

SOIL STABILIZATION BY MICROBIAL ACTIVITY

By

Arnelia N. Paulse



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Supervisor: Prof. G.M. Wolfaardt
Co-supervisor: Prof. L. van Huyssteen

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

SUMMARY

Microorganisms play an important role in the stability and maintenance of the ecosystem and in the condition of the soil. However, in their natural environment, microorganisms often experience changing and hostile conditions. They therefore need to be able to adapt physiologically and modify their micro-environment. Biofilm formation is one mechanism to establish favorable micro-environments. The extracellular polymeric substances (EPS) that are typically associated with biofilm formation may also have an impact on soil structure. The aim of this project was to evaluate the potential of microbial manipulation on EPS production and the possible impact thereof on soil structure in order to improve water retention.

Specific objectives of this study included the screening of natural environments for EPS-producers, developing techniques to observe EPS production and accumulation in the pores between soil particles, measuring the effect of EPS production on soil water hydraulic gradient, as well as determining the fate and impact of EPS-producers when introduced to naturally-occurring soil microbial communities. Several environmental samples have been screened for EPS-producing microorganisms. Soil columns were then inoculated with these EPS-producers and the passage of 20 ml- aliquots water through the columns measured at 3 or 4-day intervals. Microbes isolated from soil, through their EPS production capability proved to retain water more effectively than was the case for water-borne EPS-forming microbes. This phenomenon was further studied using flow cells, filled with soil and inoculated with the EPS-producers isolated from either soil or water. Fluorescence microscopy showed that the soil microbes produced EPS that clogged pores between sand particles more effectively. This clogging resulted in lowering the soil water hydraulic gradient. To evaluate the effect of EPS-producers on existing soil microbial communities, cell counts, BiologTM whole-community carbon utilization studies and T-RFLP (terminal-restriction fragment length polymorphism) analyses were performed. Shifts in the soil microbial community could not be readily seen by observing microbial numbers and T-RFLP-analysis, but was noticeable in carbon utilization patterns.

OPSOMMING

Mikroorganismes speel 'n belangrike rol in die stabiliteit en instandhouding van die ekosisteem en in die kondisie van die grond. In hul natuurlike omgewing ervaar mikroorganismes dikwels veranderlike en ongunstige toestande. Mikroorganismes het dus nodig om hulself fisiologies aan te pas en verander hul mikro-omgewing daarvolgens. Biofilm-vorming is een meganisme om gunstige mikro-omgewings te skep. Die ekstrasellulêre polimeriese produkte (EPP) wat tydens biofilm-vorming gevorm word, mag ook 'n impak hê op die grondstruktuur. Die doel van hierdie projek was om die potensiaal van mikrobiële manipulasie op EPP-vorming te evalueer asook die moontlike impak daarvan op grondstruktuur wat sodoende waterretensie kon bevorder.

Die spesifieke doelwitte van hierdie studie het ingesluit die isolasie van EPP-produseerders vanuit natuurlike omgewings, die ontwikkeling van verskeie tegnieke waarvolgens EPP-produksie en die akkumulering daarvan in die porieë tussen gronddeeltjies bestudeer kon word, die effek van EPP-produksie op hidrouliese gradiënt van grondwater en om die lot en impak van EPP-produseerders op natuurlike grondmikrobiële populasies te bepaal. Verskeie grond- en watermonsters was getoets vir die voorkoms van EPP-produkerende mikroorganismes. Grondkolomme is geïnkuleer met EPP-produseerders en die vloeitempo van 20 ml-volumes water deur die kolomme is gemeet met 3 of 4-dag intervalle. Grond-geïsoleerde mikrobiese kolonies het beter waterretensie tot gevolg gehad as water-geïsoleerde mikrobiese kolonies. Hierdie verskynsel was verder bestudeer deur die gebruik van vloeiende, gevul met grond of sand en geïnkuleer met EPP-produseerders geïsoleer vanuit grond of water. Fluoresensie mikroskopie het aangetoon dat grondmikrobiese kolonies EPP produseer wat die porieë tussen gronddeeltjies meer effektief verstop. Dié verstopping het gelei tot die verlaging van die grondwater se hidrouliese gradiënt wat bepaal is deur die gebruik van die konstante-vlak bepalingmetode. Om die effek van EPP-produseerders op bestaande mikrobiële populasies te bepaal, is seltellings, BiologTM heel-gemeenskap koolstofverbruik studies en T-RFLP (terminale-restriksie fragment-lengte polimorfisme) analises uitgevoer. Veranderinge in die mikrobiële populasie kon nie geredelik bloot deur die bepaling van mikrobiële getalle en T-RFLP-analise waargeneem word nie, maar wel met die koolstofverbruikspatrone.

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CHAPTER 1

CHAPTER 1

Introduction and Objectives

All ecosystems and human societies depend on a healthy and productive natural environment that contains diverse plant, animal and microbial species. The earth's biota is comprised of an estimated 10 million species of plants, animals and microbes (Pimm *et al.*, 1995). Losses in biodiversity have been escalating with the growing encroachment of human activities on ecosystems and increasing intensification of land use to meet demographic and socio-economic pressures. Recent extinction rates of species range from approximately 1000 to 10000 times higher than extinction rates calculated or observed earlier (Kellert & Wilson, 1993), and if this trend continues as many as 2 million species of plants, animals and microbes will be exterminated worldwide by the middle of the 21st century (Pimm *et al.*, 1995). This forecast is alarming because biodiversity in general, and soil biodiversity in particular, is essential for the sustainable functioning of the agricultural, forest and natural ecosystems on which humans depend.

Efforts to curb the loss of biodiversity have intensified in recent years, but they remain modest and have not kept pace with the rate of human-induced change. Furthermore, their application has been primarily focused on preserving a small number of species of large plants and animals, while neglecting the small organisms. However, the numerous small organisms that inhabit the soil such as fungi, nematodes, insects and bacteria, dominate the structure and the basic functions of natural ecosystems. Holistic strategies are therefore needed to protect whole ecosystems to conserve total biological diversity (FAO, 1996).

Questions, therefore, as to why soil biodiversity needed to be managed and conserved, led researchers to reasons focusing on the ecological, agronomic, socio-economic, ethical and moral importance of this diversity.

Altieri (1995) claimed that because soil biota and their activities are fundamental to soil fertility and agricultural productivity, management objectives should focus upon mitigating the negative impacts of agricultural practices on these organisms and promoting their beneficial effects on sustainable agronomic productivity. To add to this, Swift (1996) stated that as human interference and the use of external inputs decrease, soil biodiversity and the role of soil biological processes in maintaining soil

fertility and productivity increase, and the opportunities for soil biological management become more feasible. The imbalance between the short-term (socio-economic) and long-term (ecological) perspective when deciding how to manage agricultural production may have disastrous consequences given the immense scale of agricultural activities worldwide. According to Pimentel *et al.* (1997) approximately 50% of the terrestrial areas are devoted to agriculture, while in temperate ecosystems agriculture occupies 70% of the land. Dixon *et al.* (1994) ascribes only 9% of terrestrial areas to agriculture (figure 1.1).

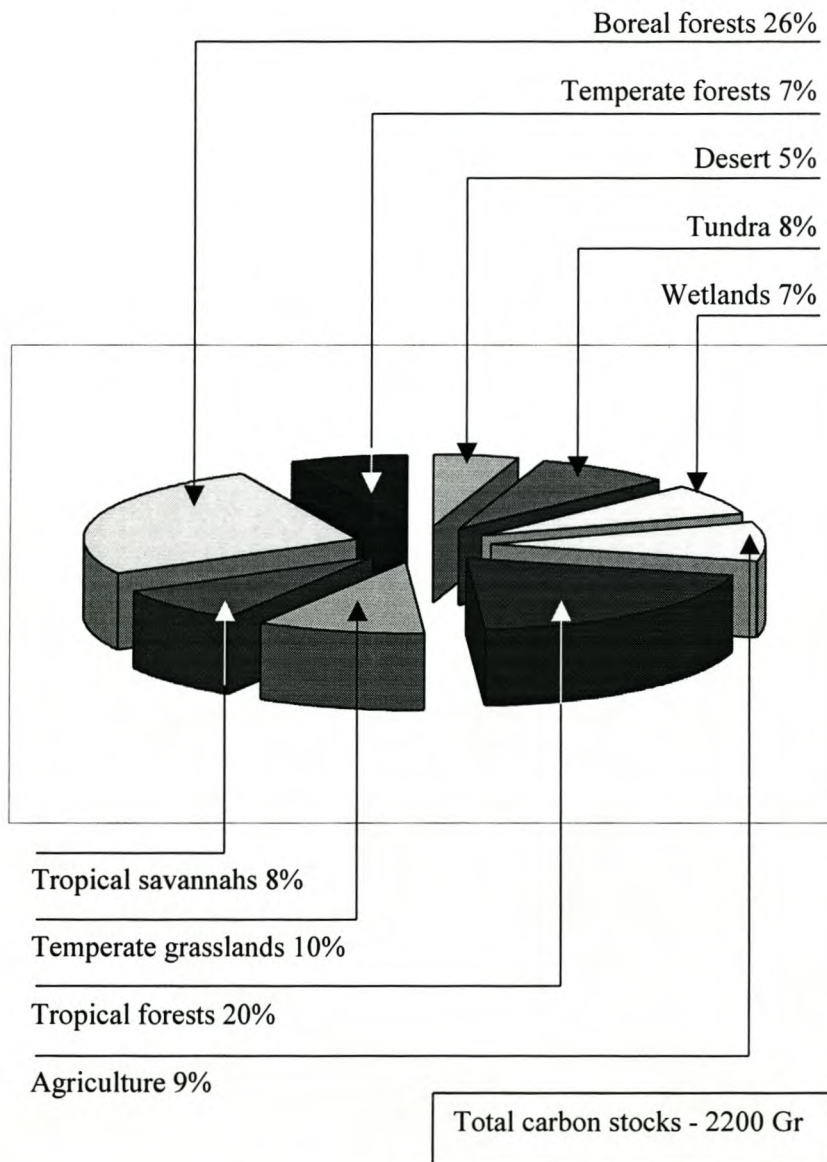


Figure 1.1 Terrestrial carbon stocks by ecosystem. Image adapted from Dixon *et al.* (1994) and Schlesinger (1997).

Ecologically, soil microbiota is responsible for regulating several critical functions in soil and the stability and resilience of the ecosystem to perturbation can be significantly affected by their activities. Soil microbiota also play an important role in regulating decomposition and nutrient cycles, soil structure, gas exchange, soil hydrological processes, control of pests and diseases, soil detoxification and plant production. The presence of a range of species and organisms capable of supporting these critical soil processes, is essential for the maintenance of healthy productive soils in the face of changing environmental conditions, which subject the system to different degrees of stress and magnitudes of shock. Thus, excessive reduction in soil biodiversity, especially the loss of key species or species with unique functions (e.g. symbiotic microorganisms) may have catastrophic ecological effects (Barros, 1999), leading to the long-term deterioration of soil fertility and the loss of agricultural productive capacity.

On the ethical or moral stance, the intrinsic value (i.e. the value in and of itself), regardless of its potential or actual use, of biodiversity has been stressed by various authors (Kellert & Wilson, 1993; Hågvar, 1994; McNeely *et al.*, 1995). It is also well recognized that, to varying degrees, most of the world's religions give intrinsic worth to the natural world, and it is unlikely that this deep-seated notion will disappear, even despite the force of the economic use values placed on biodiversity (Gaston & Spicer, 1998).

Soil, as one of the major components of the natural world, represents a highly heterogeneous environment consisting of solid, liquid and gaseous phases. The dominating soil solid phase is composed of inorganic (sand, silt and clay) and organic (humic matter) materials, which to varying degrees, complex with one another. The soil biota, including soil microorganisms such as bacteria, fungi and protozoa, are known to inhabit different sites in the soil pore matrix (figure 1.2). Organisms associate in particular with soil solids, e.g. clay / organic matter complexes, within pores between soil grains that aid in their survival (Sooksa-nguan, 1999).

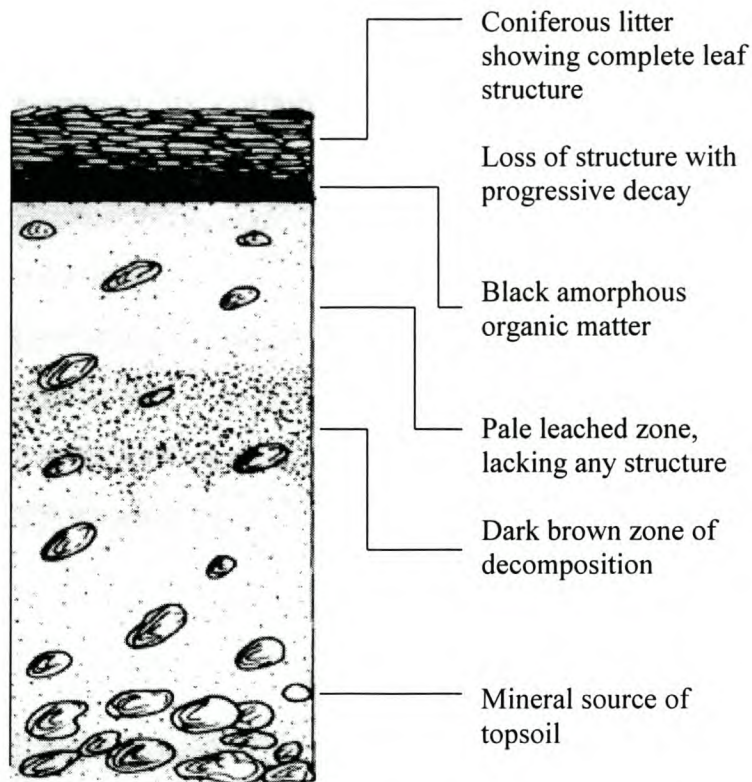


Figure 1.2 A schematic diagram of a typical soil profile (a podzolic soil) showing the different phases or zones inhabited by microorganisms. Image adopted from B.N. Richards (1987).

As previously mentioned, soil represents a favorable habitat for microorganisms and as a result, is inhabited by a wide range of microorganisms, including bacteria, fungi, algae, viruses and protozoa. These microbes are found in large numbers in soil (e.g., approximately between 1×10^6 and 10×10^6 cells per gram of soil, with bacteria and fungi being the most prevalent) (O’Gara, 1993).

However, the availability of nutrients or water is often limiting for microbial growth in soil and most soil microorganisms may not be physiologically or metabolically active in the soil at a given time. Microorganisms are important in soil as the majority of chemical transformations taking place in soil involve active contributions from microorganisms. In particular, they play an important role in soil fertility through their involvement in the cycling of nutrients essential for plant growth, such as carbon and nitrogen. For example, soil microorganisms are responsible for the decomposition of the organic matter entering the soil (e.g. plant litter) and therefore in

the recycling of nutrients in soil. Certain soil microorganisms such as mycorrhizal fungi can also increase the availability of mineral nutrients (e.g. phosphorus) to plants. Other soil microorganisms can increase the amounts of nutrients present in the soil. For instance, nitrogen-fixing bacteria can transform nitrogen gas present in the soil atmosphere into soluble nitrogenous compounds such as nitrates and ammonium that plant roots can utilize for growth (O'Gara, 1993).

The adherence of soil particles to form stable aggregates increases with the addition of monosaccharides or polysaccharide polymers to soil, either as plant (organic) residues, microbial metabolites, or as simple carbohydrates (Martens & Frankenberger, 1992).

Truly useful measurements of microbial adhesion on sediments in terms of relevant exopolymer components and associated erosion resistance are non-existent. The purpose of this research was, therefore, to study some aspects of exo- or extracellular polymers (EPS) in soil in order to evaluate the potential improvement of agricultural soils through directed microbial activity.

A major challenge in agriculture is to remain competitive without compromising ecosystem health. While intensive agricultural practices generally result in loss of desirable soil structure and a reduction of soil organic matter (Tate 1987), soil microbial communities are known to play a key role in soil improvement and stabilisation. However, the microbes do not act alone – properties of the soil ecosystems are ultimately defined by the extensive nature of interactions between its chemical, physical and biological components. The extracellular polymeric substances (EPS) produced by the microbes, as discussed earlier, act as important mediators for these interactions, especially because of their role in aggregate formation. The ultimate aim, therefore, was to evaluate controlled EPS production *in situ*, and to evaluate the effects such action may have on soil stability.

The specific objectives of this study were:

- 1) To isolate bacteria capable of EPS production from natural environments.
- 2) To determine if the selected EPS-producers in pure culture could retard water movement through soil profiles with and without the addition of an exogenous nutrient source.
- 3) To develop techniques to observe EPS production and accumulation in the pores between sand particles.
- 4) To determine the effect of EPS production on soil hydraulic conductivity.

- 5) To determine the fate and impact of introduced EPS-producers in soils with naturally-occurring microbial communities.

CHAPTER 2

CHAPTER 2

Literature Review

2.1 Soil

2.1.1 Structure and properties

According to Lascano (1997) soil is referred to as the weathered and fragmented outer layer of the earth's surface. In the physical sense the soil represents a complex disperse system consisting of three phases: solid, liquid and gaseous. The solid phase of soil consists of individual diverse particles, according to their chemical composition and dimensions. Lascano (1997) also describes soil as a heterogenous three-phase system. It is disperse because at least one of the phases can be divided into minute particles that together exhibit a very large surface area, and it is heterogenous, because it has different properties in different parts of the system. The size of soil particles fluctuates across a wide range. The different properties of the soil and its fertility depend, to a considerable extent, on the composition and size of the particles. On the other hand, the quality of the soil as an environment for microorganisms is determined not only by the size of the particles but also by the character of their distribution, in other words, by the structure of the soil (Krasil'nikov, 1958).

Soil may exist in two states: in a single particle state or in a form of aggregates. In the single particle form all properties of the soil are distributed in all directions. Such soils are called structureless. Structured soil consists of aggregates of different sizes, from less than 1 to 20 mm and more in diameter. The aggregates in the soil are more or less loose; the intervals between them are of different sizes and forms. These intervals or pores ensure the presence of water, air permeability and various foodstuffs for plants and microorganisms. In such soil the evaporation of water is diminished. The structure of soils is therefore of utmost importance. With each increase in the degree of orderliness of the structure, the physical properties of the soil such as aeration, water permeability, etc. are improved (Krasil'nikov, 1958). Due to the ability of the structured soil to absorb and hold water, as well as to its other properties, the structured soils are better able to provide conditions indispensable for the growth of microorganisms and hence, the growth and development of plants.

The various researchers investigating the role of microbes in the process of structural formation had different approaches to the problem of the mechanism of microbial action. Some of them assumed that microorganisms, by decomposing plant residues, form intermediate decomposition products that are responsible for glueing together the soil particles. Others assumed that soil particles are coalesced by the products of microbial metabolism, while plant residues serve as substrate for their nutrition (Rubashov 1949). Some scientists (Kanivets 1951) suggested that fungi such as *Trichoderma lignorum*, *Mucor intermedius* and *Mortierella isabellina* are the most important factor in the process of structure formation. Other investigators tested *Trichoderma kőningii*, *Aspergillus niger* and other fungi with positive results (Martin & Waksman 1940; Peele 1940; Martin 1942; 1945b; 1946; Peele & Beale 1941).

Kononova (1951) meanwhile assumed that the cementing substance consists of a mixture of cellulose decomposition products and protoplasm of the decomposed cellulose bacteria.

All these studies demonstrated the role of microorganisms in the formation of soil structure. However, the essence of this action remains unclear.

Chenu & Stotzky later (2002) stated that the understanding of how soil characteristics control the activity of microorganisms in soil, is essential to predict the occurrence and rate of microbially mediated functions. These functions are of agronomic and environmental importance, such as nitrogen mineralization, denitrification, biological nitrogen fixation, turnover of carbon (C) and nitrogen (N), stability of soil structure, biodegradation of organic pollutants, soil-borne pathogenicity, etc. Such knowledge is also needed to optimize the success of microorganisms purposely introduced to soil and to improve the bioremediation of pollutants.

As mentioned, soil microbiota inhabit different sites in the soil pore matrix (figure 2.1). A healthy soil consists of a variety of small particles that are structured into clumps or aggregates (as described above). Mineral and organic particles are thereby intimately mixed in soil, forming various types of these soil aggregates, which are found in association with air (i.e. the soil atmosphere) and the aqueous phase (i.e. soil water) in the pores between particles and / or aggregates. These aggregates provide a myriad of tiny caves and cracks that help hold nutrients, water and organic matter (Rillig *et al.*, 1999).

Most studies on the effects of increased atmospheric CO₂ focus on the plants in ecosystems. However, it has been demonstrated that CO₂ also affects the structure of soil, including those properties that are important in preventing erosion and maintaining fertility. These properties include soil aggregation, which is important for preventing soil loss through wind and water erosion, as well as the size-distribution and abundance of water-stable aggregates, which in turn influence a range of physical, chemical, biological and agricultural properties of soil (Rillig *et al.*, 1999).

To examine the effects of elevated atmospheric CO₂ on soil aggregates, Rillig *et al.* (1999) examined soils from three ecosystems and experimentally exposed them to a range of different CO₂ concentrations for 3 to 6 years. Their conclusion was that elevated [CO₂] appears to stimulate changes in soil structure, leading to an increased abundance, stability or size of aggregates. These workers claimed that the effect of elevated CO₂ levels (or concentrations) on soil structure creates the possibility of a whole class of indirect, but not yet studied effects of elevated [CO₂]. It was also stated that in the long run, they might find that the effect of [CO₂] on erosion and fertility, are comparable in importance to its effect on plant growth (Rillig *et al.*, 1999).

