Charophyta group filamentous algae commonly found in freshwater as well as terrestrial habitats (Pickett-Heaps, 1975 and John *et al*., 2002). *Nostoc* and *Phormidium* species are classified under Cyanophyceae and occur in terrestrial as well as aquatic habitats (Fogg *et al*, 1973, Prescott, 1978 and John *et al*., 2002). *Phormidium* species are filamentous, blue-green algae and some of these species are known for living in extreme habitats such as desert soils forming biological soil crust mats (Fogg *et al*, 1973).

Microcoleus vaginatus was included in this study as it is a common soil cyanoprokaryote known as a pioneer during the establishment of soil flora (Fogg *et al*. 1973 and Zhang *et al*., 2009), commonly found on submerged substrates (Prescott 1978 and John et al., 2002). *M. vaginatus* is also often used in soil studies (Bowker, 2007 and Langhans *et al*., 2009). *Interfilum* species, a close relative of the *Klebsormidium* species (Pickett-Heaps, 1975), was also chosen as very little information is known about this species.

4.2.1.1 Methods

Sampled mine tailings were prepared as described in Chapter 3. The algae chosen for isolation were transferred from the agar or mine tailings on to 1% Bold's Basal medium agar slants in test tubes and incubated at 20°C with continuous light intensity of 35 $\mu molm^{-2}s^{-1}$. If a distinct colony appeared it was picked up and streaked out on a fresh agar slant. This was repeated until unialgal colonies were obtained (Shields and Durrell, 1964). Algal and cyanoprokaryote cells from a unialgal colony were respectively transferred to 100 ml liquid growth medium in an Erlenmeyer flask and incubated at a temperature of 20°C. Bold's Basal medium (Stein, 1973) commonly used for green algae, was the growth media used, as it is highly enriched (Andersen, 2005 and Langhans *et al*., 2009), and GBG11 growth medium (Krüger, 1978) for the cyanoprokaryote species (Andersen, 2005 and Langhans *et al*., 2009).

4.2.1.2 Results

Unialgal cultures of *Chlamydomonas*, *Chlorococcum*, *Klebsormidium*, *Nostoc*, *Phormidium,* and *Interfilum* species as well as *Microcoleus vaginatus* were obtained.

4.2.2 Determining the growth rate of the experimental organisms

The biomass production or growth rate of an organism is a measure of biomass increase over time. It is presented in the form of a growth curve consisting of a lag, exponential, stationary, and death phase (Institute of Terrestrial Ecology, 1982) as presented in Figure 4.1.

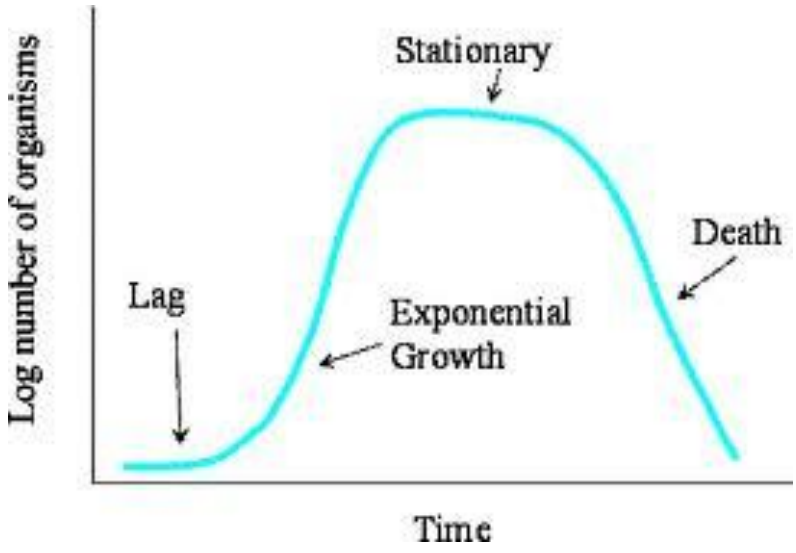


Figure 4.1: Illustration of a typical algal growth curve (http://www.ecs.umass.edu)

The lag growth phase is the phase were minimal growth occurs, as the growing culture is adapting to its new environment and medium (Institute of Terrestrial Ecology, 1982). During the exponential phase the organisms grow exponentially (Institute of Terrestrial Ecology, 1982).The stationary phase is entered when no net growth is recorded and the final phase or death phase is usually very rapid, therefore also known as 'culture crash' (Institute of Terrestrial Ecology, 1982). In the death phase some organisms will lose their pigmentation, while others will undergo lyses and still maintain its colour. Culture colour should therefore never be used as determining factor to identify the growth phase of the experimental organisms (www.marine.csiro.au).

The aim of the first experiment was to determine the best growth medium for the chosen species. The use of different media for different organisms is thus essential. It was decided to use GBG (Krüger, 1978) and BBM (Stein, 1973) media. GBG is a specialized medium, formulated specially for blue-green algae (Andersen, 2005). BBM is a more frequently used medium, mostly for green algae (Andersen, 2005).

4.2.2.1 Methods

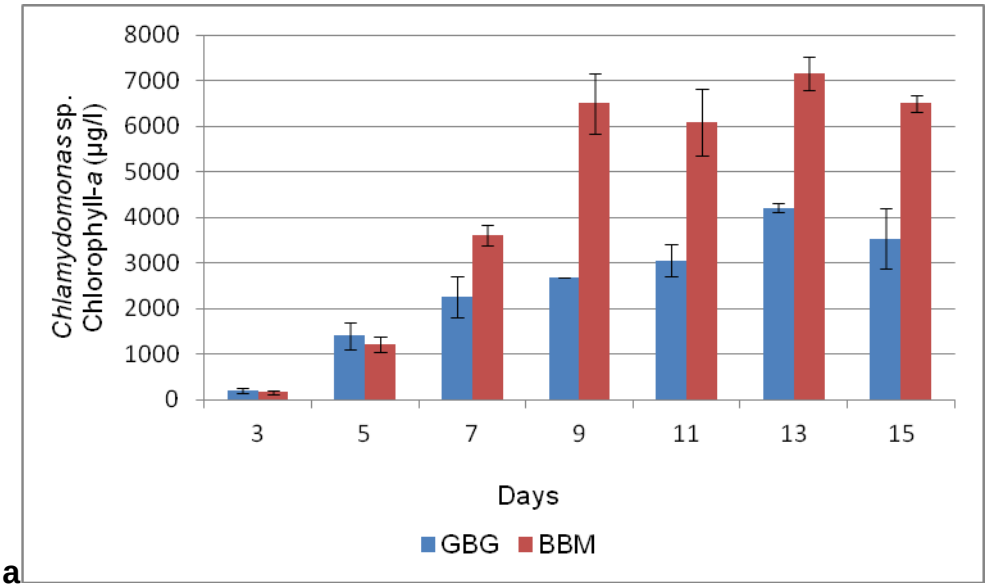
Two millilitre of the test organisms: *Chlamydomonas* sp., *Chlorococcum* sp., *Interfilum* sp., *Microcoleus vaginatus*, *Nostoc* sp. and *Phormidium* sp. was inoculated in 100 mℓ BBM as well as 100 mℓ GBG11 growth medium and incubated at a temperature of 20°C and a light intensity of 35 $\mu molm^{-2}s^{-1}$.

Chlorophyll-*a* concentration was determined with the method described by Sartory (1982) and Swanepoel *et al*. (2008). Three mℓ of the culture was filtered through a Whatman GF/C filter. The chlorophyll gathered on the filter was extracted with 10 mℓ 95% ethanol in a water bath at 78ºC for 5 minutes. The samples were removed and left in the dark to cool down. The difference in absorbance of the extract was determined at 665 and 750 nm respectively, using 95% ethanol as the blank. The difference in absorbance of the same sample was again determined 2 min after acidification with 0.1 mℓ 1N HCl. The chlorophyll-*a* concentration was calculated with the following equation: chlorophyll-*a* ($\mu g\ell^{-1}$) = [(A665-A750) – (A665a-A750a) x 28.66 x extract volume]/volume of sample, where: A665 = absorbency at 665 nm before acidification; A750 = absorbency at 750 nm before acidification; A665a = absorbency at 665nm after acidification; A750a = absorbency at 750nm after acidification; extract volume = 10 mℓ; volume of sample = 0.3 ℓ. Chlorophyll-*a* concentration measured every second to fifth day was used to compile growth curves of each of the experimental organisms.

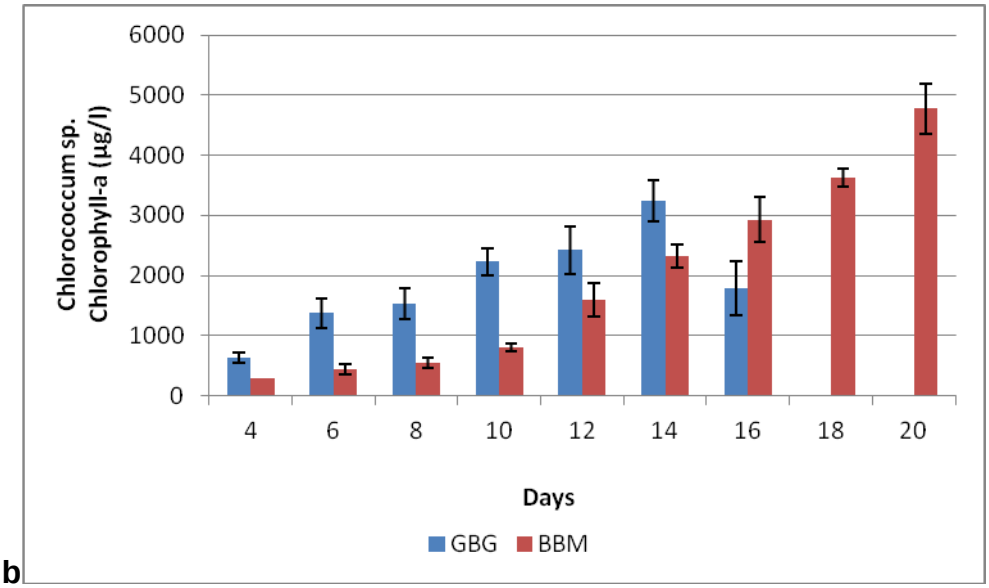
4.2.2.2 Results

The growth of each experimental organism in a liquid growth medium is shown in Figure 4.2.

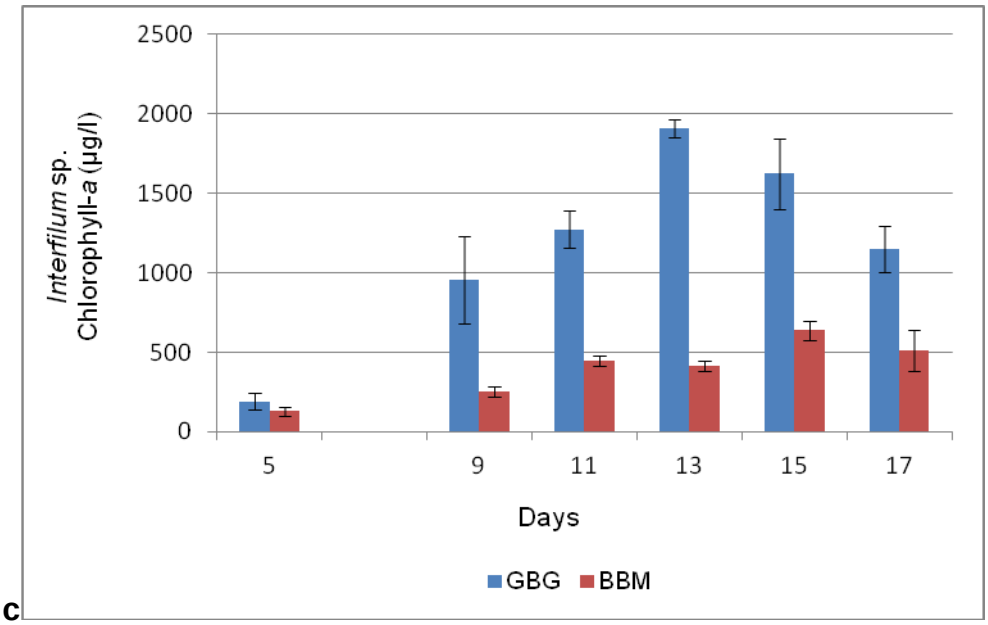
a



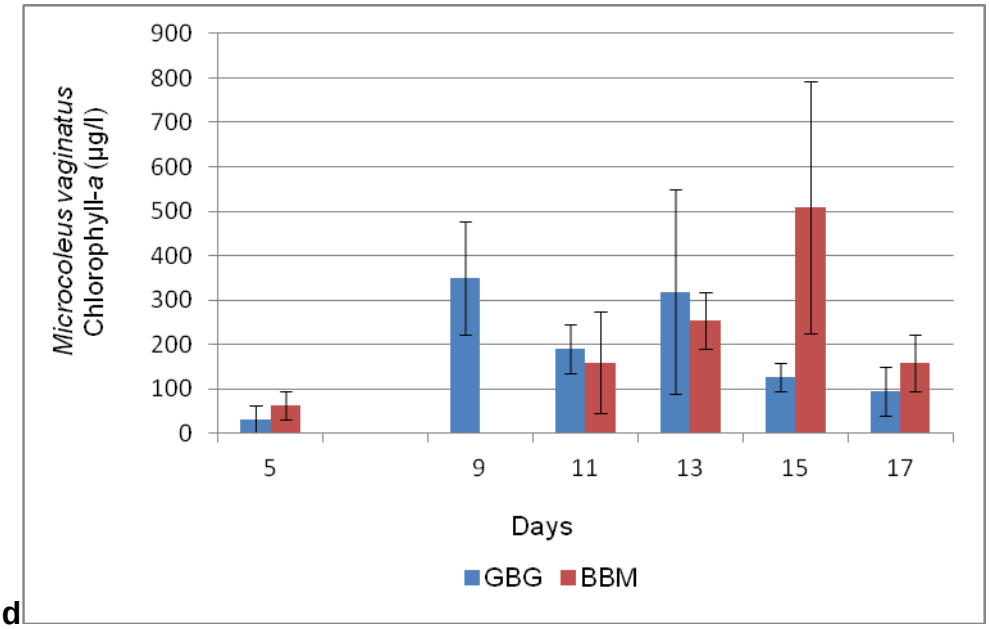
b



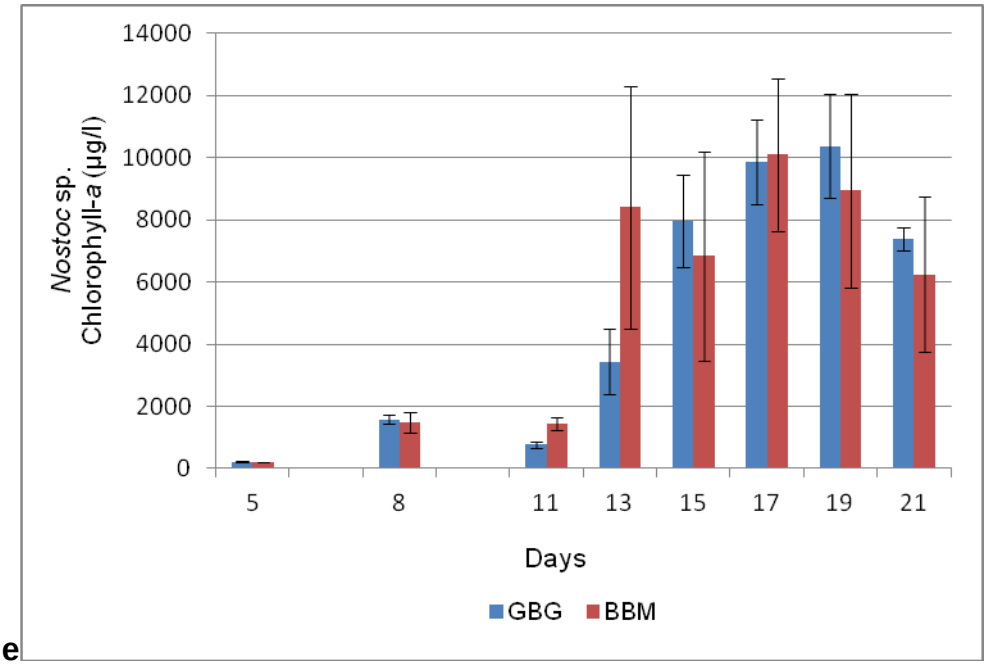
c


Interfilum sp.
Chlorophyll-a (µg/l)
2500
2000
1500
1000
500
0
5
9
11
13
15
17
Days
GBG
BBM

