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THE BIOLOG SYSTEM AS A METHOD TO DETERMINE MICROBIAL SPECIES DIVERSITY IN NATURAL HABITATS

MSc UP 1998

THE BIOLOG SYSTEM AS A METHOD TO DETERMINE MICROBIAL SPECIES DIVERSITY IN NATURAL HABITATS

by

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Submitted in partial fulfilment

of the requirements for the degree

M.Sc (Agric) Microbiology

In the Faculty of Biological and Agricultural Sciences

Department of Microbiology and Plant Pathology

University of Pretoria

Pretoria

South Africa

January 1998

I, the undersigned, declare that the thesis hereby submitted to
the University of Pretoria for the degree M.Sc. (Agric)
and the work contained therein is my own original work and
has not previously, in its entirely or in part, been submitted
to any university for a degree.

Signed: _____ this 19th day of February 1997

Ek kyk op na die berge: waarvandaan sal daar vir my hulp kom?

My hulp kom van die Here wat hemel en aarde gemaak het.

Ps. 121:1-2.

Opgedra aan my man

BEDANKINGS

Ek wil graag die volgende persone opreg bedank:

- **Prof T E Cloete,** Hoof van die Departement van Mikrobiologie en Plantpatologie, van die Universiteit van Pretoria, en promotor van hierdie studie: vir u vertroue in my, vir u leiding, raad, motivering en ondersteuning met die projek.

- **Die Water Navorsings Komissie,** vir die befondsing van die navorsingsprojek.

- **Daspoort and Zeekoegat Waterversorging,** vir hul bereidwilligheid en hulp tydens die verkryging van watermonsters.

- **Aan my familie en vriende**, vir hul belangstelling en ondersteuning.

- Vir **Marthie Ehlers,** se hulp en raad met die skryf van die tesis en eksperimentele werk

- Vir 'n besondere vriendin, **Leanne Zdyb,** vir haar hulp en aanmoediging tydens my studies.

- **Aan my Ouers**, wat in my geglo het en my die geleentheid gegun het om verder te studeer, vir hul opregte belangstelling, ondersteuning, en liefde.

- **Aan my man, Hennie Korf,** vir sy liefde, belangstelling en geduld. Dankie vir al jou goeie raad, hulp, bystand en aanmoediging.

- **Aan my Skepper en Saligmaker,** aan wie alle eer toekom, wat my krag, wysheid en insig gegee het om te volhard.

INDEX

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PROMOTOR : Prof. T.E. Cloete
DEPARTMENT : Microbiology and Plant Pathology
DEGREE : M.Sc.(Agric) Microbiology

SUMMARY

Microorganisms are an element of ecosystems. Microbial ecology is the study of interrelationships that exist between microorganisms and their biotic and abiotic environment. Microbial activities compose vital links in the chain of geochemical events that cycle nutrient elements within the biosphere and that are essential for the continuance of life.

Our knowledge of microbial biodiversity has been severely limited by relying on microorganisms that have been cultured; these represent only a tiny fraction of the microbial diversity in the environment. Recently, however, molecular- and biochemical techniques have provided methods for characterizing natural microbial communities without the need to cultivate organisms. This is because cell constituents like nucleic acids are directly extracted from environmental samples and isolation and culturing of individual species are thus not required. However, these methods

are time-consuming, complex and it is difficult to interpret the data obtained from these techniques regarding diversity of microbial communities. A need therefore exists to develop a technique which would not require culturing of organisms, and which would be less complex than the molecular techniques.

The Biolog Automated Microbial Identification System was recently introduced, mainly for the identification of microorganisms (Biolog Inc., Hayword, CA). However, microbial ecologists recently used Biolog plates to investigate carbon-source utilization patterns for microbial communities. In this project the Biolog system was used to dermine carbon source profiles, based on carbon source utilization of different microbial communities. It was determined whether these carbon source utilization profiles could give a better understanding about species diversity by using pattern recognition.

According to the results, the validity of a theoretical model was verified. This was done by classifying mixtures of pure bacterial cultures and natural microbial communities to represent different patterns of microbial communities based on their carbon source utilization profiles. Furthermore, it was determined that the carbon source utilization profile of natural microbial communities, could serve as an information system, to describe certain patterns which would reflect the species diversity of a particular habitat.

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OPSOMMING

Mikroörganismes is 'n element van ekosisteme. Mikrobe ekologie is die studie van verwantskappe wat bestaan tussen mikroörganismes en hul biotiese en abiotiese omgewing. Mikrobe aktiwiteite vorm noodsaaklike skakels in die biogeochemiese omsettings wat nutriënte in die biosfeer vrystel wat essensiëel is vir die voortbestaan van lewe.

Ons kennis oor mikrobe biodiversiteit is beperk omdat ons staatgemaak het op mikroörganismes wat kweekbaar is; hierdie organismes verteenwoordig slegs 'n fraksie van die wat in die omgewing voorkom. Huidiglik het molekulêre- en biochemiese tegnieke metodes verskaf wat natuurlike mikrobe gemeenskappe karateriseer sonder dat mikroörganismes gekweek hoef te word. Dit is omdat selbestandele soos nukleïensure direk geëkstaeer word vanuit monsters in die

omgewing sodat isolasie en kweking van individuele spesies onnodig is. Maar, hierdie tegnieke is tydrowend, kompleks en dit is moeilik om die data te interpreteer oor die diversiteit van mikrobe gemeenskappe. Daarom bestaan daar 'n behoefte om 'n tegniek te ontwikkel wat nie kweking van mikroöganismes vereis nie, en wat minder kompleks is as molekulêre tegnieke.

Die Biolog Geoutomatiseerde Mikrobe Identifikasie Sisteem wat hoofsaaklik vir die identifikasie van mikroörganismes gebruik word, het onlangs ook 'n ekologiese toepassing verkry (Biolog Inc., Hayword, CA). Mikrobe ekoloë het die Biolog plate gebruik om koolstofbronbenuttingspatrone vir mikrobe gemeenskappe te ondersoek. In hierdie projek was die Biolog sisteem gebruik om koolstofbronpatrone te bepaal wat gebasseer was op die koolstofbron benutting van verskillende mikrobe gemeenskappe. Dit was bepaal of hierdie koolstofbronbenuttingsprofiele 'n beskrywing van spesie diversiteit kon gee deur gebruik te maak van patroonherkenning.

Na aanleiding van die resultate, was die geldigheid van 'n teoretiese model bevestig. Dit was gedoen deur die mengsels van suiwer bakteriese kulture en natuurlike mikrobe gemeenskappe te klassifiseer in verskillende katogorieë, gebasseer op hul koolstofbronbenuttingsprofiele. Verder, was daar bepaal dat die koolstofbronbenuttingsprofiele van natuurlike mikrobe gemeenskappe kon dien as 'n informasie sisteem, wat sekere patrone kon beskryf, en die spesie diversiteit van daardie spesifieke habitatte kon reflekteer.

CHAPTER 1

INTRODUCTION

Microorganisms are vital to the function and maintenance of the Earth's ecosystems and biosphere. As major contributors to biogeochemical cycles, they perform unique and indispensable activities in the circulation of matter in the world, on which large organisms, including humans depend (Cloete and Muyima, 1997). Microorganisms can be used for the biomonitoring of global ecology, for climatic changes, the effects of pollutants and other habitual disturbances (Hawksworth and Colwell, 1992; Tiedje, 1994). Therefore, microorganisms are of central importance to biosphere sustainability (Tiedje, 1994).

The determination of bacterial species diversity is an important aspect of environmental studies. The maturity of a community is reflected in both its diversity and its productivity (Atlas and Bartha, 1993). A community that has a complex structure, rich in information as reflected by high species richness, needs a smaller amount of energy for maintaining such a structure (Atlas, 1984). Diversity decreases in communities under stress (Atlas, 1984). Important conclusions, about microbial community structure and function can therefore be made, by determining species diversity.

Evaluation of diversity in environmental microbial communities has been limited by the large

percentage of microorganisms that are non-culturable (Hugenholtz and Pace, 1996; Cloete and Muyima, 1997). It was reported that only 10% or less of the microorganisms in water and soil can be enumerated and, by implication, be grown on agar media (Olsen and Bakken, 1987). Using 16SrRNA as molecular markers, (Wagner and Amann, 1997; Wise, McArthur and Shimkets, 1997), DNA isolation and PCR (Hugenholtz and Pace, 1996; Yap, Soong and Davies, 1996) and biochemical techniques such as signature lipid biomarker analysis (White, Starr and Ringelberg, 1996), the limitations of traditional culturing techniques are circumvented in the assessment of the biodiversity of microbial communities. This is because cell constituents like nucleic acids are directly extracted from environmental samples and isolation and culturing of individual species are thus not required. However, these methods are time-consuming, complex and it is difficult to interpret the data obtained from these techniques regarding diversity of microbial communities. A need therefore exists to develop a technique which would not require culturing of organisms, and which would be less complex than the molecular techniques.

The Biolog Automated Microbial Identification System was recently introduced, mainly for the identification of microorganisms (Biolog Inc. CA). However, microbial ecologists recently used Biolog plates to investigate carbon-source utilization patterns for soil microbial communities (Zak, Willig, Moorhead and Wildman, 1994; Bossio and Scow, 1995; Haack, Garchow, Klug and Forney, 1995; Garland, 1996; Hitzl, Rangger, Sharma and Insam, 1997), aquatic samples (Garland and Mills, 1991) and wastewater treatment communities (Guckert, Carr, Johnson, Hamm, Davidson and Kumagia, 1996; Victorio, Gilbride, Allen and Liss, 1996). According to the results of Bossio and Scow (1995) differences in substrate utilization patterns of the soil microbial communities were significantly related to carbon and flooding treatments. The Biolog System detected considerable variation in the ability of six plant communities to metabolize different

carbon compounds (Zak *et al.*, 1994). According to Haack *et al.*(1995) soil samples from Michigan State University varied significantly in the rate and extent of oxidation of seven tested substrates, suggesting microscale heterogeneity in composition of the soil microbial community. Guckert *et al.* (1996) selected Biolog as a measure of changes in metabolic diversity of activated sludge samples and suggested that the use of GN Biolog microplates provides the reproducibility and resolution needed to evaluate changes in metabolic diversity of mixed microbial communities.

The Biolog System is based on the utilization of a large number of different organic compounds by the test organisms and oxidation of these substrates is monitored by the colometrically determinable conversion of the redox dye tetrazolium violet into a vividly purple formazan. Standardized microplates allow the simultaneous testing of 95 diagnostically significant carbon sources, against one reference well that contains no carbon substrate (Wünsche and Babel, 1996). Community physiological profiling with Biolog plates is a simple, rapid method that appears to provide information on both the density and composition of microorganisms in environmental samples through evaluation of the rate and pattern, respectively, of colour development (Garland, 1996).

The hypothesis of this study was, that the carbon source profile of natural microbial communities could serve as an information system, to describe certain patterns, which would reflect the species diversity of the habitat.

The aims of this study were therefore as follows:

- To develop a theoretical model for the interpretation of the carbon source utilization patterns of microbial communities.
- To verify the validity of this model by using the carbon source profile of mixtures of pure bacterial cultures.
- To determine whether carbon source profiles of natural microbial communities could be used to determine species diversity and whether the pattern recognition model is valid.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Microorganisms are essential components of all ecosystems (Hawksworth and Colwell, 1992). Microbial activities compose vital links in the chain of geochemical events that cycle nutrient elements within the biosphere and that are essential for the continuance of life (Atlas, Colwell. Pramer, Tiedje, Vidaver and Wodzinski, 1992).

Microorganisms are primary producers and dominate the biogeochemical cycles on our planet (Tiedje, 1994; Cloete and Muyima, 1997). Hence microorganisms have an immense functional diversity and capability and constitute a major genetic resource (information pool) to counteract environmental changes and stresses (Alexander, 1971; Cloete and Muyima, 1997). Microorganisms transform organic compounds in ways that are exceedingly important for environmental decontamination and remediation (Hawksworth and Colwell, 1992; Tiedje, 1994). Acting as biochemical incinerators , microorganisms convert pesticides, petroleum and other pollutants in soil and water to harmless products such as carbon dioxide and water (Atlas *et al.*, 1992; Cloete and Muyima, 1997). Microorganisms are thus major agents in natural ecosystems of reducing environmental stresses (Alexander, 1971).

Knowledge of the species composition of microbial biocenoses (communities) can provide insights into the ecological function of these communities and assist the choice of suitable methods for remediation of polluted systems (Wünsche and Babel, 1996). Therefore, microbial diversity is essential for a healthy earth (Fox, 1994). However, less than 5% of the world's microorganisms have yet been described, and it is not improbable that their real number exceeds even that of insects (Hawksworth and Colwell, 1992). The lack of knowledge about the diversity and function of microbial communities is due to the fact that the majority of the organisms are viable but non-culturable (Höfle, 1992; Haldeman and Amy, 1993; Hugenholtz and Pace, 1996). It was reported that only 10% or less of the organisms in water and soil can be enumerated, and by implication, be grown on agar media (Steyn and Cloete, 1989).

The rRNA approach, together with other molecular techniques (Wise *et al.*, 1996; Yap *et al.*,1996: Wagner and Amann, 1997) and biochemical techniques such as signature lipid biomarker analysis (White *et al.*,1996), enables identification and phylogenetic characterization of micro-organisms without cultivation. However, these methods are time-consuming, complex and it is difficult to interpret the data obtained from these techniques regarding diversity of microbial communities. Hence, there is a need for techniques that allow one to characterize microbial communities without the usual reliance on selective culturing and which would be less complex than the molecular techniques.

An alternative approach is to examine components of microbial biodiversity, for which there exists a reasonable chance of detecting patterns, which can be related to diversity and function, for example carbon source utilization patterns, protein profiles, DNA reannealing etc. Pattern recognition may simplify the interpretation of complex information obtained using phenotypic and

genotypic methods. This simplified information will lead to a better understanding of bacterial community structure. It is believed that the patterns in species diversity are the products of interacting forces that vary in relative importance both in time and space. These interactions are as yet not completely understood (Cloete and Muyima, 1997).

The goal of studies on biodiversity is to construct inventories of living organisms to serve as frames of reference for monitoring natural resources like rivers (Palleroni, 1994). Management of systems will be made more successful if microorganisms can be used as tools to detect, assess, restore and maintain environmental support systems (Atlas *et al.*, 1992). Pattern recognition, for example, using carbon source utilization profiles, can provide the ecologist with valuable information regarding the dynamics of ecosystems and effect of these changes. These are reflected in species diversity of ecosystems. Systems under stress normally cause species diversity to decrease and may cause an increase in numbers of the species capable of tolerating stress. Determination of species diversity is therefore, important (Atlas and Bartha, 1993). This overview will deal with interactions among microbial populations, the concept and determination of species diversity as well as the Biolog System.

2.2 Interactions among microbial populations

The microbial populations that live together at a particular location, a habitat, interact with each other to form a microbial community. The microbial community is structured so that each population contributes to its maintenance (Atlas and Bartha, 1993). Both positive and negative interactions occur between individuals within a single microbial population and between the diverse

microbial populations of a community (Allee, Emerson, $Park_a$, $Park_b$ and Schimdt, 1949). These interactions permit some populations to reach sizes optimal for the resources available within a habitat. The totality of the interactions between populations maintains the ecological balance of the community (Bull and Slater, 1982). The different types of interactions will now be discussed in more detail.

2.2.1 Interactions within a single microbial population

According to Allee's principle, both positive and negative interactions may occur even within a single population (Allee *et al.*,1949). Generally speaking, positive interactions increase the growth rate of a population, whereas negative interactions have the opposite effect (Figure 2-1) (Atlas and Bartha, 1993).

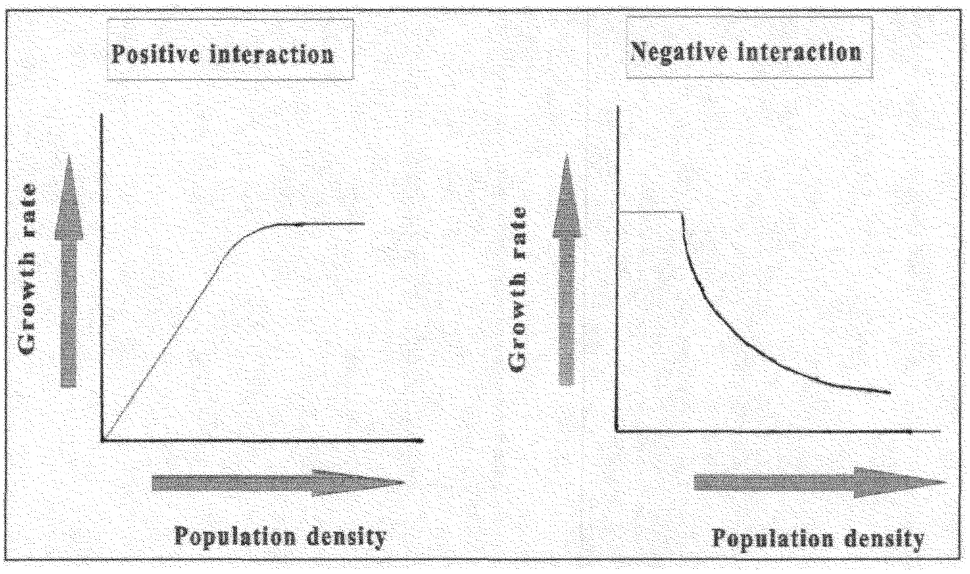

Figure 2-1 Growth rates with increasing population density, showing the effects of independent positive and negative interactions.