In addition to the potential effect of CO₂, many studies have documented that intensive tillage results in deterioration of soil structure (Dalal & Mayer, 1986; Reganold *et al.*, 1987; Havlin *et al.*, 1990). Changes in management, such as tillage reduction, or use of cover crops, have often been reported to induce improvement in soil structure without observable changes in total organic C or total carbohydrate content (Hamblin & Davies, 1977; Baldock *et al.*, 1987).

2.1.2 Agricultural considerations of soil

A soil's stability and resilience affects the stability and resilience of the remaining parts of the ecosystem. If a soil is degraded, the ecosystem is impaired. Organic matter helps stimulate microbial populations that are essential to the stability and resilience of the soil ecosystem as a whole. Organic matter also helps in revegetation and erosion control (Alexander, 1999). Examples of sources for organic matter can be found in agricultural by-products from corn (*Zea mays*) and soybeans (Glycine) (Aulakh *et al.*, 1991) or it can also come from industrial by-products such as paper mill and cardboard sludge (Xiao *et al.*, 1999). Organic matter from sewage sludge

can exert significant influence on the physical, chemical and biological properties of soils. In general, organic matter greatly contributes to soil's productive capacity. When incorporated into the soil surface, it can affect its structure, as denoted by porosity, aggregation, and bulk density, as well as causing an impact as expressed in terms of content and transmission of water, air and heat, and of soil strength. Nutrients are mineralized during organic matter decomposition. Carbon, nitrogen and cation exchange capacity increase following organic matter additions.

Other soil chemical properties such as pH, electrical conductivity, and redox potentials are also changed by organic matter decomposition. The soil biosystem can be altered by addition of new energy sources for organisms, reflected by changes in micro- and macro-biological populations, which in turn influence synthesis and decomposition of microbially-produced soil humic substances, nutrient availability, and interactions with soil inorganic components. While it is commonly recognized that organic matter imparts a desirable physical condition to soils, as well as altering chemical and biological relationships, the mechanisms by which it reacts have not been well documented (Clapp *et al.*, 1986).

2.1.3 Problems with soils

2.1.3.1 Erosion

The soil surface is subjected to vast inputs of energy from rainfall, runoff, wind and solar radiation as well as a wide range of human and biotic inputs. Particularly, in the case of energy from rainfall and runoff causing water erosion, the operative processes are destructive, both to soil structure and to its capacity to sustain biomass growth (Lal, 1998). Rainfall impact and the shearing forces of runoff disintegrate soil aggregates (Le Bissonnais & Arrouays, 1997) and transport fertile topsoil along with plant nutrients and organic matter away from eroded soil landscapes (Rogers, 1941; Massey & Jackson, 1952; Lal, 1980; Zobish *et al.*, 1995) (figure 2.1).

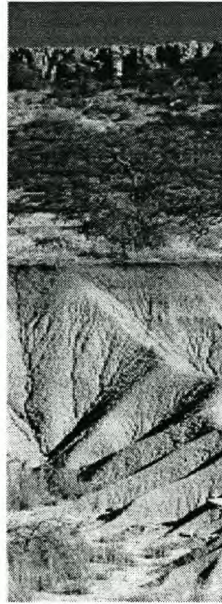


Figure 2.1 Erosion is the number one factor degrading soils globally. It is a process where wind and water facilitate the movement of topsoil from one place to another. Image adopted from State of the environment - South Africa: Terrestrial ecosystems: Overview, Department of Environmental Affairs and Tourism (1999).

The impact of erosion on net primary productivity can lead to serious reduction in agronomic yields, particularly in severely eroded soils that are not adequately fertilized (Lal, 1998). The reduction in living plant biomass that accompanies accelerated soil erosion is implicated as a causative factor in reduced soil C levels through reduced inputs of litter and root biomass to the soil organic carbon (SOC) pools (Gregorich *et al.*, 1998).

Little is known of the properties and fate of SOC that is translocated from erosion to deposition points on the landscape. The physics of particle settling would tend to suggest that the extent of erosional translocation is negatively correlated with the size and density of detached soil aggregates and primary particles. Thus, soil erosion is a highly selective process that preferentially removes the smallest and lowest density components of soil and transports them over great distances (Lal, 1995). This means that eroded agricultural soils are deprived of the bonding substances for aggregation, as these small and low density components are the most fertile of all soil particles.

2.1.3.2 Soil water-repellency

It is also important to note that soils may become water-repellent through the coating of soil particles or structural elements, with water-repellent organic substances

originating from decaying plant material. Due to the adverse and sometimes devastating effects of soil water-repellency on environmental quality and agricultural crop production, soil water repellency is receiving increasing attention from scientists and policy makers (Ritsema, 1998). Ritsema (1998) claimed that soil water-repellency often leads to severe erosion and runoff, rapid leaching of surface-applied agrichemicals, and loss of water and nutrient availability for crops. In general, soil water-repellency manifests itself when the water content of the soil drops below a critical level. Also, according to Ritsema (1998), water flow and solute transport patterns are adversely affected under such conditions.

At present many laboratory and field methods exist to determine soil-hydraulic properties, including especially the unsaturated hydraulic conductivity (Green *et al.*, 1986; Klute & Dirksen, 1986). These properties are important for determining rates of infiltration and water flow in soils. Most methods to obtain these properties are relatively time-consuming and costly. One relatively simple laboratory method for simultaneous estimation of both retention and unsaturated hydraulic conductivity data (for the past forty years) has been the evaporation method. Gardner & Miklich (1962) first introduced this method. They imposed a series of constant fluxes on one side of an initially equilibrated sample, and measured the pressure head response of two tensiometers. The flux needed to be sufficiently small to assume a constant hydraulic conductivity and diffusivity in the sample (Halbertsma & Veerman, 1994). Wind (1968) introduced an iterative graphical procedure to calculate the water retention characteristic from average water content and pressure head readings at several locations in a homogenous soil sample (Simunek *et al.*, 1998).

2.1.3.3 Soil compaction and sealing

Land degradation is a serious worldwide environmental problem. Most significant forms of land degradation are soil erosion (both by water and wind), deterioration of vegetation, soil compaction and surface sealing, water logging and salinization (Al-Dousari *et al.*, 1999). Soil crusting is a significant form of land degradation, particularly in severely degraded rangelands. Generally it occurs in poorly sorted, fine- to medium-textured soils after the deterioration of the vegetative cover, and it inhibits the water infiltration into soils and promotes runoff (Morin *et al.*, 1981). Among other land degradation indicators, surface sealing and soilcrusting are two important processes leading to physical degradation of soil (FAO, 1979). A crusting

soil type typically contains dispersible clay which, with mechanical energy, e.g. raindrop impact, breaks free of its attachment within aggregates and goes into suspension (Le Bissonnais *et al.*, 1989; Bradford & Huang, 1992; Sumner & Miller, 1992; Valentin & Bresson, 1992; Sutherland *et al.*, 1996). This clay ultimately accumulates at the surface to form a crust or translocates into the soil as an internal sealing agent of pores. In addition, these processes interact with other soil degradation processes such as water erosion (Misak & Al-Dousari, 1997).

Previous studies conclusively demonstrated that soil surface sealing and compaction decreases rainfall infiltration rates and hence increases surface runoff volume (McGinty *et al.*, 1979). Consequently, surface sealing and compaction increase runoff erosion. Johnson *et al.* (1979) claimed that the formation of a surface seal and consequently a crust reduces sheet and stream erosion rate. Bruand *et al.* (1993) reported a decrease in pore space percentages at different soils in Zimbabwe by using scanning electron microscopy. So far, there is no single method to investigate the properties of a soil crust. Few techniques in this respect are micromorphological (Evans & Buol, 1968; Miedema *et al.*, 1999), or penetration tests from above or below the crust surface (Holder & Brown, 1974).

The physical properties of soil can be enhanced by applying sewage sludge (Epstein *et al.*, 1976; Clapp *et al.*, 1986; Tester, 1990) and thus cut down the risk of erosion of degraded lands (Sort & Alcañiz, 1996). Therefore, considering the control of erosion as a priority issue in rehabilitating degraded lands, it is interesting to examine how the application of organic amendments may modify soil aggregation and its structural stability.

2.1.3.4 People and soil sustainability

Human activity has affected every part of the planet, no matter how remote, and every ecosystem, from the simplest to the most complex. Our choices and interventions have transformed the natural world, posing both great possibilities and extreme dangers for the quality and sustainability of our civilizations, and for the intricate balances of nature. Our numbers (figure 2.2) have doubled since 1960 to 6.1 billion, with growth mostly in poorer countries. We have since learned how to extract resources for our use, but not how to deal with the resulting waste: emissions of carbon dioxide, for example, grew 12 times between 1900 and 2000. In the process we are changing the world's climate. Humans have always changed and been changed

by the natural world; the prospects for human development now depend on our wisdom in managing the relationship. One of the key factors will be population growth which is on the increase and thereby affects the environment (New Scientist, 2001).

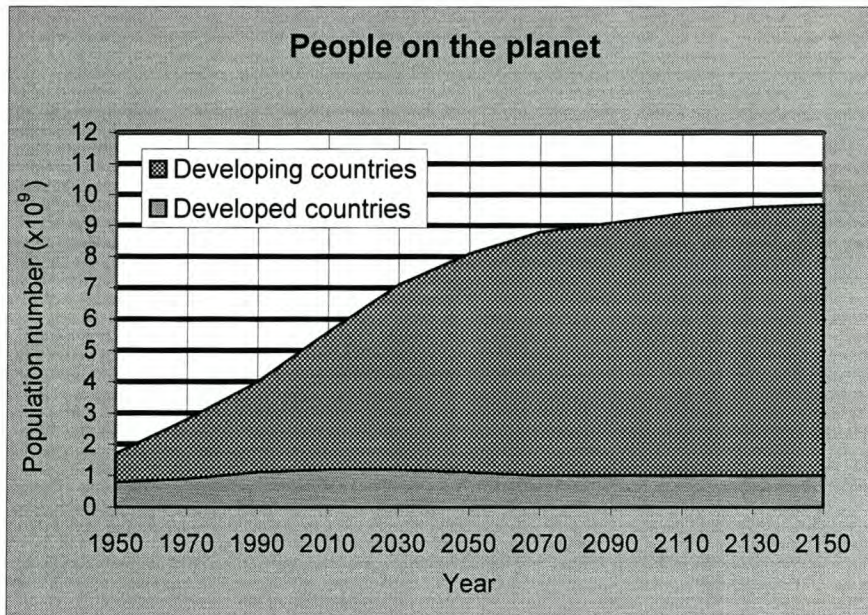


Figure 2.2 As the global population grew during the 20th century, scientists responded with new ways to feed extra mouths. A new analysis, however, predicts the global population to peak by 2070 and suggests a maximum of nine billion people, with over a third being over 60 by 2100. The y-axis represents the number of people in billions. Image adapted from the Global Population Trends Report, New Scientist (2001).

Key policy questions therefore exist as for: how can available resources of land and water be used to produce food for all; how could economic development be promoted and thereby end poverty so that all can afford to eat and, in doing so how to address the human and environmental consequences of industrialization and concerns like global warming, climate change and the loss of biological diversity (New Scientist, 2001).

More people are using more resources with more intensity than at any point in human history. Population growth, increasing affluence - with rising consumption, pollution and waste - and persistent poverty - with lack of resources and the technology to use them and lack of power to change these circumstances - are putting increasing pressure on the environment (figure 2.3).

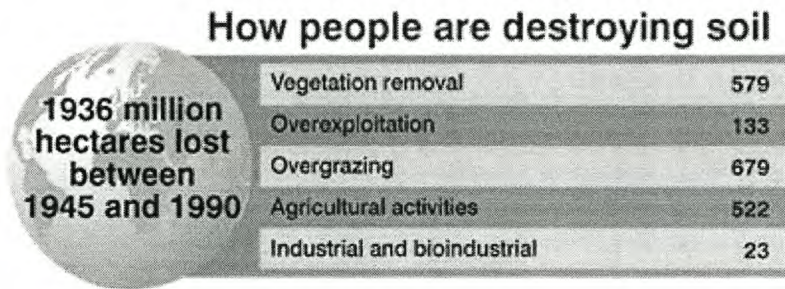


Figure 2.3 As population growth has peaked, deforestation rates have reached the highest levels in history. Two-thirds of the earth's land surface will be "destroyed, fragmented or disturbed" by human activity within 30 years, a UN report stated. Image adopted from the Global Population Trends Report, New Scientist (2001).

Never has the pressure on the world's resources been so great. Over the next 20 years, the global population is expected to grow by a quarter - that's an extra 1.5 billion mouths to feed. This at a time when, even though we grow enough to feed everyone, 800 million people are still malnourished (New Scientist, 2001).

So, what amount of soil or to what extent can we regard soil to still be arable for sustainable use and how long would it last (figure 2.4)?

2.1.3.5 Farming

In some circles, agriculture is regarded more like mining than farming. In an article that was published by the Greenpeace Organization in a New Scientist Report (2001) it is indicated that farming compromises the very earth on which all our future food needs depend. Only about 16% of the world's farmland remains free of problems such as chemical pollution. It is stated that farm chemicals such as weedkillers and chemical fertilizers have created resistant weeds, added to global warming, degraded the earth's soil, polluted drinking water and killed off wildlife such as birds and fish. Farming methods that undermine people's food security affects more than just those who go hungry. Greenpeace also claims that these farming methods undermine the environment and thereby forest wilderness and wildlife are destroyed in the search for food and land to farm. This leaves little, if any, room for future farming (figure 2.5) and hence food production. It is therefore essential to base assumptions on

scientifically obtained data when the role of farming in addressing the challenges associated with population growth (figure 2.2) is of concern.

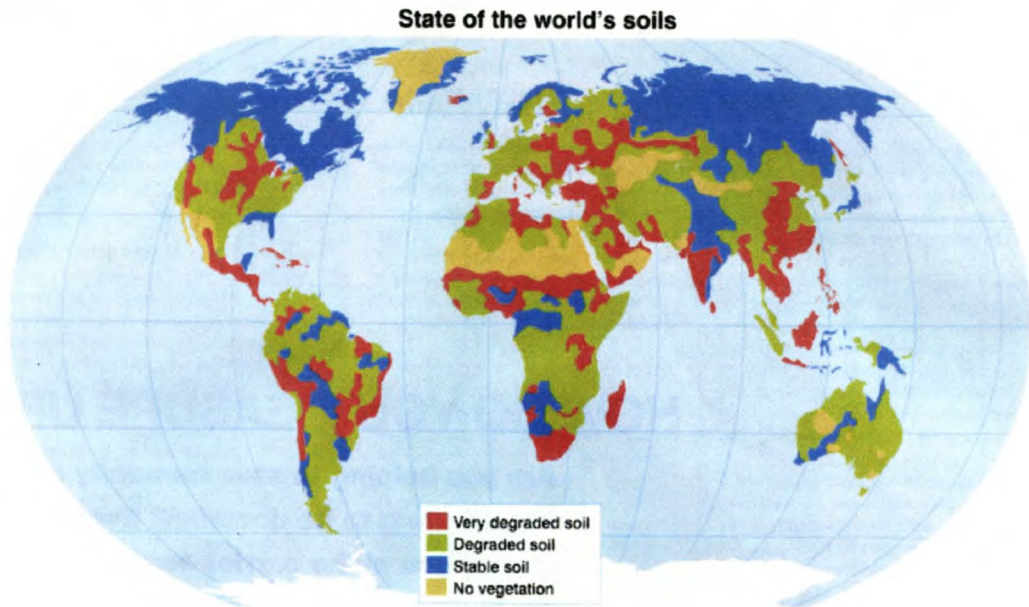


Figure 2.4 A new report highlights the overuse of natural resources and warns that by 2050 we may need two earths to live sustainably. Image adopted from the Global Population Trends Report, New Scientist (2001).

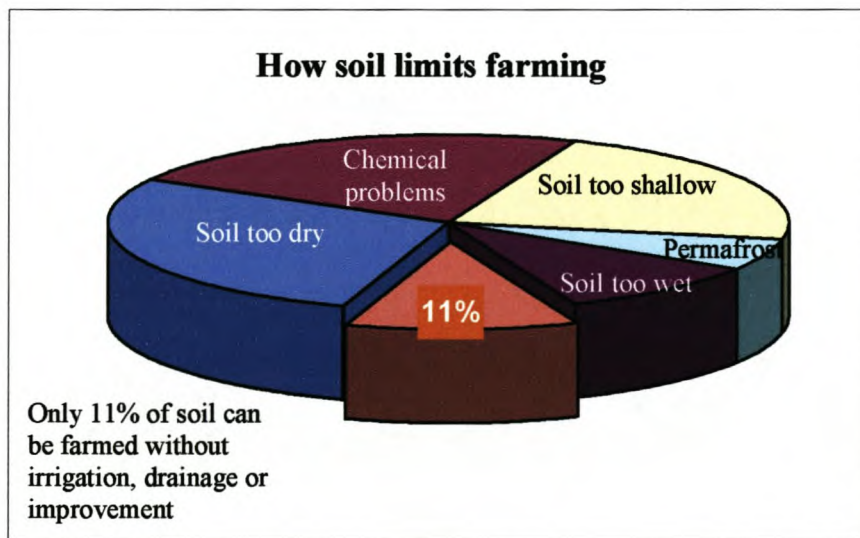


Figure 2.5 Division and percentages of the total world land area. Too dry soil takes up 28%, whereas chemical problems takes 23%, too shallow soil 22%, permafrost 6% and too wet soil 10% leaving only 11% for productive farming. Image adapted from the Global Population Trends Report, New Scientist (2001).

2.1.4 Microbially mediated soil processes

Nutrient-cycling in soil can be indexed by measurement of soil enzymes, cellulose or wood degradation, respiration and various components of the nitrogen as well as the carbon cycles (Sims, 1990). Carbon entering the soil from plant sources, (e.g. as cellulose) usually leaves as carbondioxide or methane through the processes of microbial and plant root respiration, and methanogenesis, respectively. Nitrogen cycling, on the other hand, includes mineralization, immobilization, nitrification, denitrification, and nitrogen fixation. Nitrogen fixation and nitrification are most easily disrupted. Nitrifying bacteria (e.g. *Nitrosomonas* and *Nitrobacter*) are sensitive to acidity and require aerobic conditions. Waterlogged soils can become anaerobic and may not support nitrification (Sims, 1990).

These two cycles, apart from other nutrient cycles, are probably the most important in nutrient regulation and hence, bacterial processes in soils. Successful use of C/N ratios to enhance bacterial processes requires knowledge of how the C/N ratio influences bacterial adhesion and convective transport, bacterial growth, contaminant biodegradation kinetics, and nitrogen utilization. Microbes are of significance to the soil environment, inter alia, in that microbial films that contain adhesive polymers are apparently formed by organism response to chemical or fluid stresses within the soil environment (Nickels *et al.*, 1981) or in response to predation or starvation (Dudman, 1977; Geesey, 1982).

2.2 Soil microorganisms

Despite knowledge of how soil microorganisms maintain critical processes such as carbon storage and nutrient cycling, their influence on plant species diversity (Huston, 1993), or how soil organisms participate in forming soil structure, the organisms themselves and the interactions between them remain a “black box” in our understanding of how soil systems function. Without accurate knowledge of soil biodiversity, the structure and interactions of the soil community (figure 2.6), and the relationship of soil biology to ecological processes, management of ecosystems, and the models of ecosystem functioning upon which management is based, will always be less than rigorously understood or well defined.

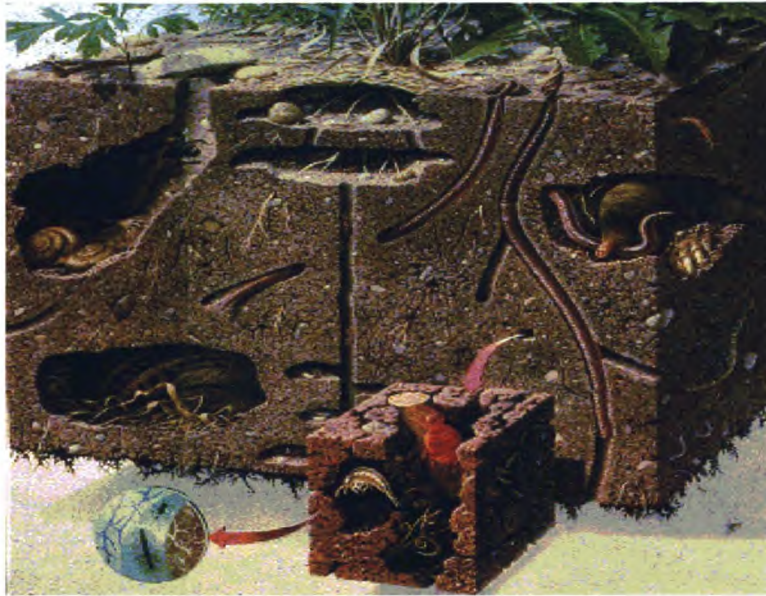


Figure 2.6 Soil biodiversity ranges from macroorganisms such as earthworms, termites and soil micro-fauna to microorganisms such as the soil actinomycetes and soil bacteria. However, each one of them plays a significant role in soil regulatory processes. Image adopted from S. Bunning, Land and Water Development Division, Food and Agriculture Organisation (2001).