d


Microcoleus vaginatus
Chlorophyll-a (µg/l)
900
800
700
600
500
400
300
200
100
0
5
9
11
13
15
17
Days
GBG
BBM

e



f

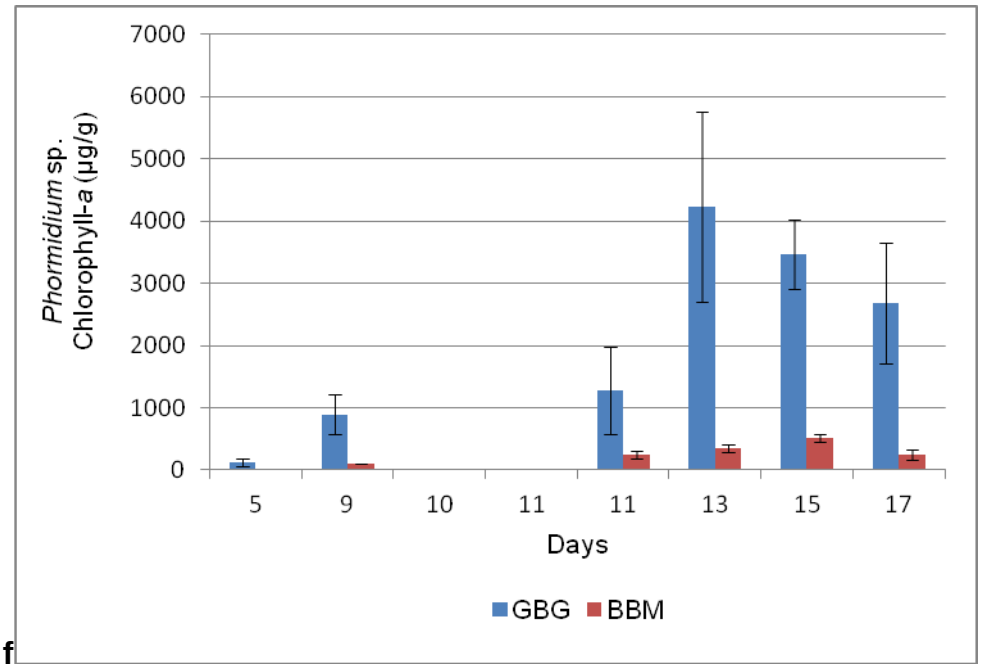


Figure 4.2: Growth of each of the experimental algal and cyanoprokaryote species, obtained by using the chlorophyll-*a* extraction method according to Sartory (1982) and Swanepoel *et al.* (2008) a= *Chlamydomonas* sp. b= *Chlorococcum* sp. c= *Interfilum* sp. d= *Microcoleus vaginatus* e= *Nostoc* sp. f= *Phormidium* sp.

4.2.2.3 Discussion

From the experimental growths curves given in Figure 4.3 the following tendencies could be seen; *Chlamydomonas* sp. showed the best growth in BBM growth medium, *Chlorococcum* sp. in BBM as well, *Interfilum* sp. in GBG, *Microcoleus vaginatus* in GBG, *Nostoc* sp. in GBG

and *Phormidium* sp. in GBG. These findings are confirmed by Andersen (2005) stating that BBM growth medium is generally used for green algae, while GBG11 growth medium is most commonly used for blue-green algae.

4.2.2.4 Conclusion

Chlamydomonas sp., a single cell organism, produced the highest biomass in the shortest period of time (Figure 4.2 (a)). It was therefore decided to use *Chlamydomonas* sp. in the rest of the protocol testing experiments as that would give the needed results in the shortest period of time. *Chlamydomonas* sp. is commonly used in growth studies as these organisms show quick adaptability and generation time (Pickett-Heaps, 1975).

4.2.3 Identifying the most efficient chlorophyll-*a* extraction method

Biomass produced by algae and other photosynthesizing organisms in the soil, is an indicator of the presence and degree of the biological crust (Castle *et al*., 2011). Various methods to measure biomass include cultural, direct, and indirect methods (Kabirov and Gaisina, 2009). When using cultural methods, the soil suspension, the soil suspension dilution, and inoculation in liquid and solid growth medium are used.

On the solid medium, every colony appearing is counted and the number of algae per 1 g of soil is also counted. The advantage of this method is that, while counting the algae, the species can be identified simultaneously (Kabirov and Gaisina, 2009).

With direct methods a weighed portion of soil is examined under a microscope. Direct methods are much more precise, as you do not only count the algae; you also measure the length of filaments, project the coverage of the algal cells and calculate the biomass and production rate of organic matter within the soil (Kabirov and Gaisina, 2009). However, these are very time consuming methods.

Indirect methods include the measurement of oxygen emission, carbon dioxide fixation and compounds such as chlorophyll and nitrogen present in the soil. The results give an indication of the algal development in the soil (Kabirov and Gaisina, 2009).

In a study by Tsujimura *et al*. (2000) the culture dilution and chlorophyll-*a* extraction methods were compared. With the culture dilution method an amount of soil is transferred distilled water, the sample is then inserted in a shaker, where after the sample is poured onto agar medium in a petri dish. The sample is then incubated for 18 to 22 days, until algal colonies

appear on the agar medium. The algal colonies are then classified and counted with the help of a stereomicroscope or an optical microscope (Tsujimura *et al*., 2000).

From the literature it is evident that the chlorophyll-*a* extraction technique proved to be the most successful, especially due to its ease of use. The pigment content and amount of algal biomass have a direct relationship (Henriques *et al*., 2007). Most studies (Nagarkar and Williams, 1997) use chlorophyll-*a* concentration to determine the algal biomass in the soil. It was decided to use chlorophyll-*a* as an indication of the algal biomass content of the soil as it is a fast and easy method.

Different authors used different solvents to extract chlorophyll-*a*. Lan *et al*. (2011) compared the use of ethanol, acetone, nitrate, N-dimethyl and dimethyl sulphoxide and found that ethanol showed the greatest efficiency and stability. Wasmund *et al.* (2006), in a similar experiment compared ethanol and acetone, in a similar experiment and also found ethanol to be more efficient. However, Hansson (1988) compared the use of methanol and acetone and found acetone the most efficient.

The aim of this experiment was therefore to find the best method to determine the growth rate of soil algae and cyanoprokaryotes. Three different chlorophyll-*a* extraction solvents and methods were compared (see paragraph 4.4.2): ethanol and a method adapted from Swanepoel *et al*. (2008); methanol and the method described by Castle (2010), and acetone with the method from Diana (2012).

4.2.3.1 Methods

Chlamydomonas sp. performed the best (see Figure 4.2 (a)) and was therefore chosen as the test organism to determine the best protocol to measure the biomass of the algae and cyanoprokaryotes in the soil. The trial was carried out in a temperature controlled glasshouse. The temperature in the glasshouse varied between 26°C during the day and 20°C at night. Light intensity changed throughout the day and ranged from 2 - 395 $\mu molm^{-2}s^{-1}$ (Figure 4.3). Rectangular trays (30 x 27.5 x 10 cm), with drainage holes at the bottom, were filled with Hutton soil (Macvicar and De Villiers, 1991) to a depth of 7.5-8 cm.

In total 15 trays were filled, 9 with unsterilized soil, and 6 with sterilized soil and inoculated with an 8 day old suspension of *Chlamydomonas* sp. with known chlorophyll-*a* concentration cultured in Bold's Basal medium over a period of 16 days at a light intensity of 35 $\mu mol^{-2}s^{-1}$.

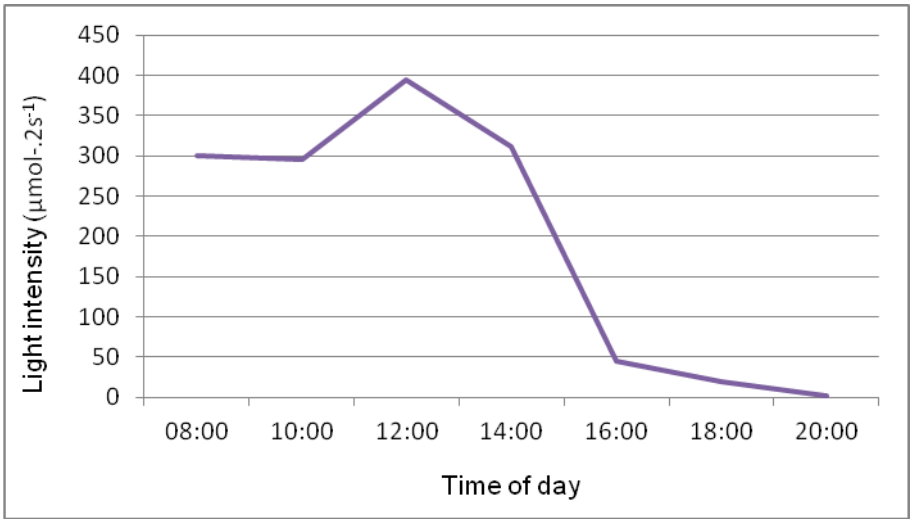


Figure 4.3: Graph to show the change in light intensity (µmolm^{-2}s^{-1}) in the glasshouse during the day.

The biomass was measured on days 3, 6, 9 and 16, using three different chlorophyll-*a* extraction solvents and methods accordingly; as follows:

Method 1: Ethanol with the method adapted from Swanepoel *et al*. (2008).

Three grams soil, collected with a teaspoon from the upper 2 cm of the soil surface (Belnap and Lange, 2003), were placed in a 15 mℓ screw-cap vial. Ten millilitre ethanol (95%) was added and placed in a shaker for 4 hours, after which the sample was filtered through a Whatman GF/C filter. The supernatant was then decanted to a clean vial, and analysed on the spectrophotometer at 665 nm and 750 nm. The extract was then acidified by adding 0.1 µℓ of 1 N HCl solution and analysed at 665 nm and 750 nm again (665c and 750c).

To calculate the chlorophyll-*a* concentrations the following equation was used:

$$\mu g.\ g^{-1}\ \text{Chl-}a = \frac{A * 28.6\mathbf{6} * s}{g}$$

Where:

A = (A665 – A750) – (A665*c* – A750*c*); *c* = absorbency after acidification with 0.1 µℓ, 1 N HCl

s = mℓ solvent used (10 mℓ was used)

g = gram of soil used (3 g was used)

Method 2: Methanol with the adapted method from Castle (2010).

Three grams soil, collected with a teaspoon from the upper 2 cm of the soil surface (Belnap and Lange, 2003), was placed in a 15 mℓ screw-cap vial. Nine millilitre methanol solution was added and placed on a shaker for 4 hours, after which the sample was put in the centrifuge for 6 minutes at 3000 rpm. The supernatant was then decanted to a clean vial and the sample analysed on the spectrophotometer at 652 nm, 665 nm and 750 nm. The values obtained through the 750 nm wavelengths were then subtracted from the values obtained through the 652 nm and 665 nm wavelengths respectively, and used in the equation.

To calculate the chlorophyll-*a* concentrations the following equation was used (Henriques *et al.,* 2007):

$$\mu g.g^{-1}\ \text{Chl-}a = \frac{(16.29 * A665) - (8.54 * A652) * s}{l * V}$$

Where:

s = volume of solvent used (mℓ)

l = the spectrophotometric cell length (cm)

V = the sample volume (g)

Method 3: Acetone with the method from Diana (2012).

Three grams soil, collected with a teaspoon from the upper 2 cm of the soil surface (Belnap and Lange, 2003), was placed in a 15 mℓ screw-cap vial. Ten millilitre 90% acetone was added and placed in a 5°C incubator for 24 hours. After the 24 hours the pH of the sample was adjusted to 9.0 and filtered through a Whatman GF/C filter. The supernatant was decanted into a clean vial and the sample analysed on the spectrophotometer at 664 nm, 647 nm and 630 nm.

To calculate the chlorophyll-*a* concentrations the following equation was used (Henriques *et al.,* 2007):

$$\mu g\ g^{-1}\ \text{Chl-}a\ \text{soil} = ([11.85(A664) - 1.54(A647) - 0.08(A630) * s)/lV$$

Where:

s = volume of solvent used (mℓ)

l = the spectrophotometric cell length (cm)

V = the sample volume (g)

4.2.3.2 Results

The growth of the different trials is illustrated in Figure 4.4 and 4.5.

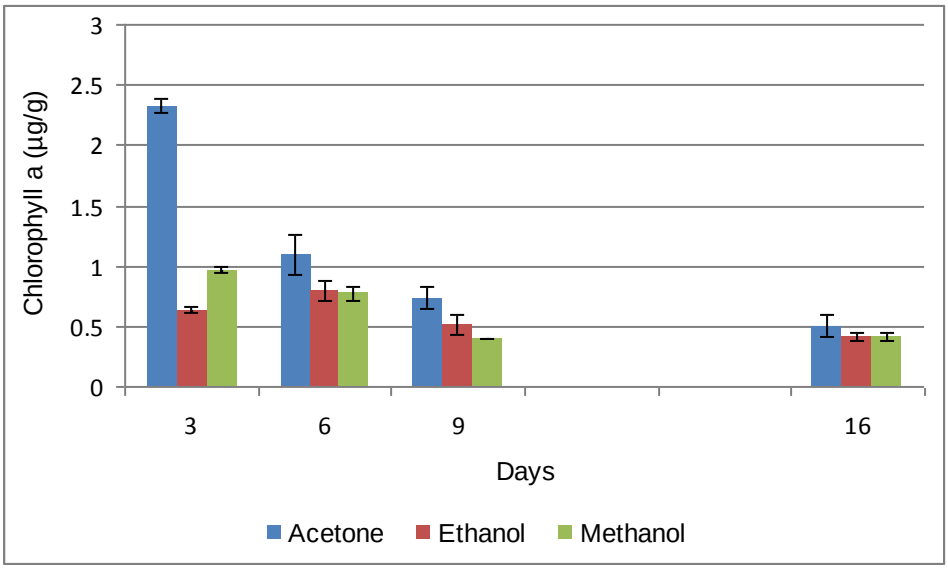


Figure 4.4: Representation of the growth of *Chlamydomonas* sp. in sterilized soil.

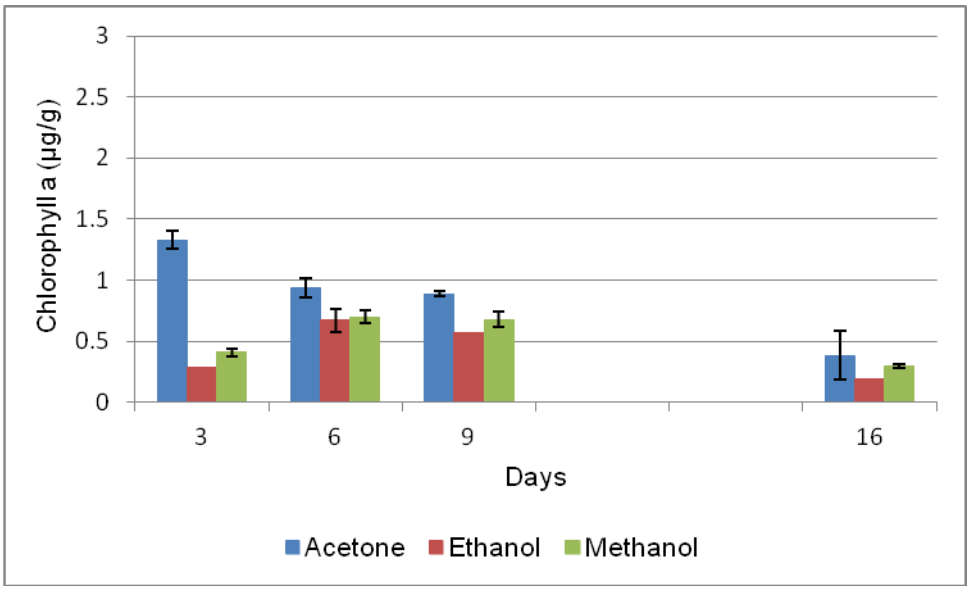


Figure 4.5: Representation of the growth of *Chlamydomonas* sp. in non-sterilized soil.

