BIOLOG FOR THE DETERMINATION OF MICROBIAL SPECIES DIVERSITY AND EVENNESS IN ACTIVATED SLUDGE SYSTEMS

J van Heerden • MM Ehlers • C Korf • TE Cloete

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BIOLOG FOR THE DETERMINATION OF MICROBIAL SPECIES DIVERSITY AND EVENNESS IN ACTIVATED SLUDGE SYSTEMS

Final Report

to the

Water Research Commission

by

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EXECUTIVE SUMMARY

BACKGROUND:

The majority of microorganisms in their natural habitat cannot be cultured and remain unidentified (Haldeman and Amy, 1993; Wagner *et al.*, 1993). This has led to a lack of knowledge on microbial community composition and function. Recently molecular techniques have been used in microbial ecology studies in an attempt to overcome the limitations of culture techniques (Wagner *et al.*, 1993; Amann *et al.*, 1995; Muyima *et al.*, 1997). These techniques require a high level of expertise and sophistication and are often tedious (Schwieger and Tebbe, 1998). Hence, there is a need for techniques that would characterize microbial communities without the reliance on selective culturing and which would be less complex than the molecular techniques. This would provide a more complete picture of community structure and function.

Microbial diversity is essential because (Tiedje, 1994):

1) it allows us to investigate the erudition (lessons) about the strategies and limits of life,
2) micro-organisms are important to the biosphere (water, land, air, etc.) sustainability,
3) microbial resources have value for biotechnology,
4) micro-organisms can be used to monitor environmental change,
5) they play a role in conservation and restoration biology of higher organisms and
6) micro-organisms represent significant models for understanding principles of ecology and evolution.

In any given system one or more species exist each performing a certain function. The more species, the more functions related to their metabolism. It can therefore be said that a specific microbial community has a specific

metabolic capacity. The objective is to relate this microbial capacity of the community to utilize certain selected substrates. The hypothesis being that the more substrates utilized the higher the diversity due to the collective action of individual species.

One possible system that affords the opportunity to generate metabolic patterns for a specific community, is the Biolog system (Garland and Mills, 1991; ; Haack *et al.*, 1994; ; Winding, 1994; Zak *et al.*, 1994; Bossio and Scow, 1995; Ellis *et al.*, 1995; Wünsche and Babel, 1995; Guckert *et al.*, 1996; ; Insam *et al.*, 1996; Smalla *et al.*, 1996; Garland, 1997; Glimm *et al.*, 1997; Heuer and Smalla, 1997; Kersters *et al.*, 1997; Engelen *et al.*, 1998). This system relies on the potential utilization of 95 substrates in a microwell plate by the community (Biolog, Inc., Hayward, CA.). Unlike traditional culture - dependent methods, which are generally selective for the component of the microbial community that has to be cultured, the Biolog method can reflect the activities of a broad range of bacteria (Zak *et al.*, 1994).

Any one organism will not necessarily utilize all the available substrates in a system, nor does the utilization of some of the substrates suggest that this is the complete set of substrates which a particular organism can use, because of:

* competition, which might suppress the activity of a particular organism,
* dominance where one organism utilizes all the substrates in such a way that the contribution of other organisms to substrate utilization is overshadowed and goes unnoticed,
* substrates might not match the metabolic activity of a particular organism (meaning that it will not show - up on the analysis),
* the system might be selective, i.e. it would only allow the metabolic activities of aerobic or facultatively anaerobic, heterotrophic and copiotrophic microorganisms capable of growing at sufficient rates on the substrates,
* depending on the abundance of each species, i.e. the organisms present

in higher numbers will be able to utilize the carbon sources easier than organisms present in low numbers,

* inoculum density has an influence on the tempo and occurance of colour development due to the growth rates of each organism on different substrates,
* incubation time influences substrate utilization of a community due to individual growth rates of the microorganisms on different substrates (Wüsche and Babel, 1995),
* antagonistic interactions between organisms, where one organism inhibits another organisms growth and therefore its ability to utilize the carbon sources and
* toxic effects of the redox dyes may inhibit the growth of the organism (Ullrich *et al.*, 1996).

The aim is not to try and detect each and every metabolic reaction of all the individuals in the community, but the collective pattern for a specific community. Since, 1) a high species diversity should lead to a higher relative number of substrates utilized, because there are more possibilities and 2) upon dilution, some organisms will be lost (causing a decrease in species diversity) from the community, depending on their abundance and the relative contribution (perhaps only one metabolic reaction in the system), reducing the number of possibilities. The extent of the reduction of the possibilities upon dilution, should theoretically reflect something about the community structure. The key lies in the interpretation of the results. We therefore do not consider the Biolog system as a culture - dependent methods, but rather as a battery of metabolic tests to use for generating a database for a specific community as discussed.