Positive interactions (cooperation) predominate at low population densities and negative ones (competition) at high population densities. Cooperation within a single microbial population is evidenced by an extended lag period or a complete failure of growth when a very small inoculum is used in routine culture transfer procedures (Atlas and Bartha, 1993). An example of cooperation is the life cycle of the slime mold, *Dictostelium,* during which individual cells migrate together and form a single structure that aids in the survival of the population (Clark and Steck, 1979). Competition takes place when individuals within a microbial population all utilize the same substrates and occupy the same ecological niche (if an individual within the population metabolizes a substrate molecule, then that molecule is not available for other members of the population). Positive and negative interactions within a single microbial population, results in an optimal population density for maximal growth rate (Atlas and Bartha, 1993).

2.2.2 Interactions between diverse microbial populations

When two different populations interact, one or both of them may benefit from the interactions, or one or both may be negatively affected by the interaction (Table 2-1).

Neutralism

Neutralism implies a lack of interactions between two microbial populations. It occurs between populations that are spatially distant from each other. It is likely at low population densities where one microbial population does not sense the presence of another. An example of this interaction is, microbial populations that occurs in marine habitats and oligotrophic lake habitats.

Table 2-1 Different interactions found between two microbial populations (Pop. = Population; no = no interaction; pos = positive interaction; neg = negative interaction).

Interaction	Effect of Interaction	
	Pop. A	Pop. B
Neutralism	no	no
Commensalism	no	pos
Synergism	pos	pos
Mutualism	pos	pos
Competition	neg	neg
Amensalism	no/pos	neg
Predation	pos	neg
Parasitism	pos	neg

Positive interactions

Commensalism

In this interaction one population benefits while the other remains unaffected, it is an undirectional relationship between two populations. Commensalism often results when the unaffected population in the course of its normal growth and metabolism, modifies the habitat in such a way that another population benefits because the modified habitat is more suitable to its needs. For

example, when a population of facultative anaerobes utilize oxygen and the amount of oxygen lowers, it creates a habitat suitable for obligate anaerobes.

Synergism

Synergism (Protocooperation), describes a relationship between two microbial populations where both populations benefit from the relationship, but unlike mutualism, the relationship is not obligatory. For example, *Streptococcus faecalis* is able to convert arginine to ornithine, which can only then be utilized by *Escherichia coli* to produce putrescine. *E.coli* can not produce putrescine without the assistance of *S.faecalis*. Once putrescine is produced, both organisms can use it (Gale, 1940).

Mutualism (Symbiosis)

This relationship can be described as extended synergism. This is an obligatory relationship between two populations that benefit both populations. It is highly specific, requires close physical proximity and one member of the association ordinarily cannot be replaced by another related species. Mutualistic relationships between microorganisms allow the microorganisms to act as if they were a single organism with a unique identity (Atlas and Bartha, 1993). The relationships between certain algae or cyanobacteria and fungi that results in the formation of lichens are an example of mutualism (Hale, 1974).

Negative interactions

Competition

Competition is a negative relationship between two populations in which both populations are

adversely affected with respect to their survival and growth (Atlas and Bartha, 1993). Competition may occur for any growth-limiting resource. Sources like, carbon, phosphate, nitrogen, oxygen, water and growth factors available, are all resources for which microorganisms may compete.

Amensalism

Microorganisms that produce substances toxic to competing populations will naturally have a competing advantage (Fredrickson and Stephanopoulos, 1981). When one microbial population produces a substance that is inhibitory to other populations, the interpopulation relationship is called amensalism (antagonism) (Atlas and Bartha, 1993). The first population may be unaffected by the inhibitory substance or may gain a competitive edge that is beneficial. The terms antibiosis and allelopathy have been used to describe such cases of chemical inhibition.

Parasitism

The parasite, in this relationship, benefits and normally derives its nutritional requirements from the population that is harmed, namely the host. This relationship has usually a long period of contact, which may be direct physical or metabolic and the parasite-host interaction is quite specific. Viruses are obligate intracellular parasites that exhibit great host cell specificity. There are viral parasites of bacterial, fungal, algal, and protozoan populations (Atlas and Bartha, 1993).

Predation

Predation typically occurs when one organism, the predator, engulfs and digests another organism, the prey. Normally this relationship is of short duration and the predator is larger than the prey (Atlas and Bartha, 1993). For example, *Didinium nasutum*, preys on *Paramecium* until the *Paramecium* population becomes extinct.

Populations can exhibit a variety of interactions. Both positive and negative interactions occur between individuals within a single microbial population and between the diverse microbial populations of a community. The totality of the interactions between populations maintains the ecological balance of the community (Bull and Slater, 1982) and eventually determines species diversity. The latter will now be discussed.

2.4 What is Species Diversity?

According to Atlas and Bartha (1993) diversity is the heterogeneity of a system; the variety of different types of organisms occuring together in a biological community. Classical concepts of diversity involve species richness or variety, evenness or equitability and composition (i.e. the numbers of different species present, the relative contribution that individuals of all species make to the total number of organisms present, and the type and relative contribution of the particular species present) (Griffiths, Ritz and Wheatly, 1997). These have been used to generate commonly used indices of diversity, such as the Shannon-Weaver index.

Species diversity indices are mathematical indices that describe the species richness and apportionment of species within a community and the assemblage of populations within a community (Pielou, 1975). Species diversity indices have rarely been applied to microbial communities because of the technical difficulties in speciating the large numbers of microorganisms, their use requires (Atlas, 1984).

There are a few diversity indexes available that utilize the number of species (species richness). Some of these indexes calculate the number of species and the equitability or evenness of the species richness of the individual's distribution (Troussellier and Legendre, 1981). The Shannon index (with slight mathematical variations of the Shannon-Weaver and the Shannon-Weiner indexes) is one of the most widely used diversity indexes, which can measure diversity in a community and is based on the phenotypic features of the members of the community (Mills and Wassel, 1980; Atlas, 1984, Atlas and Bartha, 1993). The Shannon index is sensitive to sample size, especially with small samples and includes an evenness factor (Perkins, 1983). The Shannon index represents the number of individuals (or species) in a sample divided by the total number of isolates being analized (Mills and Wassel, 1980).

The Shannon index is expressed as:

$$H' = - \Sigma P_i \log p_i \quad (i)$$

with P_i the proportion of the community belonging to the i^{th} species. The calculation of H' was simplified to the following log base 10 formula (Atlas, 1984; Atlas and Bartha, 1993; Torsvik, Sørheim and Goksøyr, 1996).

$$H' = \frac{C}{N} (N \log_{10} N - \Sigma n_i \log_{10} n_i) \quad \text{(ii)}$$

H' = diversity

C = 3.3219

N = total number of individuals

n_i = number of individuals in the group (Pielou, 1966, Hairston, Allan, Colwell,

Futuyma, Howell, Lubin, Mathias and Vandermeer, 1968; Kaneko and Atlas, 1977; Mills and Wassel, 1980; Atlas, 1984; Atlas and Bartha, 1993).

Species diversity is a complex term and consists of various factors that are related to and dependant on each other. Although diversity indexes are useful for determining environmental stress, they can be misused when one is not sure which index is appropriate or how to interpret results obtained. The major disadvantage of diversity indexes is that they do not give any information about the community stability of the different species present (Pielou, 1975). Despite the above mentioned, measurement of microbial diversity provides insights into the ecological functioning of the community (Atlas, 1984). But, more simplistic models are required to describe interactions and function of microorganisms in their specific ecosystems.

2.4 Diversity, stability and interactions of microbial communities

In ecological hierarchies, communities are the highest biological unit, consisting of populations and individuals. The structure and dynamics of natural microbial communities, their species composition over time and space, are poorly understood (Höfle, 1992). This is in contrast to the wealth of knowledge about the community structure formed by higher organisms like plants and animals (Amann *et al.*, 1995). The lack knowledge is due to the fact that distinctive morphological features are absent in microorganisms and that the bulk of natural microbial cells cannot be cultured and remain unidentified (Höfle, 1992; Haldeman and Amy, 1993; Amann *et al.*, 1995).

Microorganisms are part of interrelationships between the biotic and abiotic factors in ecosystems. The maturity of a community is reflected in its diversity and its productivity (Lovejoy, 1993; Fox, 1994). Mature ecosystems are complex; the presence of a high number of species (high diversity content or information content) allows for numerous interspecies relationships and require a lower amount of energy for maintaining the structure (Atlas, 1984; Atlas and Bartha, 1993).

When the number of species present in the community, and their abundance, remain constant over time, the community is considered to be stable (Atlas, 1984). Stability can be measured as the persistence of populations and the evenness of distribution of species abundance within the community (Ehlers, 1995). Fitness of a community (due to a high information content) is its ability to withstand changes in the environment (Ehlers, 1995). A stable community contains a high degree of information (species diversity) to recover or withstand environmental perturbations (Atlas, 1984).

Communities are subjected to unpredictable variations in their environment, but microorganisms have evolved strategies that enable them to survive and maintain themselves within communities. A stable community is one that can recover its characteristic composition and relative abundance following an environmental disturbance (Hattori, Ishida, Maruyama, Morita and Uchida, 1989). An example of a stable community is an activated sludge plant, where a large amount of information is present, due to many different species represented by a high number of individuals of each species (Ehlers, 1995). This community is best suited to overcome environmental stress, because it contains a large amount of information.

Diversity and bacterial numbers determine the ability of the community to resist stress (Atlas,

1984). The more stable the community the more it will resist a decrease in both numbers and diversity (Atlas and Bartha, 1993). Low species diversity characterize areas in which the intensity of one or more ecological factor approaches extremes that are capable of supporting life (Ehlers, 1995). Communities with low genetic diversity should be less able to withstand environmental stress, than those communities in which the genetic heterogeneity provides the elasticity needed for dealing with environmental modifications (Alexander, 1971; Rashit and Bazin, 1987).

The exception to the rule is found in extreme environments like ice seas and a high salt concentration environments where the species diversity is low and the community stable (Ehlers, 1995). The communities have adapted to these conditions and consist of large numbers of organisms, but of only a few species (Kaneko and Atlas, 1977). Community stability is determined not only by the number of species present but also by the type of environmental stress (Atlas, 1984).

Diversity generally is lower in communities under environmental stress (Atlas and Bartha, 1993). For example, diversity calculated with the Shannon Weaver index is lower in surface water bacterial communities in the Arctic Ocean than in temperate oceans where low temperatures and ice do not cause the same degree of physical stress (Atlas, 1984). Disturbances, such as the introduction of pollutants into aquatic ecosystems, places living organisms under stress, and will result in a decline in the number of species and individuals in that environment. Species which are tolerant to the stress factors will become predominant, while certain species may not survive and this will lead to a decrease in diversity (Atlas, 1984; Atlas and Bartha, 1993). Microorganisms can, therefore, be indicators of environmental stress (Atlas, 1984). Theoretically, stressed communities with low diversities are less well adapted to deal with further environmental fluctuations than

biologically accommodated communities with higher diversities.

Diversity generally decreases when one or a few populations attain high densities; high numbers signify successful competition and domination of a single population (Atlas and Bartha, 1993). Species diversity tends to be low in physically controlled ecosystems, for example acid bogs, hot springs and antarctic dessert habits, because adaptations to the prevailing physicochemical stress are of the highest priority and leave little room for the evolution of closely balanced and integrated species interactions (Atlas and Bartha, 1993). Species diversity tends to be higher in biologically controlled ecosystems, that is, where the importance of interpopulation interactions outweighs that of abiotic stress (Atlas and Bartha, 1993). In such biologically accommodated communities the physiocochemical environment allows greater interspecies adaption resulting in species-rich associations. Microbial diversity in habitats, such as soil, is normally high, but diversity is markedly low under conditions of stress or disturbance, such as in infected plant or animal tissues.

Communities which have a lower diversity will contain less information and lower bacterial numbers, are less stable and will be more affected by stress conditions than communities with high species diversity and numbers (Atlas, 1984). After removal of the ecological stress factor, a stable community containing a large quantity of information will recover to its original stable status. The relationship between diversity and stabiltiy is acceptable and useful for the examination of the short and long-term effects of stress factors on the environment. More experimental work is needed to define the functional relationships between diversity and stability of different communities (Atlas, 1984). The information content of an ecosystem is probably more important than the species diversity and it is suggested that the concept of information content by further explored in understanding microbial ecosystems (Ehlers, 1995).

2.5 Koch's postulates

The Koch-Henle postulates have not been applied to the study of the ecology of microorganisms. This is probably because it has not yet been possible to inoculate a system with a pure culture to see whether it would perform the same function after it has been isolated and studied in pure culture *in vitro*. Steyn and Cloete (1989) proposed an adaption of Koch's postulates to determine the function of a specific population in a community, hence, particularly useful for aut-ecological studies. They suggested the following:

1) A particular microorganism must always be associated with a certain phenomenon;
2) the same function(s) observed in the natural habitat must also be performed in pure culture;
3) the organism must be enumerated in the natural habitat, isolated and studied in pure culture;
4) the function must be quantified (activity per cell determined); and
5) the product of the number of individual cells and activity per cell should account for the magnitude of the phenomenon observed in the natural habitat (Steyn and Cloete, 1989).

However, the above relies on the culturability, isolation and identification of the organisms. The challenge therefore, is to obtain information about the structure and function of a community without having to culture the organisms. Molecular ecology is rapidly evolving and is giving new insight into the interrelations that exist between microorganisms and their environment (Cloete and Muyima, 1997).

2.6 Determination of species diversity

When considering microbial communities the parameters of diversity indices simply cannot be determined. There are no methods currently available, or likely to be available in the foreseeable future, that can determine the identity, richness and evenness of all microbial species present. Rather, microbial ecologists have to interpret the data that can be obtained and devise experimental approaches to overcome the technical shortcomings (Griffiths *et al.*, 1997).

Our knowledge of microbial diversity has been limited, because of the non-culturability of microorganisms using standard techniques such as the viable plate count or the most probable number (MPN) techniques (Griffiths *et al.*, 1997; Wagner and Amann, 1997). Furthermore, isolate-based techniques offer a limited, biased view of microbial communities, due to (1) selective nature of laboratory media, (2) the multiple microbial interactions and, (3) the difficulties of determining microbial function *in situ* (Kersters, Van Vooren, Verschuere, Vauterin, Wouters, Mergaert, Swings and Verstraete, 1997).

2.6.1 Phenotypic diversity

Microbial diversity has been determined by methods revealing the phenotypic features of members of the community. These features have been employed by bacteriologists as the criteria for differentiating between species. The methods are based on sampling, isolation for pure cultures and differentiation of phenotypic properties (Haldeman and Amy.1993). Thus, recognizable and

distinctive phenotypes must be expressed during *in vitro* culture (Atlas and Bartha, 1993).

The phenotypic approach has several limitations. Bacterial strains have to be isolated from the biotype and pure cultures cultivated in the laboratory before phenotypic testing can be performed (Torsvik *et al.*, 1990; Stahl, 1993). It is difficult to isolate and grow the majority of bacteria found in nature in culture and it has been reported that only 10 % or less of the organisms in water and soil can be enumerated and, by implication, be grown on agar media (Steyn and Cloete, 1989). Furthermore, less than 1% of the bacteria visible microscopically can be cultured (Leff, Dana, McArthur and Shimkets, 1995) and microorganisms constituting extremely low proportion of a community can be overlooked by plating procedures (Atlas and Bartha, 1993). Thus, only a restricted part of the genetic information is revealed through phenotypic testing and we do not even know whether these organisms are representative of natural populations and what their respective functional niche is (Torsvik *et al.*, 1990; Stahl, 1993).

2.6.2 Taxonomic diversity

Bacteria are classified according to classical bacteriological tests. (Atlas, 1984). Commercial kits are available to assist the scientist in identifying bacteria, for example the API-kits or Biolog (Haldeman and Amy, 1993). Pure cultures of unknown organisms are examined and compared with previously described reference cultures. After phenotypic characterization of each strain the results of these tests are subjected to Bayevian cluster analysis for identification (Torsvik *et al.*, 1990). The phenotypic diversity is determined by the appropriate index for example the Shannon index which can be used to determine the diversity of a community in a particular habitat (Torsvik *et al.*, 1990).