When comparing Figures 4.4 and 4.5 it is evident that the chlorophyll-*a* concentration declined rapidly in the sterilized soil, while ‘n more normal growth pattern was observed in the non-sterilized soil. This experiment was carried out in open trays and the sterilized soil could have produced a surface for opportunistic organisms such as bacteria and fungi to colonize it. In some cases the soil’s chemical and physical properties, such as the bioavailability of phosphorus, are altered during sterilization and exposure to high

temperatures (Sinegani and Sedri, 2011). The sterilization of the soil was also a very time consuming process and it was decided not to sterilize the soil or tailings material used in further studies.

Acetone proved to be the best solvent (Figures 4.4 and 4.5), but entails a time consuming process to produce a result and crystal cuvettes are needed to do the spectrophotometric measurements. Crystal cuvettes are more costly at R1, 967.99 per cuvette, where normal glass cuvettes are priced at R556.79 (http://www.sigmaaldrich.com). Therefore it was decided to use methanol as the solvent, but instead of centrifuging, the supernatant of the sample was filtered through a Whatman GF/C filter to remove debris. This decision was based on the fact that the supernatant still contains debris in suspension after centrifuging.

The chlorophyll-*a* extraction solvent and method used in further studies will thus be as follows:

Methanol method, adapted from Castle (2010).

Three grams soil, collected with a teaspoon from the upper 2 cm of the soil surface (Belnap and Lange, 2003), was placed in a 15 mℓ screw-cap vial. Nine millilitre methanol solution was added and placed on a shaker for 4 hours, after which the sample was filtered through a Whatman GF/C filter. The supernatant was then decanted to a clean vial and the sample analysed on the spectrophotometer at 652 nm and 665 nm and 750 nm. The values obtained through the 750 nm wavelengths were then subtracted from the values obtained through the 652 nm and 665 nm wavelengths respectively, and used in the equation.

To calculate the chlorophyll-*a* concentrations the following equation was used (Henriques et al., 2007):

$$\mu g.g^{-1}\ \text{Chl } a = \frac{(16.29 * A665) - (8.54 * A652) * s}{l * V}$$

Where:

s = volume of solvent used (mℓ)

l = the spectrophotometric cell length (cm)

V = the sample volume (g)

4.2.4 Identifying the most successful inoculation method

4.2.4.1 Methods

Three methods to inoculate the tailings material with the algae and cyanoprokaryotes were tested, namely: pour, spray, and slush.

The trials were done in a glasshouse, where the temperature ranged between 26°C during the day and 20°C at night. Rectangular trays (30 x 27.5 x 10 cm), with drainage holes at the bottom, were filled with Hutton soil (Macvicar and De Villiers, 1991) to a depth of 7.5-8 cm. Three replications of each of the three treatments were done (9 in total). Two hundred millilitre 8 day old *Chlamydomonas* culture was used for the treatments. The treatments were as follows:

Pour: Suspension was poured evenly over the soil surface.

Spray: Suspension was sprayed over the surface, using a 2 ℓ adjustable *Thema* spray bottle.

Slush: *Chlamydomonas* sp. was grown in a 500 ml flask with BBM with 1% agar that was poured evenly over the soil surface after 8 days.

Chlorophyll-*a* extractions were done on day 3, 6, 10, 20, 27, 34 and 41 using methanol as the extraction solvent (see paragraph 4.2.3.2)

4.2.4.2 Results

For future trials it was necessary to identify the most efficient way in which to inoculate the algae and cyanoprokaryotes into the soil. The results of the spray, pour and slush methods are shown in Figure 4.6. This experiment was done on non-sterilized Hutton type soil (Macvicar and De Villiers, 1991).

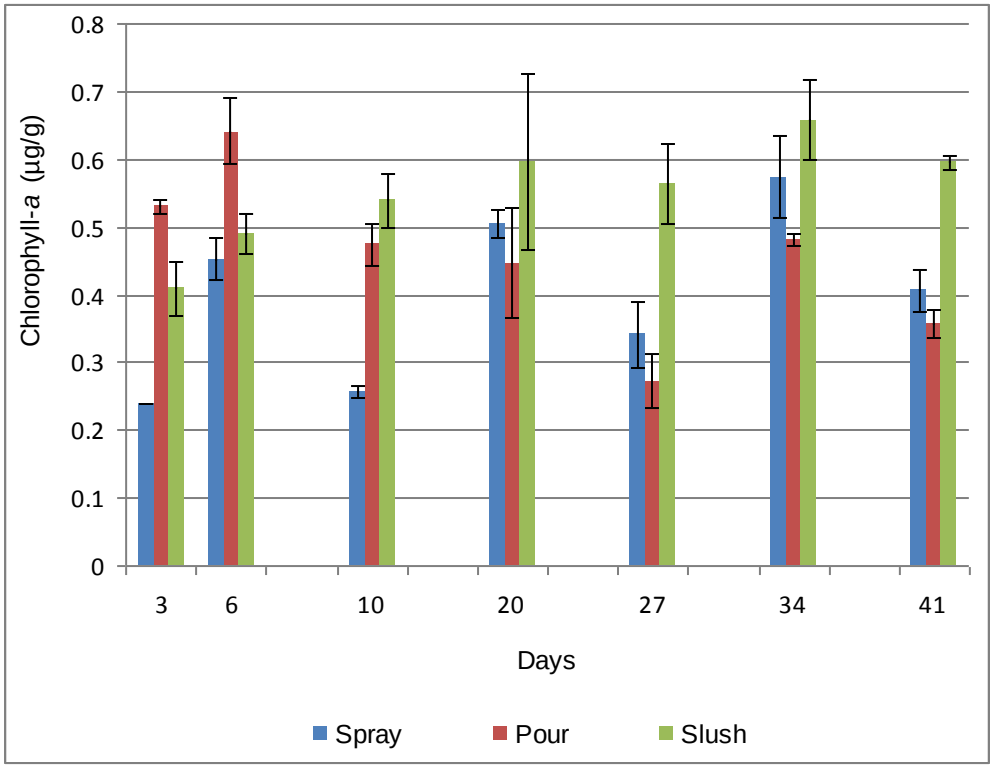


Figure 4.6: Representation of the growth of *Chlamydomonas* sp. when inoculated via the spray, pour and slush methods respectively.

According to the results in Figure 4.6 the slush method proved to be the most efficient way in which to inoculate algae and cyanoprokaryotes into the soil. On day 3 the chlorophyll-*a* concentration was 0.41 µg/g and the experiment ended on day 41 when the chlorophyll-*a* concentration of the slush method was 0.595 µg/g in comparison with 0.375 µg/g for the pour method and 0.406 µg/g for the spray method. Although the spray method delivered a more even coverage of the suspension, this method clearly had a negative effect on the organisms and caused erratic growth. The pour over method showed a high initial growth, but from day six, biomass declined. The slush method showed a continuous increase in biomass, most probably because the agar buffers the organism against the sudden harsh conditions of the soil habitat. The adaptation to the new soil environment is slower and less stressful. Therefore the slush method will be used in further studies for algalization.

4.3 Conclusion

Further experimentation will include the inoculation of the test organism onto the soil by means of the slush method and the chlorophyll-*a* concentrations of the organisms will be determined using the following method:

Adapted Methanol method, adapted from Castle (2010).

Three grams soil, collected with a teaspoon from the upper 2 cm of the soil surface (Belnap and Lange, 2003), will be placed in a 15 mℓ screw-cap vial. Nine millilitre methanol solution will be added and placed on a shaker for 4 hours, after which the sample will be filtered through a Whatman GF/C filter. The supernatant will then decanter to a clean vial and the sample will be analyzed on the spectrophotometer at 652 nm and 665 nm and 750 nm. The values obtained through the 750 nm wavelengths will then be subtracted from the values obtained through the 652 nm and 665 nm wavelengths respectively, and used in the equation.

To calculate the chlorophyll-*a* concentrations the following equation was used (Henriques *et al.,* 2007):

$$\mu g\ g^{-1}\ \text{Chl-}a\ \text{soil} = \frac{(16.29 * A665) - (8.54 * A652) * v}{l * V}$$

Where:

v = volume of solvent used (mℓ)

l = the spectrophotometric cell length (cm)

V = the sample volume (g)

4.4 References

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Chapter 5

5.1 Introduction

Gold mine tailings material are known to be very susceptible to the erosive forces of wind and water (Hattingh and van Deventer, 2004) due to the chemical and physical properties of the tailings material and the steep slope angles of the tailings dumps exacerbate the problem even more (MacVicar and De Villiers, 1991 and Haagner, 2008). In many studies it has been proven that biological soil crusts (BSC) are one of the soil covers, after plant cover, which provides the most protection for barren landscapes (Hu *et al*., 2002, Flechtner, 2007, Bowker, 2007, and Issa *et al*., 2007). The amount of protection provided by these BSCs however, is in direct correlation with the degree of the BSC development (Belnap, 2003).

Luxurious growth of soil algae and cyanoprokaryotes, as well as the amount of species present, correlates with the nutrients available in the soil (Shields and Durrell, 1964). These nutrients required for the growth of algae do not differ much from those required for plant growth (Shields and Durrell, 1964 and Fogg *et al.*, 1973) and include the elements N, P, S, K, Na, Mg, Ca, C, H, and O (Fogg *et al.*, 1973). In various studies (Mostert and Grobbelaar, 1987 and Fried *et al*., 2003) the specific influence of phosphate and nitrate on freshwater algal growth was investigated. Both of these studies found a positive correlation between elevated nitrogen and phosphate concentrations on algal growth.

The most suitable source of nitrogen is nitrate, which can be produced through nitrogen fixation by some of the soil algae and cyanoprokaryotes such as *Nostoc* sp. (Fogg et al, 1973, Belnap, 2001 and Nabors, 2004). Although phosphate is an essential element, high concentrations (exceeding 0.5%) may restrain algal growth (Metting, 1981). Cyanoprokaryotes are able to assimilate phosphorus, which they are can store in polyphosphate bodies and use when there is a phosphorus deficiency (Fogg *et al.*, 1973). Studies by Bergey (2008) showed that the addition of phosphorous significantly influences algal growth and the addition of nitrogen increased the chlorophyll-*a* content.

Magnesium is essential, as this is a component of the chlorophyll molecule. When inadequate concentrations of magnesium are present, growth is restrained (Fogg *et al*, 1973). Trace elements playing a role in algal growth include: Mn, B, Mo, Cu, Zn and Co. For nitrogen fixation, molybdenum in trace amounts is of importance (Fogg *et al*., 1973).

The aim of this study is to determine if *Chlamydomonas* sp., *Microcoleus vaginatus* as well as *Nostoc* sp. can be anthropogenically introduced to colonize gold mine tailings. As cost of

implementation is an important factor, the inputs to assist the establishment of these organisms were minimized. Therefore, treatments with only the addition of water as well as treatments with half the optimum phosphate and nitrate concentrations were tested.

5.2 Materials and Methods

The establishment of BSCs on gold mine tailings material could be a solution to the multiple problems associated with these materials, as discussed in Chapter 1. The application and establishments of BSCs should be as cost effective as possible due to the rehabilitation budgets the mining companies have to adhere to (van Wyk, 2013). In Table 5.1 a budget is given for the application of algal and cyanoprokaryote species cultured in BBM and in BBM with lower phosphate (8.1 g/ℓ) and nitrate (9.12 g/ℓ) concentrations respectively.

Table 5.1: Comparison of the costs associated with the establishment of soil algae and cyanoprokaryote species, cultured in BBM and BBM with 8.1 g/ℓ PO_4 and 9.12 g/ℓ NO_3 respectively.

Name	**R/100g**	**R/250g**	**R/500g**	**R/1ℓ**	**R/2.5ℓ**	**R/1g/1mℓ**	**R/BBM stock solution**	**1mℓ**	**BBM/ℓ**	**8.1g/ℓ PO_4**	**9.12g/ℓ NO_3**
BBM											
KH_2PO_4		227				0.91	7.96	0.015925	0.15925	0.08	
$CaCl2.2H_2O$			67			0.13	0.16	0.000325	0.00325		
$MgSO_4.7H_2O$			88			0.18	0.675	0.00135	0.0135		
NaNO3			56			0.11	1.38	0.003	0.03		0.02
K2HPO4		280				1.12	4.2	0.008	0.08	0.04	
NaCl			49			0.09	0.11	0.00022	0.0022		
$Na_2EDTA.2H_2O$			1111			2.222	22.22	0.02222	0.02222		
KOH			42			0.08	0.5	0.0005	0.0005		
$FeSO_4.7H2O$			356			0.71	3.54	0.00354	0.00354		
H_2SO_4					203	0.08/ml	0.08	0.00008	0.00008		
H3BO3			49			0.098	0.5635	0.001127	0.0007889		
Trace metal*				0.00669		0.00000669			0.00000669		
Agar			558			1.116			0.1116		
Total R/ℓ										BBM/ℓ	**0.43**
Total R/ℓ										BBM 8.1g/ℓ PO4	**0.35**
Total R/ℓ										BBM 9.12g/ℓ NO3	**0.41**

*The trace metal solution includes: H_3BO_3, $MnCl.4H_2O$, $ZnSO_4.7H_2O$, $CuSO_4.5H_2O$ and $Co(NO_3)_2.6H_2O$.

Currently 200 mℓ growth medium is used per 0.0899 m^2, thus 22246.94 ℓ will be used on one hectare. If BBM is used the cost will be R 9566.18 per hectare however if only 8.1 g/ℓ PO_4 BBM is used the cost decreases to R 7726.6 per hectare. If 9.12 g/ℓ NO_3 BBM is used the cost will be R9164.31 per hectare. It is thus evident that BBM with 8.1g/ℓ PO_4 will be a much cheaper option to use. The question is what impact the decrease in phosphate concentrations will have on the growth and establishment of algae and cyanoprokaryotes. Therefore the next step was to investigate the effect of the different phosphate and nitrate concentrations on the growth of the chosen algae and cyanoprokaryotes in a controlled area such as a glasshouse. It has to be noted that these costs are applicable for two applications of the organisms – initial application and three weeks later.

Some of the trials were also carried out without a cultured organism (control). That was done to determine if the inoculum in the atmosphere, as well as those present in the unsterilized tailings material, can be encouraged to establish without the added costs of culturing an organism and applying it to the surface. There is enough inoculum present in the atmosphere surrounding the tailings material (see Chapter 3).

5.2.1 Glasshouse trials

Rectangular trays (30 x 27.5 x 10 cm) were filled with gold tailings material from a gold mine in Stilfontein (26.48° South latitude, 26.47° East longitude). The trays were filled to a depth of 7.5-8 cm, water soaked, levelled and incubated in a glasshouse where the temperature varied between 26°C during the day and 20°C at night. The trays were watered from Monday to Saturday at 07:00, 12:00 and 17:00 for 3 minutes and at 24:00 for 1 minute by an automized sprinkler system.

Table 5.2: Soil analyses of the tailings material used in the glasshouse trials.

Soil analyses of gold mine tailings (<5% pyrite)	
pH(KCl)	5.3
pH(H_2O)	5.4
EC (msm^{-1})	193
SO_4 (mg kg^{-1})	1674
P (mg kg^{-1})	1
K (mg kg^{-1})	30
Ca(mg kg^{-1})	1793
Mg (mg kg^{-1})	94

Na (mg kg^{-1})	9
CEC ($cmol.kg^{-1}$)	1.3
Al ($cmol.kg^{-1}$)	0.04
ESP (%)	3.11
Al (%)	3.2

The Exchangeable Sodium Percentage (ESP) is the amount of sodium held in exchangeable form. These results are used to estimate the structural stability of the soil, as Na^+ ions are likely to cause dispersion of soil particles (Van de Graaff and Patterson, 2001). The Cation Exchange Capacity (CEC) is the relative measure of the soil's ability to retain nutrients (Winegardner, 1995). A high CEC value is thus desired, as the nutrients are then less likely to leach out (Hardy *et al*., 2012). The CEC is determined by adding the extractable calcium, magnesium, potassium and exchangeable acidity (Van de Graaff and Patterson, 2001 as well as Hardy *et al*., 2012). The CEC is measured in cmol(+)/kg (centimole per kg) and is revealed through dividing the concentration of a cation in units of milligrams per 100 g soil by its equivalent weight (Van de Graaff and Patterson, 2001), for example:

Exchangeable sodium percentage (ESP) = Conc. Na^+ x 100

Sum of conc. all cations

Or ESP (%) = Conc. Na^+ Equation 1

CEC

Where the units of concentration are in cmol (+) kg-1 (or meq/100 g) (Van de Graaff and Patterson, 2001)

Electrical Conductivity (EC) is the ability of a material to transmit an electrical current (Sparks, 2003, and Barbosa and Overstreet, 2013). Electrical conductivity in the soil is a measure of dissolved salts present and is measured in mS/m (milliSiemens per meter) (Aucamp, 2003 and Sparks, 2003).