OBJECTIVES:

The objectives were:

* To inoculate Biolog microplates with samples from natural and artificially created microbial communities and to interpret the metabolic (substrate utilization) patterns and to relate this to functional (species) diversity and evenness
* To inoculate Biolog microplates with samples from a number of activated sludge systems and to interpret the metabolic patterns and to relate this to functional (species) diversity and evenness within the community and to phosphorus removal

SUMMARY OF RESULTS:

In a sample where *Serratia marcescens* was used in order to simulate dominance, as might be expected in an extreme environment, our results indicated that the presence of one organism (dominance) in a community would result in the same number of carbon sources (substrates) utilized upon dilution, given that the initial inoculum density and incubation time were sufficient.

In a sample where evenly distributed mixtures of pure cultures were used, our results indicated that "absolute" evenness would result in the same number of substrates utilized upon dilution, given that the inoculum density and incubation time were sufficient.

In a sample where a mixture of organisms was representative of an uneven distribution of species, a decline in the number of substrates utilized was observed, due to the loss of some species upon dilution and therefore the loss of their contribution to substrate utilization.

The substrate utilization pattern of a hot water spring sample stayed consistent, indicating dominance as was expected, due to the fact that this is an extreme environment. Activated sludge systems representative of an environment with a high species diversity and uneven distribution of species, indicated that upon dilution some of the substrates where no longer utilized due to the loss of some of the species.

When comparing Biolog Gram-negative (GN) and Gram-positive (GP) microplate data, differences between the GN and GP data was indicated. It is therefore important, not to compare GN and GP patterns, since this would show a difference within the community structure, which is not related to the true community structure, but rather to the substrates utilized within the GN and GP microplates. Considering GN and GP microplate data in isolation indicated that the same conclusions could be made for a specific community in terms of dominance and evenness. This indicated, that a significantly different set of substrates (with a sufficient number of substrates) could be utilized and the hypothesis that upon dilution some of the members of the community will be lost and would be reflected in the metabolic pattern generated, should then be true.

The different zones of the activated sludge systems tested indicated a high initial diversity, due to the high number of substrates utilized. No specific patterns could be identified for phosphate removing systems, indicating that phosphate removal was not community structure specific.

CONCLUSIONS

In this study it was indicated that:

- The Biolog system could be used to determine functional (species) diversity and evenness

- Metabolic profiles in the activated sludge reflected a pattern resembling unevenness, indicated by some of the substrates no longer being utilized after the loss of some of the species upon dilution

- When using the Biolog system, no specific patterns could be identified for phosphate removing and non-phosphate removing systems, indicating that phosphate removal could not be related to the functionality of the aerobic heterotrophic microbial community which was determined using the Biolog system

- Different Biolog systems (GN, GP, ECO plates) could be used and yet the data generated substantiates the hypothesis, that upon dilution some members of the community will be lost and this would be reflected in the metabolic pattern (database) which is generated

- Some limitations might make it impossible to draw a valid conclusion. One such a scenario, would be where the diversity in a system is so high, that irrespective of dilution, all the substrates remain to be utilized. However, whether this type of community exists, is arguable. Therefore, should a loss of substrate utilization occur upon dilution, this technique will reflect the extent of evenness in a microbial community, as was illustrated for activated sludge in this case

- As proven by this and other studies, the use of substrate utilization profiles to characterize microbial communities have clear limitations, but this rapid technique remains a valuable tool for the comparison of microbial communities, provided the data are cautiously interpreted

RECOMMENDATIONS FOR FURTHER RESEARCH:

A thorough knowledge of the bacterial populations responsible for a functioning

activated sludge process can only originate from the combination of different approaches. Therefore, there is a need for techniques that do not necessarily identify individual species, but that can differentiate bacterial communities in terms of their constituents. These methods alleviated the need for culturing and samples were analyzed in a more direct manner which prevents the selection for specific organisms. These methods include Biolog, SDS-PAGE, molecular techniques and monoclonal and polyclonal antibodies. Population shifts could serve as early indicators of malfunctions (e.g. filamentous bacteria as indicators for sludge bulking) so that corrective actions could be taken in time. Keeping in mind the biases caused by cultivation, future studies should rely on *in situ identification of individual cells with immuno- or nucleic acid probes*. There is a need to better understand community structure and function, in order to manage wastewater treatment systems to control bulking or to improve biological phosphate removal capacity.