2.6.3 Physiological diversity

Physiological diversity is based on environmental parameters (e.g. temperature, light intensity, salinity and pollutants) and physiological functions (e.g. metabolism) within the community (Atlas, 1984). The microbial isolates are characterized by a set of binary biochemical descriptors. Identification of species is not performed (Atlas, 1984). A functional evenness index is based on the physiological characteristics of bacteria. This index measures the evenness of distribution of physiological attributes within the community. In addition to taxonomic diversity measurement, this method appears to be useful for spatial or temporal comparisons of community structure and function (Atlas, 1984).

2.6.4 Serology

Bacteria can be identified by their serological properties. Structures on the cell wall of bacteria can induce an immune response in the bodies of mammals. With this method it is possible to generate antibodies against these antigenic structures i.e. proteins, flagella and lipopolysaccharides. The bacteria can be identified through serological reactions, for example the enzyme-linked immunosorbent assay (ELISA) (Priest and Austen, 1993).

Immunological detection

Immunological laboratory methods have become powerful and indispensable research tools in biochemistry, molecular biology, microbiology, cell biology, and other related disciplines (Erasmus, 1997). Antibodies have been used to address problems that would have been difficult

or impossible to approach by other methods. The main advantage of antibodies is that they can be raised against virtually any organic substance (Erasmus, 1997). The success of immunological approaches in research strategies is based largely on the characteristics of the antibodies available with regard to their specificity, affinity and cross-reactivity (Dreckhan, Jöns and Schmitz, 1993).

Antibodies have successfully been used in many studies on microbial communities (Atlas and Bartha, 1993). Fluorescent polyclonal antibodies have been used for the detection and enumeration of particular microorganisms whereas conventional techniques are especially difficult (Cloete and Steyn, 1989). Antigenic fingerprinting as well as detailed analysis of serological variability within bacteria from pure culture and natural habitats was also successfully studied by using antibodies (Witzel, 1990).

2.6.5 Genetic diversity

Phylogenetic studies on prokaryotes are based on the comparison of homologous genetic sequences in the genetic material of species (Shleifer and Stackebrandt, 1983). A fundamental problem when using the traditional species- and physiological diversity approaches is their dependance on cultivation and phenotypic characterization of bacterial isolates (Torsvik *et al.*,1996). Thus, only a minor part of the genetic information in a population or a community is explored when applying traditional methods for diversity measurements. To circumvent the culturing problem there has been a shift from the analysis of isolates to total community analysis. For such analysis molecular techniques and especially nucleic acid analysis have been of great importance (Griffiths *et al.*, 1997). A few methods will now be discussed.

Chemosystematic analysis

In chemosystematic analysis bacterial cells are chemically characterized. Growth conditions can effect the biochemical and physiological tests used in numerical taxonomy considerably. The determination of DNA base composition, protein profiles, fatty acid profiles and phospholipid analysis have become established in groups of bacteria where morphological characteristics have failed to provide classification (Schleifer and Stackebrandt, 1983; O'Brien and Colwell, 1987; Haldeman and Amy, 1993; Griffiths *et al.*, 1997). Cell content and cell wall structure are analyzed after culturing the bacterial species under the same laboratory conditions. Chromosomal DNA and RNA are the only chemical components unaffected by growth conditions (Priest and Austen, 1993). The % GC base pairs of DNA and the determination of the genome size of bacterial species can be seen as part of these studies because they reveal some of the genetic features of an organism (Priest and Austen, 1993). Protein, phospholipid and fatty acid profiles of unknown organisms may be compared with patterns of reference strains to identify the organism (Priest and Austen, 1993

Nucleic acid hybridization

Advantages of this methodology include the ability to detect specific organisms without prior culturing of organisms and without the need for selectable markers. The hybridization method can also be used to detect multiple populations in the same analysis as well as detection of genetic rearrangements or gene transfer in natural communities (Holben and Tiedje, 1988). The principle features of nucleic acid hybridization techniques are denaturation and selective annealing of complementary strands of nucleic acid molecules. The specificity of this hybridization reaction can be controlled such that only identical or nearly identical sequences in a complex mixture of nucleic acids extracted from a population or community can anneal (Holben and Tiedje, 1988).

Another method for nucleic acid studies is the use of DNA probes to quantify the level of transcription of a gene of interest. To detect the transcription level an excess of probe DNA, identical in sequence to the gene, is bound to a filter, then hybridized to the total RNA of an organism grown under specific conditions. Usually the RNA of the organism is radioactively labelled *in vivo*. The amount of labelled RNA that hybridizes to the probe is proportional to the number of RNA molecules transcribed from that gene (Amann *et al.*, 1995).

The use of DNA and RNA probes is also known, and it is used to detect specific genes in the environment. Careful control of conditions in the process of hybridization and subsequent washing away of the unhybridized probe allows one to probe for identical sequences. Recently, DNA probe hybridization has been used for clinical identification of microorganisms. Nucleic acid hybridization techniques are also used for the phylogenetic classification of microorganisms on the basis of similarities and differences in the genes that encode for rRNA (Holben and Tiedje, 1988).

Using hybridization analysis, nucleic acids can be isolated *in situ*, which allows direct quantification and comparisons of microbial populations. Gene expression is not a prerequisite to successful monitoring of bacterial population, because a specific gene can be detected. Furthermore, several different taxa can be monitored in a single sample and selectable phenotypes (mutant derives) are not required because the probe detects a specific nucleic acid sequence.

However, nucleic acid hybridization analysis for environmental samples needs greater sophistication and is much more complex than traditional methods. Current protocols for the isolation of nucleic acids from the environment matrices are the limiting step in many analyses. Although not overly difficult, these techniques are tedious and involve many extraction and

purification steps. RNA, especially mRNA, is less stable that DNA, for Rnases are stable and ubiquitous and because the alkaline conditions employed in most DNA isolation protocols destroy RNA (Holben and Tiedje, 1988).

DNA reassociation

DNA reassociation is also used as a method to study genetic diversity in microbial communities. The given DNA of a bacterium is characterized by its length (genome size), base composition, and nucleotide sequence (Britten and Kohne, 1968). When a DNA solution is boiled, denaturation occurs, which is associated with a higher absorbency at 260 nm (Torsvik *et al.*, 1990), DNA incubated at a temperature 20-30°C lower that the Tm (thermal denaturation midpoint), both strands will reassociate (Grimont, 1988). By measuring the rate at which DNA reanneals, one can assay directly the state of the genetic diversity in communities, however, 1) extraction and purification of DNA from the environmental samples are tedious, 2) sequence determination approaches are based on analysis of material and are tedious, time consuming and cannot be applied readily to large numbers of strains (Schleifer & Stackebrandt, 1983), 3) the C_0t ½ value is an underestimation of the real species diversity of a sample, and, 4) the Shannon index in which both species richness and evenness play a role in determining the value of the index, can have the same index value for quite different communities (Ehlers, 1995).

By measuring the rate at which DNA reanneals, one can assay directly the state of the genetic diversity in communities. The use of genetic techniques in microbial ecology should provide an improved basis for understanding community structure, the true state of diversity within microbial communities and the degree of heterogeneity that must be stored within the microbial community to maintain stability (Atlas, 1984). DNA cross-hybridization gives a measure of the DNA that is

common between samples (i.e. the similarity of DNA, which is a measure of relative species composition) and also a measure of the relative diversity of the samples (Ritz and Griffiths, 1994). The diversity component cannot distinguish between species richness and evenness, and while it can determine the most diverse of the two samples compared it cannot give information about the absolute level of diversity involved (Griffiths *et al.*, 1997). The interpretation of the results is also dependent on the %G+C content of the DNA (Lee and Fuhrman, 1990). A shift in the %G+C content can be used to determine changes in microbial community structure (Leser, Boye and Hendriksen, 1995), but does not reveal any of the diversity parameters (richness, evenness and composition). The same is true for phospholipid fatty acid (PLFA) analysis, an alternative approach which also overcomes the problem of non-cultivability (Griffiths *et al.*, 1997).

2.7 The Biolog System

2.7.1 Introduction

Biolog MicroPlates are 96-well plates that contain pre-dried carbon sources and a tetrazolium violet redox dye. The Biolog system is based on the utilization of a large number of organic compounds by the test organisms. Oxidation of the substrates is monitored by the colorimetrically determinable conversion of the redox dye tetrazolium violet into a vividly purple formazan. Standardized micro plates allow the simultaneous testing of 95 diagnostically significant carbon sources; one reference well contains no carbon substrate. In addition to the carbon sources, all 96 wells of the tray contain the necessary nutrients for growth as well as the redox dye in a dry

state (Wünsche and Babel, 1996). There are several different arrays of carbon-sources designed to optimally identify Gram-negative isolates (GN Biolog plates), Gram-positive isolates (GP Biolog plates) or yeasts (YT Biolog plates). In addition, empty plates (MT Biolog plates) are available that contain no carbon source, but do contain the tetrazolium dye (Guckert *et al.*, 1996).

According to the instruction manual produced by the manufacturer, Biolog Inc., Hayward USA, the Biolog Automated Microbial Identification System ought to be particularly suitable for identifying and characterising a wide variety of environmental bacteria and yeasts. 148 strains of the phytopathogenic *Xanthomonas campestris pv. citri* originating from 24 countries, was identified by the Biolog Automated system (Verniere, Pruvost, Civerolo, Gambin, Jaquemon-Collet and Luisetti, 1993). The potential of the Biolog system was assessed for the identification of 45 unknown Gram negative isolates from the prototype of a water recycling system proposed for use on the US space Freedom (Klingler, Stowe, Obenhuber, Groves, Mishra and Pierson, 1992). Futhermore, the suitability and the limitations of the Biolog system were also investigated (release 3.50), with regard to the rapid and reliable identification of a large number of different bacterial isolates representing the bacterial biocenoses of terrestrial ecosystems exposed to different environmental conditions (Wünsche and Babel, 1996). The Biolog system provided satisfactory or even excellent results in the identification of authentic strains representing some taxa of heterotrophic, copiotrophic and aerobic or facultative anaerobic bacteria which are frequently found in soils (Wünshe and Babel, 1996). A total of 224 strains of the genus *Pseudomonas* was also studied by using 99 carbon utilization tests. Biomerieux Biotype-100 strips and the Biolog GN Micro Plate system were used, and although they did not give identical grouping of all strains, their efficiency to uncover the taxonomic diversity of the pseudomonands was comparable (Grimont, Vancanneyt, Lefevre, Vandemeulebroecke, Vauterin, Brosch, Kersters

and Grimont, 1996).

In bacterial taxonomy, the use of conventional carbon source utilization tests has been limited by the tediousness of minimal medium preparation and the constraints of replica plating. However, for diagnostic purpose, a phenotypic description of bacterial taxa is still extremely valuable (Grimont *et al.*, 1996). The development and availability of such tests in strips makes nutritional studies of any scale easier, more rapid, reliable and less time-consuming (Grimont *et al.*, 1996).

Biolog analysis was mostly done for the identification of specific cultures. It was only recently that microbial ecologists used Biolog plates to investigate carbon-source utilization patterns for mixed microbial communities to obtain more information about microbial diversity. Garland and Mills (1991) introduced Biolog GN microplates (Biolog, Inc., Hayward, CA) for the characterization of heterotrophic microbial communities. Since then, this approach was found to be effective for discriminating microbial communities from aquatic habitats (Garland and Mills, 1991), wastewater treatment systems (Guckert *et al.*, 1996; Victorio *et al.*, 1996), compost (Insam, Amor, Renner and Crepaz, 1996), rhizosphere (Garland and Mills, 1994; Garland, 1996), and soil (Winding, 1994; Zak *et al.*, 1994).

2.7.2 Using the Biolog System to evaluate species diversity

Community-level carbon source utilization (Biolog) profiles have recently been introduced as a means of classifying microbial communities on the basis of heterotrophic metabolism (Garland and Mills, 1991). Such a classification system might allow microbial ecologists to compare the metabolic roles of microbial communities from different environments without involving tedious

isolation and identification of community members (Haack *et al.*, 1995). The simplicity of the method and the commercial availability of Biolog plates are particularly attractive. Therefore, a procedure was proposed based on the Biolog identification system to quickly and effectively, assess aspects of microbial diversity, based on carbon source utilization of these communities. According to Zak *et al.*(1994), the numbers and types of substrates utilized by bacterial communities, as well as the levels of activities on various substrates and patterns of temporal development, constitute an information-rich data set from which to assess microbial diversity. They demonstrated that the Biolog System can detect considerable variation in the ability of microbial communities to metabolize different carbon compounds (Zak *et al.*,1994).

Community-level Biolog analysis is accomplished in four steps: (1) samples are processed to make a suspension of microorganisms, (2) Biolog plate(s) are inoculated with aliquots of the suspensions, (3) the Biolog plates are incubated while colour development in each well is monitored, and finally (4) the results are analysed (Guckert *et al.*, 1996).

Bossio and Scow (1995) inoculated Biolog plates with soil extracts to quantify the metabolic diversity of the soil microbial community. A dilution of 10^{-3} of the soil samples was used which gave the most desirable results, for lower dilutions contained too much clay that interfered with readings, and higher dilutions resulted in increased variability in substrate utilization among replicate micro plates. Canonical correspondence analysis was used to analyze the data. It was found that differences among microbial communities in their Biolog sole-carbon-source utilization patterns, (1) could be explained by the environmental variables imposed upon the communities,

(2) exhibit seasonal fluctuations in direct correspondence to environmental fluctuations, and (3)

showed similar patterns at two independent locations (Bossio and Scow, 1995).

It was also suggested that the Biolog assay may be a suitable approach to determine compost maturity. Biolog GN microtiter plates were inoculated with appropriately diluted suspensions of compost material (0.85% NaCl) to yield an initial cell density of 15 000 microorganisms per well. Colour development was rapid, and readings were made after 2-4 days. A dramatic shift in functional microbial community structure during the 8-week composting process was found (Insam *et al*, 1996).

All the Biolog results are interpreted as a function of the original microbial community structure from the sample. Since the community will have a single carbon-source to utilize in each well of the Biolog plate, the microbial community will likely change independently in each of these wells. However, the measured Biolog response is still related to the functional potential of the original community. Therefore, it is believed that a comparison of microbial communities based on the 95-carbon sources array available from a Biolog plate is an appropriate relative measure of the metabolic diversity of these communities (Guckert *et al.*, 1996). The rate and extent of utilization of any particular carbon source on a Biolog plate will be related to the original microbial community structure and metabolic capacity for that sample (Guckert *et al.*, 1996). The factors that affect the Biolog system will now be discussed.

2.7.3 Average well colour development (AWCD)

Average well colour development (AWCD) is the overall colour development in the microwells of the Biolog plates (Garland and Mills, 1991). For each well, the absorbance value of the control

well is subtracted from the well absorption, yielding a single difference value (SDV) and the AWCD for the plates is then calculated by summing all these SDV values and dividing by 95 (Kerster *et al.*, 1997).

The relative effects of an average rate of colour development versus the pattern of relative carbon source utilization on the classification of rhizosphere samples from different crop types were evaluated by Garland (1996). The average rate of colour development was correlated to the density of total bacterial cells and active bacterial cells inoculated into the plate. Results suggested that single-plate readings can be used to classify samples, but only if potential differences in average well colour development (AWCD) are accounted for in the data analysis. It has been reported that repeated plate readings will provide a more complete understanding of differences in carbon source utilization among samples (Garland, 1996). The resulting suspension of the rhizosphere samples was diluted 10-fold in 0.85% NaCl and then inoculated into Biolog GN plates without further dilutions. The primary aim was to evaluate the relative effectiveness of two alternative approaches for collecting and analysing community Biolog data involving either standardized inoculum density and incubation time, or continuous monitoring of plates. According to the results it was indicated that standardized inoculum density and incubation time, may be acceptable as long as differences in the extent of colour development among samples are accounted for in the data analysis. Continuous monitoring of plates appears superior based on its relative ease and the increase in information it yields regarding carbon source utilization (Garland, 1996).

2.7.4 Factors affecting carbon source utilization of microbial communities

It is suggested that the Biolog assay holds considerable promise as a rapid and simple method for characterizing microbial communities (Garland and Mills, 1994; Haack *et al.*, 1995; Garland, 1996). However, in order to interpret responses of heterotrophic microbial communities in Biolog microplates, it is important to determine factors that affect them (Kersters *et al.*, 1997).