The analyses reveal that the tailings have a low CEC, as the ideal CEC is 5-20 $cmolkg^{-1}$ (van Wyk, 2002). The low CEC is however normal for gold tailings. The high EC is typical for oxidised gold tailings (Table 5.2), and the ideal EC should be between 60 and 100 mSm^{-1} (van Wyk, 2002). The deficiency of plant nutrients is due to the low weathering potential of the primary minerals and also a lack of primary minerals which could weathered to nutrients i.e. potassium, magnesium and phosphorus (van Deventer, 2013).The possibility of dispersion is realities to be dealt with in this type of tailings material.

The following treatments were tested in the glasshouse:

1 Control; mine tailings that only received water.

2 Mine tailings treated with BBM medium with 0.1% agar added. The tailings again received 200 mℓ BBM medium after three weeks.

3 Mine tailings treated with BBM medium with 8.1 g/ℓ PO_4 and 0.1 % agar, which received the same treatment (200 mℓ) after three weeks.

4 Mine tailings treated with BBM medium with 9.12 g/ℓ NO_3 and 0.1% agar, which again received 200 mℓ 9.12 g/ℓ NO_3 BBM medium after three weeks

5 Mine tailings treated with *Chlamydomonas* sp. cultured in BBM medium with 0.1% agar. This treatment received BBM medium (200 mℓ) after three weeks.

6 Mine tailings treated with *Chlamydomonas* sp. cultured in BBM medium with 8.1 g/ℓ PO_4 and 0.1% agar. This treatment received (200 ml) BBM medium with 8.1g/ℓ PO_4 after three weeks.

7 Mine tailings treated with *Chlamydomonas* sp. cultured in BBM medium with 9.12 g/ℓ NO_3 and 0.1% agar, which received 200 mℓ 9.12 g/ℓ NO_3 BBM medium after three weeks.

Each of the above treatments was done in three replications for statistical purposes.

5.2.2 Species analysis

Before inoculation of the tailings material with the different treatments, a soil sample was taken to compile a species list of the algae and cyanoprokaryotes present in the tailings material. The methods used to determine the species present were described in Chapter 3 (Orlekowsky *et al*., 2013).

This was repeated six weeks after inoculation, when the different treatments were sampled to determine the biomass (as described in Chapter 4).

5.2.3 Penetration tests

Soil penetrometers are used to estimate the strength of the soil and in most cases the soil strength is directly related to soil compaction (Jones and Kunze, 2004). In ecological and rehabilitation studies, soil compaction influences root penetration, aeration of the soil as well as water infiltration (Jones and Kunze, 2004 and van Deventer, 2013). A soil penetrometer measures the force needed to break a soil crust to enter the soil surface (van Deventer, 2013). The unit in which penetration results are measured is kg/cm^2.

A hand operated penetrometer (Figure 5.1) was used in this experiment. The penetration tests were conducted before the tailings material was inoculated with the different treatments and approximately three weeks after the trial, on dry soil. Three penetration tests were done per replication of each treatment. The penetrometer is placed on the surface of the soil and pushed down into the soil. The small black rubber then pushes forward on the penetrometer until the crust is broken and the penetrometer enters the soil surface. The result can then be read off the penetrometer where the black rubber stopped. The higher the result (for example 2.5 kg/cm^2 vs. 0.5 kg/cm^2), the more strength is necessary to break the crust, thus the higher the soil resistance.

Figure 5.1: Illustration of the hand operated soil penetrometer (http://www.coleparmer.com).

5.2.4 Microscopy: SEM and light microscopy

For Scanning Electron Microscopy (SEM) biological soil crusts were removed with a scalped blade, six weeks after inoculation. One square centimetre crusts were lifted in 4% osmium vapour according to the method of Tiedt *et al.* (1987). Thereafter the samples were mounted on aluminium stubs with carbon tape and Leit C plus, sputter coated with gold-palladium and observed in a FEI Quanta 250 FEG SEM at 8 kV. To compare the crust formation and the colonization of different species the same experimental procedure was followed with *Microcoleus vaginatus* and *Nostoc* sp.

Biological crust thickness was measured for *Chlamydomonas* sp. by observing 1 cm cross sections of the biological soil crusts under a Leica Wild MZ8 Stereo microscope (see Figure 5.3).To compare the crust formation and the colonization of different species the same experimental procedure was followed with *Microcoleus vaginatus* and *Nostoc* sp.

5.2.5 Statistical analyses

Statistical analyses were conducted by the North-West University-Statistical Consulting Services. The Kolmogorov-Smirnov and Lilliefors tests for normality were used to determine if the data sets were distributed parametrically. The data did not meet the assumptions of normality in the distribution of all variables; therefore the Kruskal-Wallis ANOVA was used (non-parametric data) for comparing multiple independent samples to determine differences between the sampling sites and treatments.

5.3 Results and discussion

5.3.1 Glasshouse trails

5.3.1.1 Different treatments with *Chlamydomonas* species.

Figure 5.2 shows that the tailings treated with *Chlamydomonas* sp. cultured in BBM agar slush produced the highest chlorophyll-*a* concentration (6.13 µg/g) that differs significantly ($p<0.05$) from the other treatments. The presence of *Chlamydomonas* has a significant impact on biomass production when comparing BBM with agar (0.3 µg/g) to BBM with agar and *Chlamydomonas* (6.13 µg/g). The biomass measured in the treatments with the lower phosphate and nitrate concentrations did not differ significantly and it seems that the presence of the organism in the inoculum did not make a difference in this case.

The control that received only water did not grow well at all, however it was the most diverse in terms of species composition (Table 5.3) and one can only speculate that given time a biological crust may eventually develop with this treatment.

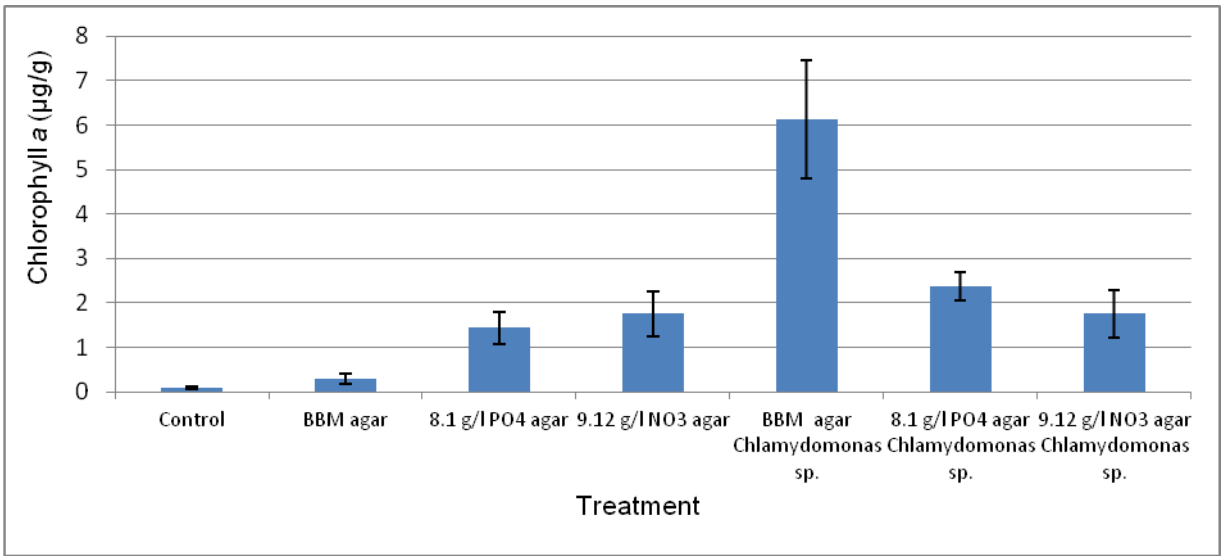

Figure 5.2: Representation of the influence of various treatments on the biomass production of *Chlamydomonas* sp. on tailings material after a trial period of six weeks.

The following species were identified in each treatment:

Table 5.3: Presence of algal and cyanoprokaryote species present in the tailings material before inoculation with *Chlamydomonas* sp. and six weeks after inoculation.

Species	Before inoculation	Control	BBM	8.1 g/ℓ PO_4	9.12 g/ℓ NO_3	BBM + *Chlamydomonas* sp.	8.1g/ℓ PO_4 + *Chlamydomonas* sp.	9.12 g/ℓ NO_3 + *Chlamydomonas* sp.
Chlamydomonas sp. 1		✓		✓	✓	✓	✓	✓
Chlamydomonas sp. 2		✓						
Chlorococcum sp.	✓	✓		✓				
Chlorosarcinopsis sp.					✓			
Calothrix sp.		✓						
Klebsormidium sp.			✓					
Lyngbya sp.								✓
*Navicula pelliculosa (*Brebisson) Hilse								✓
Nostoc sp. 1	✓		✓	✓	✓	✓	✓	✓
Nostoc sp. 2			✓					
Phormidium autumnale (Agardh) Gomont		✓			✓			
Phormidium sp. 1	✓	✓	✓	✓	✓	✓	✓	
Phormidium sp. 2	✓	✓	✓	✓	✓	✓		✓
Scytonema sp.	✓	✓						
Tetracystis aggregata Brown et Bold				✓			✓	
Total number of species	**5**	**8**	**5**	**6**	**6**	**4**	**4**	**5**

Before inoculation five species were identified. After the treatment with *Chlamydomonas* sp. cultured in BBM slush, four species were identified. *Chlamydomonas* sp. was still present but *Chlorococcum* sp. and *Scytonema* sp. were not detected after the treatment. High *Chlamydomonas* numbers from the inoculation could have outcompeted *Chlorococcum* sp. and *Scytonema* sp. *Nostoc* and *Phormidium* species were present before and after the treatment trial, this might be due to the presence of especially cyanoprokaryotes in every successional stage of BSCs (Rahmonov and Piatek, 2007; see Figure 5.2 and Table 5.3). Soil algae have the ability to withstand periods of drought remaining dormant in the soil and then regenerate when experiencing moisture conditions (Fogg *et al*., 1973). *Chlorosarcinopsis*, *Phormidium*, *Calothrix, Klebsormidium, Lyngbya, Navicula* and *Teracystis* species were absent when the species list of the tailings material was compiled before the experiment. These organisms could have been dormant in the soil or present in the atmosphere. According to Zancan *et al*. (2005) the nature of algal flora in different localities is the result of a complex influence of the local type of vegetation, soil properties and climatic conditions, but it also often depends on the input of airborne algal spores.

Table 5.4: Penetration test results in kg/cm^2 for treatments with and without the addition of *Chlamydomonas* sp.

Treatment:	**Average:**	**Standard error:**
Before inoculation	1.375	0.125
After inoculation: Control	1.25	0.14
After inoculation: BBM	1.3	0.44
After inoculation: 8.1 g/ℓ PO_4	1.58	0.22
After inoculation: 9.12 g/ℓ NO_3	0.42	0.125
After inoculation: BBM *Chlamydomonas*	**2.58**	0.3
After inoculation: 8.1 g/ℓ PO_4 *Chlamydomonas*	0.75	0
After inoculation: 9.12 g/ℓ NO_3 *Chlamydomonas*	1.5	0.14

The penetration test showed that the tailings treated with *Chlamydomonas* sp. cultured in BBM medium with a lower phosphate concentration was the most stable and had the highest reading of 2.58 kg/cm^2. This measurement differs significantly from the other treatments ($p < 0.05$).

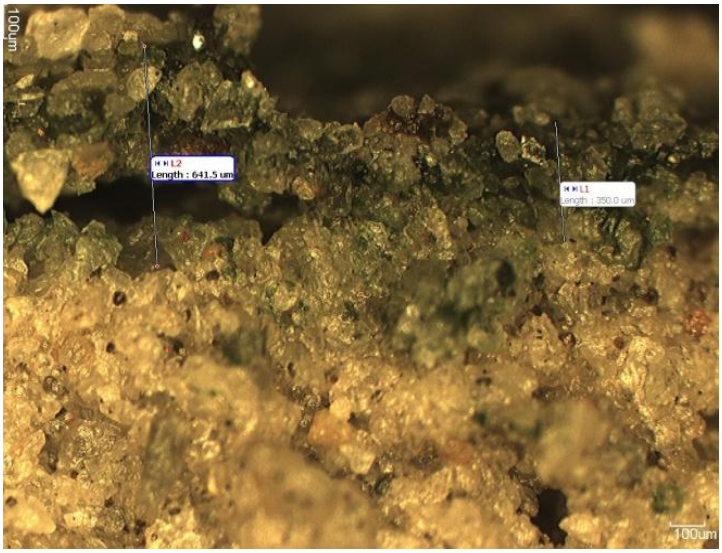

Figure 5.3: Crust thickness of treatments with *Chlamydomonas* sp. 641.5 µm and 350.0 µm

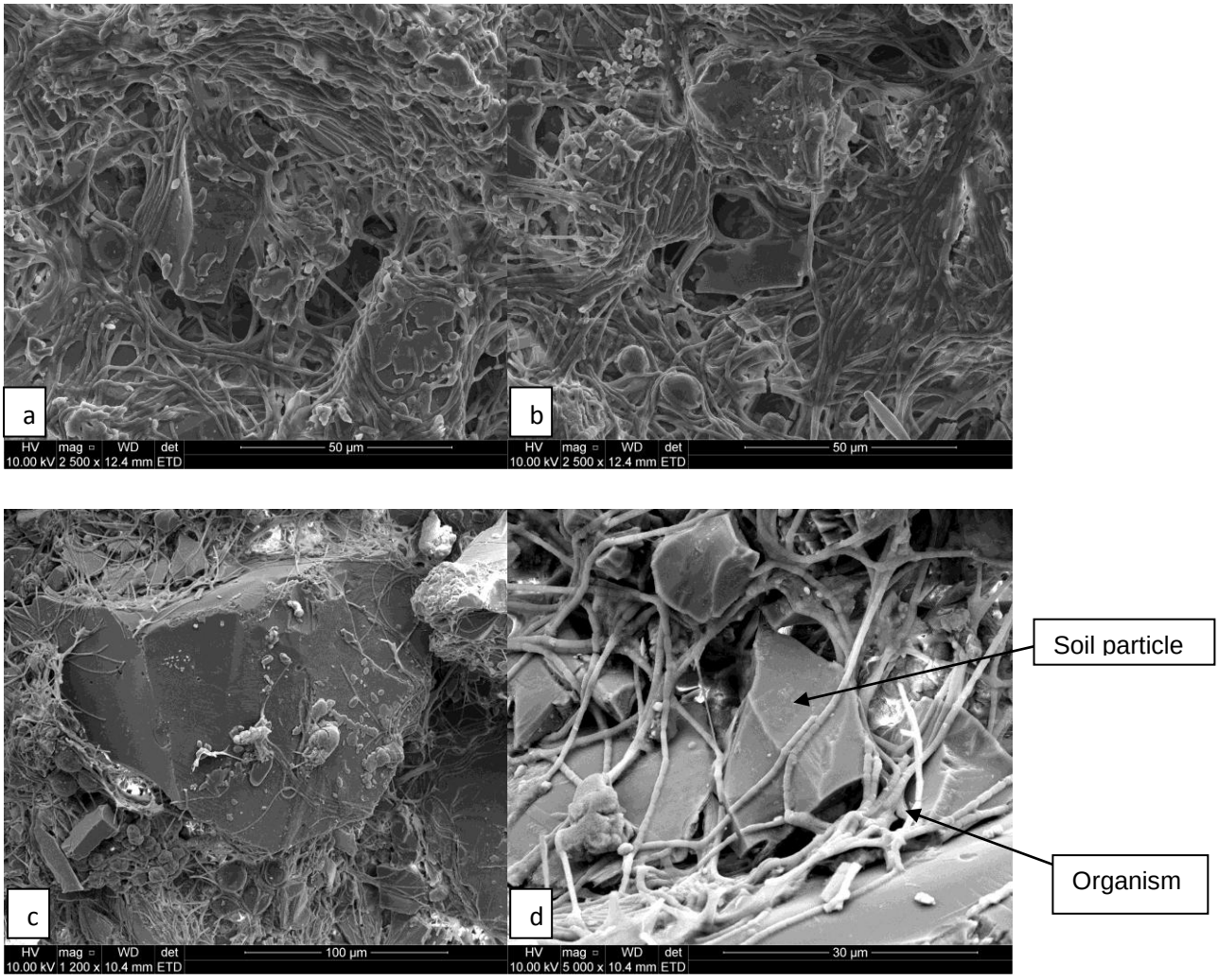

Figure 5.4: The intimate relationship formed between the soil algae and cyanoprokaryotes and the soil particles can be seen in scanning electron microscopy photos a-d

5.3.1.2 Different treatments with *Microcoleus vaginatus*

There was no significant difference between algal biomass measured in the treatments with *Microcoleus vaginatus* (BBM; BBM with lower nitrate and phosphate). Again, as with *Chlamydomonas*, there was a significant difference between treatments with the organism and treatments without ($p<0.05$). The control treatment did not grow well and had the lowest biomass production with a chlorophyll-*a* concentration of 0.1 µg/g, but again the control was the most diverse in terms of species composition (Table 5.5).