Soil organisms contribute a wide range of essential services to the sustainable function of certain ecosystems, by acting as the primary driving agents of nutrient cycling, regulating the dynamics of soil organic matter, soil carbon sequestration and greenhouse gas emission; modifying soil physical structure and water regimes, enhancing the amount and efficiency of nutrient acquisition by the vegetation, and enhancing plant health. These services are not only essential to the functioning of natural ecosystems but constitute an important resource for the sustainable management of agricultural systems (FAO, 2001).

Microbes are the most diverse group of soil organisms. These soil microorganisms are living in complex communities, where they are responsible for nutrient cycling and other functions as mentioned above. Until recently, research has focused on those organisms that can be cultured; however, a wealth of information is now being collected from both cultured and uncultured organisms. Functions of the soil microbial population impact many soil processes, and therefore soil productivity. Without microbes and their functions, no other life forms can exist (Kennedy &

Gewin, 1997). In addition, the roles of the various species are inextricably interconnected, so that, in experiments, direct and indirect treatment effects may be indistinguishable.

Soil bacteria are mostly heterotrophic. Their single-celled structure gives them access to resources and refugia unavailable to larger organisms, but it may be a disadvantage in accessing nutrients that are contained in relatively large particles (i.e. plant residues) that may contain valuable carbon, but be deficient in other nutrients. Motile bacterial types can also occur in colonies in the soil, and this may confer advantages such as improved nutrient mobilisation and absorption, and protection from drying, toxins or ultraviolet radiation (Sims, 1990).

The study of microbial diversity in soil is often hampered by limitations in isolation and culture techniques. It has been estimated that we are able to culture less than 1% of the microbes present in soil (Rozsak & Colwell, 1987). In order to overcome these problems, various methods to assess microbial diversity have been developed to circumvent the need for isolation. Many nonmolecular and molecular methods are available. These methods include fatty acid methyl ester (FAME) analysis (Zelles & Bai, 1994; Cavigelli *et al.*, 1995; Buyer & Drinkwater, 1997; Griffiths *et al.*, 1999) and phospholipid fatty acid analysis (Vestal & White, 1989; Wander *et al.*, 1995; Bossio *et al.*, 1998; Ibekwe & Kennedy, 1998; Griffiths *et al.*, 1999). Biolog-substrate-utilisation-profile analysis (Buyer & Drinkwater, 1997; Ibekwe & Kennedy, 1998; El Fantroussi *et al.*, 1999), guanine-plus-cytosine composition analysis (Harris, 1994; Griffiths *et al.*, 1999; Nüsslein & Tiedje, 1999), amplified ribosomal DNA (deoxyribonucleic acid) and restriction analysis (Fries *et al.*, 1997; Dunbar *et al.*, 1999) are commonly used. Cloning and sequencing (Borneman *et al.*, 1996) and denaturing gradient gel electrophoresis (DGGE) (Harris, 1994; McCaig *et al.*, 1999) are also used. Other methods include two more recent techniques, terminal restriction fragment length polymorphism (T-RFLP) analysis (Avaniss-Aghajani *et al.*, 1994; Bruce, 1997; Liu *et al.*, 1997) and length heterogeneity PCR (LH-PCR) (Suzuki *et al.*, 1998).

LH-PCR analysis is similar to the more commonly used T-RFLP method. The difference between these two methods is that the T-RFLP method identifies PCR fragment length variations based on restriction site variability, whereas LH-PCR analysis distinguishes different organisms based on natural variations in the length of 16S ribosomal DNA sequences (Ritchie *et al.*, 2000).

2.3 Biofilm basics

A common feature of most microorganisms, including those found in soil, is that they produce a slimy substance when they experience stressful conditions (Waksman & Martin, 1939; Martin & Waksman, 1940; Peele, 1940). During these conditions they group together to form biofilms (figure. 2.7). Biofilms have been described by various researchers as communities of microorganisms attached to a solid surface (Geesey *et al.*, 1977; O'Toole *et al.*, 2000).

Bacteria within a biofilm adhere to surfaces in aqueous environments where they excrete a glue-like substance that can anchor them to various types of materials - such as metals, plastics, medical implant materials, tissues and soil particles. This is generally referred to as extracellular polymeric substances (EPS). A biofilm can be formed by a single bacterial species, but more often biofilms consist of many species of bacteria, as well as fungi, algae, protozoa, debris and inorganic material.

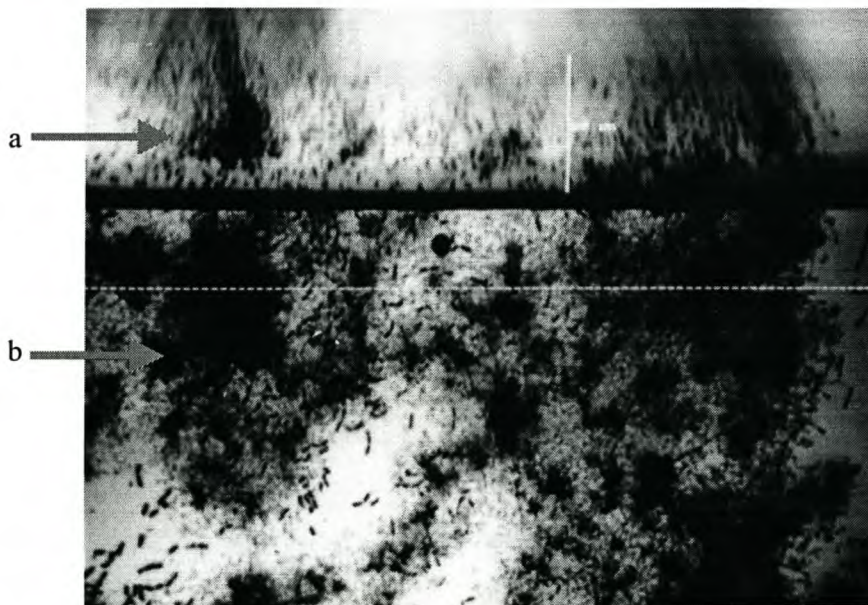


Figure 2.7 Microorganisms colonizing to form a biofilm. The arrows indicate a side view (a) and top view (b) of a biofilm community.

Essentially, biofilms may form on any surface exposed to bacteria and some amount of water. Once anchored to a surface, biofilm microorganisms carry out a variety of reactions that can be detrimental or beneficial, depending on the surrounding environmental conditions. Biofilm bacterial behaviour is often more complex than suspended cell behaviour, because biofilm bacteria, not only live in communities, but

when bacteria attach to a surface, they express a different set of genes, resulting in a significantly different community of organisms (Geesey, 1999).

Studies have revealed that there are significant differences in the level of expression of genes involved in the nutrient cycling among different members of a biofilm community exposed to the same apparent conditions. Within these communities, there appear to be a "division of labor" whereby some cells utilize available energy to turn on metabolic pathways. These pathways affect partial degradation of dead particulate matter, while other adjacent cells of the same population, utilize the degradation products to produce new cells that are dispersed in the environment (Geesey, 1999).

As previously mentioned, biofilm formation and control have become an interdisciplinary research topic. In order to increase our understanding of the formation and control of biofilms, techniques to study these processes have been developed. Some of these methods, however, involve disrupting the biofilm from the surface before the growth parameters are measured. Examples of such parameters include the heterotrophic potential, viable cell counts and total counts obtained with acridine orange staining and epifluorescence microscopy. However, many of the more recent developments in this field have included direct, non-disruptive methods that allow observations of biofilms without removing them from the surface to which they are attached (Lappin-Scott *et al.*, 1993). Such techniques include Fourier transform infrared (FT-IR) spectroscopy (Nichols *et al.*, 1985; Nivens *et al.*, 1986; Jolley *et al.*, 1989; Geesey & Bremer, 1990), confocal laser microscopy (Caldwell & Lawrence, 1989) and nuclear magnetic resonance (NMR) (Hoyle *et al.*, 1990).

The phenomenon of biofilms-research is, however, not only confined to the laboratory. In nature, most microbes also grow as organized biofilm communities on surfaces. Biofilms disrupted from surfaces do not have the same properties as those that are still attached to or intact on a surface (Murray *et al.*, 1987). Also, bacteria at the base of the biofilm have a continuing need for nutrients, so as they grow, channels are left throughout the aggregated colonies of bacteria so that materials can flow through the biofilm. This is similar to the fact that most of the natural biofilms examined to date are composed of discrete microcolonies, separated by open water channels (figure 2.8). These microcolonies that comprise the biofilm are shaped like mushrooms, or stacks, and the water channels are open throughout the sessile community. This degree of structural complexity clearly precludes random growth,

and dictates that we consider some sophisticated form of communication and control when considering the development of biofilms (Costerton *et al.*, 1995).

Growth within the biofilm can influence the morphology of certain species. The predominance of filamentous bacteria leads to a relatively low bacterial density (Picologlou *et al.*, 1980; McCoy & Costerton, 1982; Trulear & Characklis, 1982).

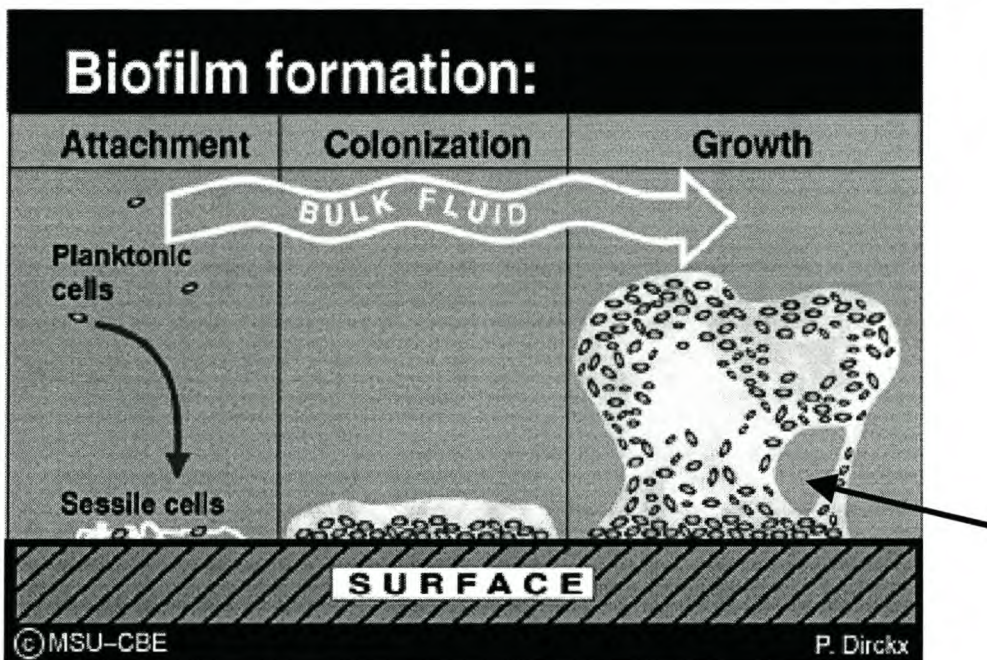


Figure 2.8 Systematic attachment, colonization and maturation of a biofilm. Free-swimming cells accumulate on a surface and attach to each other (left). New genes are expressed to synthesize slime (center). Cells communicate by exchanging signaling molecules (right). The arrow indicates a channel through which water or fluid and nutrients are transported within the biofilm. Image adopted from P. Dirckx, Center for Biofilm Engineering, <http://www.erc.montana.edu/6f-basics-99/bbasics-01.htm> (1999).

Following the general discussion of biofilms, their significance to soils can be evaluated. We know that the slimy, glue-like substances, excreted by the biofilms are, in fact, extracellular polymers of which the polysaccharides are most frequently found (Duguid & Wilkenson, 1953; Wilkenson *et al.*, 1954; Wilkenson, 1958). Several other investigators reported that these slimy substances produced by bacteria and other organisms were effective in binding soil particles into aggregates (Martin & Waksman, 1940; Peele, 1940; Waksman & Martin, 1939). It was only later shown

that the most active binding substances synthesized by bacteria were indeed polysaccharides (Martin, 1945a; 1946).

Before considering the functions of microbial extracellular polysaccharides in soil, it is necessary to establish whether or not these polysaccharides are in fact produced in such locations.

2.4 Properties of Extracellular Polymeric Substances (EPS)

Very little is known about the quantitative aspects of polysaccharide production except those under defined laboratory conditions, but it may be assumed that the same general requirements apply (Duguid & Wilkenson, 1953; Wilkenson *et al.*, 1954; Wilkenson, 1958). In dry environments, moisture may be the most important factor limiting microbial activity, including polysaccharide production (Scott, 1958; Brock, 1975). However, in moist or aquatic environments, the possibility of polysaccharide synthesis by heterotrophic organisms will be determined by the availability of suitable substrates (Poynter & Mead, 1964) or by the ratio of carbon to other nutrients (Dias *et al.*, 1968).

When grown in rich media, microorganisms may produce polymers at a high rate but release these as slime (EPS), rather than retaining the material as a distinct capsule (Christensen & Characklis, 1989).

Because of the importance of polysaccharides to the properties of soils (Mehta *et al.*, 1961, Finch *et al.*, 1971) there have been many investigations of soil polysaccharides and many attempts to determine the respective contributions of plants and microorganisms to the accumulated polysaccharide in soil. There is much evidence, both direct and indirect, that polysaccharides are mainly produced by soil-microorganisms. Radioactively labeled dextran, glucose and plant-tissues added to soil are rapidly incorporated into polysaccharides by microbial action (Keefer & Mortensen, 1963; Oades & Wagner, 1971).

The plant-derived polysaccharides, ammonium alginate, pectin and karaya gum are only moderately active while tragacanth and ghatti gums are highly effective in binding or aggregating soil particles (Martin & Richards, 1969). Most microbial polysaccharides in concentrations of as low as 0,02 – 0,2 % exert a marked binding action on the particles (Martin *et al.*, 1955; Clapp *et al.*, 1962; Martin & Richards, 1969).

Microbial polysaccharides are thus effective organic agents that promote soil aggregate formation and stability. However, the effectiveness of these polymers in stabilizing soil particles varies between microbial strains, the amount and type of polysaccharide and the prevailing environmental conditions (Martens & Frankenberger, 1992). Martens & Frankenberger (1992) conducted glasshouse and laboratory studies to determine the effectiveness of selected microbial polymers in stabilizing soil aggregates. They found that the addition and thorough mixing of 1.0 mg microbial polymer C g⁻¹ soil of different microorganisms to a coarse-loamy soil resulted in stimulated soil respiration, increased aggregate stability and decreased soil bulk density. Statistical analyses showed that the glucose content of the added polymers was significantly correlated with soil aggregation, but the extractable soil saccharides were not significantly correlated with increased aggregate stability or decreased soil bulk density during that study. Soil properties therefore influence the binding action of some polysaccharides in soils. Allison (1947) and Hepper (1975) stated that in natural anaerobic soil the presence of polysaccharides caused a reduction in permeability by blocking the soil pores, leading to conditions, which could be detrimental to plant growth.

Even though EPS contain uronic acids in addition to other polysaccharides, proteins, humic acids, DNA and cell fragments, Clapp *et al.* (1962) concluded that uronic acid groups were not essential for the binding action of polysaccharides in soils. As the concentration of uronic acid units in the polysaccharides increased, the binding action increased with increasing acidity and decreased with increasing base saturation especially with increasing exchangeable sodium (Na) or potassium (K). This could be related to the binding of the uronic acid-containing polymers to the clays by means of iron (Fe) and aluminium (Al) in the acid soils. It can also be related to a reduction of the negative charge of the uronic acid groups that would favor hydrogen bonding (Martin & Aldrich, 1955). The binding action of polysaccharides, not containing uronic acid structural units, was little affected by changes in exchangeable cations.

Fazio *et al.* (1982) concluded that uronic acid components of extracellular materials appear to correlate most closely with materials of cell cytoplasmic membranes and that uronic acid components may provide good measures of intensity of sediment binding by bacterial extracellular products.

Previous studies (Martens & Frankenberger, 1992) have shown that when microbial extracellular polymers were added to soil, only a transient increase in soil stability

was measured upon decomposition of the added saccharides. This finding suggests that the stabilization of soil aggregates is a result of other microbial processes or metabolites rather than the direct binding effects of the added polysaccharides. Two aggregate-stabilizing mechanisms have been proposed for the improvement in soil aggregation and structure following organic additions to soil (Martin *et al.*, 1955). They include a physical entanglement of soil particles with living bacteria and fungal hyphae, and/or the binding of soil particles with gelatinous organic materials produced by microbial decomposition of the added organic materials. Experiments to test these two mechanisms have shown that the addition of organic amendments or simple carbohydrates to sterile soil results in little or no increase in the percentage of soil aggregation, confirming a biotic contribution to soil stabilization (Chesters *et al.*, 1957).

Tiessen & Stewart (1988) found that cells of microorganisms frequently bridge two or more soil aggregates or particles and electron micrographs showed that this attachment was still effective after the cells have disappeared. The addition of microbial polysaccharides (5 mg.g^{-1} soil) of known composition (alginate and xanthan gum) to air-dried crushed soil increased the water-stable aggregates without microbial activity (≤ 1 day incubation). Martin *et al.* (1965) found that the extracellular polymers produced by *Azotobacter chroococum*, *Chromobacterium violaceum*, *Bacillus subtilis*, *Bacillus polymyxa*, *Agrobacterium radiobacter*, and several *Azotobacter indicus* strains, when added at a concentration of 5 mg.g^{-1} soil, were highly effective in stabilizing soil particles as water-stable aggregates when incubated for up to 60 days. The production of extracellular polymers (figure 2.9) is a common property of many soil microorganisms (Allison, 1968).

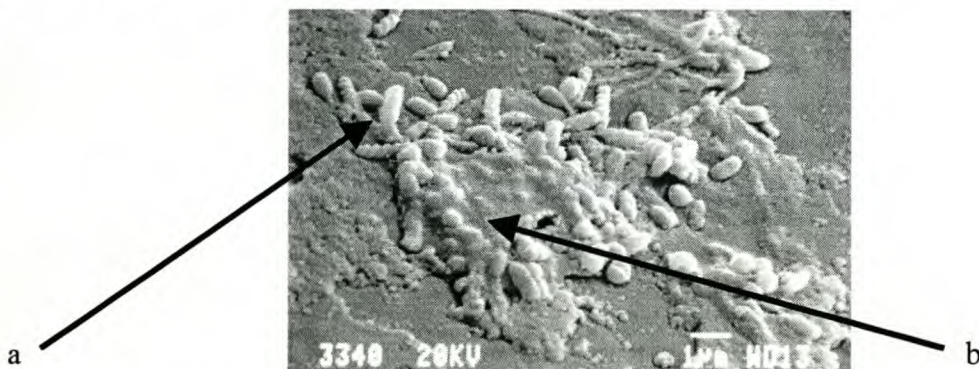


Figure 2.9 A scanning electron microscopy (SEM) image of the polymeric substance excreted by bacterial cells in a biofilm; (a) bacterial cell; (b) slime or polymeric substance (EPS).

The effectiveness of extracellular polymers in stabilizing soil aggregates may be related to composition and quantity of saccharides composing the polymer. Martens & Frankenberger (1991) found that the saccharide composition of 10 microbially produced extracellular polymers (soil isolates) varied greatly and ranged from homopolysaccharides (*Bacillus subtilis*, fructose polymers; *Hansenula holstii*, mannose polymers) to glycoproteins (*Pseudomonas* strains). However, with the exception of CO₂ evolution studies (Martin & Richards, 1963; Martin *et al.*, 1965), little is known about the stability and fate of these saccharides released from extracellular polymers in the soil environment.

It is also known that the effect of polysaccharides on soil physical properties may be greatly influenced by metal ions. If allowed to react with plant and microbial polysaccharides containing uronic acid groups before addition to soil, aluminium (Al) and iron (Fe) ions greatly reduced the binding action of most polymers while copper (Cu) and zinc (Zn) slightly reduced, exerted little effect or even increased binding (Martin & Richards, 1969). If the polysaccharides were allowed to react with the soil before treatment with the metal ions, little effect was noted. It can be concluded that it should be possible to partially manage soil physical properties by the addition of labile or inexpensive nutrients to an environment, which will result in the production of polysaccharides by the soil microbial community.

In general, most EPS related work was performed in aqueous systems. In these studies it was demonstrated that contact with a solid surface triggers the expression of a panel of genes involved in the production of bacterial enzymes, which catalyze the formation of the sticky polysaccharides that promote colonization and protection. These extracellular polysaccharides significantly influence the physical properties of the microorganisms in a biofilm, including diffusivity and thermal conductivity. EPS, irrespective of charge density or its ionic state, has some of the properties of diffusion barriers, molecular sieves and adsorbents, thus influencing the physicochemical processes such as diffusion and fluid frictional resistance (Wimpenny *et al.*, 1993).

Chemical compositions of extracellular polysaccharides reportedly changed during bacterial growth. The vast majority of bacterial EPS are polysaccharides. Common sugars such as glucose, galactose, mannose, fructose, rhamnose, N-acetylglucosamine, glucuronic acid, galacturonic acid, mannuronic acid and guluronic acid are typical constituents of bacterial polysaccharides (Christensen & Characklis, 1989; Christensen, 1989). Uhlinger & White (1983) noted that the amount of

galactose in the exopolymer fraction recovered from a batch culture of *Pseudomonas atlantica* decreased during the growth cycle relative to the other monosaccharides. The high relative amount of galactose observed occurred only very early in the growth cycle, when the absolute amounts of other sugars were low. Later, the ratio between the different sugars was essentially constant. The excess galactose observed initially might have been due to incomplete separation of the polymers from the medium, which contained galactose as the major carbon source.