Production batch of Biolog plates

According to Kersters *et al.* (1997) the production batch of Biolog GN microplates significantly affected the colour development for both pure cultures and microbial communities. According to Insam *et al.* (1996) the response of Biolog plates depends on incubation time and inoculum density. The Biolog substrate oxidation responses and AWCDs often exhibit a lag phase, a exponential phase, and a stationary phase. This nonlinearity implies that the substrates to be most significant in discriminating microbial communities may change over the course of the experiment (Kersters *et al.*, 1997).

Incubation time and Inoculum density

The Biolog substrate utilization responses and AWDC's often exhibit a lag phase, an exponential phase and a stationary phase (Haack *et al.*, 1995; Kerster *et al.*, 1997). The response of Biolog GN plates is also correlated with the inoculum density. More diluted inocula resulted in protracted rates of colour development for both pure cultures and heterotrophic communities (Garland and Mills, 1994; Haack *et al.*, 1995; Kersters *et al.*, 1997). Wünsche, Bruggeman and Babel (1995) found that communities with cell densities ranging from 10^6 to 10^8 cfu.ml^{-1} utilized nearly the same percentage of substrates after prolonged incubation. However, according to Kersters *et al.* (1997),

inoculum-dependent differences among the Biolog patterns for both pure cultures and heterotrophic microbial communities persisted even after extended incubation periods.

Reliable use of the Biolog system requires samples of approximately equivalent inoculum densities (Garland and Mills, 1991; Haack *et al.*, 1995) and if this criterion is not met, community comparisons will be compromised by the different responses of inocula with different cell densities (Kersters *et al.*, 1997). In order to overcome the influence of inoculum density on the colour response date Garland and Mills (1991) suggested (1) the dilution or concentration of samples to achieve equivalent inoculum densities, (2) the use of multiple readings over a time course of incubation, or (3) the transformation of the data by dividing each single difference value (SDV) by the AWCD of the plate. According to Kersters *et al.* (1997) dilution of samples seems to be the most efficient method since the above mentioned transformation of the data sets did not reduce significantly the influence of inoculum density.

It was found that the pattern of responses to multiple substrates were highly reproducible for pure cultures and for environmental samples (Haack *et al.*, 1995; Kersters *et al.*, 1997). Differences in the response patterns to the 95 substrates in Biolog GN plates will thus most probably reflect real differences in community composition if inoculum density is not the influencing factor (Kersters *et al.*, 1997). The question remains however, whether differences in Biolog profiles are due to the response of a single member of the community or of the entire heterotrophic community (Kersters *et al.*, 1997). If carbon source profiles of natural microbial communities could serve as an information system, certain patterns could be described which would reflect the species diversity of the habitat.

2.7.5 Conclusion

Interpretation of Biolog analysis of microbial communities and of the relevance of Biolog patterns to ecological questions is still problematic. The significance of changes in utilization of specific substrates is difficult to interpret without a better understanding of how the interactions among the organisms constituting a community affect substrate utilization patterns (Bossio and Scow, 1995). A further problem with Biolog patterns, is the dependance of the patterns on inoculum density and the fact that different microbes using the same substrate may use them to different extents and that summation of the individuals does not predict the collective kinetic response of those individuals. It was also demonstrated that certain bacterial strains are unable to oxidize Biolog substrates and that their presence thus cannot be detected (Haack *et al.*, 1995). The existence of organisms that are unable to metabolize substrates on Biolog plates, many of which are likely to be present in soils, will complicate any attempt to extrapolate from community Biolog profiles to *in situ* community metabolic capacity (Haack *et al.*, 1995). Nonetheless, the study of Bossio and Scow (1996) and others (Garland and Mills, 1994; Haack *et al.*, 1995; Kersters *et al.*, 1997) found Biolog patterns for specific communities to be highly producible.

The Biolog system clearly shows limitations when applied for characterization of heterotrophic microbial communities, including the sensitivity to inoculum densities, the nonlinear nature of colour production and the poor reproducibility of the control well colour development (Kersters *et al.*, 1997). These limitations, however, should not refrain the potential user of the Biolog assay for the comparison of microbial communities from different environments since the methodology is simple, rapid, reproducible and commercially available.

2.8 Summary

Microorganisms are vital to the function and maintanance of the Earth's ecosystems and biosphere. As major contributors to biogeochemical cycles, they perform unique and indispensable activities in the circulation of matter in the world, on which large organisms, including humans depend (Cloete and Muyima, 1997). Microorganisms can be used for the biomonitoring of global ecology, for climatic changes, the effects of pollutants and other habitual disturbances (Hawksworth and Colwell, 1992; Tiedje, 1994). Therefore, microorganisms are of central importance to biosphere sustainability (Tiedje, 1994).

Species diversity is a complex term and consists of various factors that are related to and dependant on each other. There is no clear definition to describe diversity, but stability in communities are dependent on a high species diversity to overcome stress situations and to shift back towards their original state of equilibrium (Atlas and Bartha, 1993). Species diversity describes the assembly of species within a community structure and reflects the status of interrelationships between organisms and their environment (Atlas and Barhta, 1993).

Studies of bacterial diversity differ from each other in the procedure for obtaining isolates, mode of characterization, clustering methods used for grouping of identification, the level of similarities or distances used to define a species of biotype, and the diversity measures used. Hence, diversity measured by one method of characterization cannot be readily compared with that measured by other modes of characterization (Watve and Gangal, 1996).

The lack of knowledge about the diversity and function of microbial communities is due to the fact that the majority of the organisms are viable but non-culturable (Höfle, 1992; Haldeman and Amy,

1993; Amann *et al.*, 1995; Hugenholtz and Pace, 1996). Molecular techniques (Wise *et al.*, 1996; Yap *et al.*, 1996: Wagner and Amann, 1997) and biochemical techniques (White *et al.*, 1996), enables identification and phylogenetic characterization of micro-organisms without cultivation. However, these methods are time-consuming, complex and it is difficult to interpret the data regarding microbial diversity. Hence, there is a need for techniques that allow one to characterize microbial communities without the usual reliance on selective culturing and which would be less complex than the molecular techniques.

An alternative approach is to examine components of microbial biodiversity, for which there exists a reasonable chance of detecting patterns, which can be related to diversity and function, for example, carbon source utilization patterns. During pattern recognition, one would ideally want to determine into which category of community structure a specific habitat will fall. This will enable the researcher to draw valuable conclusions regarding microbial community structure and function in a particular habitat. Community-level carbon source utilization (Biolog) profiles have recently been introduced as a means of classifying microbial communities on the basis of heterotrophic metabolism (Garland and Mills, 1991). Such a classification system might allow microbial ecologists to compare microbial communities from different environments without involving tedious isolation and identification of community members (Haack *et al.*, 1995). This method is easy to use and commercially available.

Efforts to increase the amount of biological information we gather, and to make what we have more accessible and comparable, would be a crucial first step, furthermore, increasing our understanding of how natural systems work and how the pieces interact with each other is also crucial (Lovejoy, 1993).

CHAPTER 3

THEORETICAL MODEL FOR THE INTERPRETATION OF CARBON SOURCE UTILIZATION PATTERNS OF MICROBIAL COMMUNITIES

3.1 Abstract

Although much is known on the ecology of macro-organisms, information on how microorganisms function in nature is not well known. The lack of knowledge on microbial communities is due to the fact that distinctive morphological features are absent in microorganisms and that the bulk of natural occurring microorganisms cannot be cultured and therefore, remain unidentified. Many methods have been used to obtain more information with regards to microbial community and function. The challenge is to obtain information about the structure and function of a community without having to culture the organisms. Pattern recognition may be one way to get a better understanding of species diversity in a system. These patterns can include DNA reannealing, 16S rDNA, protein profiles, carbon source utilization patterns etc. Models were postulated to simplify information in order to deal and interpret it with regards to species diversity and function.

Key words: models, pattern recognition, species diversity, function, carbon source utilization

3.2 Introduction

Microbial ecology is the study of interrelationships that exist between microorganisms and their biotic and abiotic environments. No environmental species exists in isolation, it is supported by a system that provides it with food, shelter and physical conditions conducive to growth (Lovejoy, 1993). Microorganisms in communities possess the ability to co-exist, a property which emerges as a result of the interactions between different species, or between organisms and the abiotic environment (Hattori *et al.*, 1989; Cloete & Muyima, 1997).

Although much is known on the ecology of macro-organisms, information on how microorganisms function in nature is not well known. The lack of knowledge on microbial communities is due to the fact that distinctive morphological features are absent in microorganisms and that the bulk of natural occurring microorganisms cannot be cultured and therefore, remain unidentified (Höfle, 1992; Haldeman & Amy, 1993). Many methods have been used to obtain more information with regards to microbial community and function. Phenotypic methods for studying microbial diversity include: taxonomical identification using API or Biolog (Haldeman and Amy, 1993), physiological (Atlas, 1984), chemo-systematic (Haldeman and Amy, 1993), serological analysis (Priest and Austen, 1993) and molecular techniques (Lee and Fuhrman, 1990; Stahl, 1993; Palleroni, 1994; Leff *et al.*, 1995). However, no complete inventory of the microbial species of environmental samples exists due to the lack of suitable technology to determine microbial community structure and function (Cloete & Muyima, 1997). The integration of microbial ecology and molecular biology has given new insight into the interrelationships that exist between microorganisms and their environment. Recombinant DNA and molecular

phylogenetic techniques have provided methods for characterizing natural microbial communities without the need to cultivate microorganisms. The characterizing of an organism in terms of its phylotype requires only a gene sequence, not a functioning cell (Hugenholtz & Pace, 1996).

However, all these methods are highly complex and generate an enormous amount of information which is difficult to interpret. Although, these methods generate a vast amount of data, they fail to explain the microbial community structure or function of the microbes in their natural environment. In order to understand these complex communities, indices have been developed. There are a few diversity indexes available which can measure diversity in a community. These are normally based on the phenotypic features of the members in the community (Atlas, 1984). The major disadvantage of these diversity indexes is that they do not give any information about the community stability or the different species present (Pielou, 1975). Thus, more simplistic models are required to describe interactions and functions of microorganisms in their specific ecosystems.

Steyn and Cloete (1989) proposed an adaptation of Koch's postulates to determine the function of a specific population in a community, hence, particularly useful for aut-ecological studies. They suggested the following: i) A particular microorganism must always be associated with a certain phenomenon; ii) the same function(s) observed in the natural habitat must also be performed in pure culture; iii) the organism must be enumerated in the natural habitat, isolated and studied in pure culture; iv) the function must be quantified (activity per cell determined) and v) the product of the number of individual cells and activity per cell should account for the magnitude of the phenomenon observed in the natural habitat (Steyn & Cloete, 1989).

However, the above relies on the culturability, isolation and identification of the organisms. The challenge therefore, is to obtain information about the structure and function of a community without having to culture the organisms. In this paper we postulate that pattern recognition may be one way to get a better understanding of species diversity in a system. These patterns can include DNA reannealing, 16S rDNA, protein profiles, carbon source utilization patterns etc.

In this paper simplified models will be presented in order to deal with pattern recognition and interpretation with regards to species diversity and function.

3.3 Proposed model for pattern recognition

In this model (Figure 3-1) I will discuss the categories of microbial habitats based on species diversity. A category "A" (Figure 3-1) would indicate "a sterile situation" and directly outside square "A" - a pioneer community with only one species present in low numbers as can be found in the gastrointestinal tract of newborn animals. A category "B" (Figure 3-1) would indicate an increase in the number of different species (species diversity), but low numbers of each species as can be found in the microbial populations of deep sea fish. The low number of individuals of the various species, is due to the limited nutrient content in the environment. A category "C" (Figure 3-1) would indicate a stable community, like an activated sludge plant, with a large amount of information due to many different species represented by a high number of individuals of each species. This community is best suited to overcome environmental stress, because it

contains a large amount of information. When a few populations attain high density, diversity decreases (Atlas, 1984). Communities which have a lower diversity will contain less information and lower bacterial numbers, are less stable and will be more affected by stress conditions than communities with high species diversity and numbers (Atlas, 1984). After the removal of the ecological stress factor, a stable community containing a large quantity of information will recover to its original stable status. A category "D" (Figure 3-1) would indicate extreme environments where a few species, with a high number of individuals (i.e. halophiles) in a salt lake.

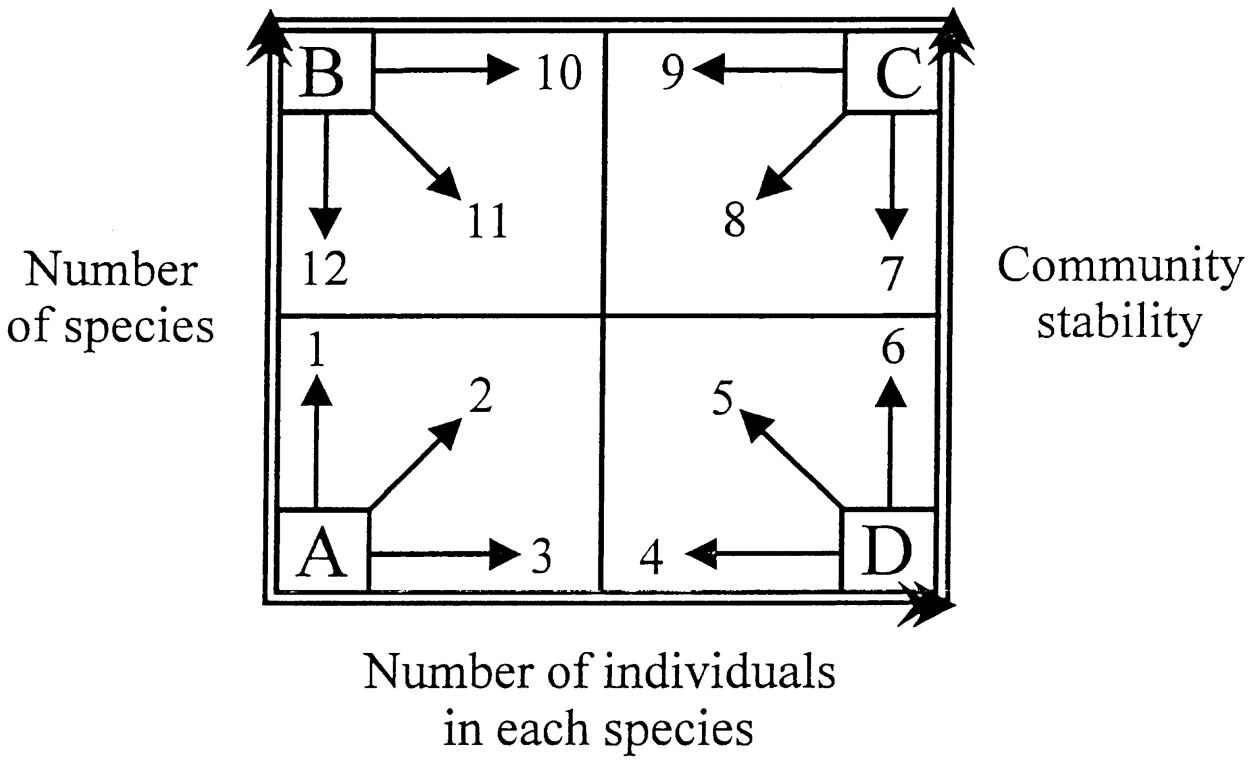

Figure 3-1 A theoretical model indicative of different categories of microbial habitats based on species diversity.

The larger part of each theoretical category A, B, C and D represents the different microbial communities (Figure 3-1):

A1 - Is the increase in species diversity, whilst numbers of each species are limited by some environmental stress factor (eg. limited availability of nutrients), but with low numbers of each species present, moving in the direction of situation B.

A2 - Indicates an increase in both the numbers of different species and the number of cells of each species. Arrow no. 2 indicates "movement" in the direction of situation "C", a stable community. Normally as a result of a favourable nutrient rich environment.

A3 - Indicates an increase in the number of bacterial cells with only a few species present (situation D). A few species start to dominate due to an extreme environment.

D4 - Indicates a decrease in a high number of cells, moving back to situation A (a pioneer to sterile environment). A stress situation can result in the decrease of cell numbers. Only a few species are present in the environment and they do not have a high enough information content to resist the stress situation.

D5 - The situation indicated by the arrow shows an increase in the number of different species but decreasing cell numbers. This community moves towards the B situation due to a decrease in nutrients or other stress factors.

D6 - This community indicates an increase in the number of different species with already high cell numbers. The community is moving to a more stable situation (C). The environment is becoming more favourable with enough nutrients for different species to colonize.

C7 - The community shows a decrease in different species but the number of cells of each species are still high. The decrease can be caused by an environmental stress situation, responsible for change to an extreme environment (situation D).

C8 - A severe stress situation results in a sterile or pioneer environment (situation A) with one (or none) species present.