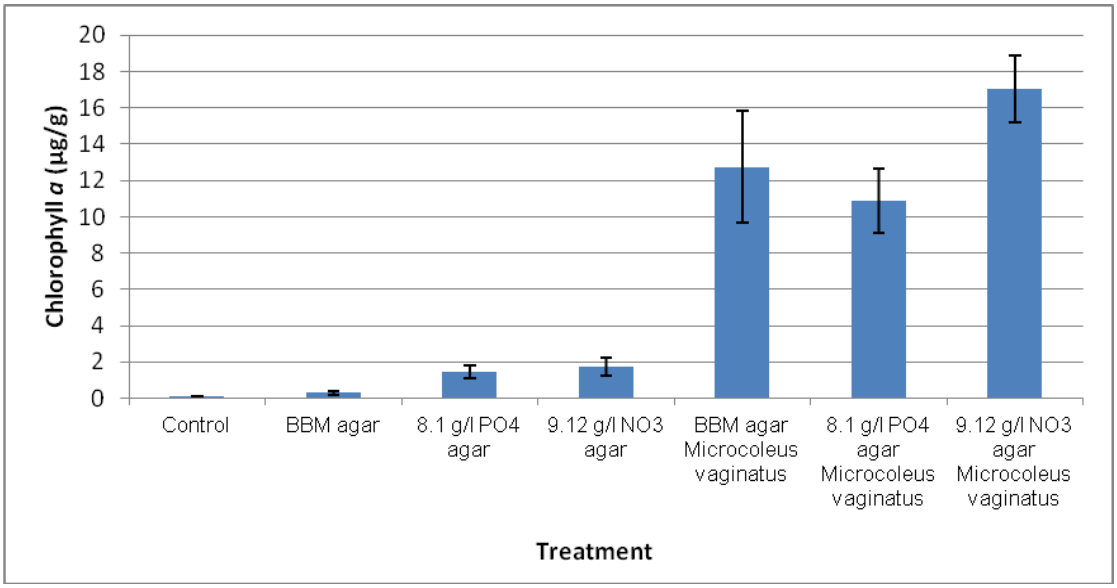

Figure 5.5: Representation of the influence of various nutrient treatments on the biomass production of *Microcoleus vaginatus* over a trial period of six weeks.

The highest chlorophyll-*a* concentration of 17.05 µg/g was measured for the treatment with *Microcoleus vaginatus* grown in BBM with a lower nitrate concentration. This was much higher than the highest chlorophyll-*a* concentration of 6.12 µg/g measured for the *Chlamydomonas* treatments.

The following species were identified in each treatment:

Table 5.5: Algal and cyanoprokaryote species identified before inoculation with *Microcoleus vaginatus* and six weeks after inoculation.

Species	Before inoculation	Control	BBM	8.1 g/ℓ PO_4	9.12 g/ℓ NO_3	BBM + *Microcoleus vaginatus*	8.1g/ℓPO_4 + *Microcoleus vaginatus*	9.12 g/ℓ NO_3 + *Microcoleus vaginatus*
Arthrospira sp.						✓		
Chlamydomonas sp. 1		✓		✓	✓			
Chlamydomonas sp. 2		✓						
Chlorococcum sp.	✓	✓		✓		✓		
Chlorosarcinopsis sp.					✓			
Calothrix sp.		✓						
Klebsormidium sp.			✓					
Microcoleus vaginatus (Vaucher) Gomont						✓	✓	✓
Navicula pelliculosa (Brebisson) Hilse								
Nostoc sp. 1	✓		✓	✓	✓	✓		
Nostoc sp. 2			✓					
Phormidium autumnale (Agardh) Gomont		✓			✓	✓	✓	✓
Phormidium sp. 1	✓	✓	✓	✓	✓	✓		
Phormidium sp. 2	✓	✓	✓	✓	✓			
Scytonema sp.	✓	✓						
Stichococcus bacillaris Nägeli								✓
Tetracystis aggregata Brown et Bold				✓			✓	
Tetracystis texensis Brown et Bold							✓	
Total number of species	**5**	**8**	**5**	**6**	**6**	**6**	**4**	**3**

Five species were identified before inoculation and three, four and six species were identified after the different treatments with 9.12 g/ℓ NO_3, 8.1 g/ℓ and Bold's Basal medium respectively. In each of the treatments with *Microcoleus vaginatus,* the species were present after the experiment as well. The pioneer species *Nostoc* and *Phormidium* were present before and after the treatments, showing that crust development is still in the early stages (Belnap, 2001). *Tetracystis* species were present in both treatments with lower phosphate concentration (9.12 g/ℓ PO_4). We can thus assume that the green algae *Tetracystis aggregata* as well as *T. texensis* were less affected by lower phosphate concentrations than lower nitrate concentrations. *Arthrospira* sp., a filamentous cyanoprokaryote was only present in the treatment with *Microcoleus vaginatus* cultured in BBM medium. It is interesting to see that most of the species identified after the trial were cyanoprokaryotes species, which generally fulfil the pioneer position, as they are filamentous, moving between the soil particles, forming soil aggregates (Fogg *et al*., 1973 and Belnap, 2001).

Table 5.6: Penetration test results in kg/cm^2, with and without the addition of *Microcoleus vaginatus.*

Treatment:	Average:	Standard error:
Before inoculation	1.375	0.125
After inoculation: Control	1.25	0.14
After inoculation: BBM	1.3	0.44
After inoculation: 8.1 g/ℓ PO_4	1.58	0.22
After inoculation: 9.12 g/ℓ NO_3	0.42	0.125
After inoculation: BBM *Microcoleus*	1.92	0.17
After inoculation: 8.1 g/ℓ PO_4 *Microcoleus*	1.83	0.46
After inoculation: 9.12 g/ℓ NO_3 *Microcoleus*	2.08	0.36

No statistical significant differences ($p>0.05$) could be seen between the penetration tests with different treatments, which was disappointing as *M. vaginatus* is a pioneer species that is known to glue sand particles together (Rogers and Burns, 1994). The highest penetration reading was measured for of *Microcoleus* growing in BBM with the lower nitrate concentration (2.08 kg/cm^2).

Figure 5.6: *Microcoleus vaginatus* produced a smooth, hard crust.

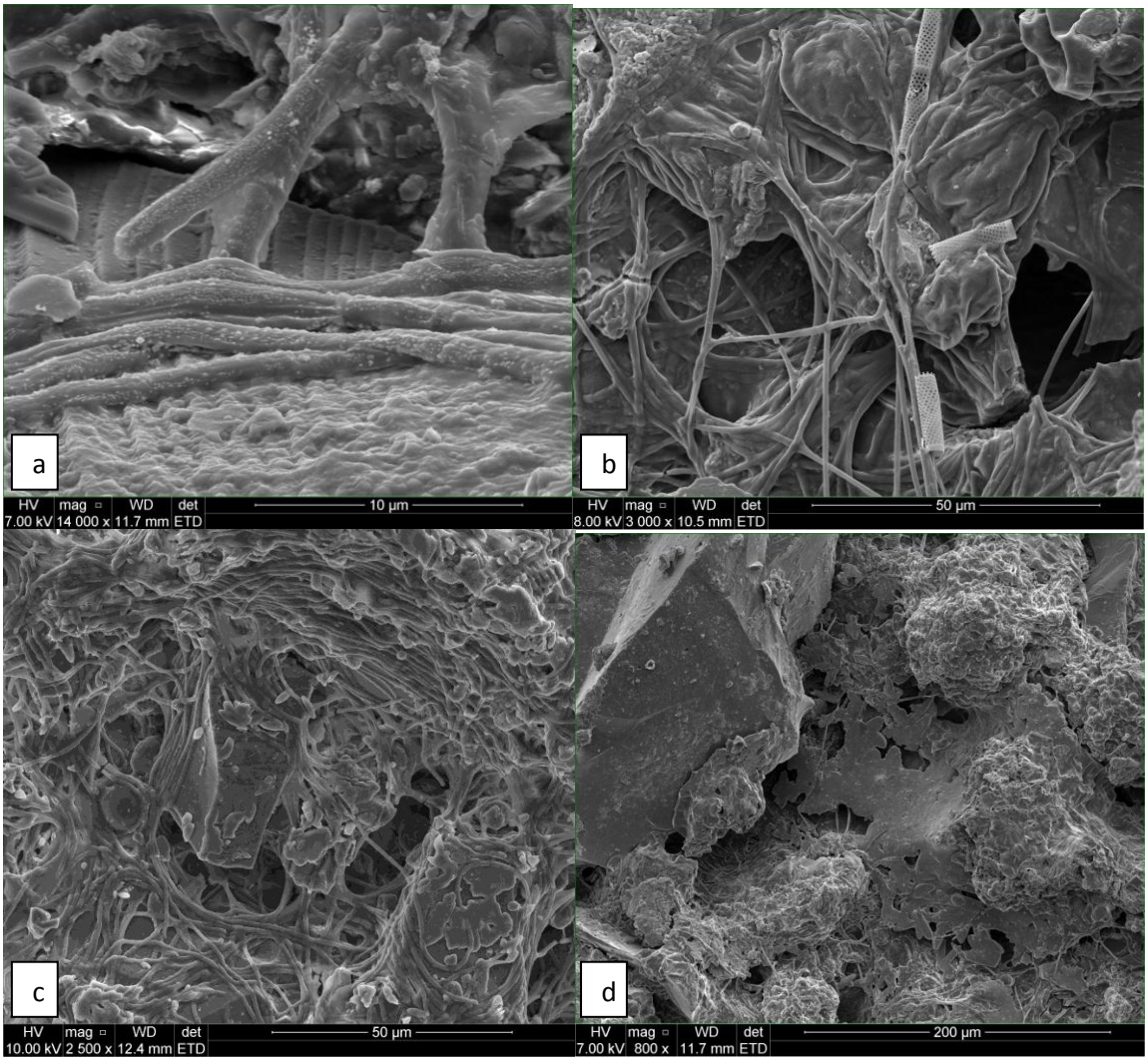

Figure 5.7: Scanning electron microscopy photographs indicate the **b**iological crust growth dominated by *Microcoleus vaginatus. S*oil particles seemed to be cemented together by the slime produced by this cyanoprokaryotes.

M. vaginatus is one of the most commonly used cyanoprokaryote species to research BSCs as it is a pioneer species with bundles of filaments surrounded by slime sheaths (Zhang *et al*., 2009). When the soil is moist the filaments slide out of the thick slime sheaths and move to the upper parts of the soil, when drying occur these filaments move back to the lower soil parts and form new slime sheaths (Belnap *et al*., 2001). Slime sheaths which are mainly polysaccharides are very effective in the aggregation process due to their macromolecular structure which glues particles together (Rogers and Burns, 1994).

Many algal-bacterial complexes were present in the treatment where *Microcoleus vaginatus* was cultured in normal BBM. A study by Belnap (2001) also found bacteria associated with *Microcoleus vaginatus* cultures, and found them to assist in nitrogen fixation, by scavenging oxygen and creating anaerobic conditions.

5.3.1.3 Different treatments with *Nostoc sp.*

The highest biomass production was seen in the treatment where *Nostoc* sp. was inoculated in BBM growth medium as well as BBM with the lower phosphate concentration (see Figure 5.6). *Nostoc* sp., growing in BBM with a lower nitrate concentration, also grew well. The fact that *Nostoc* sp. has heterocysts and can fixate nitrogen (Belnap, 2001) could be a factor that could have contributed to this. As with the other experiments (*Chlamydomonas* and *Microcoleus*) the treatments containing the organism out performed those without the organism. The treatments with *Nostoc* sp. produced more biomass than the treatments with *Chlamydomonas* or *Microcoleus*, during the study period. The highest biomass result with *Nostoc* sp. was obtained in the treatment where *Nostoc* sp. was cultured in BBM medium and 34.44 μg/g biomass was measured. The highest biomass with *Microcoleus vaginatus* (17.05 μg/g) was obtained when cultured in BBM with half the NO_3 concentration, while that of *Chlamydomonas* sp. (2.37 μg/g) was obtained in BBM with half the PO_4 concentration.

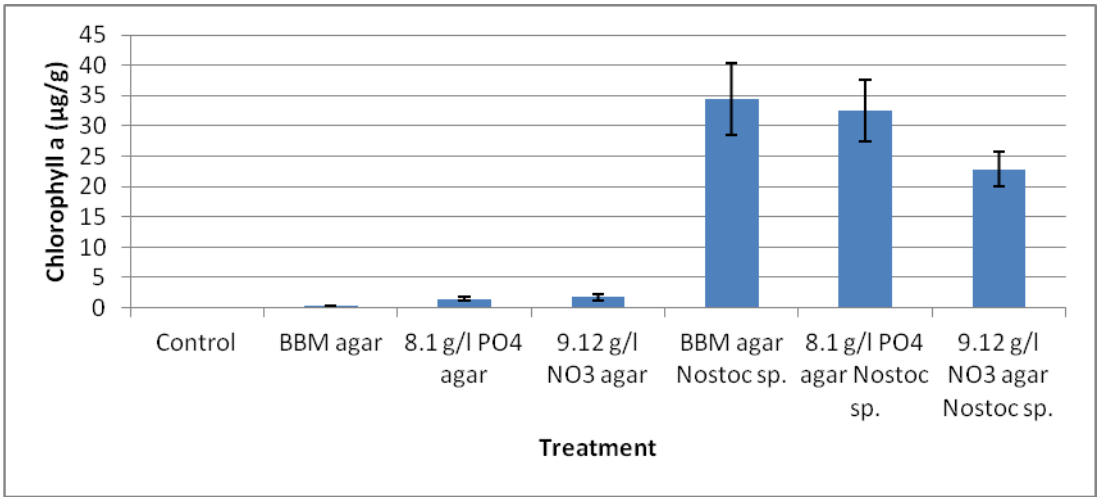

Figure 5.8: Representation of the influence of various nutrient treatments on biomass production of *Nostoc* sp. over a trial period of six weeks.

The following species were identified in each of the treatments:

Table 5.7: The presence of algal and cyanoprokaryote species before inoculation with *Nostoc* sp. and six weeks after inoculation with six different treatments.