LIST OF PRODUCTS:

Degree:

MSc Microbiology - J van Heerden

MSc Microbiology - C Korf

Articles:

BIOLOG FOR THE DETERMINATION OF DIVERSITY IN MICROBIAL COMMUNITES. J. van Heerden, M. M. Ehlers, C. Korf and T.E. Cloete. *Submitted:* Water Research (June 1999).

BIOLOG FOR THE DETERMINATION OF MICROBIAL DIVERSITY IN ACTIVATED SLUDGE SYSTEMS. J. van Heerden, M. .M. Ehlers and T.E. Cloete. *Accepted:* Water Research and Technology (Oct 1999).

COMPARISON OF TWO KINDS OF BIOLOG MICROPLATES (GN AND ECO) IN THEIR ABILITY TO DETERMINE THE MICROBIAL COMMUNITY DYNAMICS IN ACTIVATED SLUDGE SYSTEMS. J. van Heerden, M.M. Ehlers and T.E. Cloete. *Submitted:* Journal of Microbiological Methods (Oct 1999).

Presentations:

Poster:

BIOLOG FOR THE DETERMINATION OF SPECIES DIVERSITY IN PHOSPHATE -REMOVING AND NON-PHOSPHATE REMOVING ACTIVATED SLUDGE SYSTEMS. J. van Heerden, M. M. Ehlers and T.E. Cloete. *At:* The American Society of Microbiology (ASM) 99^{the} General Meeting, Chicago, Illinois, USA, 1999.

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LIST OF ABBREVIATIONS:

Aer:	aerobic
Anaer:	anaerobic
Anox:	anoxic
AWCD:	Average well colour development
BVP:	Baviaanspoort Water Treatment Plant
CENT:	Centurion Water Treatment Plant
CLPP:	Community-level physiological profiles
COD:	Chemical Oxygen Demand
CWCD:	Control well colour development
DGGE:	Denaturing Gradient Gel Electrophoresis
DSP:	Daspoort Water Treatment Plant
EBPR:	Enhanced Biological Phosphate Removal
ERWAT:	East Rand Waterboard
FISH:	Fluorescent *in situ* hybridisation
GN:	Gram Negative
GP:	Gram Positive
HBFT:	Hartebeesfontein Water Treatment Plant
HDLB:	Heidelberg Water Treatment Plant
INT:	Iodonitrotetrazolium
PAO:	Phosphate Accumulating Organisms
PHA:	Poly-ß-hydroxyalkonoates
PHB:	Poly-ß-hydroxybutyric acid
RWL:	Rooiwal Water Treatment Plant
SDV:	Single difference value
TSKN:	Tsakane Water Treatment Plant
UPGMA:	unweighted pair group method of arithmetic averages
VFA:	Volatile fatty acid
VLKP:	Vlakplaats Water Treatment Plant
ZKG:	Zeekoegat Water Treatment Plant

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CHAPTER 1

INTRODUCTION

The lack of effective methods for studying the distribution of microbial communities has limited the understanding of the microbial community structure (Griffiths *et al.*, 1997). Methods based on describing the distribution of individual micro-organisms are time-consuming and biased due to their reliance on cultural practices (Wagner *et al.*, 1993). Community-level approaches based on direct extraction and analysis of biochemicals such as phospholipid fatty acids (PFLA) (Tunlid and White, 1992; Frostegard *et al.*, 1993), DNA (Lee and Fuhrman, 1990; Torsvik *et al.*, 1990) and RNA (Schmidt *et al.*, 1991) eliminate the bias associated with culturing micro-organisms, but require a high level of expertise, are often time-consuming and results are difficult to interpret (Schwieger and Tebbe, 1998). Hence, there is a need for techniques that would characterize microbial communities without the reliance on selective culturing and which would be less sophisticated than the molecular techniques.

Microbial diversity is essential because:

1) it allows us to investigate the erudition (lessons) about the strategies and limits of life,
2) micro-organisms are important to the biosphere (water, land, air, etc.) sustainability,
3) microbial resources have value for biotechnology,
4) micro-organisms can be used to monitor environmental change,
5) they play a role in conservation and restoration biology of higher organisms and
6) micro-organisms represent significant models for understanding principles of ecology and evolution (Tiedje, 1994).