Little information is available on the effects of nutrient availability on microbial EPS production in soil. Nitrogen availability has been found to affect EPS production by microorganisms in liquid culture (Roberson, 1991). High C/N ratios (i.e. carbon to nitrogen ratios, low nitrogen availability) stimulate polysaccharide production by microbial cultures (Chenu, 1995). This happens only in some systems (Elliott & Lynch, 1984; Auer & Seviour, 1990), while in other systems EPS production is maximal when N-availability is high (Williams & Wimpenny, 1977).

The carbon to nitrogen ratios in some biofilms is considerably higher (approximately five times) than in microbial cells. The high ratios probably reflect a large proportion of EPS (generally low in nitrogen), or a preponderance of carbonate salts if the total carbon rather than organic carbon, is measured (Christensen & Characklis, 1989).

2.5 Functions of extracellular polysaccharides in soil

Interest in soil polysaccharides has largely been concerned with studies of their role in the stabilization of soil aggregates. Statistical analyses have indicated (Rennie *et al.*, 1954; Chesters *et al.*, 1957; Salomon 1962; Webber 1965), that there is often a correlation between aggregation and soil polysaccharide content.

The great majority of polysaccharide-producing microorganisms appear to be unable to utilize their own extracellular polysaccharides as carbon sources and these compounds thus do not serve as reserve sources of carbon and energy (Dudman, 1977). Soil carbohydrates are thought to be partially composed of EPS produced by soil microorganisms (Oades, 1984). Microbial extracellular polysaccharides, thereby, have proved to stabilize soil aggregates (Clapp *et al.*, 1962; Greenland & Oades, 1967; Moloje *et al.*, 1987). Interest in the use of polysaccharide-producing organisms to improve structure and infiltration in agricultural soils has been increasing (Barclay & Lewin, 1985; Metting, 1986). Emphasis was therefore placed on the properties and

origins of the polysaccharides that were effective soil aggregate stabilizers and on the mechanisms by which stabilization could be brought about.

Chenu and Stotzky (2002) stated that extracellular polysaccharides constitute the outermost surface of those microorganisms that produce them. The adsorption of EPS to mineral surfaces causes or strengthens the attachment of bacteria to soil particles. Exudation of EPS results in the establishment of a porous hydrated continuum between cell and its mineral surroundings (Chenu, 1995). The rates of water loss and the rates of decrease in water potential of pure clay-polysaccharide or sand-polysaccharide complexes with desiccation are less with than without EPS (Chenu, 1993). Furthermore, the diffusion of substrates is slow through EPS (Chenu & Roberson, 1996). Consequently, bacterial microaggregates, in which the bacteria are surrounded by EPS and clay, are presumed to be microenvironments that are well buffered against fluctuations in physical and chemical conditions in soils but in which the rate of movement of nutrients and substrates would be released.

The major unanswered question about EPS though, is the quantitative importance of their production in soils. There is no quantification of either the proportion of bacteria that produce EPS in soils or of the amounts produced, because there are no chemical criteria that enable the distinction of EPS from other soil polysaccharides (Chenu, 1995).

2.6 Water retention in soils

2.6.1 Water binding in biofilms

Water represents the main component of biofilms and other microbial aggregates such as flocs and sludge. It is assumed that the matrix is arranged in a sponge-like structure (Decho, 1990), which traps the major proportion of the water in pores and channels. The mobility and viscosity of water is crucial for the biological processes in biofilms as these factors have a strong impact on the transport of substrates and products although very little research on the role of water has been performed. Although a part of the water in biofilms is intracellular and thus trapped by cell walls, the EPS bind most of the water. They represent a highly hydrated matrix with a water content of up to 98% (Christensen & Characklis, 1989). It is known that the EPS matrix is very heterogeneous (De Beer *et al.*, 1994) and that its components can vary strongly according, e.g., to the organisms (Costerton *et al.*, 1994), the nutrient conditions (Uhlinger & White, 1983) and the hydrodynamic conditions (Christensen

& Characklis, 1989). The EPS hold the key position by forming the space, in which the microorganisms are immobilized, as well as keeping them in their three-dimensional arrangement, allowing the formation of stable micro-consortia. All components present in the EPS will influence water binding and water properties to a certain extent and, thus, also influence the biological processes of biofilm organisms (Schmitt & Flemming, 1999).

It is well known that microorganisms play a vital role in a number of issues key to agriculture, such as nutrient cycling, soil stabilization and thus, water retention.

Water provides a major transport mechanism for nutrient flux within and between terrestrial ecosystems. It is a prerequisite for all life, and participates in geochemical cycles by weathering geological substrates, by leaching materials to groundwater and by moving ions and particles through the soil profile. Plants and other organisms within soil alter the various solutes in percolating water, which reach the groundwater system. A dynamic equilibrium is maintained in soil because different types of substances are exchanged and stored. The pore space, which is not occupied by minerals, organic matter and living organisms is filled with air and water. There is a dynamic equilibrium between water and air content within a soil, and when water enters the soil, it displaces air from some of the pores (Juma, 1997).

2.6.2 Water processes

In order to investigate the water properties of EPS, non-destructive methods are preferred as they reflect the actual situation and processes to be observed *in situ*. Among such methods, attenuated total reflectance Fourier transform infrared spectroscopy (FTIR-ATR) provides a suitable technique to analyze bacteria, bacteria-polymer mixtures, digester samples and microbial biofilms (Nichols *et al.*, 1985; Bremer & Geesey, 1991).

However, other methods for studying the properties of EPS include the extraction of the EPS from the biomass. As the EPS consists mainly of polysaccharides, proteins, uronic acids, humic acids, DNA and cell fragments, different methods for the EPS extraction have been evaluated in the past (Brown & Lester, 1980). The crucial step of all methods is the destruction of the matrix without causing cell lysis. Therefore, different complexing agents are used to diminish electrostatic interactions and thus remove bridging cations bound to EPS. After this treatment the polymeric substances are separated by centrifugation. The efficiency of each method could be evaluated by

determining the content of protein, carbohydrates and uronic acids in the EPS fraction (Späth *et al.*, 1998).

A cation exchange resin has been applied successfully to extract water-soluble EPS from biofilm material present in activated sludge (Rudd *et al.*, 1983; Frølund *et al.*, 1996). However, this method is not appropriate for the quantitative examination of sorbed organic or inorganic substances in biomass, because of the sorption properties of the resin itself. Extracellular polymers can also be released from cells by blending and centrifugation in the presence of complexing agents such as EDTA (Platt & Geesey, 1985).

2.6.3 Soil hydraulic properties

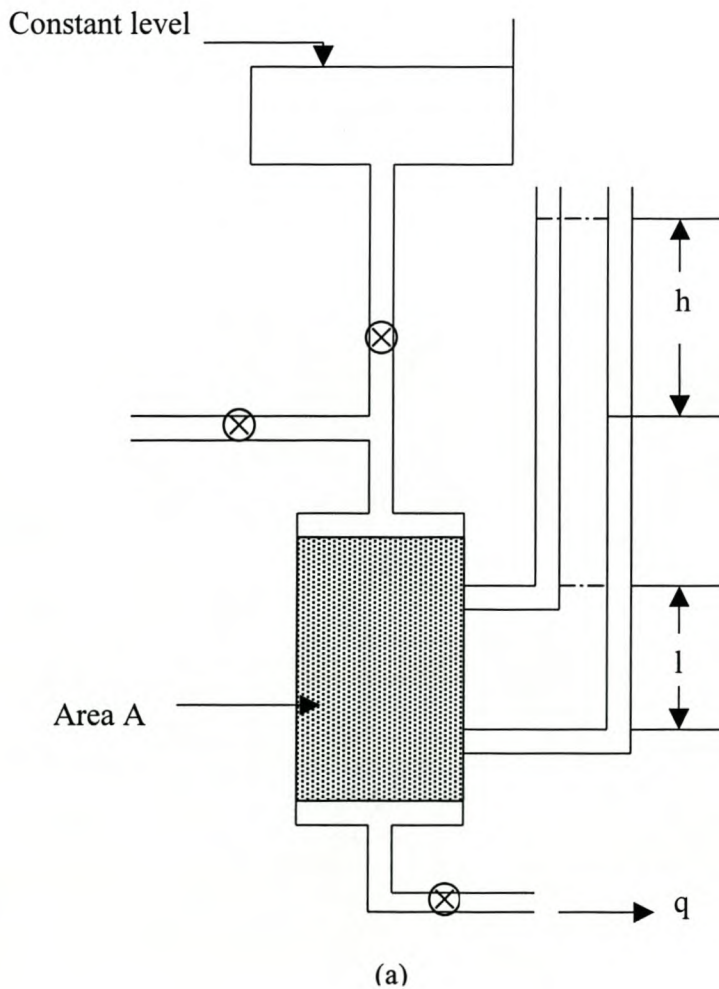
Knowledge of soil hydraulic properties are required to fully understand and predict soil water distribution. These properties include a soil water curve (the relation between volumetric water content $[\theta]$ and pressure head $[h]$ $\theta(h)$, hydraulic conductivity (K), and water diffusivity (D). Usually the hydraulic conductivity and the soil water characteristic curve are considered to be two of the most important hydraulic properties (Shao & Horton, 1998). The permeability of soil, however, plays just an important role in water flow and chemical transport in subsurface environments. A quantitative understanding of the relationship between increase in microbial biomass (as this influences soil permeability) and reduction in hydraulic conductivity is of critical importance to the simulation of the fate and transport of biologically reactive contaminants in soil and groundwater systems (Wu *et al.*, 1997).

2.6.4 Determination of soil permeability

All soils are permeable (Craig, 1983). Permeability is the capacity of soil or rock to conduct or direct liquid or gas. It can also be seen as the proportionality constant between flow velocity and hydraulic gradient. Also referred to as flow porosity, it is the portion of the total porosity that is interconnected and contributing to the overall flow of fluid through the sample. The coefficient of permeability for coarse-grained soils can be determined by the constant-head permeability test (figure 2.10a). The soil-specimen, at the appropriate density, is contained in a Perspex cylinder of cross-sectional area A : the specimen rests on a coarse filter or wire mesh. A steady vertical flow of water, under a constant total head, is maintained through the soil and the

volume of water flow per unit time (q) is measured. Markings from the side of the cylinder enable the hydraulic gradient (h/l) to be measured.

For fine-grained soils the falling-head test (figure 2.10b) should be used. In the case of fine-grained soils, undisturbed specimens are normally tested and the containing cylinder in the test may be sampling tube itself. The length of the specimen is l and the cross-sectional area A . A coarse filter is placed at each end of the specimen and a standpipe of internal area a , is connected to the top of the cylinder. The water drains into a reservoir of constant level. The standpipe is filled with water and a measurement is made of the time (t_1) for the water level (relative to the water level in the reservoir) to fall from h_0 to h_1 . At any intermediate time t the water level in the standpipe is given by h and its rate of change by $-dh/dt$. At time t the difference in total head between the top and bottom of the specimen is h (Craig, 1983).



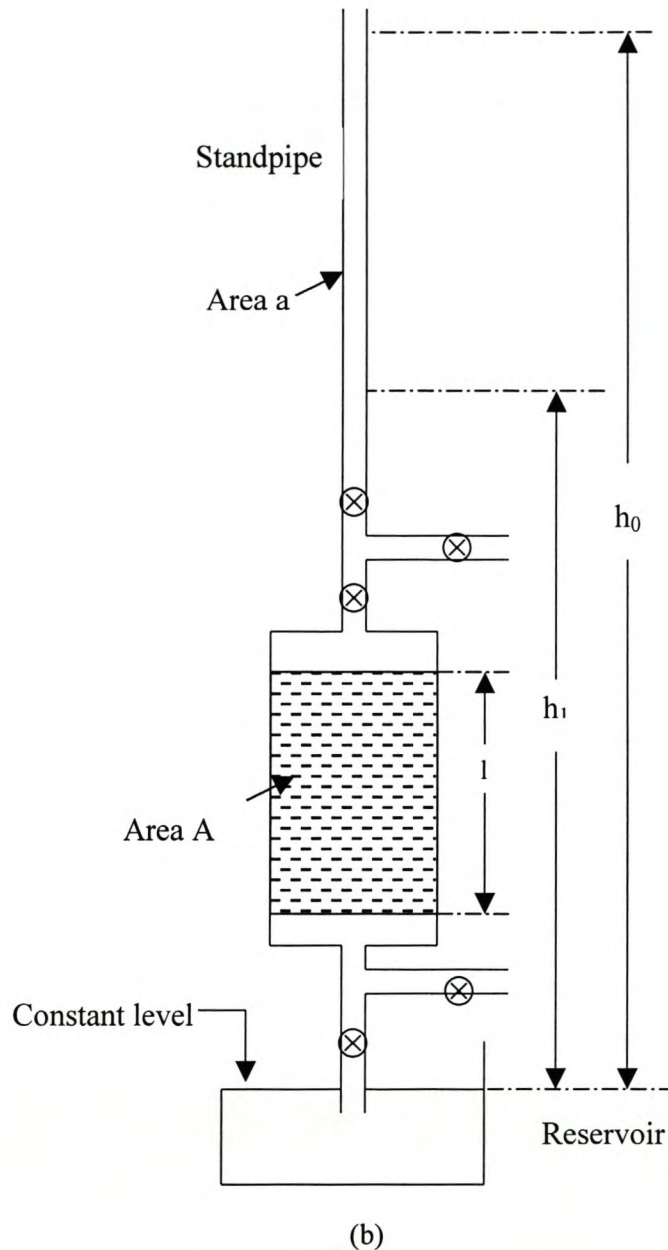


Figure 2.10a-b Examples of the laboratory permeability tests for coarse-grained soils, the constant head permeability test (a) and the falling head permeability test (b) to determine permeability of finer soils. Images adapted from R.F. Craig (1983).

2.6.5 Hydraulic conductivity

The hydraulic conductivity of a soil is a measure of the ease at which water moves through the soil. It can be obtained by measuring the flux density of water passing through a soil and is the proportionality constant which, when multiplied by the driving force (or gradient in total potential) causing water to move, gives the flux density of water. If the potential is defined in terms of a unit weight of water, then the

gradient in total head has no dimensions and the conductivity has units of length per unit time just as the flux density does (Nofziger, 1998). Reliable estimates of saturated hydraulic conductivity are a prerequisite for accurate estimations of water flow and chemical transport through soil profiles. While informative, mean estimates of flow and transport have been shown to be inadequate for modeling purposes (Bosch & West, 1998).

Earlier observations indicated that microbes attached to individual grains could be important to determining mechanical properties of sediments (Meadows & Anderson 1966); since then, many studies concerned with microbially -induced sediment stability have appeared but none have satisfyingly quantified microbial effects. More recent studies have demonstrated that intertidal sand stability is due in part to sediment binding by benthic diatom assemblages that vary in time and space (Grant *et al.*, 1986; Vos *et al.*, 1988; Paterson 1989). Studies attempting simultaneous measurements of microbial adhesion and erosion resistance include those of Rhoads *et al.* (1979) and Grant & Gust (1987).

CHAPTER 3

CHAPTER 3

Materials and Methods

3.1 Screening of vineyard soils for microbial extracellular polymeric (EPS) producers and evaluating the effect it has on water retardation within soil.

During the initial screening of soil samples, conventional techniques such as cultivation on complex growth media, were used in order to cultivate and isolate EPS-producing microorganisms. The growth media primarily used was tryptic soy agar (TSA) for isolation of EPS-producers and tryptic soy broth (TSB) for consequent cultivation of isolated EPS-producers.

3.1.1 Strain selection.

Bacteria from soil and aqueous samples were selected for EPS production. Composite samples were aseptically collected from the top soil layers of vineyards in Stellenbosch, South Africa, and weighed in 10 g quantities, each added and thoroughly mixed with 90 ml sterile tap water. The resulting slurry was then left to stand for one hour in order for the larger soil sediments to settle to the bottom. Water samples were collected from irrigation furrows in the Stellenbosch area. A dilution series was prepared and plated onto TSA using the spread-plate technique. The plates were incubated at 26°C for 3 to 5 days and were regularly observed and screened for EPS-producing colonies. Colonies with a slimy appearance were isolated from the plates, streaked onto fresh TSA plates to ensure that pure cultures were obtained, labeled and kept for subsequent experimentation.

3.1.2 Soil columns to measure the effect of EPS production on soil-water retention.

Glass columns (20 mm in diameter and 150 mm in length) were filled with well-mixed soil collected in vineyards. The column set-up (figure 3.1) allowed addition of known volumes of water to the soil and collection thereof after it flowed through the soil profile at the bottom to determine soil permeability. To obtain a uniform grain size the soil was sieved using a 425 μm sieve. Triplicate columns were prepared for

3.2 Biofilm development in soil-filled flow cells and the subsequent production of EPS by bacteria within a biofilm.

The purpose of this experiment was to view, by means of epifluorescence microscopy, the production of EPS by bacteria in a biofilm on soil particles. Perspex flow cells (figure 3.2) were filled with soil and sterilized with 3.5% (m/v) sodium hypochloride. These soil-filled flow cells were connected to a reservoir containing 10% TSB as growth medium. A Watson Marlow 205S peristaltic pump was used to pump medium at a constant rate from the reservoir to the flow cells. The flow rate was set at 0.0014 ml.s^{-1} (figure 3.3). The flow cells were then inoculated with microorganisms isolated from soil and water and allowed to grow for one week.

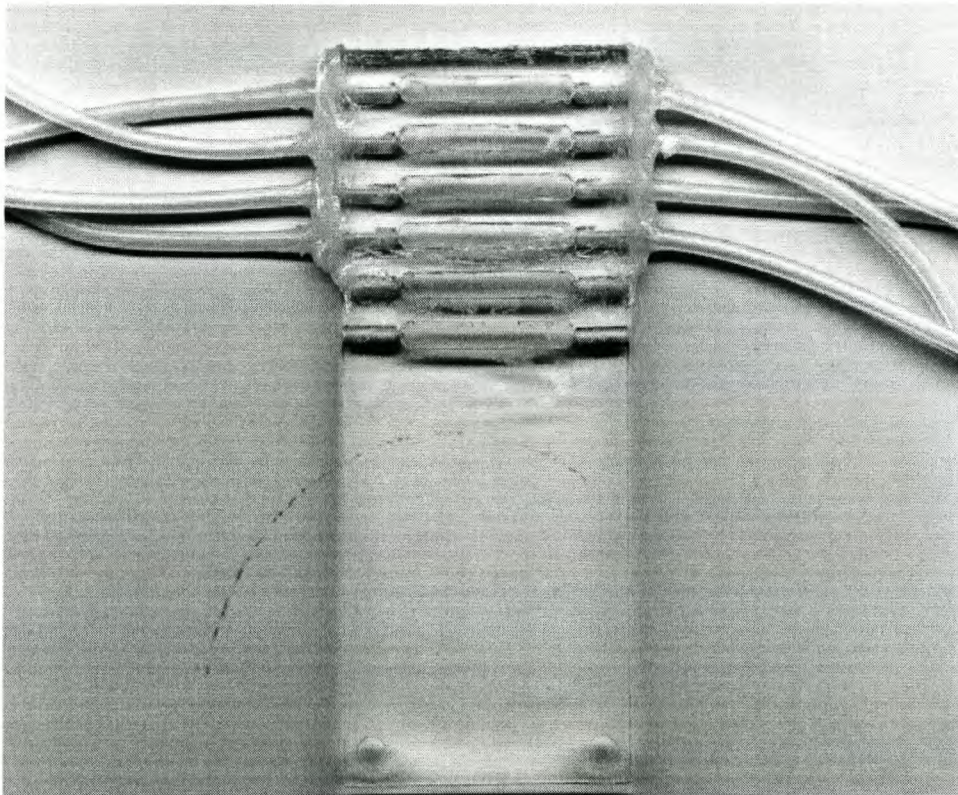


Figure 3.2 An example of a flow cell used in this study. A microscope coverslip (50 mm x 75 mm) was used to cover each channel (5 mm x 33 mm) and to allow microscope examination of microbial colonization of soil particles and plugging of pores by EPS.

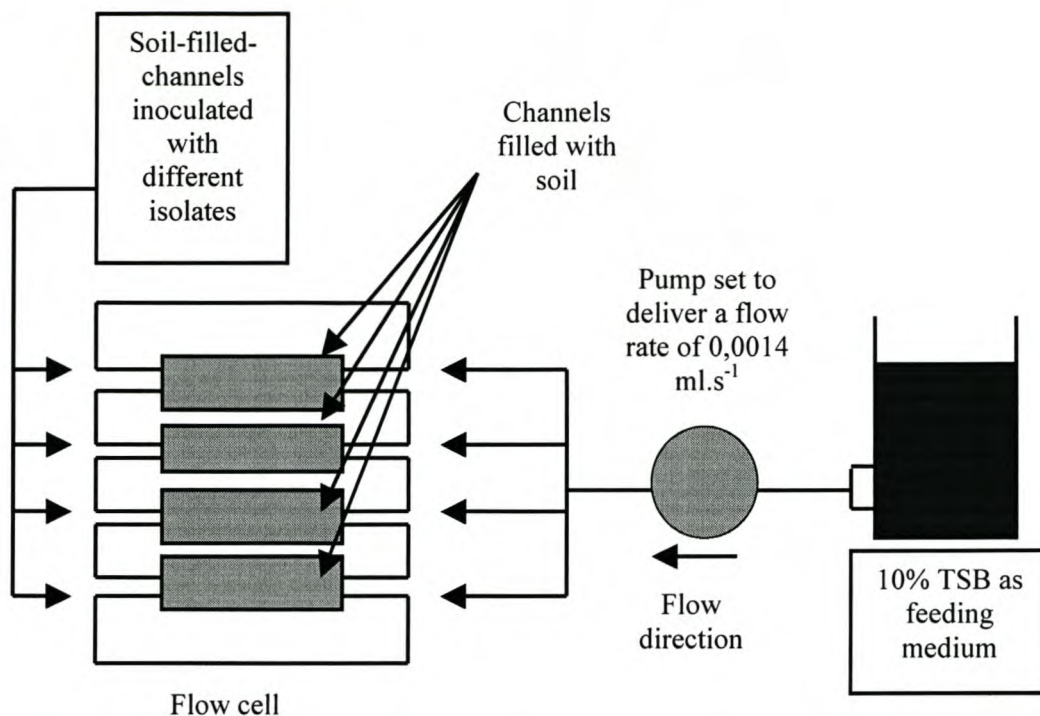


Figure 3.3 Schematic diagram of a flow cell experiment. Soil-filled flow cells were inoculated with previously isolated microorganisms and constantly fed with TSB. The cells were stained with Nile red and subsequent growth of microorganisms viewed with epifluorescence microscopy.