Species	Before inoculation	Control	BBM	8.1 g/ℓ PO_4	9.12 g/ℓ NO_3	BBM + *Nostoc* sp.	8.1g/ℓ PO_4 + *Nostoc* sp.	9.12 g/ℓ NO_3 + *Nostoc* sp.
Chlamydomonas sp. 1		✓		✓	✓	✓		✓
Chlamydomonas sp. 2		✓						
Chlorococcum sp.	✓	✓		✓			✓	
Chlorosarcinopsis sp.					✓			
Chlorella sp.								✓
Chlorolobion lunulatum Hindák							✓	
Calothrix sp.		✓						
Klebsormidium sp.			✓					
Lyngbya sp.								
Navicula pellioulosa (Brebisson) Hilse								
Nostoc sp. 1	✓		✓	✓	✓	✓	✓	✓
Nostoc sp. 2			✓					
Phormidium autumnale (Agardh) Gomont		✓			✓			
Phormidium sp. 1	✓	✓	✓	✓	✓	✓	✓	✓
Phormidium sp. 2	✓	✓	✓	✓	✓			
Scytonema sp.	✓	✓						
Tetracystis aggregata Brown et Bold				✓		✓		
Total number of species	**5**	**8**	**5**	**6**	**6**	**4**	**4**	**4**

Five algae and cyanoprokaryote species were present in the tailings material before inoculation with different treatments (see Table 5.7). After inoculation four species were identified in each of the samples. *Nostoc* sp. 1, *Phormidium, Scytonema* and *Chlorococcum* species were present before and after inoculation. Again the control was the most diverse in terms of species diversity and the treatments were organisms where added had the least amount of species. The reason can be that the organism that is inoculated dominated the surface and do not give other species the chance to establish.

Table 5.8: Penetration test results, measured in kg/cm^2, with and without the addition of *Nostoc* sp.

Treatment:	**Average:**	**Standard error:**
Before inoculation	1.375	0.125
After inoculation: Control	1.25	0.14
After inoculation: BBM	1.3	0.44
After inoculation: 8.1 g/ℓ PO_4	1.58	0.22
After inoculation: 9.12 g/ℓ NO_3	0.42	0.125
After inoculation: BBM *Nostoc*	**1.75**	0
After inoculation: 8.1 g/ℓ PO_4 *Nostoc*	0.92	0.17
After inoculation: 9.12 g/ℓ NO_3 *Nostoc*	0.75	0

The treatment with *Nostoc* growing in BBM with agar had the highest penetration reading (1.75 kg/cm^2). This is lower than the highest measurement of the *Chlamydomonas* treatments (2.58 kg/cm^2) and *Microcoleus* treatments (2.08 kg/cm^2). A clear distinction can be made between the biological crust produced and the soil surface (see Figure 5.7). From the penetration values it can be established that treatment with *Nostoc* sp. does not contribute significantly to the soil surface strength ($p>0.05$). *Nostoc* filaments usually lay on top of the soil particles and do not penetrate the soil surface (Belnap, 2001).

When comparing all the treatments with one another a statistical significant difference between all the treatments with *Nostoc* sp. and *Chlamydomonas* sp. cultured in 9.12 g/ℓ NO_3 can be seen ($p<0.05$). There is also a significant difference between *Nostoc* sp. cultured in BBM and 8.1 g/ℓ PO_4 and *Chlamydomonas* sp. cultured in 8.1 g/ℓ PO_4 ($p=0.026$ and $p=0.016$ respectively). However, the three treatments of *Nostoc* (cultured in BBM, BBM with 9.12 g/ℓ NO_3 and BBM with 8.1 g/ℓ PO_4) performed the best on the tailings material and had the highest average biomass with a chlorophyll-*a* concentration of 34.44 µg/g, 32.52 µg/g and 22.81 µg/g respectively. It was therefore puzzling that the treatment with *Chlamydomonas* sp. which had the highest penetration reading also had the lowest biomass.

Figure 5.9: *Nostoc* sp. produced a flaky crust, which easily detaches from the soil surface.

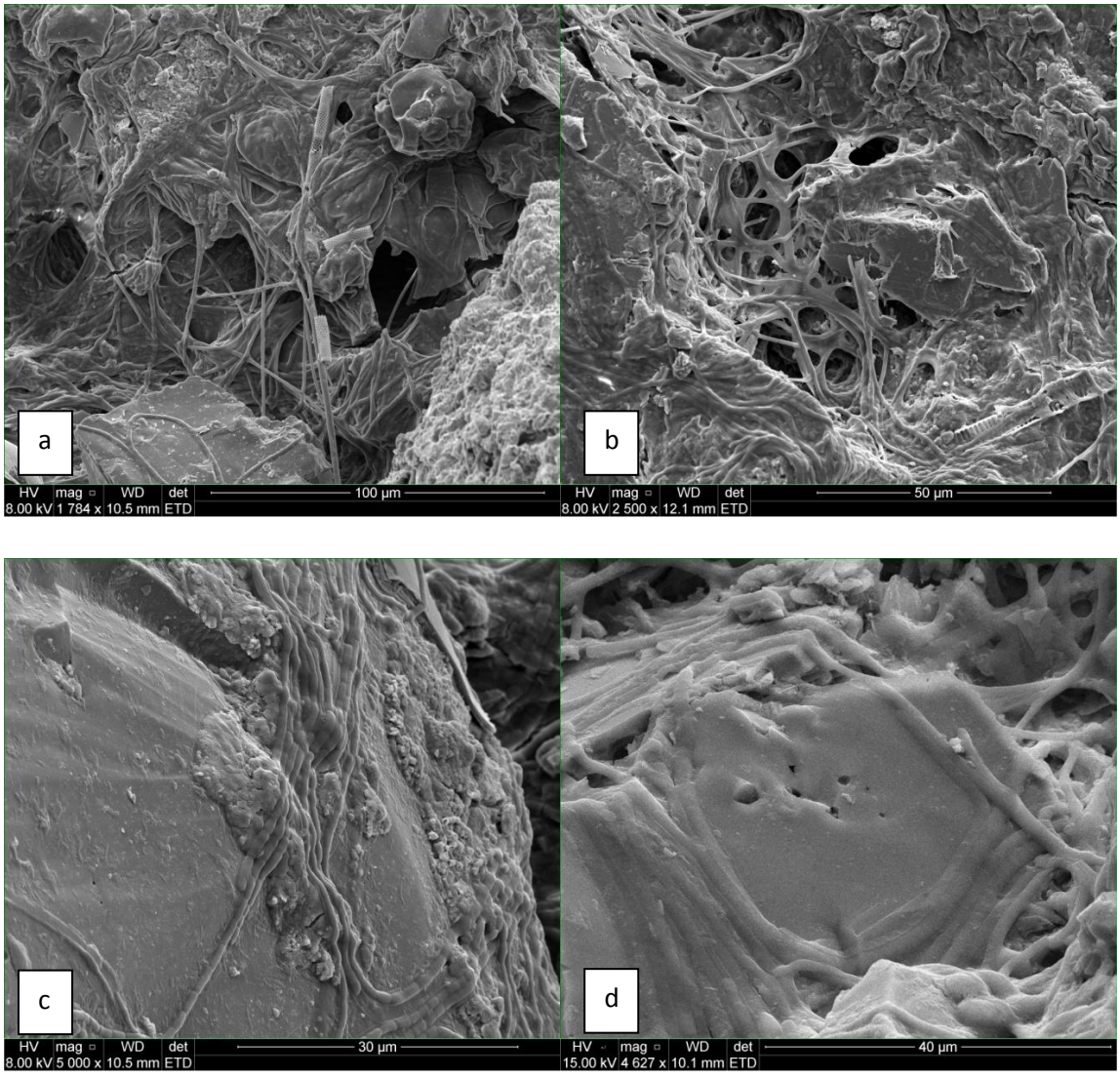

Figure 5.10: Scanning electron microscopy photographs depicting the relationship between *Nostoc* sp., other soil algae and cyanoprokaryotes and the soil particles.

5.4 Conclusion

The results showed clear differences between the biomass productions of the treatments where organisms were added and treatments where organisms were not added. It is therefore important that an inoculum is provided when attempting restoration with micro-organisms. According to a study by Zancan *et al*., (2005) the natural establishment of BSC is dependent on airborne spores and this could be seen in the control that received only water but was the most diverse in terms of species composition. Even the treatments without the added organisms had a high diversity. Given time (from 6 years to centuries according to Bowker, (2007)), and moisture, natural BSCs may develop. However this will not work for restoration and assisted recovery must be induced to be economically viable.

Nostoc sp., cultured in BBM medium, showed the highest average biomass with 34.44 µg/g chlorophyll-*a* in the treatment, but very low soil surface strength with the highest penetration result being 1.75 g/cm^2. *Microcoleus vaginatus* cultured in 9.12 g/ℓ NO_3, produced average chlorophyll-*a* concentrations of 17.05 µg/g, and the highest penetration result obtained was 2.08 kg/cm^2. The strong threshold impact velocity of *M. vaginatus*, in spite of the lower biomass production in comparison with *Nostoc* sp., may be attributed to the thinner crusts having the largest biomass per gram soil (Hu *et al*., 2002).

The glasshouse trials therefore indicate that *Nostoc* is a good candidate to test in field trials. Money can also be saved by using the growth medium with lower phosphate levels. Soil algae and cyanoprokaryotes, especially *Microcoleus vaginatus* are known to quickly colonize substrates which are inhabitable for many other organisms (Shields and Durrell, 1964). The secret to success of *M. vaginatus* is their ability to move around within the soil. When the soil is wet they can move towards the surface, while they retract deeper into the soil when the soil dries out (Belnap, 2003). The continuous movement of the filaments in the soil ensures that sheath material is left behind in the soil layers thereby joining loose sand particles (Belnap, 2003), forming a biological soil crust on the soil surface.

As seen in Figure 5.9, there is an array of different biological soil crusts that can form (Rosentreter *et al*., 2007). Smooth crusts, as those produced by *M. vaginatus*, are found in hyper arid regions (Belnap, 2003 and Rosentreter *et al.*, 2007). Smooth crusts, however reduce pores within the soil, and therefore may decrease water infiltration (Belnap, 2003). The establishment of a smooth crust, such as with *M. vaginatus*, should thus be seen as the first step in succession to produce a well-developed crust which contain lichens and mosses which can almost completely protect the bare tailings from water and wind erosion (Belnap, 2003 and Zhang *et al*., 2009).

Figure 5.11: Illustration of different forms of biological soil crusts (Rosentreter *et al*., 2007).

There are quite some contradicting statements concerning the time frames for the development of BSCs. Hu *et al*. (2002) found that crusts only one year old already had the same stability than crusts that were up to 42 years old, whereas Bowker (2007) found that BSC development is influenced by abiotic factors and of course ecological factors as mentioned previously and can take from 6 years to millennia. Bowker however also stated that quicker establishment might be a reality when assisted (Bowker, 2007), by supplying adequate amounts of water (Shields and Durrell, 1964). Within six weeks crusts with the ability to withstand impacts of up to 2.75 kg/cm^2 were produced. It would thus seem that the addition of the correct organism for the habitat might speed up the formation of crusts that can assist protection against wind and water erosion. Filamentous cyanoprokaryotes are proposed in the case of mine tailings material, as these organisms are known to colonize sandy soils (Belnap, 2003). These organisms protect soil surfaces better than green algae, as they are longer and larger and thus connect soil particles with ease (Belnap, 2003).

The mine tailings that were investigated during this study, especially the freshly dumped tailings, consisted of very fine sand (see Chapter 3; Table 6 in paper, page 290). After a one year restoration period with higher plants, *Nostoc* sp. and *Phormidium* sp. were some of the dominant cyanoprokaryotes identified in the material. *Microcoleus* sp. was only found in tailings material that was restored 10 and 15 years ago.

5.5 References

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Chapter 6

Field trials

6.1 Introduction

Rogers and Burns (1994) investigated the inoculation of soil surfaces with cyanoprokaryotes as a way to improve soil stability by improving the soil structure. The importance of algalization with cyanoprokaryotes lay in their ability to produce polysaccharide sheaths, which bind soil particles together, producing aggregates and thereby assisting in counteracting the erosive forces of wind and water (Rogers and Burns, 1994, Belnap, 2003, Hu *et al*., 2003, Belnap and Lange, 2003, Zancan *et al*., 2005, Flechtner, 2007, Bowker, 2007 and Issa *et al*., 2007). In an attempt to test the feasibility of the results that were obtained in the glasshouse trials (Chapter 5), the practical application of BSCs in field conditions was done during February/March 2013.

In Chapter 3 soil algal and cyanoprokaryotes species present and biologically dominant in the mine tailings, as well as in the atmosphere surrounding the mines, were identified. *Chlamydomonas* sp., *Nostoc* sp. and *Microcoleus vaginatus* were dominant in some of the sites and were therefore chosen as test organisms for the glasshouse trials. Results obtained from glasshouse trials (Chapter 5) showed that *Nostoc* produced the highest average biomass (34.44 µg/g), but low crust strength (only 1.75 kg/cm2). One of the trials with *Chlamydomonas* sp. (cultured in BBM) produced the most stable crust during the study period (2.58 kg/cm2), but had low biomass results (6.13 µg/g). *Microcoleus vaginatus* produced a crust with strength of 2.08 kg/cm2 and the highest biomass results were 17.05 µg/g. it was therefore decided to use *Nostoc* sp. (producing high biomass) and *Microcoleus vaginatus* (with high crust strength and biomass) in the field experiments.

6.2 Material and methods

6.2.1 Description of the study site

The study was conducted on a gold mine tailings dam in Stilfontein (Figure 6.1), located in the North West Province of South Africa, with coordinates approximately 26°48’ S, 26°47’ E. Experimental plots were located on the North-Eastern and Southern slopes that have not been rehabilitated for four years. This area is in a summer rainfall region, characterized by a mean annual precipitation of 592.2 mm, usually in the form of isolated thundershowers (see

Figure 6.2). The mean annual temperature in this region is 17.8°C, with minimum temperatures of as low as 1° C and maximum temperatures reaching up to 28°C (see Figure 6.2).

Figure 6.1: Illustration of the tailings storage facility in Stilfontein where the sampling plots were set out (google maps and http://www.weatherbase.com).

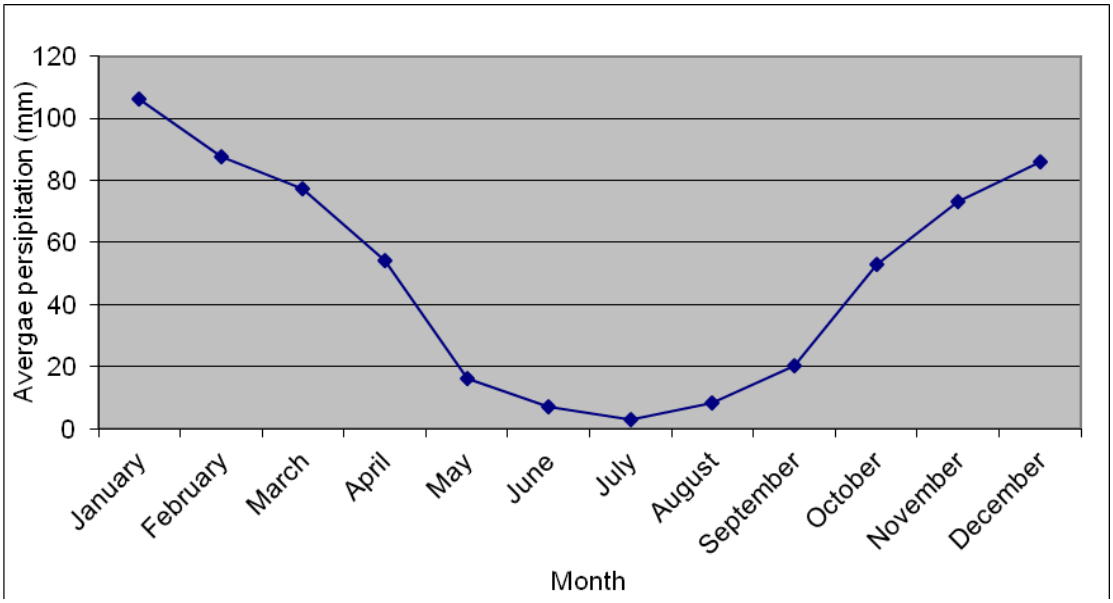

Figure 6.2: Illustration of the average rainfall for the Stilfontein area (http://www.weatherbase.com) over a period of 12 months.