Individual level approaches for analyzing microbial communities rely on (a) isolation of micro-organisms, (b) characterization and/or identification of the microbe and (c) description of the microbial community based on the diversity or relative abundance of the different micro-organisms (Garland, 1997). Garland and Mills (1991) approach involved direct inoculation of environmental samples into the Biolog microplates and use of the resulting response to describe differences in microbial communities. The response, or community-level physiological profiling (CLPP), involves (a) the overall rate of colour development, (b) the evenness (or diversity) of response among wells and (c) the pattern, or relative rate of utilization, among wells. The hypothesis being that a high species diversity should lead to a higher relative number of substrates utilized, due to the collective action of the individual species and upon dilution some organisms will be lost (causing a decrease in species diversity), depending on their abundance and the relative contribution (perhaps only one metabolic reaction in the system) to substrate utilization.

The community level physiological profiling (CLPP) system unlike traditional culturing methods, which are selective for the members of the community that could be cultured, can reflect the metabolic activities of a broad range of bacteria (Zak *et al.*, 1994). The Biolog system is therefore not considered as a culture-dependent method in this study, but rather as a collection of metabolic tests (database) used to generate a recognizable pattern for a specific microbial community.

A specific organism will not necessarily utilize all the available substrates in a system, nor does the utilization of some of the substrates suggest that this is the complete set of substrates which the particular organism can use, due to:

* competition, which might suppress the activity of a particular organism
* dominance where one organism utilizes all the substrates in such a way that the contribution of the organisms to substrate utilization is overshadowed and goes unnoticed

* substrates might not match the metabolic activity of a specific organism (meaning that it will not show up on the analysis)
* the system might be selective, i.e. it would only allow the metabolic activities of aerobic or facultatively anaerobic, heterotrophic and copiotrophic micro-organisms capable of growing at sufficient rates on the substrates
* depending on the evenness of each species, i.e. the organisms present in higher numbers will be able to utilize the carbon sources easier than organisms present in low numbers
* inoculum density has an influence on the tempo and occurrence of colour development due to the growth rates of each organism on different substrates
* incubation time influences substrate utilization of a community due to individual growth rates of each organism on different substrates (Wüsche and Babel, 1995)
* antagonistic interactions might occur, where one organism inhibits another organisms growth and therefore its ability to utilize the carbon sources and
* toxic effects of the redox dyes may inhibit the growth of some organisms (Ullrich *et al.*, 1996).

The objectives for this study were therefore:

* To inoculate Biolog microplates with samples from a number of activated sludge systems and to interpret the metabolic patterns and to relate this to functional (species) diversity and evenness within the community and to phosphorus removal.
* To compare different Biolog systems to detect if there is any differences in the effectiveness of these techniques for studying activated sludge system microbial community structure dynamics.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Microbial ecology is the study of the interrelationships that exist between organisms and their biotic (living) as well as abiotic (non-living) environment (Atlas *et al.*, 1992). Studies of species composition of microbial communities can provide insights into the ecological function of these communities (Atlas, 1984). The small size and rapid growth rate of micro -organisms allow for complex community interactions that can be studied more readily than with plants or animals. It is essential to understand the microbial community structure, diversity and the relationship between environmental factors and ecosystem functions (Torsvik *et al.*, 1996). Ecosystem functions can be used to evaluate the effect of environmental stress such as pollution, agricultural exploitation, *etc.* on ecosystems (Torsvik *et al.*, 1996).

Micro - organisms (i.e. bacteria) play an important role in our ecosystem by determining the composition of the atmosphere, soil and water on earth (Atlas *et al.*, 1992). These micro -organisms (i.e. bacteria and fungi) respond to their environment at a faster rate than other organisms, i.e. insects or animals, due to their shorter generation time (Atlas, 1984). Micro-organisms oxidize and reduce different inorganic compounds of phosphorus (P), nitrogen (N), sulphur (S) and other elements (Atlas *et al.*, 1992). Therefore, micro-organisms can act as biochemical incinerators converting pesticides, petroleum and other pollutants in water and soil to harmless products, such as carbon dioxide (CO_2) and water (H_2O). Microbes contribute to waste treatment (i.e. composting) and self purification processes by which aquatic (water) and terrestrial (earthly) ecosystems are purified of unwanted residues (i.e. P-removal in activated sludge systems) (Atlas *et al.*, 1992).