C9 - Indicates a stable community moving towards the B situation. A stress situation/factor could cause a decrease of individuals present, with the species diversity remaining high. A stable community moving towards situation B having and abundance of information due to the high number of different species, even though the relative numbers of each species are decreasing. This community can move back to it's original stable situation after the stress situation has been removed.

B10 - Indicates a community with a high number of different species, but with low cell numbers. The information content of such a community is sufficient to overcome the stress situation, due to the high number of species.

B11 - This arrow indicates an increase in the number of bacterial cells, but a decrease in the number of species. This can result in a D situation representing an extreme environment. The information content is low, with high numbers of bacterial cells of a few species.

B12 - An environment with a high number of species but with low numbers of cells. This can lead to a sterile situation as represented by situation A.

During pattern recognition, one would ideally want to determine into which of the above categories of microbial community structure a specific habitat will fall. This will enable the researcher to draw some valuable conclusions with regards to microbial community structure and function in a particular habitat.

3.4 A hypothetical example of pattern recognition using Biolog profiles to categorise microbial communities according to the above model (Figure 3-1)

The hypothesis is that:

- the more species present, the more carbon sources would be utilized in the Biolog system, because of the contribution of each species in terms of its carbon source utilization;
- pre-dominance would be indicated by the same number of carbon sources utilized upon dilution of the sample, since only the number of the organisms would change and not its carbon source utilization profile, as long as the inoculum size is sufficient;
- equitability would be indicated by the same number of carbon sources utilized upon dilution of the sample, since the organisms are diluted at the same ratio;
- a linear decline in the number of carbon sources utilized, would indicate an unequal distribution of the species present in the sample, since some of these will be diluted out and their contribution to carbon source utilization would disappear.

Community-level Biolog analysis is accomplished in four steps: (1) samples are processed to make a suspension of microorganisms, (2) Biolog plate(s) are inoculated with aliquots of the suspensions, (3) the Biolog plates are incubated while colour development in each well is monitored, and finally (4) the results are analysed (Guckert *et al.*, 1996). Thus, by inoculating Biolog microplates with microbial samples, carbon source utilization profiles are obtained. If these carbon source profiles of natural microbial communities could serve as an information system, certain patterns could be described, which would reflect the species diversity of the

habitat. Therefore, a theoretical model for the interpretation of the carbon source utilization patterns of microbial communities was developed (Figure 3-2).

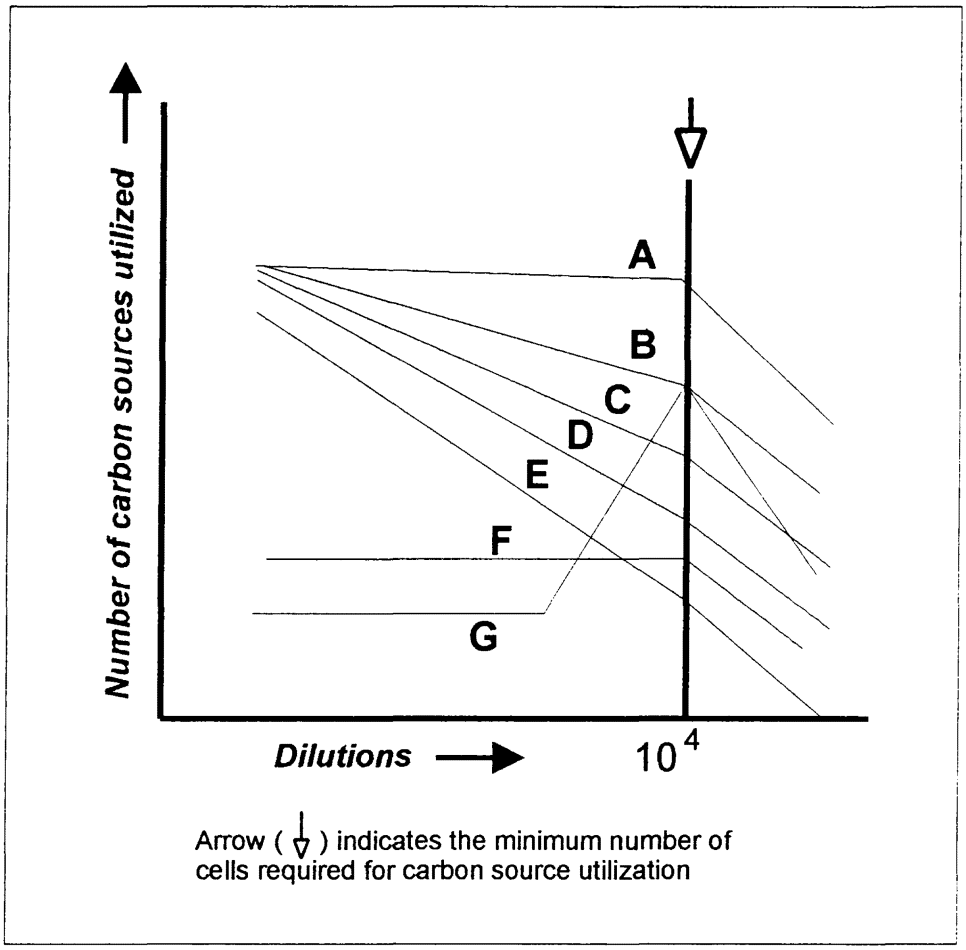

Figure 3-2 Predicted carbon source utilization patterns of diluted bacterial samples using the Biolog system

In Figure 3-2, theoretically pattern "A" indicates equitability, because the contribution of each species in the community remains the same upon dilution, since all the species present are diluted in the same ratio. However, theoretically pattern "A" could also indicate pre-dominance, according to our hypothesis. Absolute equitability is however highly unlikely to occur in natural microbial communities, therefore, pattern "A", would rather be indicative of pre-dominance and

CHAPTER 4

BIOLOG FOR THE DETERMINATION OF MICROBIAL DIVERSITY USING MIXTURES OF PURE CULTURES

4.1 Abstract

The lack of knowledge about the diversity and function of microbial communities is due to the fact that the bulk of microorganisms in nature cannot be cultured and remain unidentified when phenotypic methods are used. These methods include: taxonomical identification using API or Biolog, physiological, chemo-systematic, serological and molecular techniques. An alternative approach is to examine components of microbial biodiversity, for which there exists a reasonable chance of detecting patterns, which can be related to diversity and function. In this study the Biolog system was evaluated, using mixtures of pure cultures, to determine whether this method could be used to do diversity studies on bacterial communities. Carbon source patterns of diluted suspensions of a pure culture and mixtures of pure cultures, suggested that the Biolog system could be used for bacterial diversity studies. Different mixtures of pure cultures, based on different carbon source profiles, could be categorised according to the hypothesis of pattern recognition.

Keywords: Biolog system, carbon source utilization, pattern recognition, pure bacterial cultures.

4.2 Introduction

Evaluation of diversity in environmental microbial communities has been limited by the large percentage of microorganisms that are non-culturable. It was reported that only 10% or less of the microorganisms in water and soil can be enumerated and, by implication, be grown on agar media (Olsen and Bakken, 1987). Using 16SrRNA as molecular markers, (Wise *et al.*, 1996; Wagner and Amann, 1997), DNA isolation and PCR (Yap *et al.*,1996) and biochemical techniques such as signature lipid biomarker analysis (White *et al.*,1996), the limitations of traditional culturing techniques are circumvented in the assessment of the biodiversity of microbial communities. This is because cell constituents like nucleic acids are directly extracted from environmental samples and isolation and culturing of individual species are thus not required. However, these methods are time-consuming, complex and it is difficult to interpret the data obtained from these techniques regarding diversity of microbial communities. A need therefore exists to develop a technique which would not require culturing of organisms, and which would be less complex than the molecular techniques.

Microbial ecologists recently used Biolog plates to investigate carbon-source utilization patterns for soil microbial communities (Garland & Mills, 1991; Zak *et al.*, 1994; Haack *et al.*, 1995; Garland, 1996;), aquatic samples (Garland and Mills, 1991) and wastewater treatment communities (Guckert *et al.*, 1996; Victorio *et al.*, 1996).

Bossio and Scow (1995) inoculated Biolog plates with soil extracts to quantify the seasonal differences in soil microbial communities receiving different inputs of carbon and water. Soil samples were diluted to 10^{-3} to obtain appropriate inoculums to give the most desirable results

(Bossio and Scow, 1995). Substrate utilization patterns were significantly related ($P<0.001$) to carbon and flooding treatments. Zak *et al.* (1996) demonstrated that the Biolog System could detect differences between six soil microbial communities. Dilutions of the soil samples were made from 10^{-1} to 10^{-4}. 100µl aliquots of the 10^{-4} dilution were used to inoculate the Biolog microwell plates. Inoculated microwell plates were examined every 12h, for a period of 72h. Cluster analysis (based on the presence or absence of utilized substrates), revealed a consistent relationship among the six communities regardless of the duration of incubation (Zak *et al.*,1996). However, according to Insam *et al.* (1996) the response of Biolog plates depends on incubation time and inoculum density. Nevertheless, Zak *et al.* (1994) found that the Biolog system effectively distinguished among different soil communities using multivariate analysis of carbon source profiles.

According to Haack *et al.* (1995) different microbes use the same substrates to different extents. This may be due to bacterial cells that are less metabolic active at certain dilutions when the inoculum is prepared, and therefore cannot be detected on the Biolog microwell plate. Kersters *et al.* (1997) indicated that inoculum-dependent differences among Biolog patterns for both pure cultures and heterotrophic microbial communities persisted even after extended incubation periods. In order to overcome the influenced inoculum density on the colour response data, their results indicated that dilution of samples seems to be the most efficient method. Thus, by inoculating Biolog microwell plates with different dilutions of the mixtures of pure cultures and incubating these plates for periods of 24h, 46h and 72h, the impact of inoculum density and time of incubation could be circumvented. Furthermore, in this study we wanted to prove the hypothesis, that microbial communities can be classified into different categories, using Biolog profiles, based on carbon source utilization patterns (Figure 4-1).

According to Figure 4-1, theoretically, pattern "A" indicates equitability, because the contribution of each species in the community remains the same upon dilution, since all the species present are diluted in the same ratio. However, theoretically pattern "A" could also indicate pre-dominance, according to our hypothesis. Absolute equitability is however highly unlikely to occur in natural microbial communities, therefore, pattern "A", would rather be indicative of pre-dominance and not equitability. In Figure 3-2 theoretically pattern "B" would indicate an uneven distribution of species according to the above hypothesis, since some of the species will be diluted out upon dilution, and their contribution to carbon source utilization would disappear. Accordingly a decline in equitability is represented in Figure 3-2 in the following order "A">"B">"C">"D">"E". Hence, the larger the gradient of the lines in Figure 3-2, the more uneven the distribution of species. Pattern "F" reflects pre-dominance but with a low percentage of carbon sources utilized by the organisms. Pattern "F", however, does not reflect a high species diversity and equitability, due to the low number of carbon sources utilized in contrast to pattern "A" which is also a straight line.

Pattern "G" indicates a microbial community where the dominant organism is suppressed by non-fermentative organisms in that community. As soon as the competitive organisms have been diluted out, the pre-dominant organism will start to utilize carbon sources and, hence, the increase in the number of carbon sources utilized upon dilution as indicated by pattern "G" (Figure 4-1).

The objective of this study was to verify the validity of the theoretical model by using the carbon source profile of mixtures of pure bacterial cultures, representative of the different patterns in the model described above.

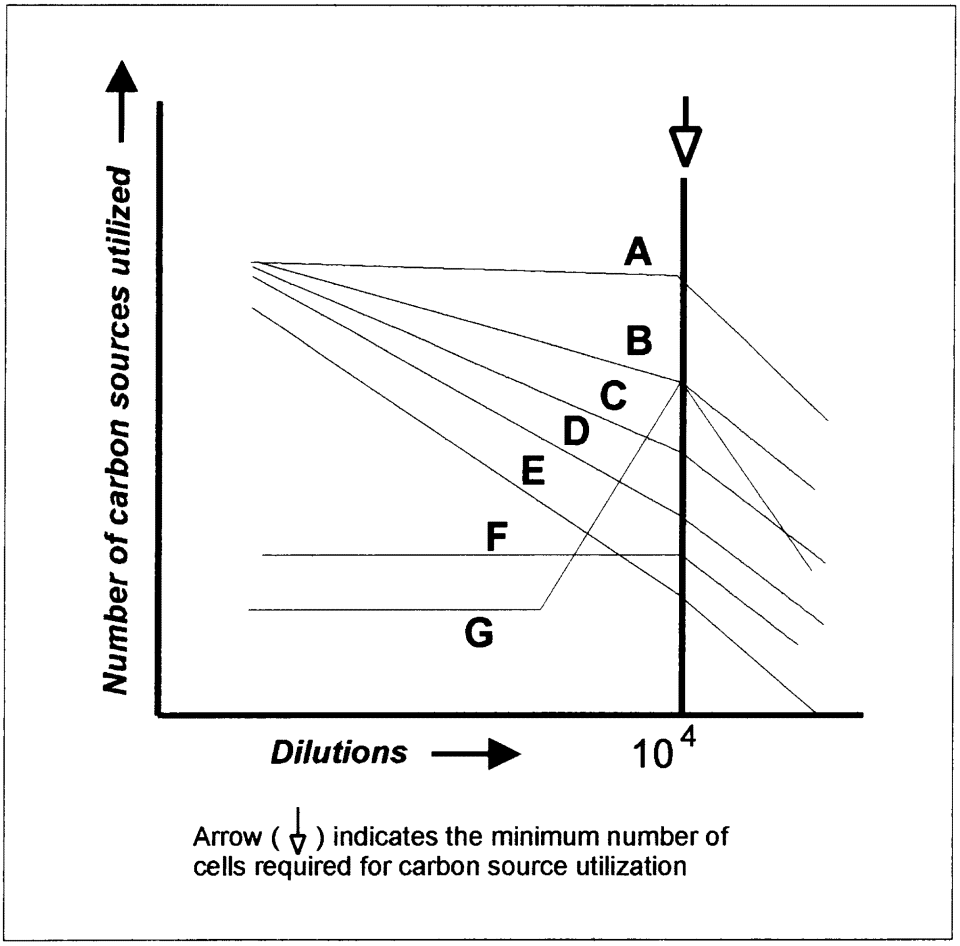

Figure 4-1 Predicted carbon source utilization patterns of diluted bacterial samples using the Biolog system.

4.3 Materials and Methods

Biolog plates

Biolog GN microwell plates (Biolog Inc., Hayward, CA) were used in this study. The Biolog GN microplates contain 95 wells with different carbon sources and one control well without a carbon source. Each of the 96 wells is also preloaded with a complex buffered nutrient medium and a tetrazolium violet redox dye (Kersters *et al.*, 1997).

Bacterial strains

Studies were performed using the following pure cultures. *Serratia marcescens* (DSM 30121), *Staphylococcus aureus* (ATCC 196E), *Pasteurella multocida* (LMG 2851), *Alcaligenes eutrofus* (DSM 531), *Acinetobacter calcoaceticus* (DSM 588), *Rhodococcus equi* (LMG 5358) and *Micrococcus luteus* (DSM 20030). LMG cultures were obtained from the LMG culture collection (Gent, Belgium), and DSM cultures from Germany and ATCC cultures from the USA. The percentage carbon sources utilized by the recommended cell density (10^8 cell.ml^{-1}) (Biolog Inc., Hayword, CA) of each culture is summarized in Table 4-1.

Table 4-1 Percentage carbon sources utilized by the different bacterial strains used in this study.

Bacterial strains	Percentage carbon sources utilized (%)
Serratia marcescens	60
Staphylococcus aureus	17
Pasteurella multocida	25
Alcaligenes eutrophus	35
Acinetobacter calcoaceticus	31
Micrococcus luteus	5
Rhodococcus equi	4

Plate counts

Viable counts were determined by the spread plate procedure. Samples were serially diluted in 0.85% NaCl and 100µl of the dilutions were plated on the appropriate media. *Pasteurella multocida* was enumerated on Brain Heart Infusion agar (BHI, Merck) and the other cultures on

Nutrient agar (Merck). The plates were incubated at 28°C and colonies were counted after 24h (*S.marcescens, P.multocida, Alc.eutrophus, Acin. calcoaceticus*) and 48h (*S.aureus, Rod.equi* and *M.luteus*).

Inoculation and incubation of Biolog microplates

Pure cultures inocula for microplates were obtained by growing pure cultures on Nutrient agar and *P.multocida* on BHI agar. *S.marcescens, P.multocida, Alc.eutrophus* and *Acin. calcoaceticus* were collected after 24h and *S.aureus, Rod.equi* and *M.luteus* after 48h growth. Pure cultures were suspended in sterile saline (0.85% NaCl). Biolog GN microplate wells were inoculated with 150µl of the cell suspensions. Microplates were incubated in the dark at 28°C without agitation. Colour formation in the individual cells of the microtiter plates was measured with an Anthos reader 2001 (Anthos Labtec Instruments) at 620nm. Readings of the microplates were made in duplicate after 24h, 48h and 72h of incubation. Readings were also made at 405nm (Zak *et al.*, 1994) to compare it with readings at 620nm and plates were also visually studied. According to our results better absorbance readings were obtained at 620nm.