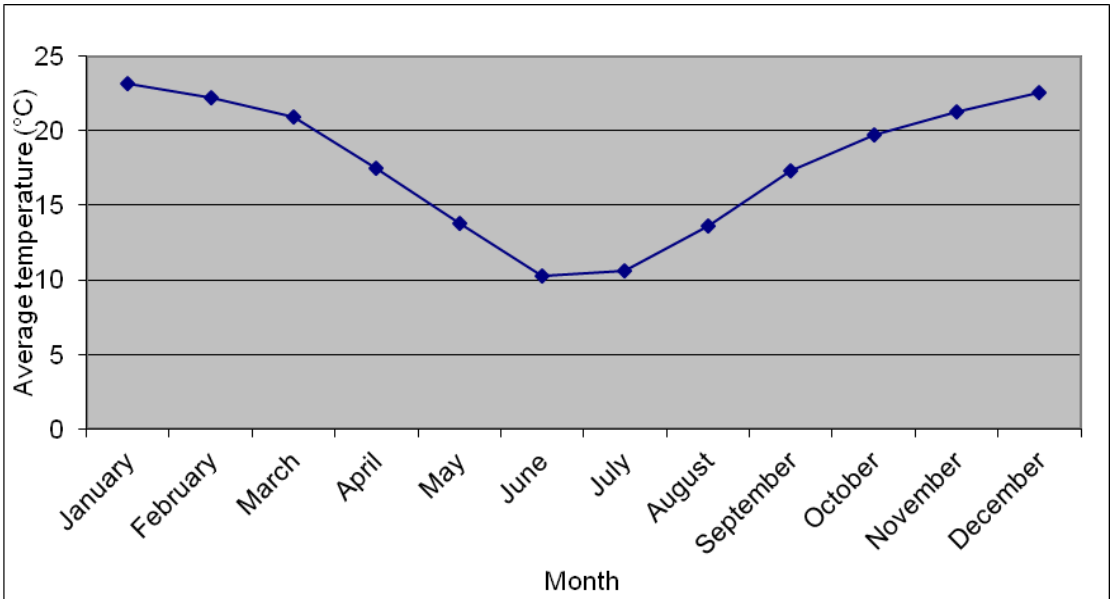

Figure 6.3: Illustration of the average minimum and maximum temperatures measured in the Stilfontein area (http://www.weatherbase.com) over a period of 12 months.

6.2.2 Experimental procedure

The trials were conducted on the top slopes of the North-Eastern and Southern slopes of the tailings dam (see Figure 6.1) and commenced on 5 February 2013. Plots of 40 x 50 cm,

which were comparable to the trays used in the glasshouse (Chapter 5), were placed out with different treatments as follows;

8 Control: mine tailings that only received water.

9 Mine tailings treated with BBM medium with 0.1% agar added. This treatment again received 200 mℓ BBM medium after three weeks.

10 Mine tailings treated with *Microcoleus* sp. cultured in BBM medium with 0.1% agar. This treatment received BBM medium (200 mℓ) after three weeks.

11 Mine tailings treated with *Nostoc* sp. cultured in BBM medium with 0.1% agar. This treatment received (200 mℓ) BBM medium after three weeks.

12 Mine tailings treated with *Microcoleus* sp. cultured in BBM medium with 0.1% agar and covered with mulch. This treatment received BBM medium (200 mℓ) after three weeks.

13 Mine tailings that only received water, but were covered with mulch.

14 Plots where Hutton soil (Macvicar and De Villiers, 1991) was placed in rectangular trays (30 x 27.5 x 10 cm) and buried to level in the tailings material. These plots only received water. This was also a control.

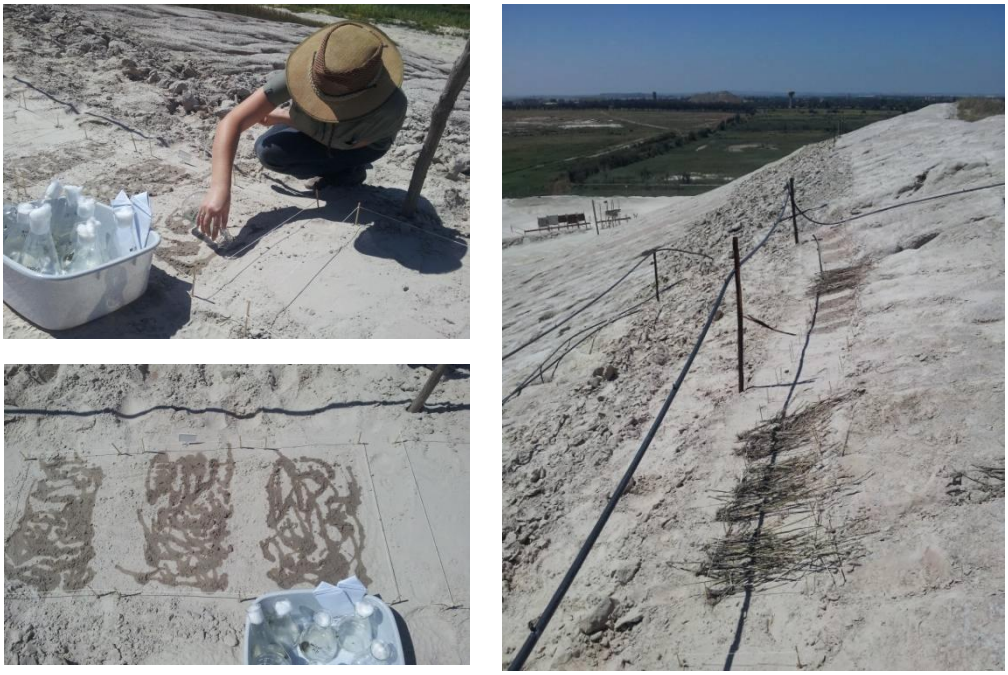

Figure 6.4: Application of various treatments to experimental plots, on the top slopes of the North-Eastern and Southern slopes of the tailings storage facility in the Stilfontein area, on 5 February 2013.

All of the above treatments received water through a sprinkler system on a daily basis. The mulch was used as a measure to investigate the possible influence of a microhabitat (Shields and Durrell, 1964 and Belnap, 2003) in the form of shade on the growth of BSCs, as much higher species diversity was seen on undisturbed soil, in the shade (Chapter 3).

Table 6.1: Soil analyses of the Hutton soil (Macvicar and De Villiers, 1991) used in the field trials as the natural soil control treatment.

Soil analyses	**Hutton Soil**
pH(KCl)	4.1
pH(H_2O)	4.1
EC (msm^{-1})	13
SO_4 ($mg\ kg^{-1}$)	12
P ($mg\ kg^{-1}$)	4
K ($mg\ kg^{-1}$)	62
Ca($mg\ kg^{-1}$)	88
Mg ($mg\ kg^{-1}$)	49
Na ($mg\ kg^{-1}$)	1
CEC ($cmol.kg^{-1}$)	1.4
Al ($cmol.kg^{-1}$)	0.31
ESP (%)	0.32
Al (%)	22.5

After three weeks (26 February 2013) follow-up nutrients were applied to treatment numbers 2, 3, 4 and 5. On the site it was seen that some of the plots on the northern side were affected by erosion problems, as seen in Figure 6.5. The erosion problems were mainly caused by a thunder storm on 5 February 2013.

Figure 6.5: Photographs indicating that some of the treatment plots were severely affected by water erosion problems.

On 20 March 2013, three weeks after the nutrients were applied, soil samples for biological crusts were taken on all the treatment plots. As with the glasshouse trials the total duration of the experiment was 6 weeks. Three samples per plot, thus nine samples per treatment, were taken (Figure 6.6). Visually very little growth could be detected, and the mulch had been blown away by the wind in all the treatments where it was applied.

Figure 6.6: Photograph indicating that three soil samples per plot, thus nine samples per treatment, were taken.

The samples were stored in containers to keep them cool and transported to the university were chlorophyll-*a* analyses were done and species present were identified, as stipulated in Chapter 4.

6.2.3 Species Analyses

Before the tailings material was inoculated with the different treatments, a soil sample was taken to compile a species list of the algae and cyanoprokaryotes present in the tailings material, as well as in the Hutton soil (Macvicar and De Villiers, 1991). The methods used were the same as those described in Chapter 3 (Orlekowsky *et al*., 2013). This was repeated six weeks after inoculation, when the different treatments were sampled to determine the biomass (described in Chapter 4).

6.2.4 Penetration Tests

A hand operated penetrometer was used to measure the BSC strength, as described in Chapter 5.

6.2.5 Statistical Analyses

Statistical analyses were conducted by the Northwest University Statistical Consulting Services and the same tests were conducted, as described in Chapter 5.

6.3 Results and Discussion

6.3.1 Biomass production

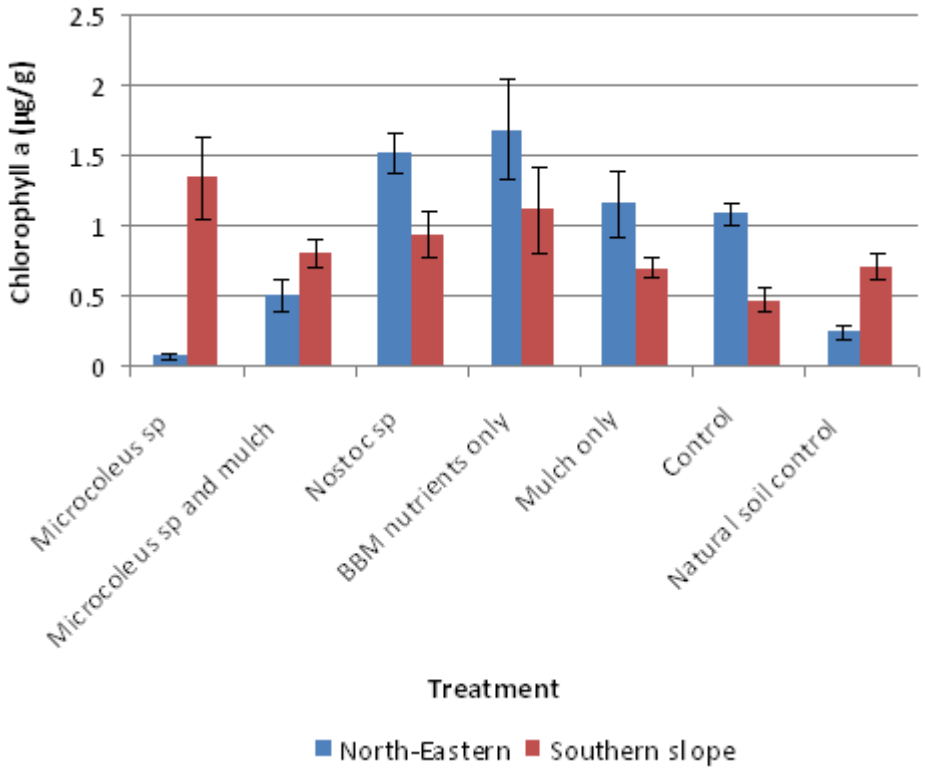

Figure 6.7: Representation of the biomass on each of the different treatment plots on the North-Eastern and Southern slopes.

Figure 6.7 shows that, in spite of the problem with erosion, higher biomass were measured on the North-Eastern slope than on the southern slope, except for the treatments with *Microcoleus vaginatus* and the natural soil treatment. There are however, no significant statistical differences between the biomass of the different treatments on either slopes ($p>0.05$). It is interesting that the there was no significant differences between the control, only mulch, only nutrients and the treatment with *Nostoc* added. This means results can be obtained without much input which will ultimately save money. The mulch should have provided a microhabitat but it blew away and the effect could not be tested. It is important to include this treatment in future tests.

6.3.2 Species Analyses

Table 6.2: Soil algal and cyanoprokaryote species present after the six weeks inoculation period on the North-Eastern slope.

Species	Classes	Before inoculation	BBM *Microcoleus*	BBM *Microcoleus* Mulch	BBM *Nostoc*	BBM	Mulch	Control	Before inoculation	After inoculation
		Mine tailings							Hutton soil	
Bracteacoccus minor (Chodat) Petrová	Chlorophyceae					✓	✓			✓
Chlamydomonas sp.	Chlorophyceae									
Chlorella minitissima Fott et Nováková	Trebouxiophyceae									
Chlorella vulgaris Beijerinck	Trebouxiophyceae					✓				✓
Chlorococcum sp.	Chlorophyceae				✓				✓	✓
Chlorosarcinopsis aggregata Arce et Bold	Chlorophyceae	✓			✓	✓				
Leptosira terricola (Bristol) Printz	Trebouxiophyceae			✓	✓	✓	✓	✓		✓
Myrmecia sp.	Trebouxiophyceae			✓						
Navicula pellioulosa (Brebisson) Hilse	Bacillariophyceae								✓	
Nitzschia palea (Kutzing)	Bacillariophyceae				✓		✓			

W. Smith										
Nostoc commune Vaucher	Cyanophyceae				✓	✓				
Phormidium sp.									✓	
Phormidium automnale (Agardh) Gomont	Cyanophyceae								✓	
Phormidium foveolarum Rabenhorst ex Gomont	Cyanophyceae	✓		✓		✓	✓	✓	✓	✓
Scottielopsis terrestris (Reisigl) Puncocharova et Kalina	Trebouxiophyceae									✓
Tetracystis aggregate Brown et Bold	Chlorophyceae			✓		✓	✓			✓
Total number of species		2	0	4	5	7	5	2	5	7

Only 2 species was identified in the tailings material on the North-Eastern slope before inoculation. The low diversity of this site was expected as no rehabilitation has been done on the site. Five species was identified on the Hutton soil before inoculation (Table 6.2) as the soil characteristic is more conducive to algal growth than the tailings material (see Appendix 1). This is comparable to the finding in Chapter 3 where the undisturbed site also had high species diversity (34 algal and cyanoprokaryote species).

The treatments with BBM and the natural soil control had the highest diversity after inoculation (7 species). The natural soil control plot might have enhanced the growth of algae and cyanoprokaryotes. As the Hutton soil has higher levels of phosphate, the soluble metals such as arsenic, lead and uranium are present at significant lower levels in the Hutton soil as in the tailings material (see Appendix 1). Tailings material was blown over the Hutton soil plot and could have influence soil characteristics as well as algal and cyanoprokaryote growth. Figure 6.8 shows the fine layer of tailings material that had settled on the natural soil control treatment during the trial period.

Microcoleus vaginatus was absent before and after the trial period. This might be due to the rainstorm on the afternoon of application, as well as the fact that the *M. vaginatus* trial plot was most severely affected by water erosion (see Figure 6.5). *M. vaginatus* forms clumps in culture due to its slime sheaths, thereby not forming a homogeneous suspension and the clumped sheaths can thus more easily be washed away (Fogg et al., 1973).

All the plots on the North-Eastern side were affected by the thunder storm and some or most of the inoculum could have been washed away. Six of the species present in the soil sample, were identified in the air (see Chapter 3). It seems that the treatments of the plots had a positive effect on the establishment of diaspores on the plots.

In the glasshouse trials, it was found that the biodiversity of the plots inoculated with an organism was lower, but that the biomass as well as soil stability was higher. It is therefore important that the effect of inoculum should be tested in future trials.

Figure 6.8: Photographs indicating a thin layer of mine tailings material settled on the soil surface of the natural control treatment.

Table 6.3: Soil algal and cyanoprokaryote species present after six weeks inoculation period on the Southern slope.

Species	Classes	Before inoculation	BBM *Microcoleus*	BBM *Microcoleus* Mulch	BBM *Nostoc*	BBM	Mulch	Control	Natural soil control
Bracteacoccus minor (Chodat) Petrova	Chlorophyceae	✓		✓					
Chlorella minutissima Fott et Novakova	Trebouxiophyceae			✓				✓	
Chlorella vulgaris Beijerinck	Trebouxiophyceae	✓		✓		✓	✓		
Chlorococcum sp.	Chlorophyceae	✓						✓	✓
Chlorosarcinopsis aggregate Arce et Bold	Chlorophyceae	✓			✓	✓	✓	✓	
Hantzschia sp.	Bacillariophyceae								✓
Leptosira terricola (Bristol) Printz	Trebouxiophyceae			✓				✓	
Navicula mutica Kutzing	Bacillariophyceae								✓
Nitzschia sp.	Bacillariophyceae		✓				✓	✓	
Nostoc commune Vaucher sensu Elenk	Cyanophyceae			✓	✓				
Nostoc punctiforme (Kutzing) Hariot	Cyanophyceae		✓		✓		✓		
Phormidium automnale	Cyanophyceae		✓						

(Agardh) Gomont									
Phormidium foveolarum Rabenhorst ex Gomont	Cyanophyceae	✓		✓	✓	✓	✓		✓
Phormidium sp.	Cyanophyceae								✓
Scottielopsis sp.	Trebouxiophyceae						✓		
Stichococcus basillarius Nageli							✓		
Total number of species		5	3	6	4	3	7	5	5

The species diversity on the Southern slope was higher before inoculation, at the mulch treatments and the control than on the same treatments on the North-Eastern slope. The Southern slope is cooler than the Northern slope and it could be that moisture retention was better on the Southern slope, however we did not measure it.