Diversity and dynamics of microbial communities in activated sludge have been analyzed by culture-dependent methods, which excludes the majority of endogenous microbes due to the selective nature of the media (Parkasam and Dondero, 1967). Hence, the majority of micro-organisms in their natural habitat cannot be cultured and remain unidentified (Haldeman and Amy, 1993).

Numerical taxonomic studies either use profiles of cellular constituents or phenetic characteristics of isolates (Sneath and Sokal,1973; Kaneko and Atlas, 1977; Mallory and Saylor, 1984). Phenotypic diversity determinations include the culturable microbial species (Torsvik *et al.*, 1990). Community-level approaches and molecular techniques based on direct extraction and analysis of phospholipid fatty acids, DNA and RNA have been performed to overcome the limitations of culture-depended techniques. These methods require a high level of expertise and are unfortunately technically sophisticated and time-consuming (Lee and Fuhrman, 1990; Torsvik *et al.*, 1990; Schmidt *et al.*, 1991; Tunlid and White, 1992; Frostegard *et al.*, 1993; Moyer *et al.*, 1994). Alternative methods are therefore required to study the microbial ecology in natural systems.

Another approach to translating the information in a microbial ecosystem would be to determine the metabolic diversity within the system. Community-level carbon source utilization patterns, have been introduced for classifying microbial communities on the basis of their metabolism and for comparison of microbial communities from different habitats (Garland and Mills, 1991). The aim of this study was to use Biolog microplates to determine the metabolic patterns within natural environments as well as between different activated sludge plants (i.e. P-removing and non-P-removing systems) and to relate these results to microbial diversity, abundance and evenness.

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diversity, abundance and evenness.

2.2. Phosphate removal in activated sludge

Phosphorus is present in wastewater in both the inorganic and organic forms. Phosphorus present in wastewater is often converted to orthophosphate (Toerien *et al.*, 1990; Bitton, 1994; Ehlers, 1998). An effluent of high quality is produced within biological phosphate removal systems in order to meet the Department of Water Affairs and Forestry of South Africa's standard (< 1 mg(orthophosphate).l^{-1}) at the lowest possible cost (Toerien *et al.*, 1990; Bitton, 1994). In many systems the latter standard is not met and chemical phosphate precipitation is still required (Toerien *et al.*, 1990; Bitton, 1994).

Bacteria grown in low nutrient environments accumulate similar quantities of phosphate per cell than those in high nutrient environments, it would appear that cells have a limit to the amount of phosphate that can be accumulated per cell irrespective of nutrient availability (Momba, 1995). Under conditions of increased nutrient (PO_4^{3-}) availability, total phosphate removal increases, possibly due to the increase in biomass (Bosch, 1992). This suggests that total biomass may be more important, than individual species or specific populations in terms of phosphate removal.

2.2.1 Enzyme activities in activated sludge

The rate of hydrolysis under different electron acceptors reveals three important points:

1) there is a lack of consensus regarding the rate of hydrolysis under different electron acceptor conditions,
2) most of the studies have concentrated on single enzyme systems and

3) there are no concrete efforts in the studies performed until now to understand the underlying reason for the change or no change in hydrolysis rate under different electron acceptor conditions (Goel *et al.*, 1998).

Goel and colleagues (1998) indicated that the enzyme activities linearly correlated with the biomass concentration. Considering that the non limiting substrate concentration in a particular assay remains the same, the enzyme activities from different samples can be compared. The observed enzyme activities did not change under different electron acceptor conditions for both alkaline phosphatase and acid phosphatase (Goel *et al.*, 1998). While, protease activity under anaerobic conditions was found to be 40 - 75% higher than that under aerobic conditions (Goel *et al.*, 1998). Dehydrogenase activity was observed to be higher by about 40 - 60% under anaerobic conditions (Goel *et al.*, 1998). Lower dehydrogenase activity under aerobic conditions may be attributed to the competition between iodonitrotetrazolium (INT) and O_2 as the final electron acceptor (Goel *et al.*, 1998). The increase of enzyme activity of a-glucosidase is higher under aerobic incubation as compared to anaerobic incubation. Protease activities seem to be marginally affected by the electron acceptor conditions (Goel *et al.*, 1998). Protease activities were observed to be stable. The relative change in the specific enzyme activities will be smaller than the change in enzyme activities due to the increase in the biomass concentration during the batch incubation (Goel *et al.*, 1998). It was indicated that inactivation rates were different for different enzymes and the typical period for 10% reduction in alkaline phosphatase and ∝-glucosidase activities were approximately calculated to be 46 h and 1 h, respectively (Goel *et al.*, 1998).