The flow cells were then stained with the fluorochrome, Nile red. The dye was left within the flow cells for 20 minutes, washed out with the irrigation medium and the subsequent growth of the microorganisms viewed with epifluorescence microscopy. Soil-filled flow cells were also inoculated with selected isolates and allowed to grow and produce EPS for approximately 14 days. The flow cells were stained with the fluorescent probes, concanavalin A and DAPI, respectively to visualize EPS.

3.3 Determining the binding efficiency of EPS.

Previously isolated EPS-producing bacteria were suspended in 10% TSB and grown overnight at room temperature. Glass columns (25 mm in diameter and 150 mm long) (figure 3.4) were filled with glass beads (2 mm in diameter), saturated with the bacterial suspension and allowed to grow within the columns for approximately 30 days, after which the clump or cluster size of attached glass beads was measured.

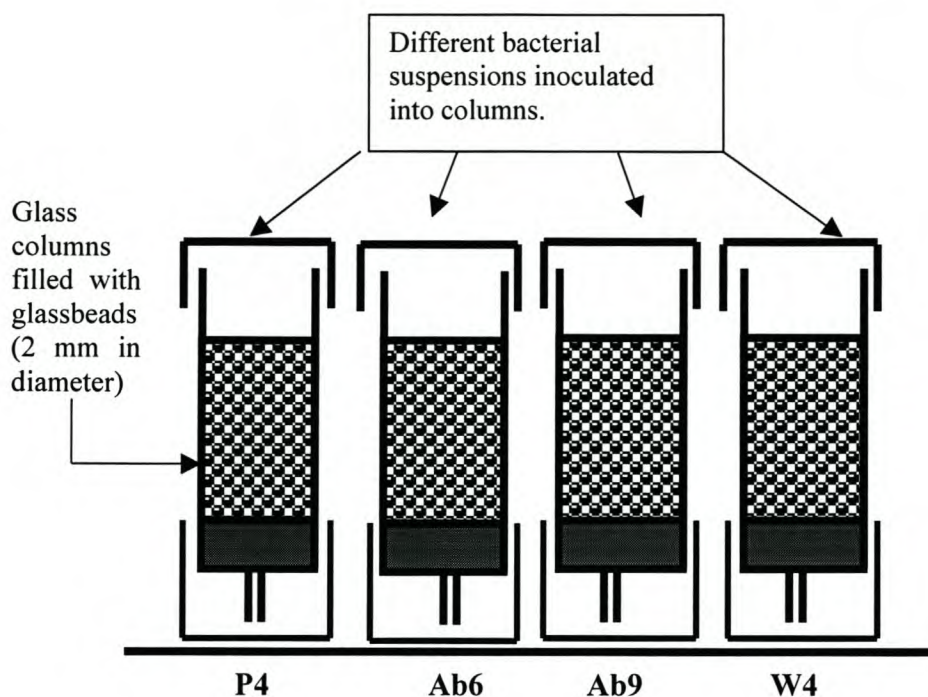


Figure 3.4 Schematic diagram of glass columns, filled with glassbeads. Columns were inoculated with different EPS-producing microbes (P4, Ab6, Ab9, W4), isolated from soils obtained from different areas in the Western Cape and allowed to grow for 30 days. One millilitre full-strength TSB was added to the columns every 4 days to maintain growth of the isolates.

3.4 Determination of hydraulic gradient or soil permeability coefficient.

3.4.1 Constant head permeability test.

The aim of this experiment was to determine the permeability of soil before and after the inoculation of soil with selected EPS-producers.

Soil columns were, at the one end connected to an empty glass tube into which water or TSB could freely flow (figure 3.5). These two tubes were on the other end connected to a peristaltic pump, which, at first pumped sterile distilled water at a constant rate ($0.0014 \text{ ml}\cdot\text{s}^{-1}$), from the reservoir. After a constant hydraulic head was obtained, the water was replaced with 1% TSB. At the other end, the soil column ran out into an outflow system (Erlenmeyer flask), which regulated the level of medium in the glass tube.

After a constant flow rate was obtained with the TSB, the different soil columns were inoculated with previously isolated microorganisms such as Ab9, K1, and W3. The column was then monitored for growth of the isolate and EPS production, which would be easily visible as a white/yellowish material between the soil grains. The hypothesis was that if the EPS successfully clogged the pores between the soil grains, it would become more difficult for the aqueous medium to flow through the soil column, with a resulting difference of the water level in the glass tube.

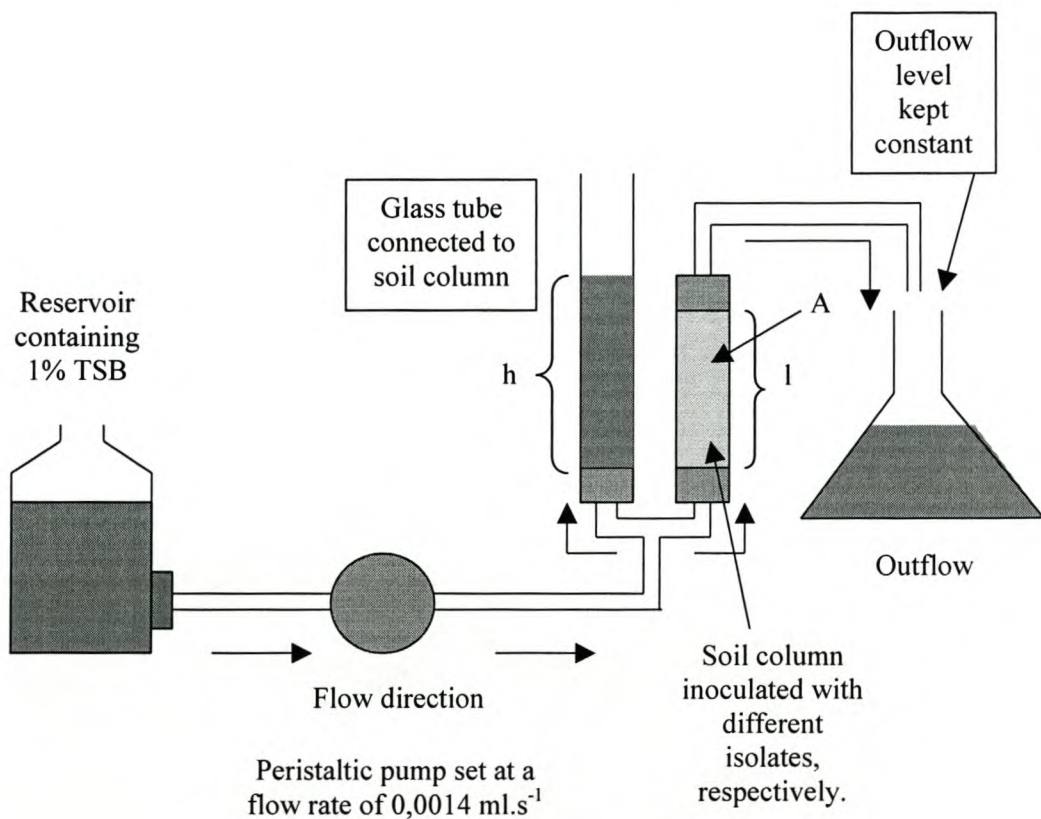


Figure 3.5 Schematic diagram of hydraulic gradient estimation procedure.

3.4.2 Hydraulic head-monitoring device and flow cell technique.

The aim of the hydraulic head-monitoring device in this experimental study was to observe and determine change in hydraulic head, while observing the subsequent effect that EPS production has on the change in head by means of the flow cell technique.

The head-monitoring device (figure 3.6) consisted of vertical columns (2 mm diameter) that was at the one end connected to two reservoirs containing sterile dH₂O and 10% TSB, respectively (figure 3.7). At the opposite end it was connected to a soil-filled flow cell, inoculated with the EPS-producing microorganism, Ab9 and a natural soil community, respectively. For this particular study, the flow rate was at first, kept constant at 0,0014 ml.s⁻¹ and later changed to a flow rate of 0,0024 ml.s⁻¹ using a Watson Marlow peristaltic pump (figure 3.7). Flow was then maintained for 7 days and regularly observed for EPS production within the flow cell and consequent change in hydraulic head of isolate, Ab9 and the natural soil community with 10% TSB and water. The flow cell was stained with concanavalin A and observed with fluorescence microscopy to view EPS production.

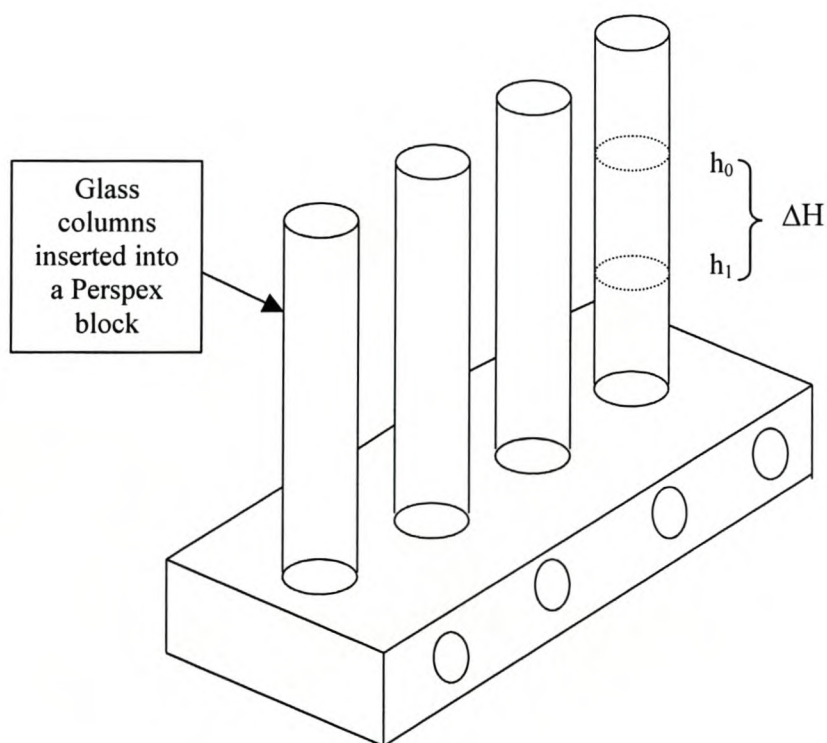


Figure 3.6 Simplified diagram of the hydraulic head monitoring device

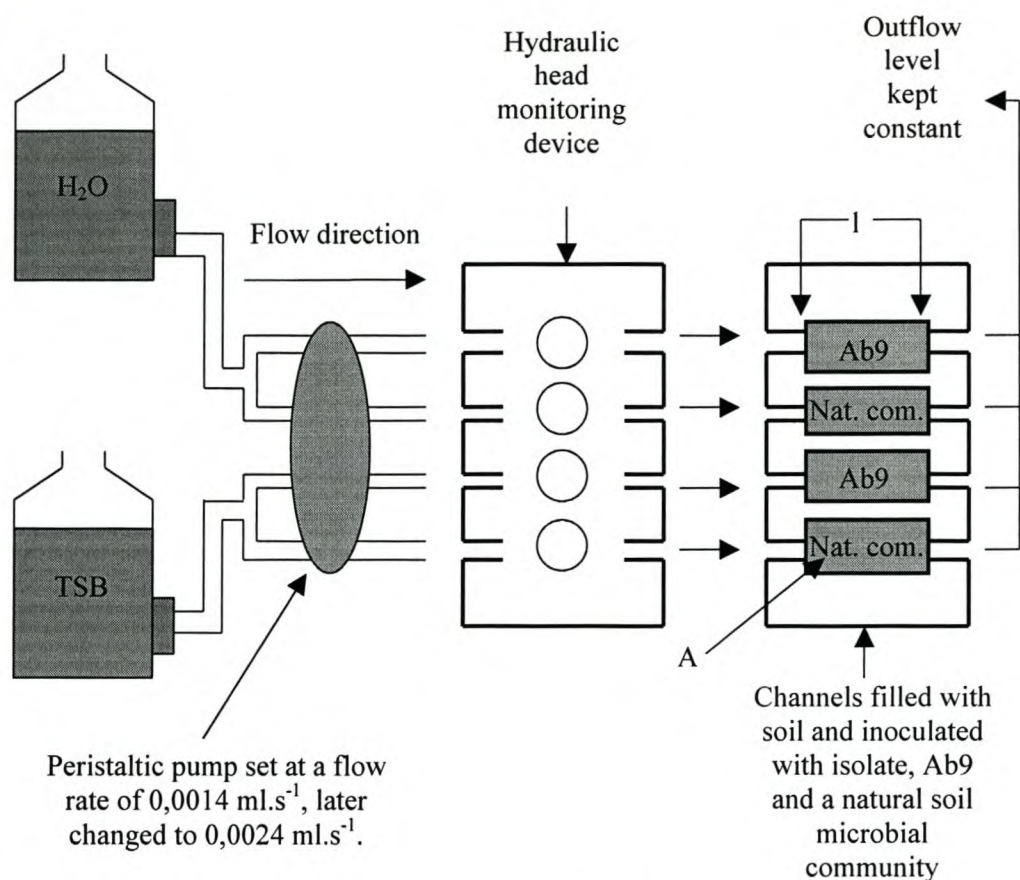


Figure 3.7 Schematic diagram of hydraulic conductivity gradient estimation procedure using the hydraulic head monitoring device and flow cell technique. Here A represents the area and l represents the length of the channel within the flow cell.

3.5 Effect of EPS-producing bacteria when introduced into microcosms and their impact on community composition.

Diversity and shifts of the microbial community within soil were compared following different approaches, including conventional cultivation of bacteria (to estimate numbers), community-level carbon substrate utilization studies using Biolog plates, and direct amplification and analysis of 16S rRNA (rDNA) by use of the T-RFLP technique.

3.5.1 Microbial counts. Soil microcosms were prepared by placing 2.5 kg quantities soil into plastic bags. Overnight cultures of EPS-producers were centrifuged at 10000 rpm for 30 minutes at room temperature. Pelleted cells were then resuspended in

peptone buffered water. The soil microcosms were inoculated with the isolates and left for approximately 14 days in order for isolates to adjust to and grow within the microcosm. This was done in triplicate for each isolate. Another set of soil microcosms was prepared into which only nutrients (excluding isolates), were added. The control contained a sterile peptone solution. After the 14-day growth period serial dilutions and consequent bacterial counts were performed in order to compare possible shifts, if any, within the natural microbial community after an EPS-producing microbe was introduced to this microbial community.

Counts were performed on two types of growth media, which includes TSA, a versatile medium suitable for general growth of many fastidious organisms without the addition of a serum, as well as actinomycete-isolation media, a selective medium for the growth of actinomycetes. TSA contains 17g tryptone, 3g soy peptone, 5g sodium chloride, 2.5g di-potassium hydrogen phosphate, 2.5g dextrose and 12 to 15g bacteriological agar per liter of distilled water. The actinomycete-isolation media contained 0.2g casein hydrolysate (made up in 100ml distilled water and at pH 6.7), 0.5g K₂HPO₄, 0.2g MgSO₄, 0.01g FeCl₃ and 12 to 15g bacteriological agar per liter distilled water. The medium was adjusted to a pH of 6.5 to 6.7. All microbial counts were done in triplicate.

3.5.2 Whole-community carbon source utilization. 10 g of soil taken from each microcosm was thoroughly mixed with 100 ml dH₂O and allowed to stand for approximately 1 hour in order for soil sediments to settle to the bottom. BIOLOGTM Ecoplates were inoculated with the soil-water suspension, incubated for 48 hours at 22°C and utilization of the carbon sources in the different wells were recorded.

3.5.3 Whole-community genetic analysis

3.5.3.1 DNA extraction from soil. DNA was extracted according to the method used by Zhou *et al.* (1996). The procedure was as follows:

0.35g of each soil-microcosm was weighed and transferred to a 2 ml-ependorf tube to which 1ml DNA extraction buffer was added. This buffer contained 100 mM Tris-HCl (at pH 8.0), 100 mM EDTA, 100 mM Na Phosphate (monobasic), 1.5 M NaCl and 1% CTAB. The soil-buffer mixture was subjected to 3 freeze-thaw cycles (-70/+65) in order to break open any Gram-positive cells. 200 µl TE (Tris-EDTA) and

200 µl PCI (Phenol-Chloroform-Isoamylalcohol) were then added to the mixture after which it was centrifuged at 13800 g for 5 minutes. 5 µl of proteinase K (20 mg/ml) was added, mixed well and the sample incubated at 37°C for 30 minutes while shaking at 225 rpm. 150 µl of 20% SDS was added and incubated at 65°C for 2 hours (mixing it at 20 minute intervals). The sample was then centrifuged at 6000 g for 10 minutes. The supernatant was transferred to a clean, chloroform resistant polypropylene tube (i.e. first supernatant). The soil pellet was re-extracted by adding 1ml DNA extraction buffer and 150 µl of 20 % SDS. The sample was vortexed for 1 minute, heated to 65°C for 10 minutes and centrifuged at 6000 g. This supernatant was then transferred to the first supernatant. An equal volume (~ 3 ml) of chloroform / isoamyl alcohol (24:1) was added to the combined supernatants and mixed well. The sample was centrifuged at 3000 g for 3 minutes and the aqueous layer transferred to a clean tube. A 0.6 volume (~ 1.8 ml) of cold isopropanol was added, the sample mixed well and left overnight at -20°C. The overnight sample was then thawed and centrifuged at 16000 g for 20 minutes and the isopropanol removed. The pellet was washed with 1ml of ice cold 70% ethanol, dried completely and finally dissolved in 100-200 µl of TE (Tris 10 mM, EDTA 0.1 mM). The sample was then run on a 0.8% agarose gel to confirm the presence of DNA. The obtained DNA was purified by using MicroSpin™ S-300 HR columns (Amersham pharmacia Biotech Inc.).

3.5.3.2 Polymerase Chain Reaction (PCR). The purified DNA was firstly amplified using universal bacterial primers in order to determine whether or not the purified DNA was indeed suitable for PCR. The primers used were fDD2 5' CCGGATCCGTCGACAGAGTTTGATCITGGCTCAG 3' 34-mer and rPP2 5' CCAAGCTTCTAGACGGITACCTTGTTACGACTT 3' 33-mer (Rawlings, 1995). The PCR Master Mix consisted of 50 units/ml *Taq* DNA Polymerase (supplied in a proprietary reaction buffer at a pH of 8.5), 4 dNTP's (i.e. dATP, dGTP, dCTP, dTTP) each consisting of a final concentration of 400 µM; and finally 3 mM MgCl₂ (obtained from Promega). Each PCR reaction, with a final volume of 50 µl, contained 25 µl PCR Master Mix, 2 µl DNA template, 2.5 µl of each primer (i.e. primer 1 and primer 2) and 18 µl Nuclease-free water. PCR reactions were carried out in a Perkin-Elmer, Gene Amp 2400 PCR system or thermocycler. Reactions involved an initial denaturation step conducted at 94°C for 2 minutes, followed by 30 cycles of

amplification (i.e. 1 minute at 94°C, 1 minute at 57°C and 2 minutes at 72°C). This was followed by a final extension step of 72°C for 10 minutes. PCR products for each sample were run on a 0.8% ethidium bromide agarose gel, molecular grade agarose # D1-LE supplied by Whitehead Scientific.

3.5.3.3 T-RFLP and DNA profiling. For the T-RFLP technique, the previously purified DNA was labelled with the oligonucleotide primers FAM63F (3'-CAGGCCTAACACATGCAAGTC-5') and HEX1389R (5'-ACGGGCGGTGTGTA CAAG-3'). These primers were previously labelled at the 5' end with the phosphoramidite dyes 6-FAM and HEX respectively (Osborn *et al.*, 2000). Each PCR reaction, as with the previous PCR procedure, with a final volume of 50 µl, contained 25 µl PCR Master Mix, 2 µl DNA template, 2.5 µl of each primer (i.e. FAM63F and HEX1389R), 0.6 µl BSA (bovine serum albumin) and 17.4 µl Nuclease-free water. The PCR products were subsequently purified using Nucleospin® Extract columns (Macherey-Nagel) in order to obtain a higher concentrated DNA.

The restriction enzyme, Msp 1, was used to digest each of the PCR products. Each 30 µl digestion reaction mixture consisted of 20 µl of the PCR product, 1 µl of Msp 1, 3µl restriction digest buffer and 6 µl sterile distilled water. The mixtures were incubated at 37°C overnight after which they were removed and placed at 70°C for 15 minutes in order to terminate the digestion reaction. The digested DNA was mixed with a DNA size standard, itself labelled with a distinct dye, and the DNA fragments analysed on an ABI 3100 genetic analyser.