Impact of inoculum density

Suspensions of the pure cultures were inoculated in Biolog GN microplates at different cell densities. The number of viable cells was varied by diluting the suspensions with sterile saline. Carbon source profiles of *S.marcescens* were obtained after inoculation of 10^5 cells.ml^{-1}, 10^6 cells.ml^{-1}, 10^7 cells.ml^{-1} and 10^8 cells.ml^{-1}. *Serratia marcescens* was used as a representative of a pure culture using at least 60% of the carbon sources in the Biolog GN microwell plate. Profiles of mixtures of pure cultures were obtained by inoculating GN Biolog plates with different mixtures of pure cultures (Table 4-2 , 4-3 and 4-4).

Table 4-2 Mixtures of pure cultures used to determine the effect of pre-dominance of an organism (*Serratia marcescens*) which utilizes a large percentage of carbon sources.

Mixtures of pure cultures *	*Staphylococcus aureus* (cells.ml^{-1})	*Pasteurella multocida* (cells.ml^{-1})	*Alcaligenes eutrophus* (cells.ml^{-1})	*Acinetobacter calcoaceticus* (cells.ml^{-1})	*Micrococcus luteus* (cells.ml^{-1})	*Rhodococcus equi* (cells.ml^{-1})	*Serratia marcescens* (cells.ml^{-1})
MS0	10^6	10^6	10^6	10^6	10^6	10^6	10^8
MS-1	10^5	10^5	10^5	10^5	10^5	10^5	10^7
MS-2	10^4	10^4	10^4	10^4	10^4	10^4	10^6
MS-3	10^3	10^3	10^3	10^3	10^3	10^3	10^5

* Mixtures of pure cultures used as inoculum for GN Biolog microwell plates in the 2nd experiment.

Table 4-3 Mixtures of pure cultures used to determine the effect of equitability.

Mixtures of pure cultures *	*Staphylococcus aureus* (cells.ml^{-1})	*Pasteurella multocida* (cells.ml^{-1})	*Alcaligenes eutrophus* (cells.ml^{-1})	*Acininetobacter calcoaceticus* (cells.ml^{-1})	*Micrococcus luteus* (cells.ml^{-1})	*Rhodococcus equi* (cells.ml^{-1})
ME0	10^7	10^7	10^7	10^7	10^7	10^7
ME-1	10^6	10^6	10^6	10^6	10^6	10^6
ME-2	10^5	10^5	10^5	10^5	10^5	10^5
ME-3	10^4	10^4	10^4	10^4	10^4	10^4
ME-4	10^3	10^3	10^3	10^3	10^3	10^3

*Mixtures of pure cultures used as inoculum for GN Biolog microwell plates in the 3rd experiment.

Table 4-4 Mixtures of pure cultures used to determine the effect of unequally distributed microorganisms.

Mixtures of pure cultures *	*Staphylococcus aureus* (cells.ml^{-1})	*Pasteurella multocida* (cells.ml^{-1})	*Alcaligenes eutrophus* (cells.ml^{-1})	*Acininetobacter calcoaceticus* (cells.ml^{-1})	*Micrococcus luteus* (cells.ml^{-1})	*Rhodococcus equi* (cells.ml^{-1})
MU0	10^5	10^4	10^5	10^6	10^4	10^5
MU-1	10^4	10^3	10^4	10^5	10^3	10^4
MU-2	10^3	10^2	10^3	10^4	10^2	10^3
MU-3	10^2	10^1	10^2	10^3	10^1	10^2

*Mixtures of pure cultures used as inoculum for GN Biolog microwell plates in the 4th experiment.

To substantiate the hypothesis of pattern recognition illustrated in Figure 4-1, the different mixtures of pure cultures summarized in the above tables were used as inocula for the Biolog microwell plates to represent the different recognition patterns (Table 4-5) (Figure 4-1).

Table 4-5 Mixture of pure cultures representative of recognition patterns illustrated in Figure 4-1.

Pure culture mixture	Representative of pattern
Serratia marcescens, SM8, SM7, SM6, SM5	"A"
Mixture of pure cultures (*Serratia marcescens* present), MS0, MS-1, MS-2, MS-3	"A"
Mixture of pure cultures, representative of species equally distributed, ME0, ME-1, ME-2, ME-3, ME-4	"A"
Mixture of pure cultures, represents species which are unequally distributed, MU0, MU-1, MU-2 and MU-3	"B"

Data handling

Overall colour development in Biolog plates was expressed as average well colour development (AWDC) (Garland and Mills, 1991). For each well, the absorbance value (OD_{620}) of the control well was subtracted from the well absorption, yielding a single difference value (SDV) and the AWDC for the plates was then calculated by summing these SDV values and dividing by 95 (Kersters *et al.*, 1997). Carbon source utilization patterns were obtained by determining the percentage carbon sources utilized. The number of carbon sources utilized was divided by 95 and expressed as a percentage value which represented carbon source profiles (Figures 4-3, 4-5, 4-7 and 4-9).

4.4 Results and Discussion

The influence of cell density and incubation time on pattern development

Colour development in each well of the GN Biolog micro plate reflects the ability of the bacterial community to utilize that specific carbon source. The influence of cell density and incubation time on the substrate utilization patterns, as measured by the AWCD values (colour development in the wells of the GN biolog microwell plate) is shown in Figures 4-2, 4-4, 4-6 and 4-8.

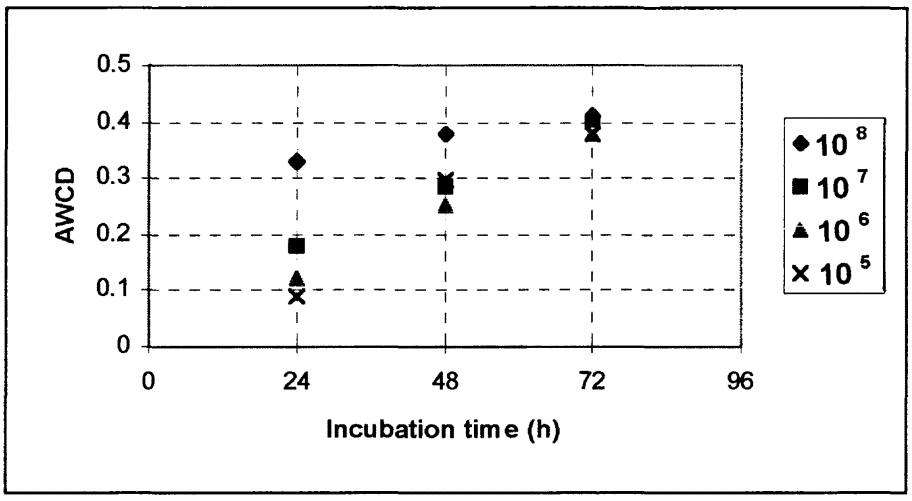

Figure 4-2 Average well colour development (AWDC) in Biolog GN microplates after inoculation with *Serratia marcescens* at different initial cell densities.

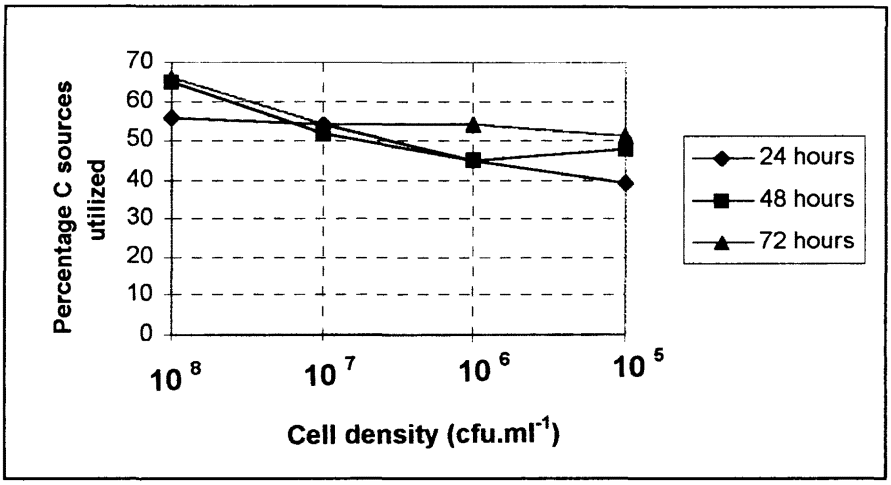

Figure 4-3 Percentage of carbon sources utilized by *Serratia marcescens* representative of pre-dominance.

The *S.marcescens* samples were incubated for 24h, 48h and 72h. Twenty four hour incubation was not sufficient to allow complete development of the colour, as a result of carbon source utilization, excepting where a high number of cells were present (10^8 cells.ml^{-1}) (Figure 4-2). This was indicated by the fact that complete colour development was noted after 48h and 72h incubation as long as their was a sufficient cell density (>10^4 cells.ml^{-1}) (Figure 4-2). This substantiates the findings of Insam *et al.* (1996) who indicated that 10^4 cells were sufficient as an inoculum density. Dilution of *S.marcescens* did not result in a decline in the number of carbon sources utilized and therefore, a linear curve representative of pattern "A" was obtained (Figure 4-1 and 4-3). This substantiates the validity of our hypothetical model, that pre-dominance would be indicated by the same number of carbon sources utilized upon dilution of the sample (Figure 4-1).

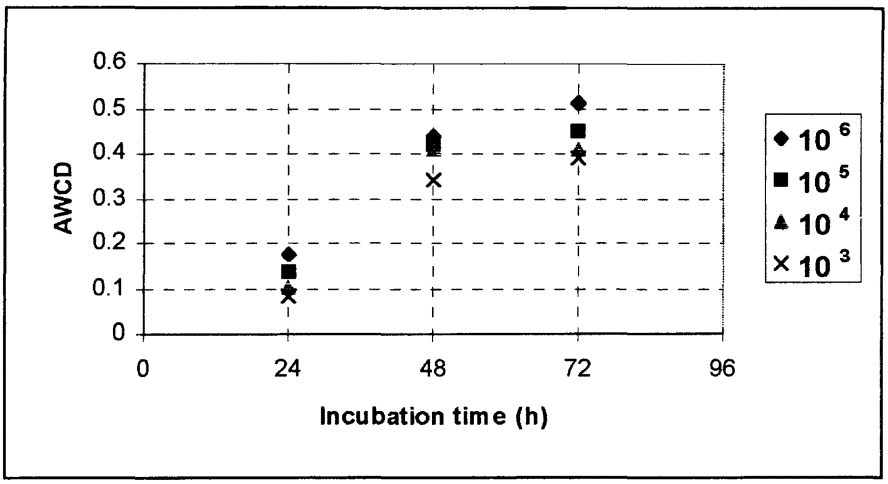

Figure 4-4 Average well colour development (AWDC) in Biolog GN microwell plates after inoculation of mixtures of pure cultures at different initial cell densities (Table 4-2).

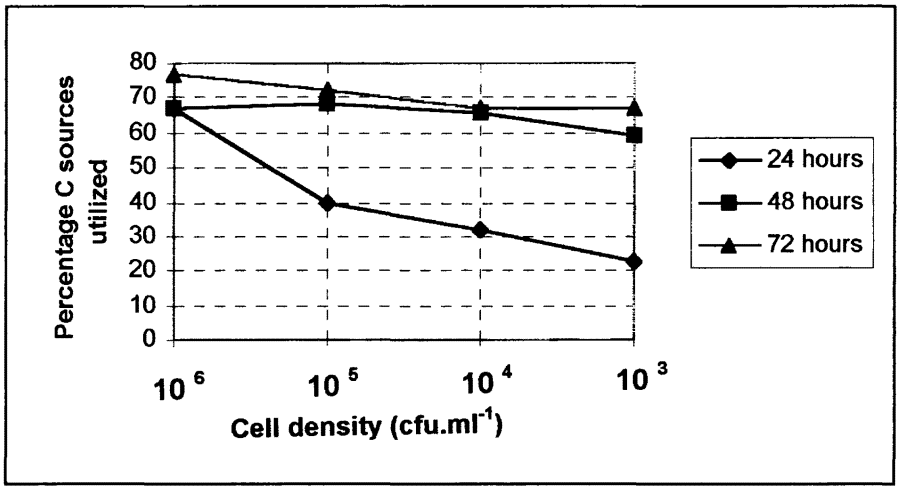

Figure 4-5 Percentage of carbon sources utilized by the mixtures of pure cultures to represent equitability in the presence of a pre-dominant organism using >60% of the carbon sources (Table 4-2).

MS0, MS-1, MS-2 and MS-3 (mixture of pure culture where *S.marcescens* was present) indicates a decline in the number of carbon sources utilized, after 24h incubation (Figure 4-5). This was expected due to results previously obtained, indicating that 24h was an insufficient incubation

period. AWCD of the different cell densities of the mixture of pure cultures, increased with incubation time and showed little variation after 48h and 72h incubation as was found with *S.marcescens* (Figure 4-2 and 4-4). Therefore, the response of Biolog plates depends on incubation time and inoculum density as was found by Insam *et al.* (1996). This phenomenon indicates pre-dominance of *S.marcescens* and/or equitability. This substantiates the validity of our hypothesis that pre-dominance and/or equitability would be indicated by the same number of carbon sources utilized upon dilution of the sample. The results also indicate that the presence of one organism which utilizes a large percentage of the substrates would mask the effect of sample dilution, given that this organism occurs in large enough numbers to ensure a sufficient cell density upon dilution to utilize the substrates in the Biolog microwell plate.

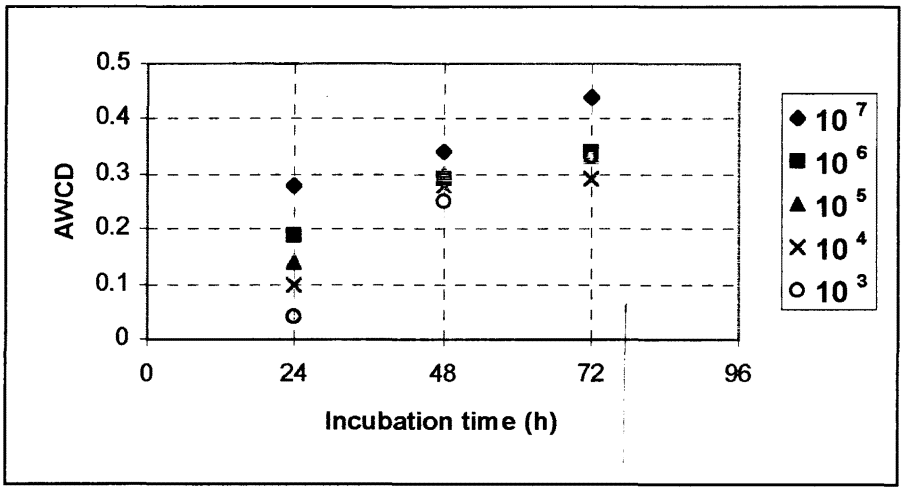

Figure 4-6 Average well colour development (AWDC) in Biolog GN microplates after inoculation with mixtures of pure cultures, representative of species equally distributed (Table 4-3).

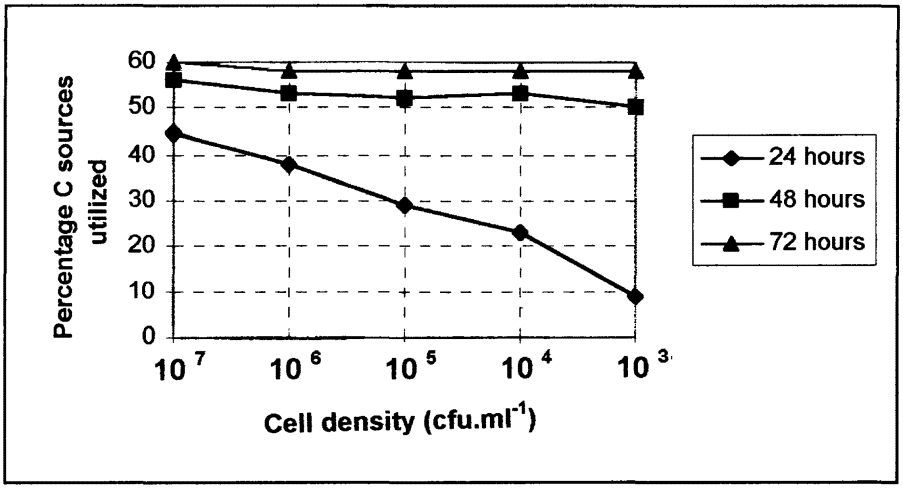

Figure 4-7 Percentage of carbon sources utilized by the mixture of pure cultures representative of equitability (Table 4-3).