6.3.3 Penetration Tests

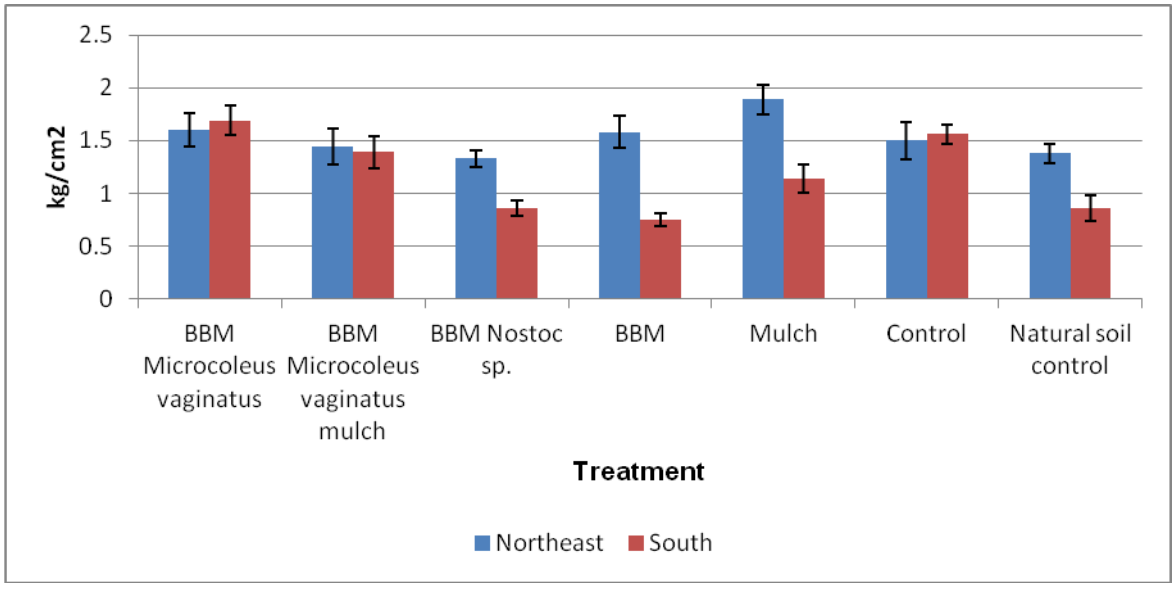

Figure 6.9: Illustration of the biological soil crust strength measured on the North-Eastern and Southern trial slopes with the penetrometer.

There were no statistical significant differences seen in the penetration tests for the different treatments on the North-Eastern slope ($p>0.05$). From Figure 6.9 and the p-values it is seen that there was a significant difference between the penetration test results of the BBM *Microcoleus vaginatus* treatments and the BBM, BBM *Nostoc* sp. and natural soil control treatments on the Southern slope. This is a very interesting result, as the BBM *M. vaginatus* treatment had the lowest species diversity and M. vaginatus was probably washed away.

However, *Phormidium autumnale* and *Nostoc punctiforme* were two filamentous cyanoprokaryotes present and could attribute for the crust strength, due to a phenomenon known as cyanoprokaryote layering (Belnap, 2001). *N. punctiforme* is present in the top soil layers, whereas *Phormidium autumnale* occurs in the lower soil layers (Belnap, 2001). Different soil layers are therefore stabilized by the presence of only two cyanoprokaryote

species. This phenomenon was seen in the glasshouse trials as well (see Chapter 5), where both *Nostoc* sp. and *Phormidium* sp. were simultaneously identified.

6.4 Conclusion

The biomass results seen in the field trials correlate with the results in the glasshouse where no organisms were added to the soil (Chapter 5). The maximum average biomass with organisms added in the field trials were 1.52 µg/g on the North-Eastern slope and 1.1 µg/g on the Southern slope, whereas the maximum average biomass without organisms added in the glasshouse trials were 1.76 µg/g. Roger and Kulasooriya (1980) found that controlled plot trials develop better than field experiments. Due to a severe thunder storm on the afternoon of application, where 50 mm of rain was measured within a period of 20 minutes, some of the inoculum as washed away together with the top layer of the soil.

Although very little growth was visible in the field trials, high species diversity was identified in the soil (see Table 6.2 and Table 6.3). The ability of these microorganisms to stay dormant in the soil during drought periods assures establishment during periods of high moisture content (Fogg *et al*., 1973, Belnap, 2003 and Mary and Hui, 2012). High diaspore concentration in the atmosphere surrounding the TFS also helps with increased diversity. The species diversity on the North-Eastern slope totals 10 species, with that on the Southern slope consisted of 11 species.

In Chapter 3 only five species were identified on the site that has not been rehabilitated for 15 years, whereas with just one year rehabilitation 25 species were identified. It would thus seem that active ecological rehabilitation plays a significant role in the success of the establishment of these crust organisms.

It has to be pointed out that irrigation sprinklers form part of the mentioned rehabilitation methods. The moisture content of the tailings might be the critical variable, but should be investigated in more depth. A study by Shields and Durrell (1964) shows that the optimum moisture content for luxurious soil algal growth is about 40 – 60% of the soil moisture holding capacity. As in the words of Belnap and Lange (2003); “Water is everything to the poikilohydric organisms that dominate soil crusts.”

Due to the thunder storm on the day of application it is difficult to make any conclusions based on the different treatments. The most important result is that, if moisture is provided for even the harshest terrain, algae and cyanoprokaryotes will be able to grow on it.

6.5 References

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Chapter 7

7.1 Conclusions

From a wide range of studies (Hoffman, 1989, Bowker, 2007, Flechtner, 2007 and Langhans *et al*., 2009,) it is evident that soil algae and cyanoprokaryote species in biological soil crusts (BSC) play a significant ecological role within the community they are present. These BSC may function as a bio indicator for soil quality, contribute to nitrogen and carbon fixation, and most importantly assist in soil stability (Rogers and Burns, 1994, Belnap, 2003 and Zancan *et al*., 2005)

Soil and air samples from gold mine tailings material that has been rehabilitated for different periods of time were collected to identify the algal and cyanoprokaryote species present within these areas. Diverse algal and cyanoprokaryote species were identified in the tailings material, as well as in the air surrounding these gold mine tailings facilities. The species list comprised of 40 different species identified from the tailings material and 30 different spores from the air. Many of the species identified in the tailings material were present in the air samples; it is therefore evident that the inoculum in the air plays a significant role in the species that colonize the tailings material (Whitton and Potts, 2000, Zancan *et al*., 2005).

Both single celled and filamentous algae and cyanoprokaryotes were identified in the tailings material. The data would therefore suggest that the BSC formation already shows signs of succession, as filamentous algae are usually pioneer species, followed by single celled species such as *Chlamydomonas* sp. and *Chlorococcum* sp. (Belnap *et al*., 2003). Cyanoprokaryotes are seen throughout all successional stages, as shown by the species list compiled with the glasshouse trials (Chapter 5), and confirmed in a study by Rahmonov and Piatek (2007). It was decided to use both cyanoprokaryotes and single celled organisms for further investigation in this study. *Chlamydomonas* sp. was chosen due to the organisms' quick adaptability to new growth conditions as well as generation time (Fogg *et al*., 1973 and Pickett-Heaps, 1975). *Nostoc* sp. was chosen because it was one of the species producing the highest biomass, as identified through growth curves in Chapter 4. *Microcoleus vaginatus* was made part of further trials, as it is a cosmopolitan soil cyanoprokaryotes, playing a major role in soil stabilization (Fogg *et al*., 1973, Belnap, *et al*., 2003, Belnap, 2003 and Zhang *et al*., 2009).

Before commencing glasshouse trials, there was a need to develop protocols on how to measure algal activity in the soil, and how to reintroduce the organisms into the soil. Various methods exist to measure algal activity in the soil by measuring the biomass production of

the photosynthesizing organisms (Nagarkar and Williams, 1997, Tsujimura *et al*., 2000 and Kabirov and Gaisina, 2009). From these methods the chlorophyll-*a* extraction method was chosen, due to its ease of use. The chlorophyll-*a* extraction method makes use of an extraction solvent such as ethanol, methanol, acetone or dimethyl sulphoxide (Lan *et al*., 2011). With the protocol determination it was decided to use methanol, as it proved to be the most efficient extraction solvent, see Chapter 4.

Three methods were tested for soil algalization (Yanni and Abdallah, 1990) or the reintroduction of soil algal and cyanoprokaryotes species into the soil namely: pour, spray and slush. The spray method caused a severe decline in organism activity in the soil, most probably due to stress of the organisms. The pour and slush methods are the same concept but 0.1% agar is added to the slush method, which also proved to be the most effective. This result is probably due to the agar growth medium buffering the organisms from the dramatic change from liquid medium to the new harsher growth conditions.

In order to optimize protection against wind and water erosion provided by BSC, the degree of BSC development must be sufficient (Belnap, 2003). In an attempt to optimize soil algal and cyanoprokaryote growth, with as little input costs as possible, the effect of different nitrogen and phosphate concentrations in BBM growth medium (Stein, 1973) on the biomass of organisms were investigated. In a controlled environment of a glasshouse where the temperature and water application was controlled, a pot trial commenced. Rectangular trays (30 x 27.5 x 10 cm), filled with gold tailings material from a gold mine in Stilfontein, were used for the trial. A control treatment that only received water and three treatments that received nutrients (BBM, BBM with half the original NO_3 concentration and BBM with half the original PO_4 concentration) were compared to treatments where *Chlamydomonas, Nostoc* species and *Microcoleus vaginatus* were cultured in BBM growth medium, BBM with half the original NO_3 concentration and BBM with half the original PO_4 concentration. *Chlamydomonas* sp. performed the best when cultured in BBM medium, but there was no statistical difference with both *Nostoc* sp. and *Microcoleus vaginatus* cultured in the different treatments (see Chapter 5).

The addition of the organisms to the substrate made a difference in terms of the tested criteria. The highest average biomass production of the treatment with half the NO_3 concentration where organisms were not added was 1.7 µg/g, whereas the addition of an organism caused the biomass production to increase to 34.44 µg/g (*Nostoc* sp.). *Nostoc* sp. performed the best with a maximum average biomass of 34.4 µg/g measured, *Microcoleus vaginatus* 17.05 µg/g and *Chlamydomonas* sp. 6.12 µg/g. The glasshouse trials showed that the addition of inoculum increases the biomass over the study period. However, species

analysis showed that the control had the highest diversity. The reason for the lower diversity present in the treatments where organisms were added may be attributed to the fact that large quantities of the selected organisms are inoculated on the surface giving it competitive advantages.

In order to test the viability of the results that was obtained through glasshouse trials it was decided to replicate the trials in field conditions. *Nostoc* sp. and *Microcoleus vaginatus* produced the most successful results with the glasshouse trials and was therefore chosen for the field trials. The field trials commenced on a tailings storage facility in the Stilfontein area on the same tailings material that was used for the glasshouse trials. The treatments were as follows: Control; mine tailings that only received water, mine tailings treated with BBM medium with 0.1 % agar added; mine tailings treated with *Microcoleus* sp. cultured in BBM medium with 0.1% agar; mine tailings treated with *Nostoc* sp. cultured in BBM medium with 0.1% agar; mine tailings treated with *Microcoleus* sp. cultured in BBM medium with 0.1% agar and covered with dry wheat as mulch, mine tailings that only received water, but were covered with mulch. Plots where Hutton soil was placed in rectangular trays (30 x 27.5 x 10 cm) and buried to level in the tailings material were also used as a control treatment, and received only water.

After six weeks the different treatments were sampled to determine the biomass and to compile a species list and penetration tests were done to test the crust strength of the BSC produced. Overall three of the treatments (*Nostoc* sp. cultured in BBM medium, tailings material that was covered by mulch and mine tailings that only received water) produced significantly higher biomass on the North-Eastern slope than on the Southern slope. This was an unexpected result as a severe thunderstorm (>50mm in 1 hour) on the day of application caused erosion problems on the northern slope. The sprinkler system on the tailings facility for the trial was also very poorly managed and days went by without water available for irrigation.

The highest mean biomass with the field trials was 1.6 µg/g where only BBM medium was applied. Compared to the glasshouse trials, the maximum biomass in the treatments where no organisms were added was 1.76 µg/g where BBM with half the original NO_3 concentration was applied. The highest biomass in treatments where organisms were added was 1.52 µg/g on the northern slope. In the glasshouse trials treatments with *Nostoc* sp. biomass results of 34.44 µg/g was obtained. Treatments with *Microcoleus vaginatus* produced a highest mean biomass of 17.05 µg/g. From these results it can be seen that the application of organisms to field conditions did not have an influence on the development of the BSC, most probably due to the thunder storm washing away the top soil layer where the applied

organisms were still present (Belnap, 2001). The higher biomass measurements were expected in a controlled experiment. Roger and Kulasooriya (1980) also found that pot experiments developed better than in field conditions.

Despite the challenges of the uncontrolled environment of the field experiment it shows the potential for growth if only moisture is available. There was no significant difference between the treatments on the Northern slope with *Nostoc*, nutrients, mulch or only water. This differs from the glasshouse trials that clearly showed the inclusion of an inoculum was necessary to achieve a high biomass. I would therefore recommend that a longer field trial must be carried out to investigate the influence of only irrigation on the soil stability of tailings material.

7.2 Recommendations

From the outcomes of the study, the following recommendations can be made:

1. Tailings type specific studies should be given attention to; this includes investigating the presence of soil algae and cyanoprokaryotes on different types of tailings material such as platinum, gypsum etc.

2. Re-apply the field trials with the addition of selected organisms; give special attention to water availability and application.

3. Test mixed cultures with algae and cyanoprokaryotes from different successional stages and with different biological functions such as a pioneer (*Microcoleus vaginatus* or *Nostoc*) and sub climax species (*Chlamydomonas* sp.)

4. Investigate the cyanoprokaryote layering phenomenon by applying mixed cultures with specifically selected species such as *Nostoc* sp. and *Phormidium* sp.

5. Investigate alternatives for mulch, such as fresh cut grass or hessian cover. Alternatively use an addition to keep the dry wheat mulch in place.

6. It is also important to investigate the long term effect of moisture. What will the effect be on ground cover and soil stability if tailings material is irrigated without the input of inoculum or higher plants over a period of time?

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Appendix 1

Soil chemical specifications and soluble heavy metal concentrations for the tailings material and Hutton soil used during the study.

Table 1: Soil chemical specifications

Growth medium	pH(KCl)	pH(H_2O)	EC	SO_4-S	P(Bray 1)	K mg/kg^{-1}	Ca mg/kg^{-1}	Mg mg/kg^{-1}	Na mg/kg^{-1}	CEC cmol.kg^{-1}	Al %	ESP %
Tailings material	5.3	5.4	193	1674	1	30	1793	94	9	1.3	3.2	3.11
Hutton soil	4.1	4.1	13	12	4	62	88	49	1	1.4	22.5	0.32

Table 2: Soluble heavy metals

Growth medium	Tailing material	Hutton soil
Heavy metals (ppm)		
Be	0.15	0.17
B	0.06	0.13
Al	4178	5268
P	410	339
Ti	37.6	60.8
V	5.6	13.3
Cr	25.5	70.4
Mn	191.8	128.0
Fe	7182	8457
Co	12.52	3.86
Ni	32.9	20.9
Cu	23.9	5.4
Zn	39.5	4.4
As	33.0	1.1
Se	0.52	0.37
Sr	6.44	0.92
Mo	0.78	0.19
Pd	0.22	0.19
Ag	0.13	0.06
Cd	0.09	0.0
Sb	0.33	0.22
Ba	15	13
Pt	0.12	0.14
Au	0.26	0.21
Hg	0.02	0.0
Pb	14.7	2.0
U	10.69	0.33