CHAPTER 4

CHAPTER 4

Results, Discussion and Conclusions

4.1 Screening of vineyard soils for microbial extracellular polymeric (EPS) producers and evaluating the effect they have on water retardation within soil.

The screening of a variety of samples provided sufficient EPS producing microbes for subsequent studies to determine the extent to which EPS production can be manipulated or altered.

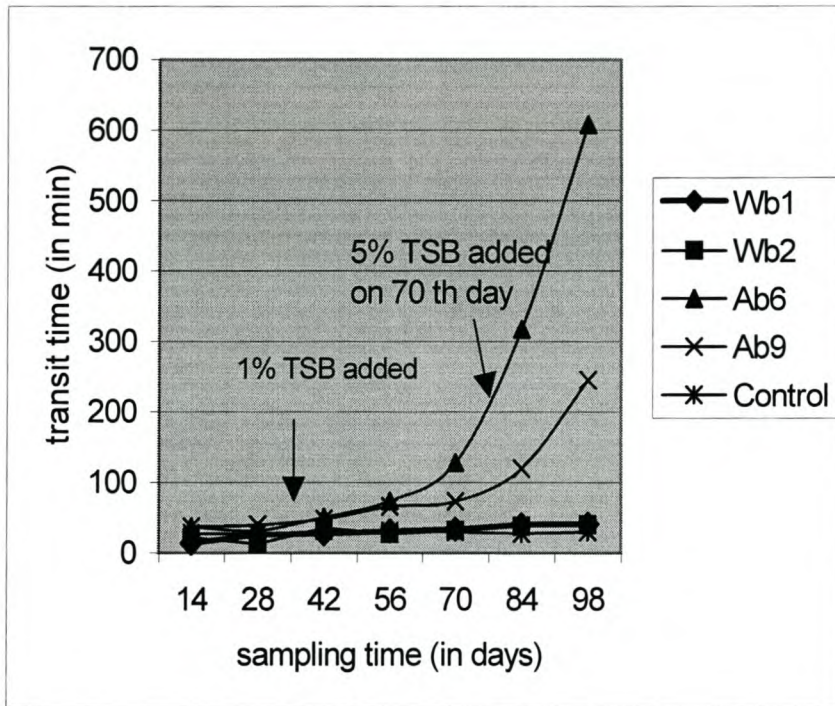
4.1.1 Selection of EPS-producers

EPS-producing strains were selected on the basis of their mucoid or 'slimy' appearance as viewed on the TSA plates. Also, by touching the single colony forming units (cfu's) with an inoculating loop, the difference between the mucoid and non-mucoid organisms could be distinguished as the mucoid organisms had a glue-like or slimy structure. These strains produced EPS to different degrees.

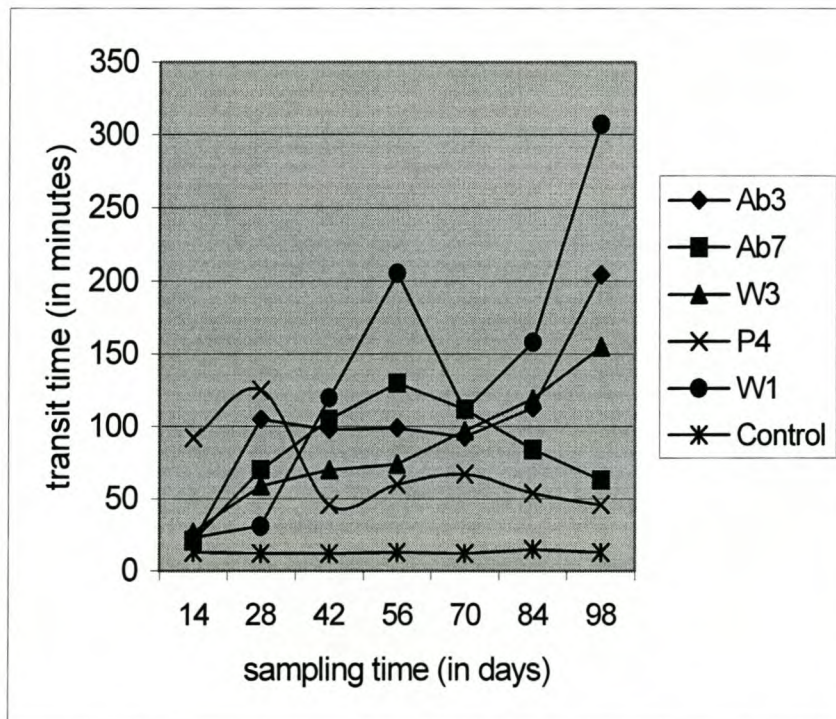
4.1.2 Effect of introduced EPS-producers on water movement through soil

The isolated microorganisms were used to determine the rate of flow of water (transit times) through soil, which was an indication of the soil's permeability or its water-holding capabilities. The different isolates had varying impacts on the water retention capabilities of the soil (figure 4.1a-f). Some of the isolates were able of retaining water for longer, suggesting different quantities and / or types of EPS produced by the different isolates. The increased production of EPS probably resulted in a better clogging of pores within the soil matrix.

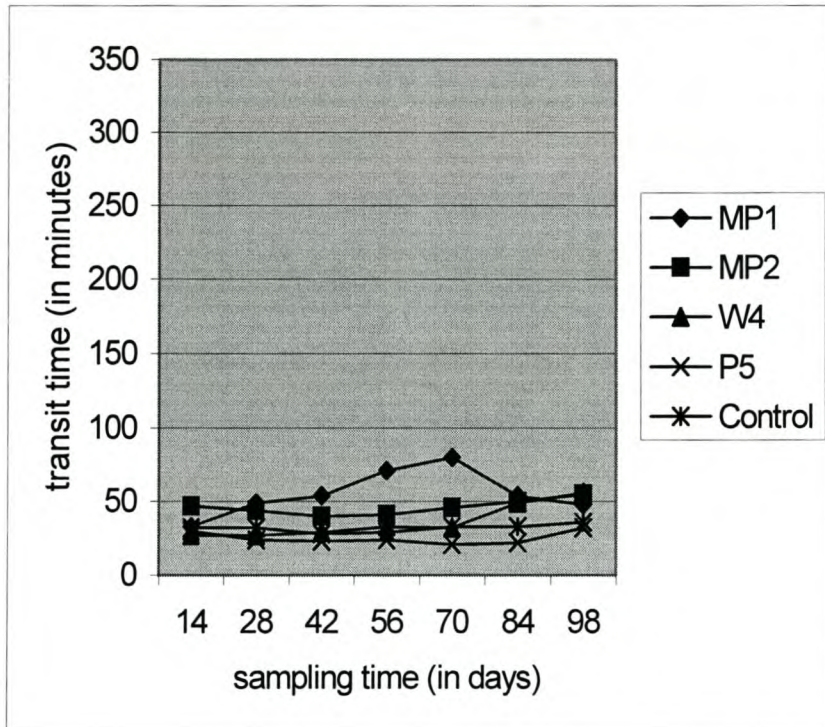
The data presented in figures 4.1a-e indicated that several isolates caused increased water retention, e.g. Ab6 and Ab9 in figure 4.1a as well as W1 in figure 4.1b and K1 in figure 4.1e. When the isolates causing the most water retention were compared (fig. 4.1f), isolate K1 retained water the best. This isolate, together with Ab9 (which produced EPS well in excess when grown on TSA) was used for further studies.



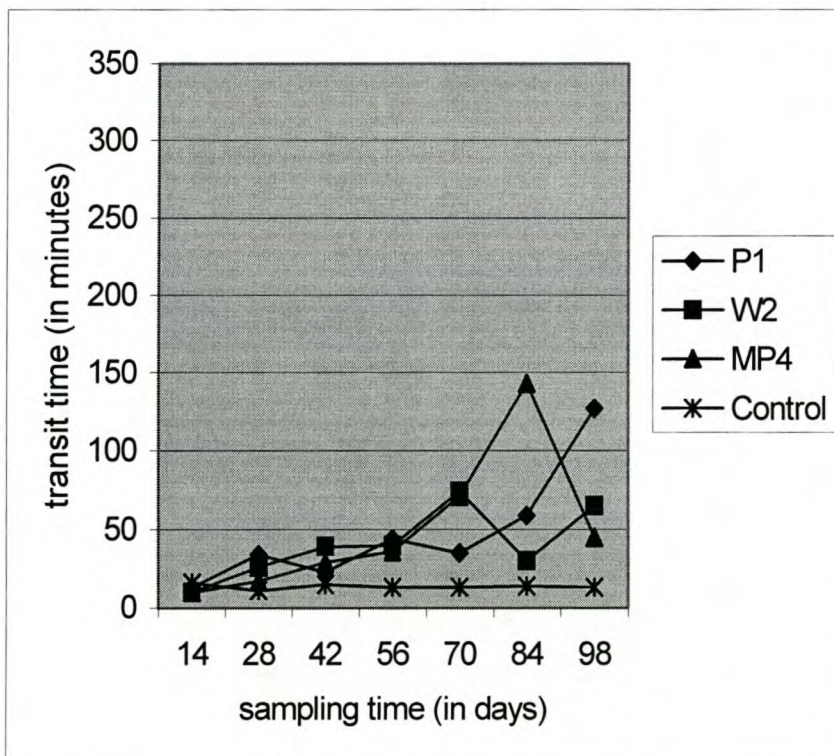
a



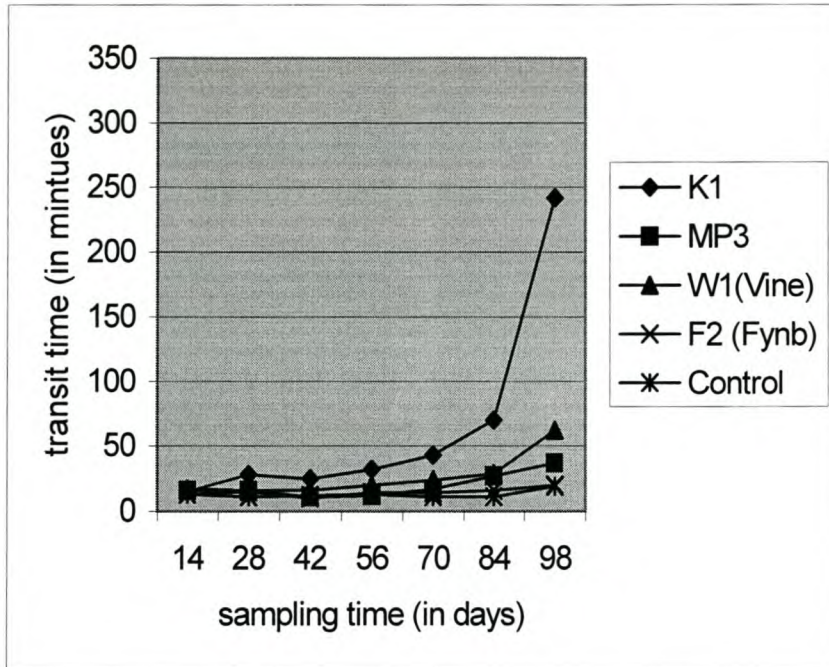
b



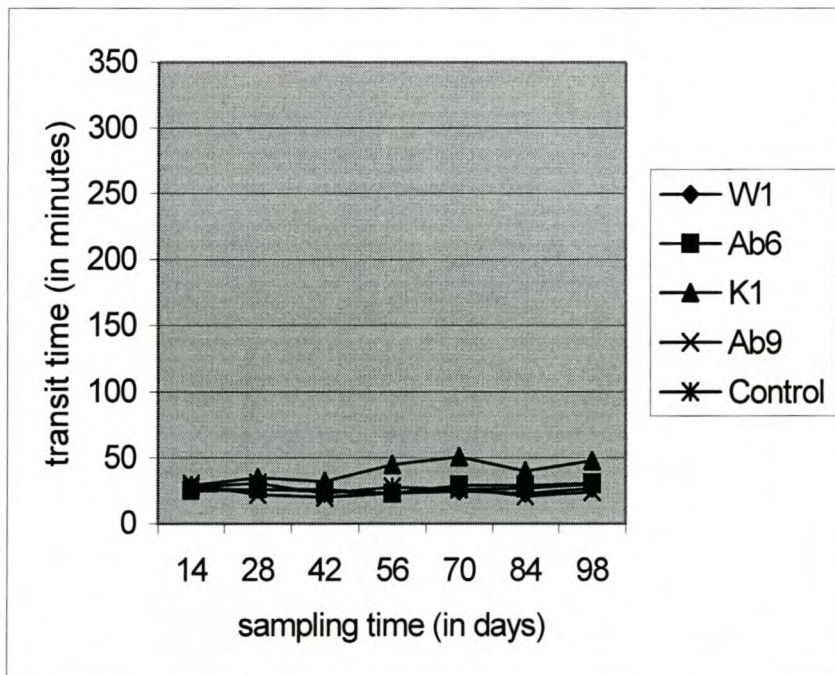
c



d



e



f

Figure 4.1a-f Microorganisms previously isolated from soil were cultivated in pure culture and inoculated into a soil environment (soil columns) in order to determine their EPS producing abilities and hence, water holding (water retention) capabilities. Transit time indicates time in minutes it took water to run through the soil column. Note the differences in scale on the y-axis. Also note that 1% and 5% TSB were only added as is indicated on the first graph. This was due to the fact that the additions had no effect on the EPS production of the isolates in the other experiments (fig. 4.1 b-f).

4.2 Biofilm development in soil-filled flow cells and the subsequent production of EPS by bacteria within a biofilm.

There was a notable difference between the production of EPS by the soil-isolated- and water-isolated microorganisms. It is a common fact that EPS, which acts as adhesins, attach particles (e.g. bacterial cells) to each other as well as to substrates. The EPS produced by the soil microbes tended to accumulate and attach more readily on the surfaces of soil grains (figure 4.2a and b).

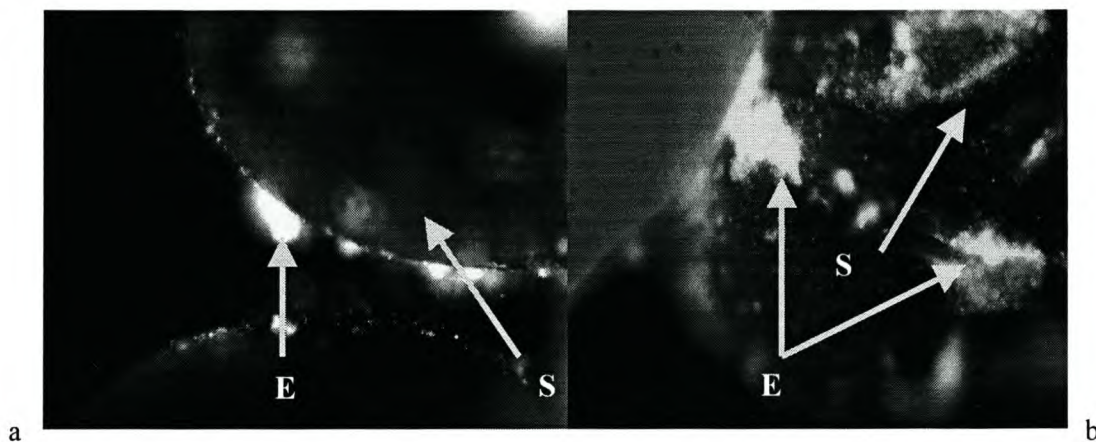


Figure 4.2a-b EPS produced by soil-isolated microbes attaches to the surface of the individual soil grains and the pores between the grains. Arrows indicate the soil grains (S) and EPS (E).

In contrast to the close association of the soil microbes with the soil surfaces, those isolated from water produced EPS that primarily accumulated in the pores between grains. The microbes isolated from water produced EPS that floats loosely in the liquid medium. This could be due to adaptation to an aqueous environment (figure 4.3a and b).

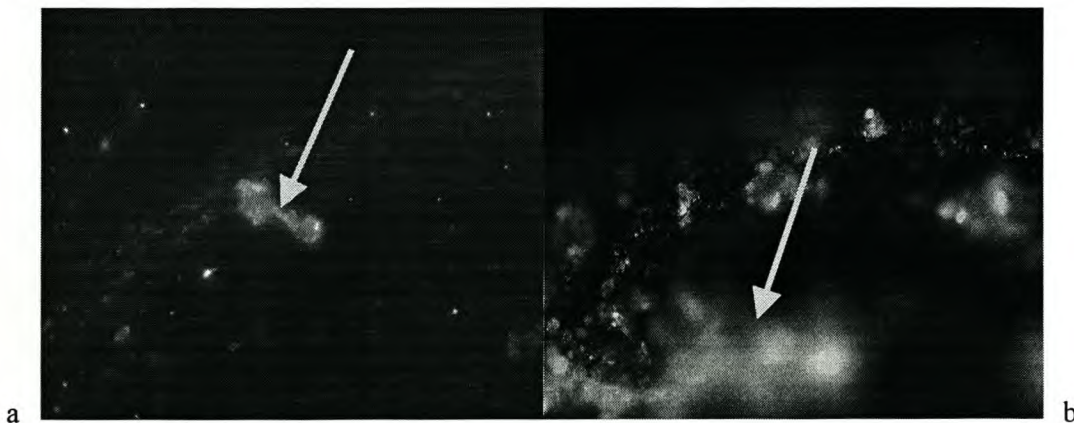


Figure 4.3a-b EPS produced by waterborne isolates was either floating within the liquid medium surrounding the soil grains or loosely associated with some of the soil particles.

The focus of subsequent studies was therefore on soil-isolated microorganisms, which proved to clog the pores between the soil grains more effectively with produced EPS than those produced by the waterborne isolates. Also, it suggested that even though waterborne isolates produced EPS just as effectively as soil-isolated microbes, they would not aid in achieving the objectives set out for this experimental study.

Various fluorescent probes at different incubation conditions and concentrations have been applied in this study for the visualization of soil-microbe interactions (data not shown). Nile red was found to be most effective and was therefore used in subsequent studies. It was demonstrated that this dye does not affect cell activity, and is therefore suitable as a probe for *in situ* visualization of microbial cells and their EPS. Nile red is formed by boiling Nile blue A with sulphuric acid. The chemical structure for Nile red is shown in figure 4.4.

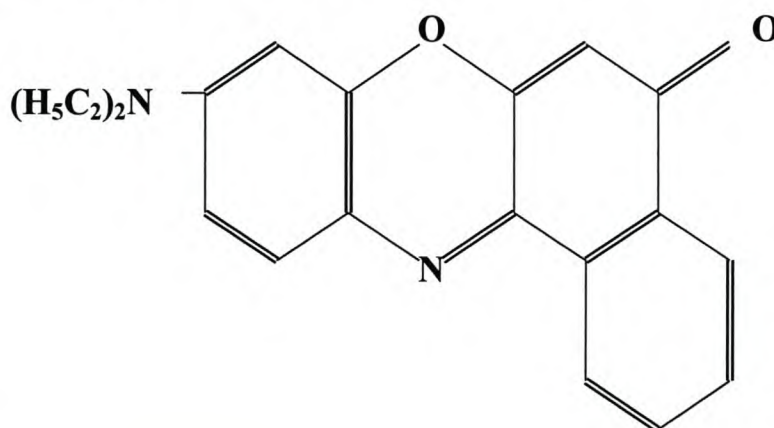


Figure 4.4 Structure of Nile red, a non-toxic fluorochrome or dye used to stain microorganisms. This dye does not appear to inhibit bacterial growth and was used to view microbial behaviour in the sand-filled flow cell channels.

Nile red can also be described as an uncharged hydrophobic molecule whose fluorescence is strongly influenced by the polarity of its environment. It interacts with many, but not all, native proteins, including beta-lactoglobulin, kappa-casein, and albumin, with a wide range of spectral changes for different proteins. The dye is photostable, the working wavelength range is broad and removed from those at which many biomolecules absorb, the fluorescence is unaffected by pH between 4.5 and 8.5, the quantum yield is high, and hydrophobic sites on proteins may be investigated in

dilute solutions (Sackett & Wolff, 1987). The absorption and emission profiles of Nile red is depicted in figure 4.5.

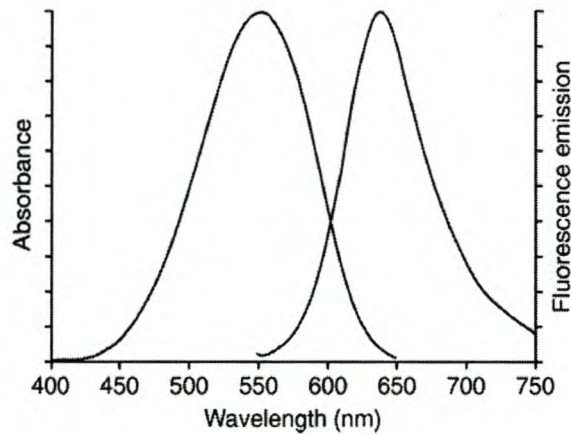


Figure 4.5 Excitation and emission profiles of Nile red. Image adopted from the Handbook of Fluorescent Probes and Research Products (8th edition), Molecular Probes, Inc. (1996).

Concanavalin A (Con A) was first crystallized by Sumner & Howell (1936). It has proven to be an interesting and useful lectin. Lectins are proteins that react with specific terminal sugar residues and are useful probes in studying carbohydrates of cell surfaces. The molecular structure of Con A (figure 4.6) has been reported by Becker *et al.*, 1975, 1976; Cunningham *et al.*, 1975; Reeke *et al.*, 1975 and Wang *et al.*, 1975.