AWCD of the equally distributed mixtures of pure cultures (ME0, ME-1, ME-2, ME-3, ME-4) increased with incubation time as with the previous experiments (Figure 4-6). In this case, inoculum-dependent differences among Biolog patterns for the equally distributed mixtures of pure cultures persisted even after extended incubation periods, as was found by Kersters *et al.* (1997). No decline in the number of carbon sources utilized by the equally distributed mixtures of pure bacterial cultures was observed after 48h and 72h incubation (Figure 4-7). This verifies the validity of our hypothetical model that equitability would be indicated by the same number of carbon sources utilized upon dilution of the sample. However, it is unlikely that one would find this sort of equitability in nature. Nevertheless, equitability of this nature, as hypothesized, could be substantiated.

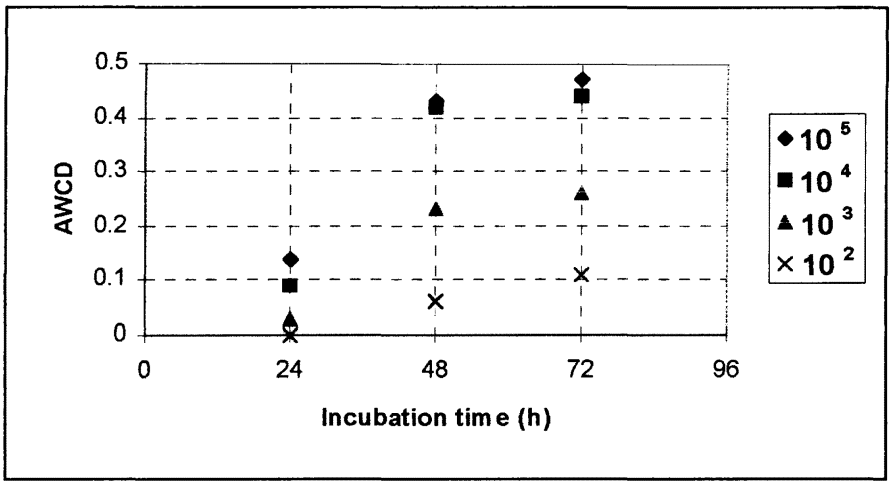

Figure 4-8 Average well colour development (AWDC) in Biolog GN microplates after inoculation with mixtures of pure cultures with different initial cell densities (Table 4-4).

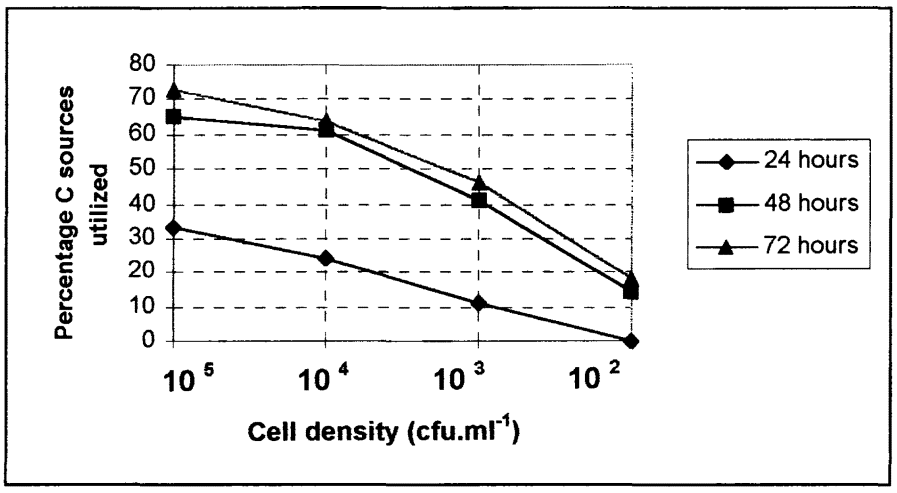

Figure 4-9 Percentage of carbon sources utilized by the third mixture of pure cultures representative of an unequal distribution of microorganisms (Table 4-4).

AWCD of the mixture of unequally distributed species (MU0, MU-1, MU-2 and MU-3) increased with incubation time as with the previous experiments, but the colour development of MU-3 showed little variation after 48h and 72h incubation, because the cell density was insufficient (10^2 to 10^3

cells.ml^{-1}) (Figure 4-8) (Insam *et al.*, 1996). In this mixture where the organisms were representative of an unequal distribution of species, a decline in the number of carbon sources utilized was observed (Figure 4-9). This verifies the validity of the model, that a linear decline in the number of carbon sources utilized, would indicate an unequal distribution of the species present in the sample, since some of these will be diluted out and their contribution to carbon sources utilization would disappear.

4.5 Conclusion

According to the results, different mixtures of pure cultures were classified into different categories, based on their carbon source utilization profiles (Figure 4-1). The results generated using known mixtures of pure bacterial cultures, prove the validity of the hypothesis that the carbon source profile could serve as an information system, to describe certain patterns which would reflect the species diversity of the mixture. This suggested that the hypothesis could be valid for microbial communities in their natural habitat and required further investigation.

CHAPTER 5

BIOLOG FOR THE DETERMINATION OF MICROBIAL DIVERSITY IN DIFFERENT NATURAL HABITATS

5.1 Abstract

The lack of knowledge about the diversity and function of microbial communities is due the fact that a need exists for effective methods that evaluates microbial community structure. Isolate-based techniques have often been used but they offer a limited, biased view of microbial communities. An alternative approach is to examine components of microbial biodiversity, for which there exist a reasonable chance of detecting patterns, which can be related to diversity and function. In this study, different carbon source profiles were generated by inoculating Biolog GN microwell plates with different dilutions of microbial communities from different habitats. These different microbial communities, based on different carbon source profiles, could be categorised according to the hypothesis of pattern recognition, which suggested that the Biolog system could be used for bacterial diversity studies.

Keywords: activated sludge, Biolog system, microbial diversity, pattern recognition.

5.2 Introduction

The lack of knowledge about the diversity and function of microbial communities is due the fact that a need exists for effective methods that evaluates microbial community structure. Isolate-based techniques have often been used but they offer a limited, biased view of microbial communities, due to (1) selective nature of laboratory media, (2) the unculturability of many micro-organisms, (3) the multiple microbial interactions and (4) the difficulties of determining microbial function *in situ* (Kersters *et al.*, 1997). The rRNA approach, together with other molecular techniques (Wise *et al.*, 1996; Yap *et al.*,1996: Wagner and Amann, 1997) and biochemical techniques such as signature lipid biomarker analysis (White *et al.*,1996), enables identification and phylogenetic characterization of micro-organisms without cultivation. However, these methods are time-consuming, complex and it is difficult to interpret the data obtained from these techniques regarding diversity of microbial communities. Hence, there is a need for techniques that allow one to characterize microbial communities without the usual reliance on selective culturing and which would be less complex than the molecular techniques.

Garland and Mills (1991) introduced Biolog GN microplates (BIOLOG, Inc., Hayward, CA) for the characterization of microbial communities. Since then, this approach was found to be effective for discriminating microbial communities from aquatic habitats (Garland and Mills, 1991), wastewater treatment systems (Guckert *et al.*, 1996; Victorio *et al.*, 1996), compost (Insam *et al.*,1996), rhizosphere (Garland and Mills, 1994; Garland, 1996), and soil (Zak *et al.*, 1994; Haack *et al.*, 1995).

In Chapter 4, I indicated that our hypothesis was valid for mixtures of pure cultures under controlled laboratory conditions. The question however, remains whether this hypothetical model would also be valid for natural microbial communities. Therefore, the objective of this study was to test the hypothesis, that natural microbial communities can be classified into different categories, using Biolog profiles which are based on carbon source utilization patterns (Figure 4-1).

5.3 Materials and Methods

Biolog plates

Biolog GN microplates (Biolog Inc., Hayward, CA) were used in this study. The Biolog GN microplates contain 95 wells with different carbon sources and one control well without a carbon source. Each of the 96 wells is also preloaded with a complex buffered nutrient medium and a tetrazolium violet redox dye (Kersters *et al.*, 1997).

Sample sources and inoculum preparation

Mixed liquor samples were drawn from the aerobic, anoxic and anaerobic zones of a five stage Bardenpho activated sludge plant at Daspoort Water Works (Pretoria) as well as from an activated sludge plant at Zeekoegat Water Works (Pretoria region). Hot water springs was collected from Warmbad (Pretoria region) and damwater from LC dam (Pretoria). All samples were collected in sterile screw-capped glass bottles and samples were used in less than 12h after collection to obtain maximum viability.

Plate counts

Viable counts were determined by the spread plate procedure. Samples were serially diluted in 0.85% NaCl and 100µl of the dilutions were plated on Nutrient agar media (Merck). The plates were incubated at 28°C (activated sludge and damwater), 37°C (hot water springs) and colonies were counted after 24h incubation.

Inoculation and incubation of Biolog microplates

Biolog GN microplates were inoculated with undiluted and diluted bacterial samples. The aquatic sample and hot water springs samples were pipetted directly into the Biolog plates. All the microbial suspensions were diluted with sterile saline and used as inocula for Biolog GN microwell plates. The different cell densities of the bacterial samples used for Biolog analysis are summarized in Table 5-1. Biolog GN microplate wells were inoculated with 150µl of the cell suspensions. Microplates were incubated in the dark at 28°C (activated sludge water and aquatic sample) and 37°C (hot water springs) without agitation. Colour formation in the individual cells of the microtiter plates was measured with an Anthos reader 2001 (Anthos Labtec Instruments) at 620nm. Readings of the microplates were made in duplicate after 24h, 48h and 72h of incubation and microwells were also visually studied.

Table 5-1 Bacterial samples used as inoculum for Biolog GN microwell plates and the different initial cell densities used for determination of the AWCD and percentage of carbon sources utilized.

Bacterial samples	Cell density (cells.ml^{-1})	Cell density (cells.ml^{-1})	Cell density (cells.ml^{-1})	Cell density (cells.ml^{-1})
DAS - Aerobic (AE)	10^6	10^5	10^4	10^3
DAS - Anoxic (AX)	10^7	10^6	10^5	10^4
DAS– Anaerobic (AN)	10^6	10^5	10^4	10^3
Hot water springs (HWS)	10^6	10^5	10^4	10^3
Damwater (DW)	10^4	10^3	10^2	10^1

DAS = Daspoort activated sludge samples

Data handling

Overall colour development in Biolog plates was expressed as average well colour development (AWDC) (Garland and Mills, 1991). For each well, the absorbance value (OD_{620}) of the control well was subtracted from the well absorption, yielding a single difference value (SDV) and the AWDC for the plates was then calculated by summing these SDV values and dividing by 95 (Kersters *et al.*, 1997). Carbon source utilization patterns were obtained by determining the percentage carbon sources utilized. The number of carbon sources utilized was divided by 95 and expressed as a percentage value which represented carbon source profiles (Figures 5-4, 5-5, 5-6, 5-7, 5-8, 5-9 and 5-11).

5.4 Results and Discussion

Influence of inoculum density and incubation time on pattern development where aerobic, anoxic and anaerobic activated sludge of Daspoort Water Works and Zeekoegat Water Works represented the natural microbial community studied (Figure 5-2 to Figure 5-9)

The plate counts for the aerobic-, anoxic- and anaerobic sludge from Daspoort Water Works ranged from 1.25 x 10^6 to 3 x 10^7. Colour development (expressed as AWCD) of the activated sludge of Daspoort Water Works increased with incubation time and was affected by the number of culturable cells in the inocula (Figures 5-1, 5-2 and 5-3). This is in agreement with our previous findings (Chapter 4) and also with findings of Kersters *et al.* (1997). AWCD values were always higher for the initial cell densities of the activated sludge (10^6 and 10^7 cell.ml^{-1}) (Daspoort) than for the dilutions (10^5, 10^4 and 10^3 cells.ml^{-1}) after 24h, 48h and 72h incubation (Figures 5-1, 5-2 and 5-3).

The percentage of carbon sources utilized after 24h incubation of the activated sludge samples of Daspoort and Zeekoegat Water Works, decreased significantly, as the aerobic-, anoxic- and anaerobic samples were diluted (Figures 5-4, 5-5, 5-6, 5-7 and 5-8). This suggested an uneven distribution of species according to our hypothesis (Figure 4-1). However, when considering the 48h and 72h incubation periods, for the Daspoort samples, the decrease was less substantial as indicated by the smaller gradient (Figures 5-4 to 5-6). Hence, the 24h Biolog profile was a time effect (too short incubation) rather than an uneven distribution (Figure 5-7 and 5-8). This indicates the importance of a sufficient incubation time. The number of carbon sources utilized, nevertheless, showed a decline indicating an uneven distribution of species according to our hypothesis (Figures 5-4, 5-5 and 5-6).

This substantiates the validity of the hypothetical model described in Chapter 3 and 4, that a linear decline in the number of carbon sources utilized, would indicate an unequal distribution of species present in the sample. This is because some of the species in the sample will be diluted out and their contribution to carbon source utilization would disappear.

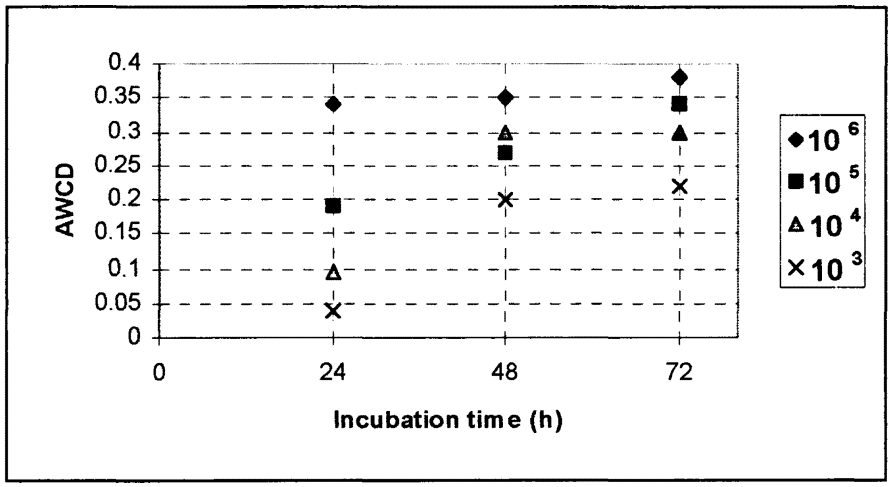

Figure 5-1 Average well colour development (AWCD) in Biolog GN Microplates after inoculation of aerobic activated sludge water from Daspoort Water Works at different dilutions.

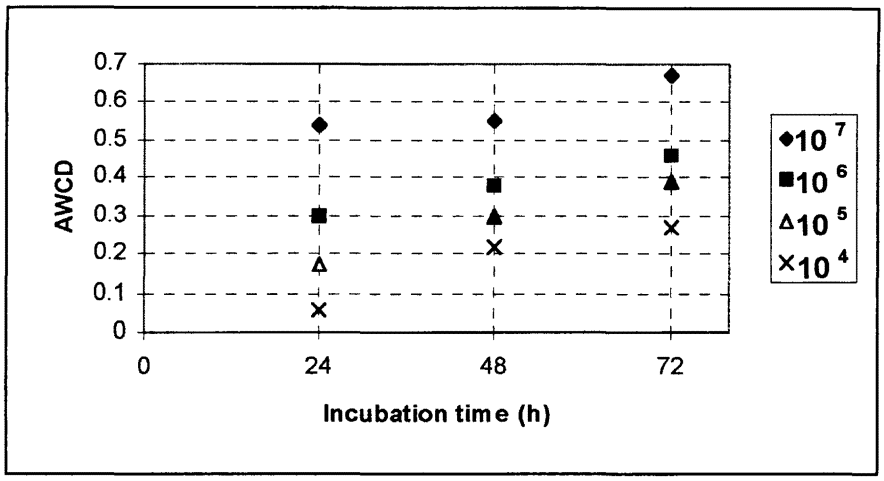

Figure 5-2 Average well colour development (AWCD) in Biolog GN Microplates after inoculation of anoxic activated sludge water from Daspoort Water Works at different dilutions.