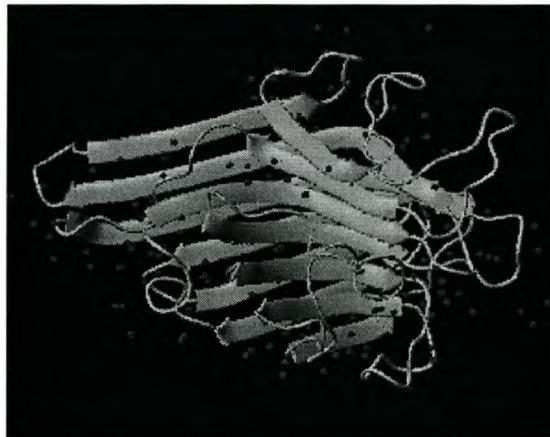


Figure 4.6 Molecular structure of concanavalin A, a 237-amino-acid lectin from the jack bean (*Canavalia ensiformis*), react with specific terminal sugar residues and are useful probes in studying carbohydrates of cell surfaces. Image adopted from EMBnet/CNB.

Con A binds two metal ions per monomer: a transition metal, nominally Mn^{2+} (Becker *et al.*, 1975) at site S1 and Ca^{2+} at S2. Both must be present for saccharide binding (Goldstein *et al.*, 1965; Agrawal *et al.*, 1968; Greer *et al.*, 1970; Poretz & Goldstein, 1970; Jack *et al.*, 1971; Grimaldi & Sykes, 1975). The absorption and emission profiles of con A can be seen in figure 4.7.

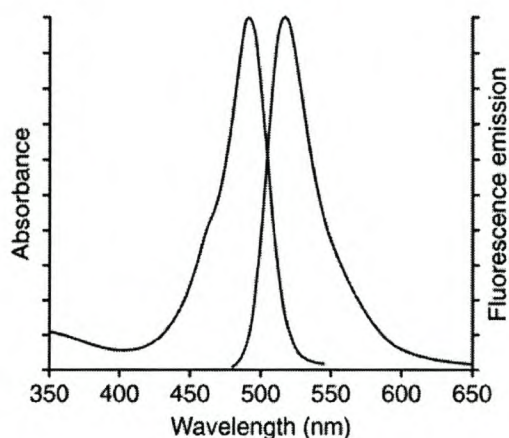


Figure 4.7 Spectrum represents a product, which is closely related, but not identical to Con A. Differences between spectra displayed and those of con A are not expected to be significant. Image adopted from the Handbook of Fluorescent Probes and Research Products (8th edition), Molecular Probes, Inc. (1996).

The fact that Con A specifically binds to sugar residues, as well as the fact that EPS contains sugar moieties, was the rationale for using Con A to visualize EPS in this study. Figures 4.8a and b show examples of EPS probing of isolate Ab6 with Con A. Although a few cells can be seen, it is clear from these images that mostly EPS are stained with this probe, thereby demonstrating the use of this probe to visualize the EPS-dominated biofilm matrix.

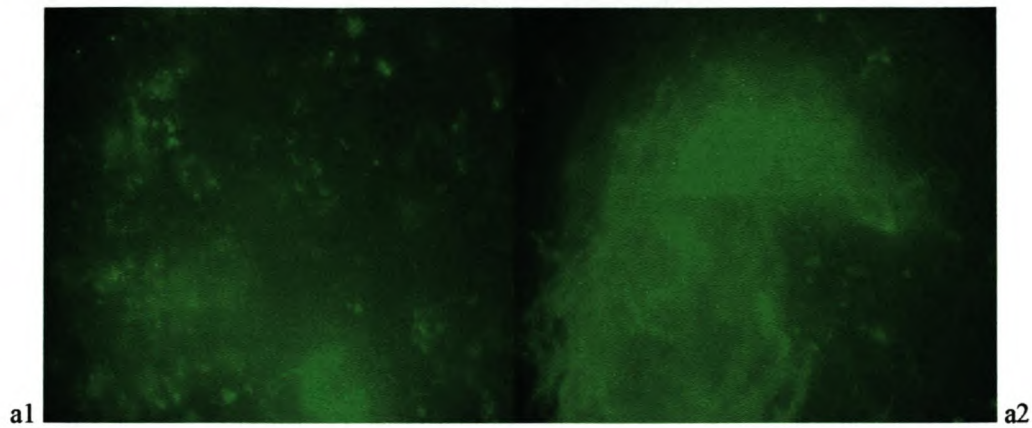
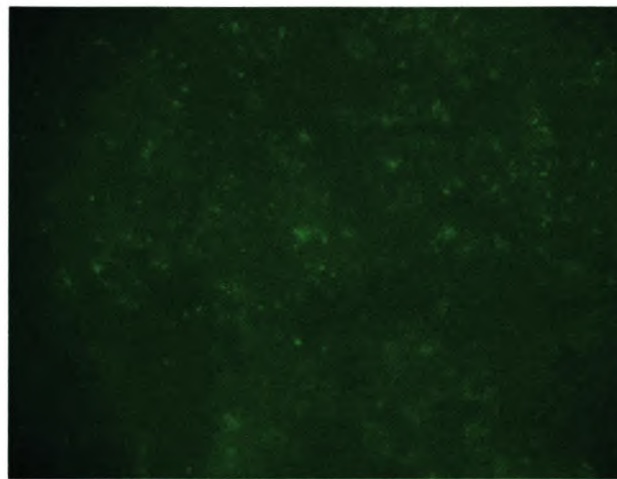


Figure 4.8a1-2 Isolate, Ab6 stained with Con A and viewed under 600x magnification. The con A stains the extracellular polymeric substance (EPS) that the microorganism produce, green.



b

Figure 4.8b Isolate, Ab6 viewed under 100x magnification.

In contrast to Con A that binds to EPS surrounding cells, the blue fluorescent DAPI (4', 6-diamidino-2-phenylindole) nucleic acid dye preferentially stains dsDNA. Binding of DAPI to dsDNA produces a ~20-fold fluorescence enhancement, apparently due to the displacement of water molecules from both DAPI and the minor groove. DAPI also binds RNA, however in a different binding mode. DAPI is a popular nuclear counterstain for use in multicolor fluorescent techniques. Its blue fluorescence stands out in vivid contrast to green, yellow or red fluorescent probes of other structures. The molecular structure of DAPI is shown in figure 4.9, and its excitation and emission spectra in figure 4.10.

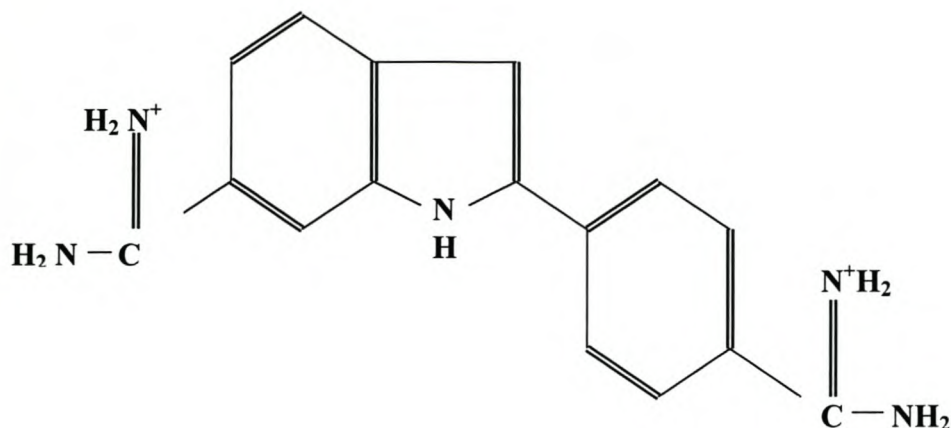


Figure 4.9 Molecular structure of 4', 6-diamidino-2-phenylindole (DAPI), a nucleic acid stain used to stain dsDNA. The excitation maximum for DAPI bound to dsDNA is 358 nm, and the emission maximum is 461 nm (figure 4.10). DAPI can be excited with a xenon or mercury-arc lamp or with a UV laser.

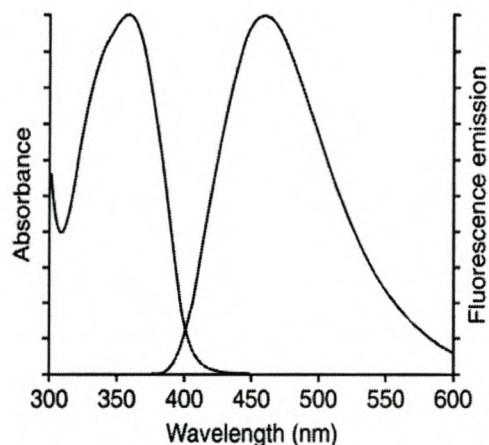


Figure 4.10 Excitation and emission profiles of DAPI bound to dsDNA. Image adopted from the Handbook of Fluorescent Probes and Research Products (8th edition), Molecular Probes, Inc. (1996).

In situ observations of microbial cells, and especially biofilm material often lack the clarity that can be achieved when cells from a pure culture are immobilized on a microscope slide and stained with these dyes. Figure 4.11 shows the typical result that was obtained in this study when biofilms established on sand grains were inspected. Generally, these observations revealed that there was extensive biofilm

formation on the sand grains, and explain the results shown in figure 4.1. For instance, in figure 4.1a, it is shown that isolate Ab6 notably retarded water transit through the sand profiles, while the microscopic examination in figure 4.2a shows the reduction in pore diameter as a result of biofilm formation and subsequent EPS production on the grain surfaces.

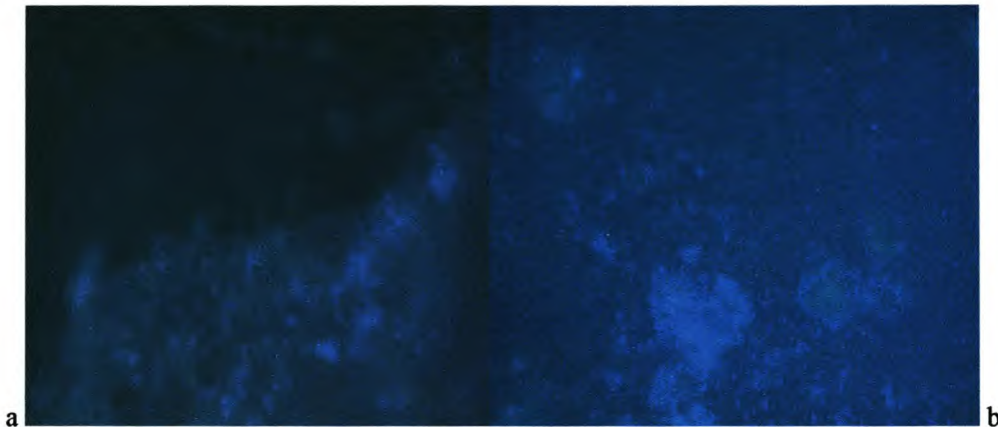


Figure 4.11a-b Isolate, Ab6 stained with 4', 6-diamidino-2-phenylindole (DAPI), and viewed under 200x magnification. The cells are stained blue.

4.3 The binding efficiency of EPS

After 30 days incubation, clusters of various sizes were noticeable in the columns filled with glassbeads. Figure 4.12 shows examples of the clusters produced by four of the isolates. The produced EPS is clearly visible between adjacent glassbeads as well as on the surface of individual glassbeads. Interestingly, there was no significant difference between the average number of beads per cluster formed by the different isolates. Considering that these four isolates were among those that caused the largest increase in water retention, as shown in figure 4.1, this result supports the previous observations that EPS has a definite effect on the retardation of waterflow by clogging the pores between adjacent soil particles which, in this experimental study, was represented by the glassbeads.

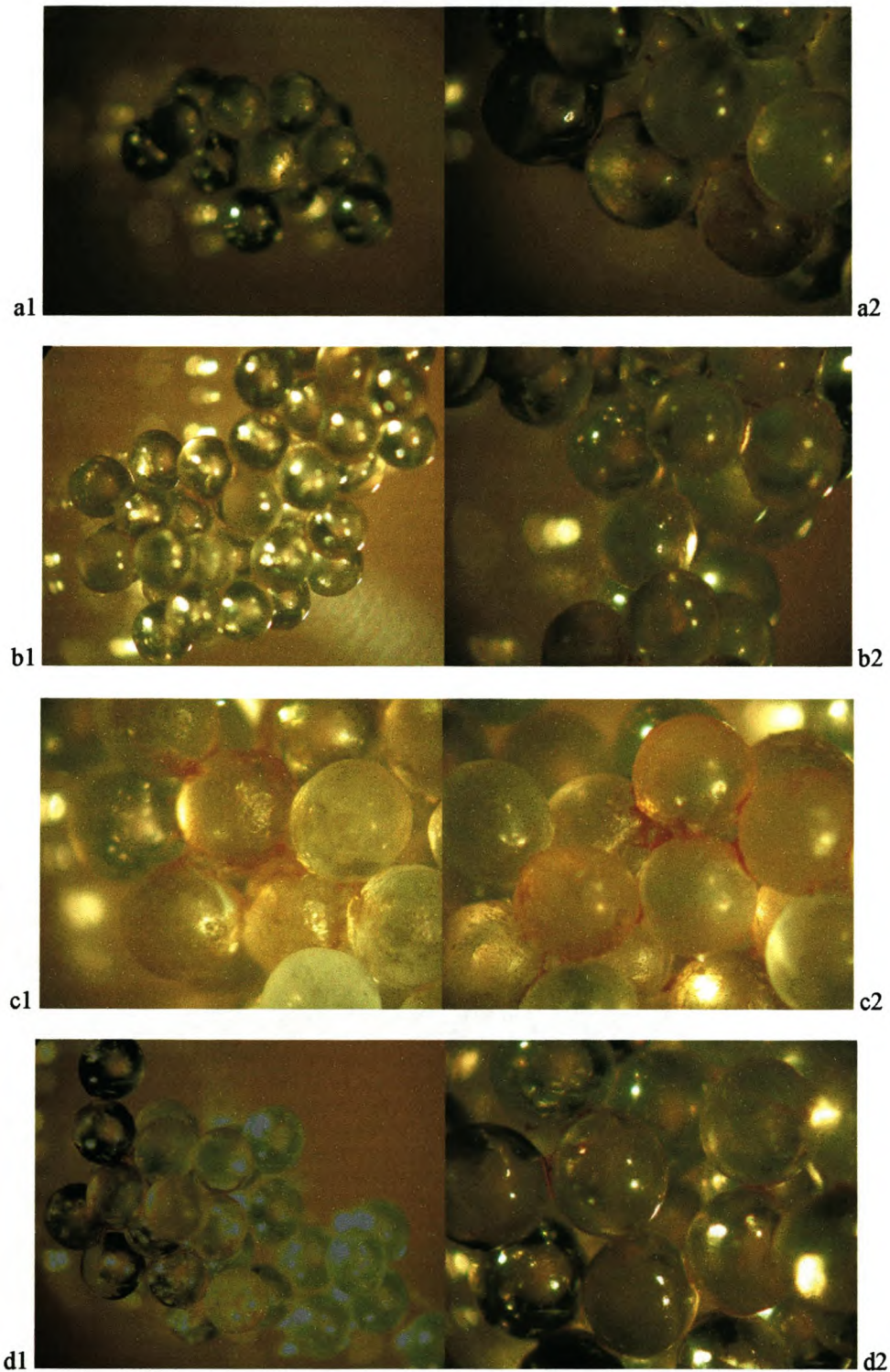


Figure 4.12 Images to demonstrate the binding capability of EPS produced by isolates (a) Ab6, (b) Ab9, (c) P4, and (d) W4. As shown here, the EPS bound 30 and more 2 mm diameter glassbeads per cluster.

It has been suggested that the high binding activity of polysaccharides is either related to their length and linear structure which allow them to bridge the space between soil particles, or their flexible nature which allows a large number of points of close contact so that Van der Waal's forces between particles can be more effective. Binding affinity can, however, relate to the large number of hydroxyl (OH) groups which may be involved in hydrogen bonding, and to acid groups, primarily carboxyl (COOH), which may allow ionic binding through di- and trivalent cations to ion exchange sites on clays or of anion adsorption to positively charge sites on clay edges (Ruehrwein & Ward, 1952; Martin *et al.*, 1955; Greenland, 1965; Harris *et al.*, 1966).

4.4 Permeability of soil

4.4.1 Constant head permeability test.

There was no significant change in hydraulic head in the control columns with H₂O without nutrient supplement. However, when the solution was supplemented with 10% TSB, a visible increase in EPS production by some of the isolates, e.g., Ab9 in fig. 4.1a, as well as the natural soil community was noticeable. In these instances, an EPS matrix clogging the pores between soil grains within the soil column was visible. This correlated well with the hypothesis that if EPS is produced, layers of EPS around bacteria can be observed in the soils and within the rhizosphere (Chenu *et al.*, 2001). Isolates such as K1 and W1 showed only a slight change in head relative to their respective controls (Table 4.1). Readings were taken 3 days after initial inoculation of columns. The hydraulic gradient (k) could then be calculated by utilizing the equation from Darcy's law (Craig, 1983):

$$k = \frac{ql}{Ah} \quad (\text{equation 1})$$

Where h = change in hydraulic head (mm) = h₀ - h₁.

l = length of the specimen (mm) = 110 mm

A = cross-sectional area of the specimen (mm²) = 314 mm²

q = water flow per unit time (mm³.s⁻¹) = 1.4 mm³.s⁻¹

Permeability can be related to the capacity of soil or rock to conduct or direct liquid or gas. It is the proportionality constant between flow velocity and hydraulic gradient. Soil permeability, therefore, is closely related to the conduction of liquid, e.g. water through soil. This conduction of water through soil is fundamental to the way in

which soils transport nutrients as well as pollutants into groundwater. The derivation of relations between waterflow and void structure has relied on the implicit assumption that water flows through aligned unconnected cylindrical capillary tubes. A wide range of effects depends on hydraulic conductivity. Primarily, hydraulic conductivity determines the rate of flow of water through soil. The results presented here (Table 4.1 and 4.2) show that EPS production by microorganisms can have an impact on hydraulic conductivity, and therefore water flow through soils. These k -values for the different isolates varied, as was the case for water retention by the different isolates as shown in figure 4.1.

Table 4.1 Examples of change in hydraulic gradient to demonstrate the effect of a labile nutrient on EPS production (and as a result, permeability) by some of the isolates and a natural soil microbial community.

Isolated sample	Hydraulic gradient (mm.s⁻¹)
Ab9	0.0025
K1	0.2452
W4	0.1635
P4	0.0234
Natural soil community	0.0035

4.4.2 Hydraulic head monitoring device.

In this experimental technique there was distinct differences in change in hydraulic heads over time between isolate, Ab9 and a natural microbial soil community, as in the previous hydraulic-gradient-determinations. The hydraulic gradient was calculated using equation 1 as in section 4.4.1 (Table 4.2). However, here

$$h = h_0 - h_1$$

$$l = 30 \text{ mm}$$

$$A = 63 \text{ mm}^2$$

$$q = 8.7 \text{ mm}^3 \cdot \text{s}^{-1}$$

Table 4.2 Examples of change in hydraulic gradient to demonstrate the effect of a labile nutrient on EPS production (and as a result, permeability) by isolate, Ab9 and a natural soil microbial community, using the hydraulic head monitoring device.

Isolated sample	Hydraulic gradient (mm.s ⁻¹)
Ab9 (H ₂ O)	0.0090
Natural soil community (H ₂ O)	0.0179
Ab9 (TSB)	0.0635
Natural soil community (TSB)	0.0212

The change in hydraulic head is a clear indication of how microbial growth and subsequent EPS production affect the flow of water through the soil profile within the flow cell (figure 4.13). In figure 4.13a microbial biomass is relatively low, with H₂O

as nutrient supplement as compared to figure 4.13b when the nutrient supplement was substituted with TSB. In 4.13c and 4.13d however, the change in nutrient supplement did not seem to significantly affect the microbial numbers.

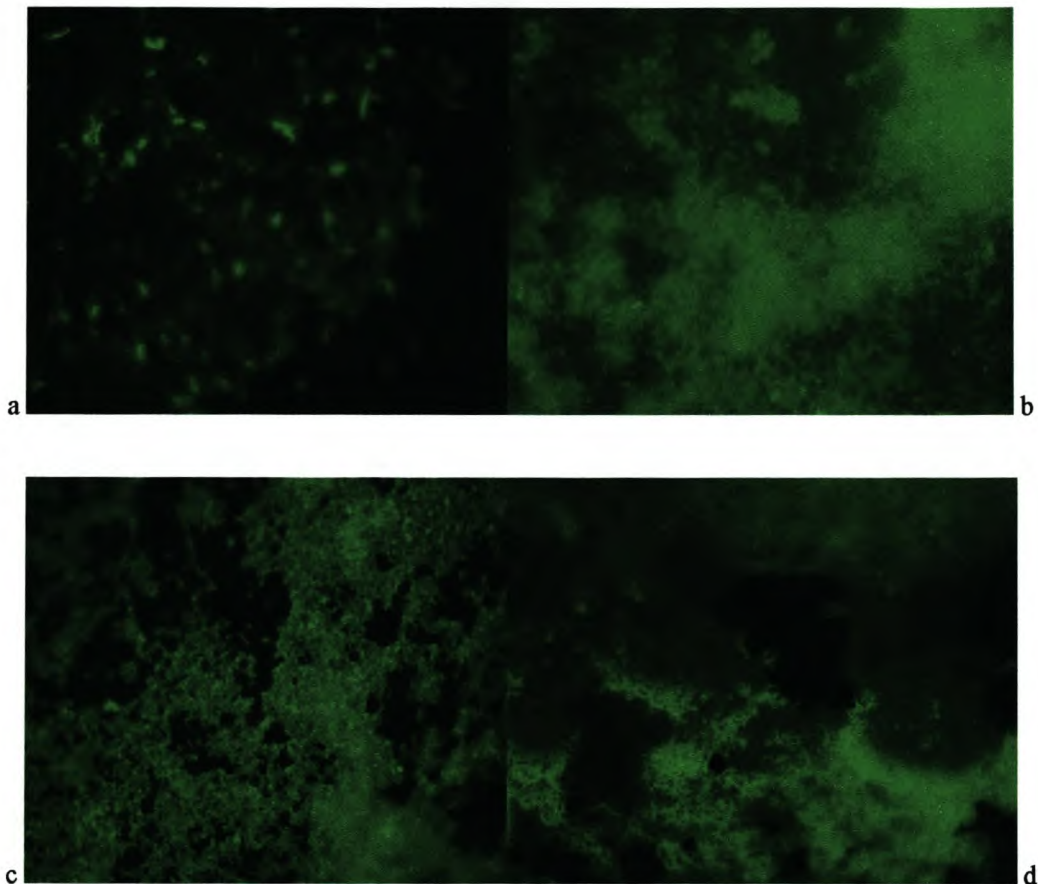
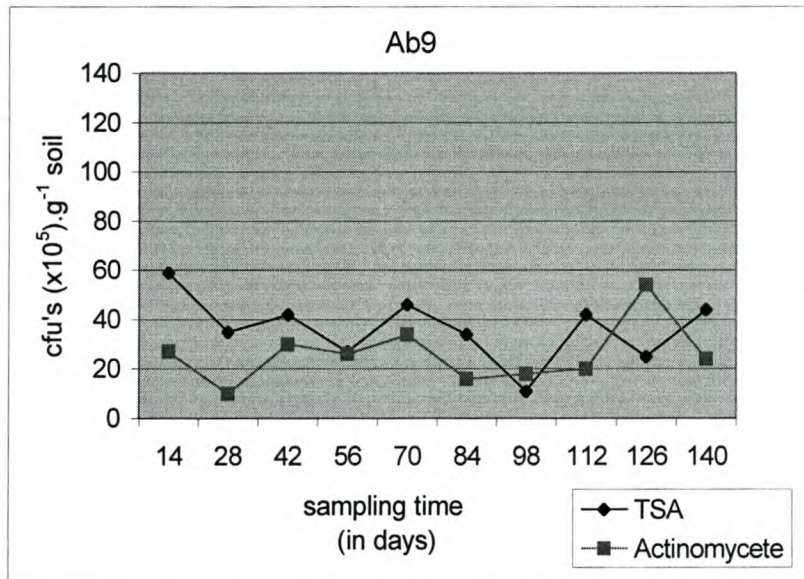


Figure 4.13 Epifluorescent images of con A stained biofilms to demonstrate microbial growth and EPS production within a soil-filled flow cell. The amount of EPS produced, clearly affects the permeability and water flow through the soil profile.

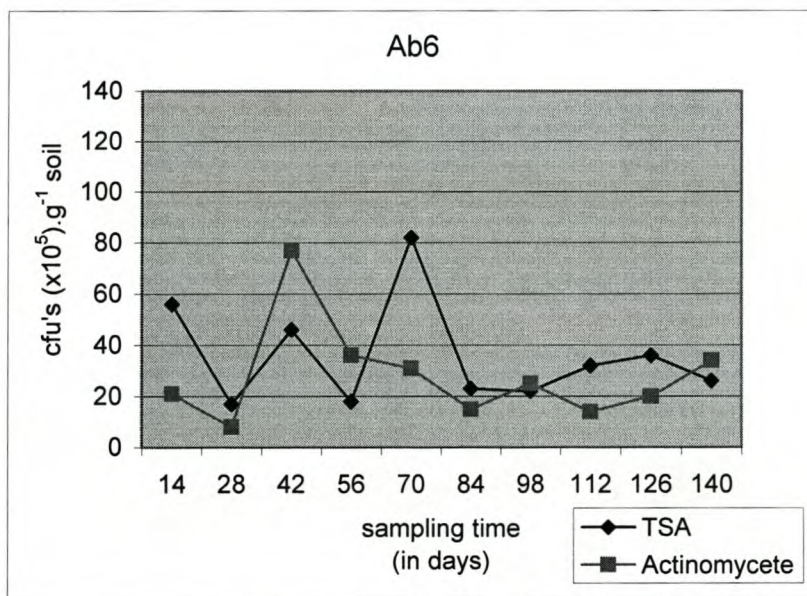
4.5 Effect of EPS producing bacteria when introduced into microcosms and their impact on community composition.

4.5.1 Microbial counts. The results shown in figure 4.14 indicate that microbial numbers remained relatively constant over the 140-day incubation period, with and without the addition of the EPS-producing isolates. In addition, there was little difference between the total aerobic heterotrophic counts (grown on tryptone soy agar, TSA) and actinomycete counts. These results demonstrate the ability of soil

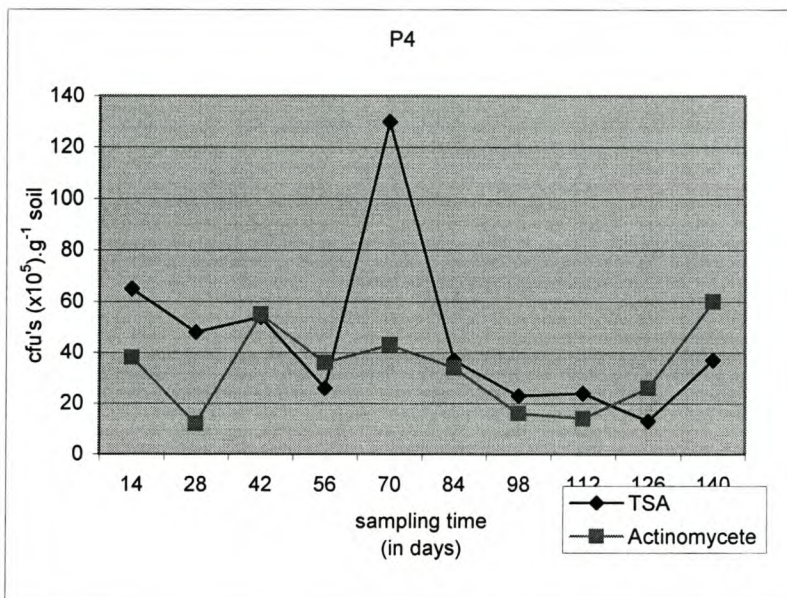
environments to support relatively stable microbial communities in terms of cell numbers.



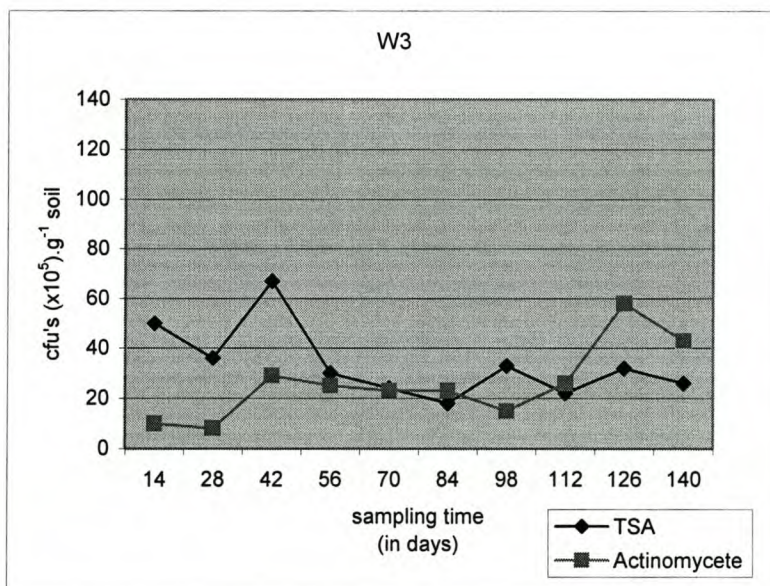
a



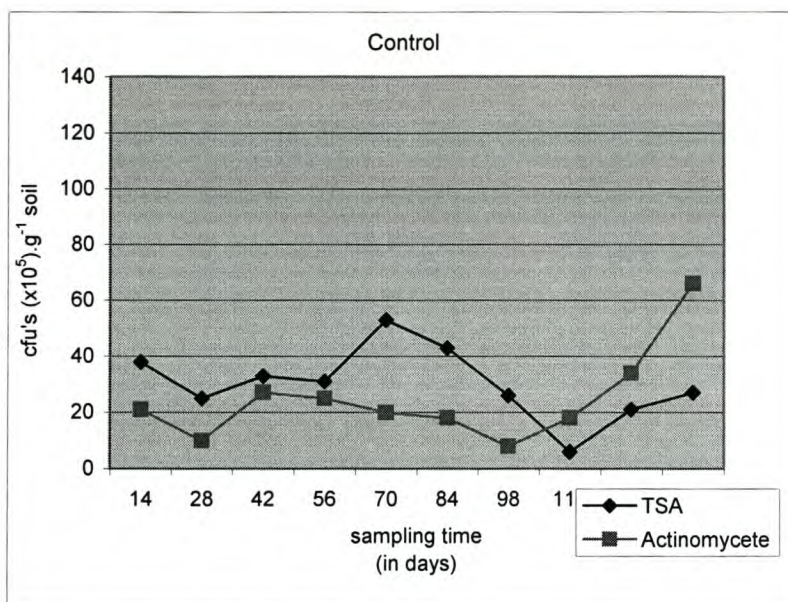
b



c



d



e

Figure 4.14 Microbial numbers, as colony forming units (cfu's) over time in microcosms containing a natural soil microbial community and inoculated with selected EPS-producers (a-d), and controls to which no isolates were introduced (e).

Based on phenotypic and genotypic characteristics, the clustering of isolates into operational taxonomic units allows comparisons of the following three elements of diversity in a sample: the types of bacteria present (composition), the number of types of bacteria (richness), and the frequency distribution or relative abundance of types (structure). The evaluation of these elements for collections of cultivated isolates provides relative measures of community diversity. These, however, do not give accurate descriptions of community diversity *in situ* (Dunbar *et al.*, 1999). It cannot be deduced from the data presented in figure 4.14 whether the introduction of an EPS producer had an impact on community composition or species diversity. Therefore, additional methods based on whole-community metabolic and molecular profiles were applied to compliment this data.

4.5.2 Whole-community carbon source utilization. There was a significant difference between the degree of carbon utilization by microbial isolates obtained from the different soil microcosms by comparing results from the Biolog Ecoplates (figure 4.15).

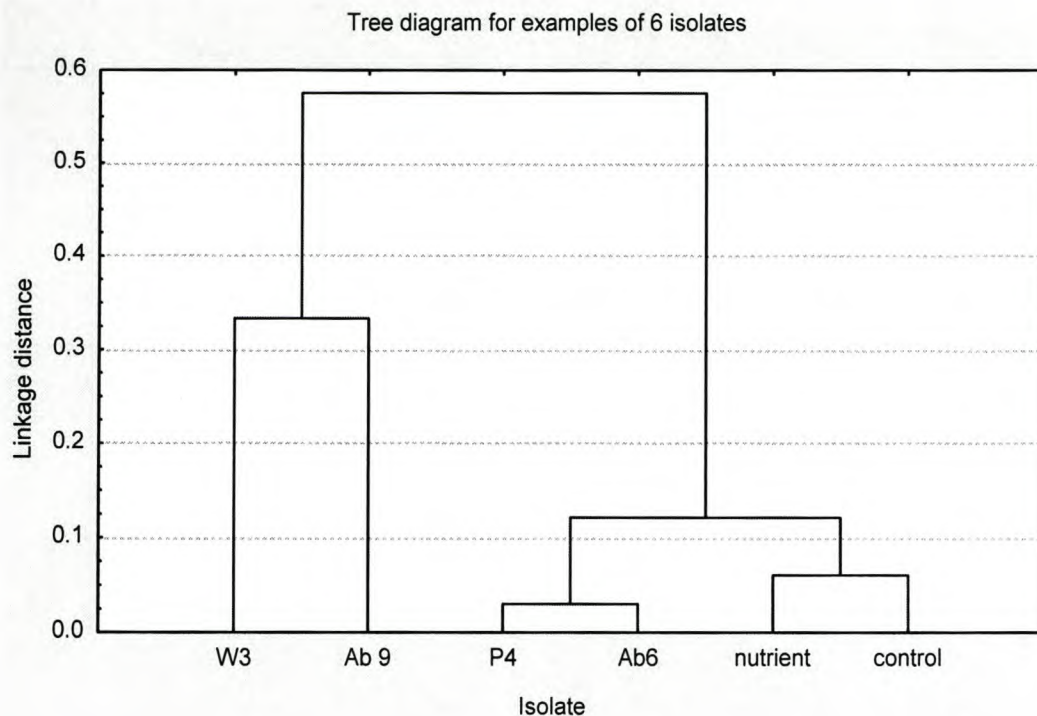


Figure 4.15 An example of a dendrogram of the metabolic fingerprint patterns generated by EPS-producing microorganisms when introduced into soil microcosms containing a natural microbial community. The microorganisms were isolated from different environments and re-introduced into individual soil microcosms as pure cultures (i.e. Ab9, Ab6, P4 and W3). The relative linkage distances give an indication of the degree of shifts that occurred within the soil microbial community after the addition of EPS-producers to the soil. The control represents the natural community without the addition of any EPS-producers, with and without nutrient supplementation.

From figure 4.15 it seems possible that the isolates P4 and Ab6 are related, because they had a similar impact on the whole-community metabolic profile. This impact was relatively small, compared to W3 and Ab9. It is therefore likely that isolates P4 and Ab6 did not have a significant effect on the natural environment. In contrast, isolates W3 and Ab9 were distinctly different from P4 and Ab6 and clearly had an effect on the community's metabolic profile over the extent of the experimental period (i.e. 140 days). It was interesting to observe that there was a relative small difference between the metabolic profiles of the community with and without the addition of

nutrients. This suggests that nutrient addition did not favour a single species or a group of related species to the disadvantage of other species or groups, but rather resulted in an increase in numbers of most of the dominant species in the community. As stated in 4.5.1, assessments based solely on culturing techniques may not have the ability to show differences in community composition. The characterization of microbial communities based on community level sole-carbon-source utilization patterns was reported by Garland & Mills (1991). Carbon substrate utilization patterns have found increasing use in environmental and ecological microbiology over the past five years, and was therefore considered in this study. For instance, ninety six-well microtiter plates are utilized by BIOLOG (Biolog Inc., Hayward, CA) and contain various carbon substrates permit these patterns to be determined quickly, economically and effectively. When the purpose is species identification, ninety five carbon sources (plus one control well) are used, whereas three identical sets of substrates, each containing thirty one carbon sources are used for whole-community analysis, as was used in this study. The use of these patterns to characterise and differentiate strains isolated from the environment has been very effective in providing information on the culturable fraction of the microbial community (Konopka *et al.*, 1998).

4.5.3 Whole-community genetic analysis

4.5.3.1 DNA extraction from soil.

The purified DNA samples were run on a 0.8% agarose gel to confirm presence of the DNA (fig. 4.16).

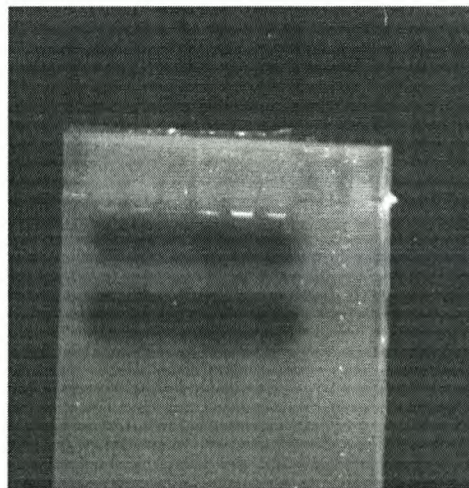


Figure 4.16 An example of purified DNA run on ethidium bromide agarose gel, indicating the presence of DNA from isolated soil microorganisms.

4.5.3.2 Polymerase Chain Reaction (PCR).

Figure 4.17 shows typical examples of the PCR-products that were obtained from soils containing the *in situ* microbial community with and without the supplementation of nutrients and/or the EPS-producing isolates.

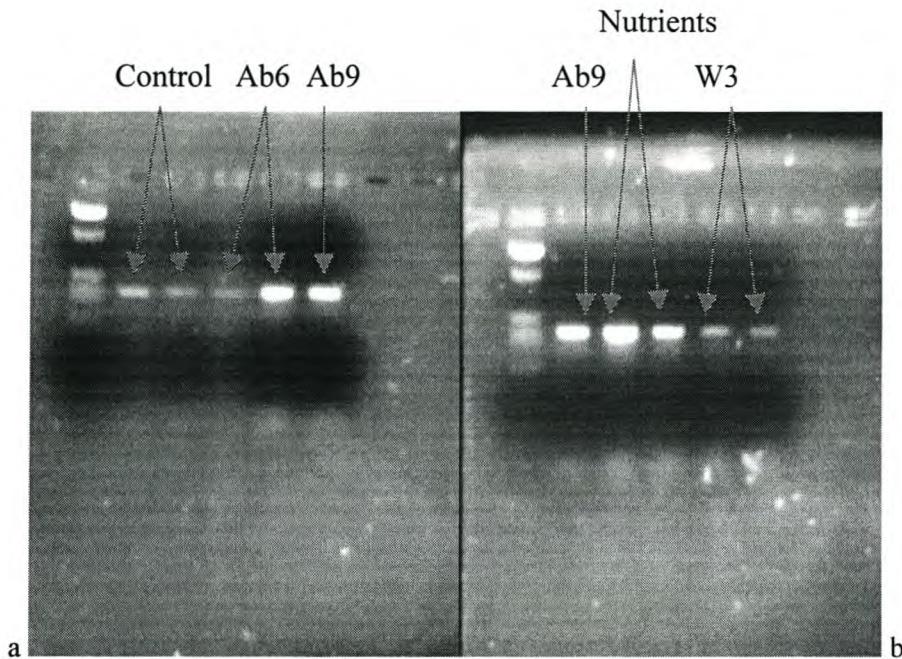


Figure 4.17a-b Amplified DNA product (PCR technique)

Direct extractions of total DNA from soils or sediments typically result in coextraction of other soil components. These include humic acids or other humic substances, which negatively interfere with DNA detection and biochemical reactions (Steffan *et al.* 1988). Initially, no PCR amplification products could be obtained in this study, which was likely the result of the coextracted contaminants described above. These contaminants can, for example, inhibit *Taq* Polymerase in PCR (Tsai & Olson, 1992a,b; Smalla *et al.*, 1993; Tebbe & Vahjen, 1993) or restriction endonucleases (Porteous & Armstrong, 1991; Tebbe & Vahjen, 1993). Humic materials, even in quantities as small as 1ng, have been shown to inhibit PCR (Bej & Mahbubani, 1994a,b).

In order to minimize the impact of coextracted contaminants on PCR, bovine serum albumin (BSA) was added to the DNA samples prior to amplification. BSA is a large, 66000 dalton, globular protein (Peters, 1985). The use of high concentrations of BSA in the hot-start PCR has therefore been shown to decrease the sensitivity of the

reaction against components in the soil extracts and to make PCR product formation reproducible (Romanowski *et al.*, 1993).

4.5.3.3 T-RFLP and DNA profiling.

Although it was possible to extract DNA from the soil (fig. 4.16) and to amplify this DNA (fig. 4.17), subsequent profiling did not result in peaks representative of fragments that were notably different from the background. It was therefore not possible to confirm the data obtained with whole community metabolic profiles (BIOLOG) with T-RFLP analysis, or to add information to that obtained through determining cell numbers. Throughout T-RFLP analysis, the peaks in the DNA profiles which represented the terminal fragments (or base pair sequence of a specific species) were found below the preferred standard or baseline and were therefore regarded as insignificant.

4.6 Concluding remarks.

In this study, a number of EPS-producers were isolated from surface waters and vineyard soils. When introduced to soil columns, these EPS-producers reduced soil permeability, as measured by their impact on water movement through the soil, as well as the hydraulic gradient as measured by the change in pressure (hydraulic heads) generated by the production of EPS. Microscopic analyses revealed that the EPS either attached to the soil surface, or accumulated in the pore throats, thereby restricting flow.

Experiments with 2 mm diameter glassbeads demonstrated the role of EPS in aggregate formation where clusters consisting up to ≥ 30 glassbeads were formed.

Total aerobic, heterotrophic and actinomycete counts showed that bacterial numbers remained constant over a 140-day period following introduction of the EPS-producers. T-RFLP analysis could not demonstrate notable differences in microbial shifts within the microcosms. In contrast, distinct differences were observed in the whole-community metabolic profiles in the presence and absence of additional nutrients.

The results from this study support earlier observations on the role of EPS-producing bacteria in the modification of soil structure and permeability. Further studies are needed to evaluate the potential for manipulating EPS production *in situ*. Furthermore, almost all the studies to date focussed on EPS production by bacteria. Recent observations suggested that soil fungi might also constitute notable properties to the microbial EPS in soil. Given the wide distribution of fungi in the near-surface soil layers, the role of fungal EPS in soil binding should be assessed.

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