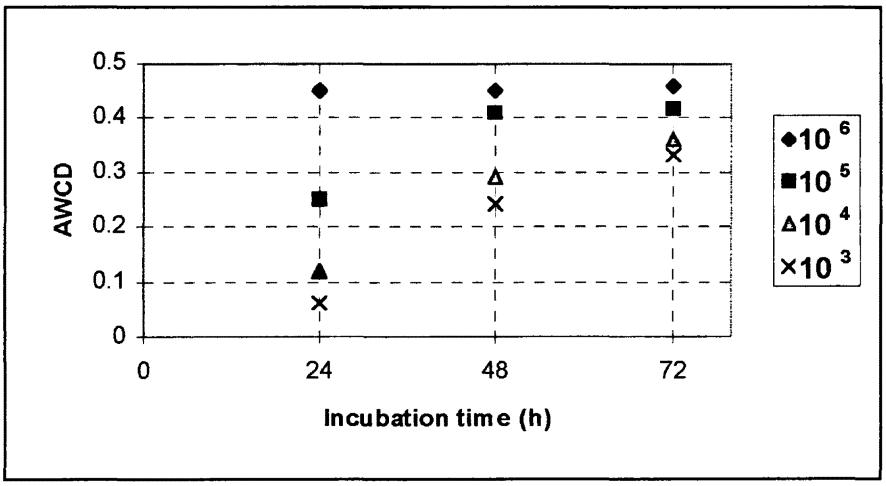

Figure 5-3 Average well colour development (AWCD) in Biolog GN Microplates after inoculation of anaerobic activated sludge water from Daspoort Water Works at different dilutions.

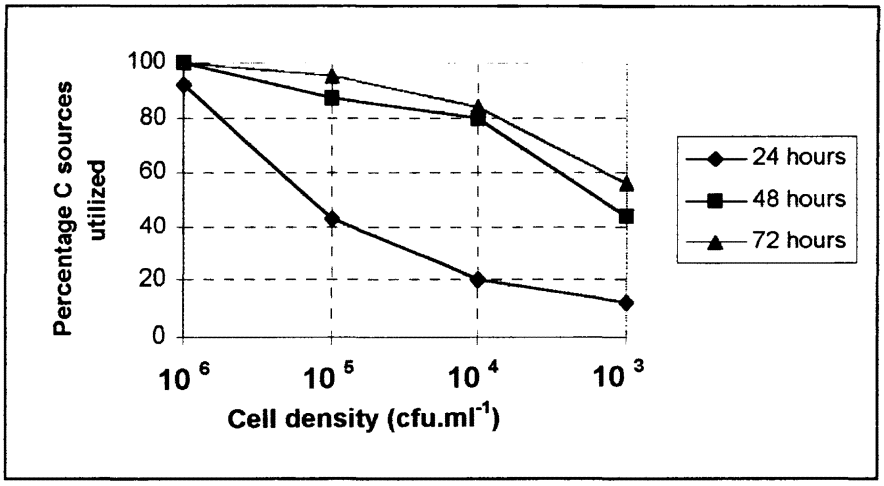

Figure 5-4 Percentage of carbon sources utilized in Biolog GN Microplates at different dilutions of aerobic activated sludge water from Daspoort Water Works.

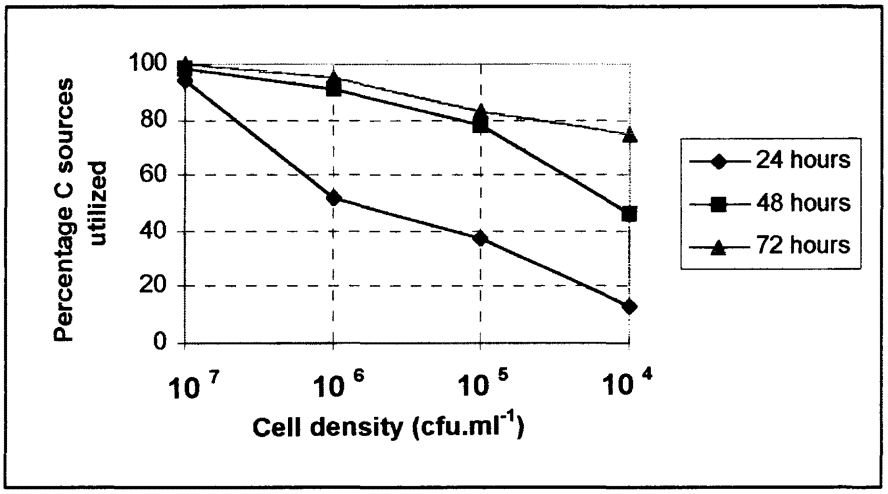

Figure 5-5 Percentage of carbon sources utilized in Biolog GN Microplates at different dilutions of anoxic activated sludge water from Daspoort Water Works.

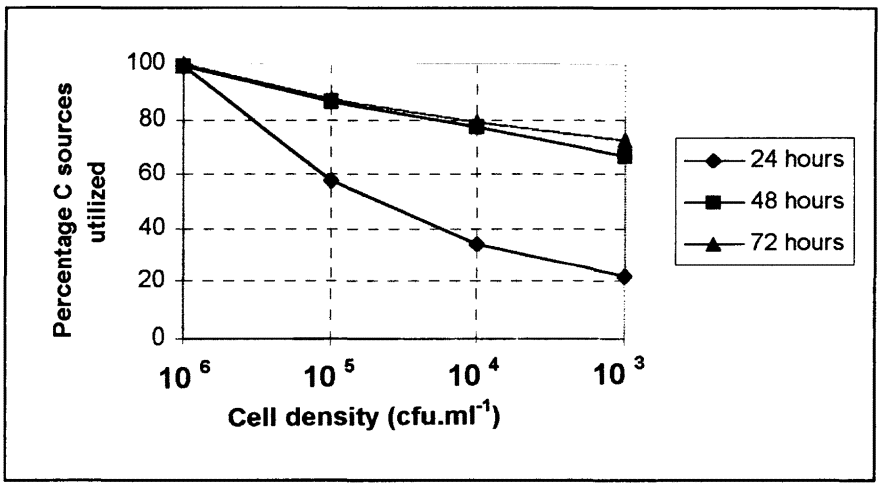

Figure 5-6 Percentage of carbon sources utilized in Biolog GN Microplates at different dilutions of anaerobic activated sludge water from Daspoort Water Works.

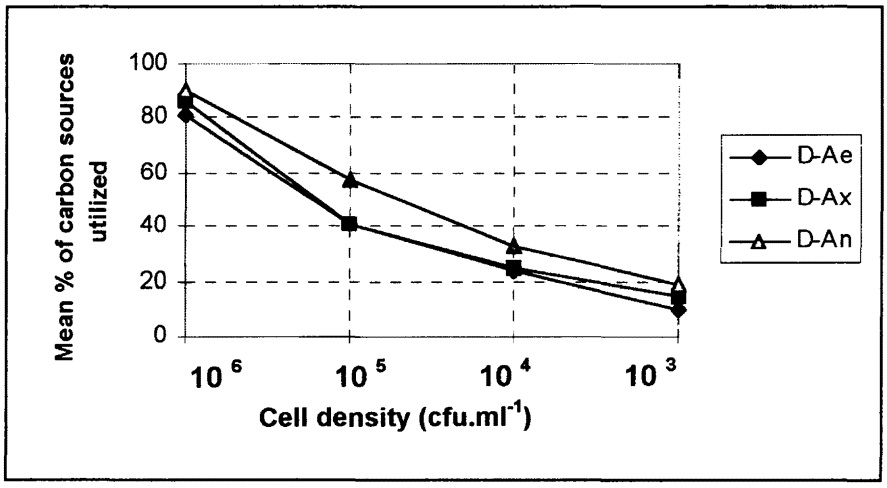

Figure 5-7 Mean percentage of carbon sources utilized, after 24h incubation, in Biolog GN Microplates of activated sludge from Daspoort Water Works. D-Ae = Daspoort aerobic-; D-Ax = Daspoort anoxic- and D-An Mean = Daspoort anaerobic zone.

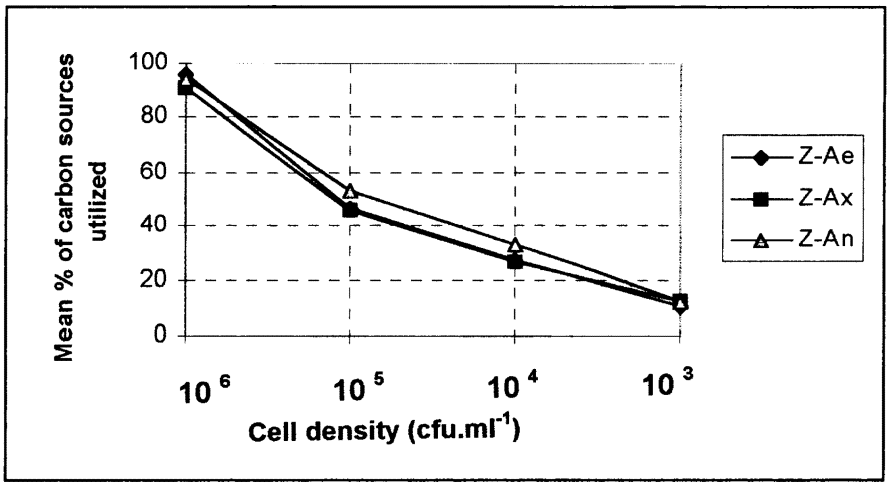

Figure 5-8 Mean percentage of carbon sources utilized, after 24h incubation, in Biolog GN Microplates of activated sludge from Zeekoegat Water Works. Z-Ae = Zeekoegat aerobic-; Z-Ax = Zeekoegat anoxic- and Z-An = Zeekoegat anaerobic zone.

Mean percentage of carbon sources utilized by the damwater (DW)(Pretoria)

Plate counts for the Damwater ranged from1.1 x 10^4 to 1.8 x 10^4. The number carbon sources utilized by the damwater samples showed a decline after 24h, 48h and 72h incubation (Figure 5-9). This suggested an unequal distribution of species in the sample according to our hypothesis. However, the decline in the number of carbon sources utilized, was due to an insufficient cell density (10^1 and 10^2 cells.ml^{-1}) as shown in Figure 5-9 and not an uneven distribution as the results suggest. This is furthermore substantiated by the small difference between the 24h, and the 48h and 72h incubation periods (Figure 5-9).

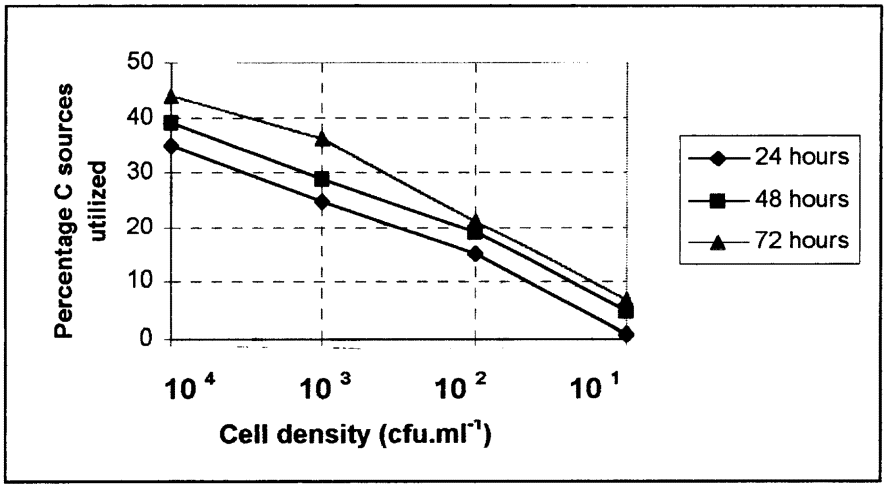

Figure 5-9 Mean percentage of carbon sources utilized in Biolog GN Microplates of damwater.

Influence of inoculum density and incubation time on pattern development where Hot Water Springs represents the natural microbial community

Plate counts for the hot water springs ranged from 5.1 x 10^6 to 7 x 10^6. AWCD of the hot water springs was significantly lower after 24h incubation although the percentage of carbon sources utilized was consistent (65%) (Figures 5-10 and 5-11). AWCD values between the different dilutions of the hot water springs, showed little variation after 48h and 72h incubation, and the percentage carbon sources utilized increased consistently after prolonged incubation (Figure 5-10 and 5-11). Thus, irrespective of the different initial cell densities of the hot water springs, the number of carbon sources utilized was consistent. This verifies the validity of the theoretical model (Chapter 3 and 4) that pre-dominance or equitability would be indicated by the same number of carbon source utilized upon dilution. In this case, due to the extreme environment, pre-dominance was expected and indicated.

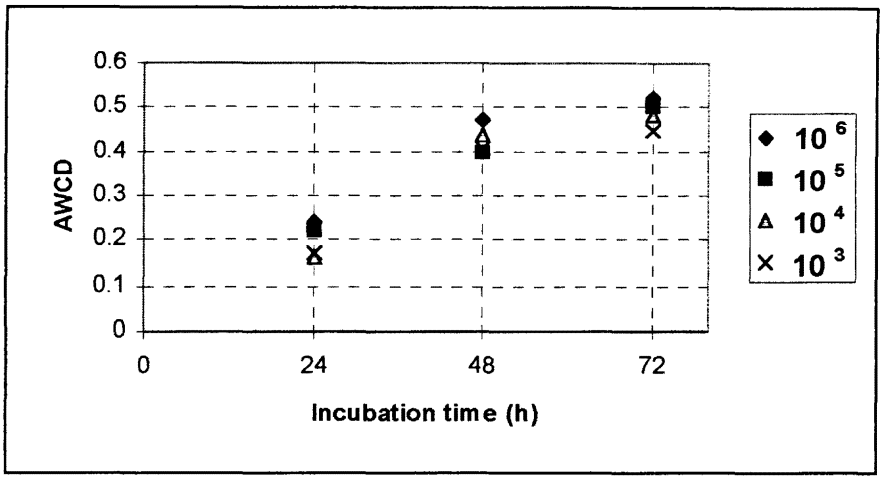

Figure 5-10 Average well colour development (AWCD) in Biolog GN Microplates of hot water springs (HWS).

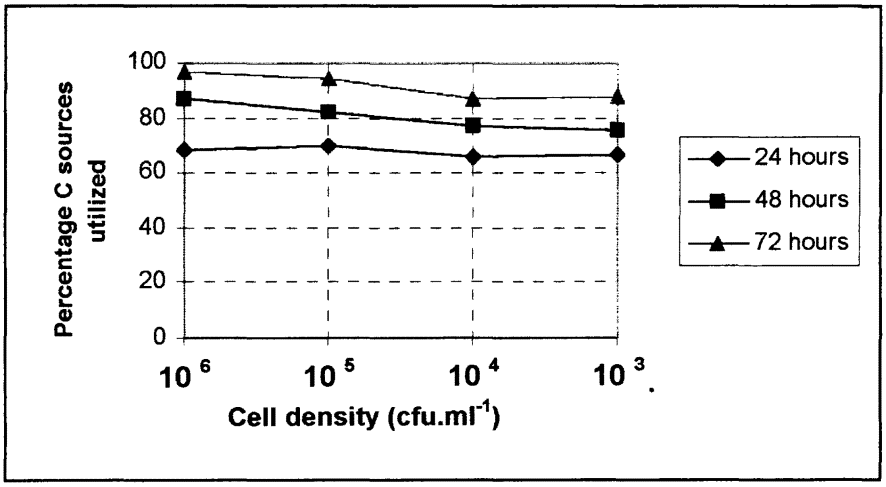

Figure 5-11 Percentage of carbon sources utilized in Biolog GN Microplates of hot water springs (HWS).

5.5 Conclusion

According to our results, natural microbial communities could be classified into different categories, based on their carbon source utilization profiles (Figure 3-1). The results prove the validity of the hypothetical model when natural microbial communities are used. Furthermore, it was determined that the carbon source utilization profile of natural microbial communities, could serve as an information system, to describe certain patterns which would reflect the species diversity of the habitat.

CHAPTER 6

CONCLUSIONS

- A theoretical model was developed for the interpretation of the carbon source utilization patterns of microbial communities.
- Using carbon source utilization profiles of mixtures of pure bacterial cultures, and classifying these into different categories, substantiated the validity of this model regarding pure bacterial cultures.
- Furthermore, a theoretical model was verified, by classifying natural microbial communities into different categories, based on their carbon source utilization profiles.
- According to the results obtained from the pure bacterial cultures and the natural microbial communities, an inoculum density of 10^4 cells as indicated by Insam *et al.* (1996) is necessary for utilization of the carbon sources in the Biolog microplate. An insufficient cell density gives insufficient patterns, which is reflected in the number of carbon sources utilized as was obtained with the activated sludge and damwater samples.
- Twenty four hour incubation was not sufficient to allow complete colour development, as a result of carbon source utilization of the activated sludge, excepting where a high number of cells were present (10^8 cells.ml^{-1}). This was indicated by the fact that complete colour development was noted after 48h and 72h incubation irrespective of cell density as indicated by *S.marcescens*, the mixtures of pure bacterial cultures and the natural microbial communities.

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