2.2.2 Phosphate removing bacteria

In enhanced biological phosphate removal (EBPR), groups of polyphosphate-accumulating bacteria are enriched in the activated sludge by recycling of the sludge

in anaerobic and aerobic zones. In the anaerobic step, the polyphosphate-accumulating bacteria take up short-chain fatty acids and store them in granules as polyhydroxyalkanoates (PHA).

2.3 Microbial ecology of activated sludge

According to Grady and Lim (1980), the expression "activated sludge" refers to a slurry of micro-organisms that removes organic compounds from wastewater and these micro-organisms are removed by sedimentation under aerobic conditions. Activated sludge subjected to alternate anaerobic and aerobic conditions in sewage treatment plants store more phosphorus (P) than necessary for normal growth (Barnard, 1976). The activated sludge micro - organisms can be divided into two major groups:

i) decomposers, which are responsible for biochemical degradation of polluting substances in wastewater (i.e. Phosphate). Bacteria, fungi and colourless cyanophyta are represented in this group, and

ii) consumers, which utilize bacterial and other microbial cells and substrates. This group belongs to the activated sludge microfauna and consists of phagotrophic protozoa and microscopic metazoa (Cloete and Muyima, 1997).

The microbial population of activated sludge consists of 95% decomposers, predominantly bacteria (Cloete and Muyima, 1997). In studies conducted on activated sludge water, attention had been directed toward the aerobic heterotrophs, since they are considered the most important in degradation and final stabilization of organic matter (Parkasam and Dondero, 1967). Parkasam and Dondero (1967) stated that many of the sewage bacteria were there by chance and do not survive long.

2.4 Microbial population dynamics

Species diversity can be expressed as species richness, which is an index expressing the ratio between the number of species and the number of individuals in a community (Odum, 1971; Atlas, 1984). Species diversity is a function of the number of species present (species richness or species abundance) and of the evenness with which the individuals are distributed among these species (species evenness) (Troussellier and Legendre, 1981). According to Odum (1971) climax (stable) communities support the highest possible biodiversity, with numerous species interactions and a balanced biomass production to respiration ratio, as well as a low nutrient loss. No environmental species exists in isolation, it is supported by a system that provides it with food, shelter and physical conditions (Lovejoy, 1993). The maturity of a community is reflected in both its diversity and its productivity (Atlas and Bartha, 1993). Diversity increases through succession in a community to a maximum climax (Atlas *et al.*, 1991). Diversity of microbial communities is an indicator of stress conditions (Atlas *et al.*, 1991).

Functional diversity can be determined by Biolog, which is based on substrate utilization (Garland and Mills, 1991). Results obtained from the Biolog system depends on the number of potential substrates used by a sample, the presence or absence of substrate utilization as well as the utilization rate of each substrate (Griffiths *et al.*, 1997). Griffiths *et al.* (1997) indicated that there could be changes in microbial community structure with no changes in function, but that function was affected below a certain level of species diversity (richness).

Microbial diversity is essential because (Tiedje, 1994):

1) it allows us to investigate the erudition (lessons) about the strategies and limits of life,
2) micro-organisms are important to the biosphere (water, land, air, etc.)

sustainability,

3) microbial resources have value for biotechnology,
4) micro-organisms can be used to monitor environmental change,
5) they play a role in conservation and restoration biology of higher organisms and
6) micro-organisms represent significant models for understanding principles of ecology and evolution.

The aim of studies on biodiversity is to construct inventories of living organisms to serve as reference for monitoring natural resources like oceans or activated sludge systems (Palleroni, 1994). According to Palleroni (1994) the discovery of new organisms living under extreme environmental conditions or capable of degrading recalcitrant organic compounds has stimulated studies in industrial and environmental microbiology.

Studies of bacterial species diversity differ in the method of isolation, mode of characterization, clustering methods used for grouping or identification, level similarities or distances used to define a species or biotype and type of diversity measures used (Watve and Gangal, 1996). According to Mills and Wassel (1980) species diversity will never become a total answer to the determination of the low-level stress effects. When species diversity is coupled with methods such as activity measurements, biomass evaluations and other descriptors of community structure, the property may be a valuable ecological tool for microbial ecologist (Mills and Wassel, 1980).

Bacterial communities with low genetic diversity should be less able to withstand environmental stress, than communities where the genetic heterogeneity provides the means to cope with environmental changes (Atlas and Bartha, 1993). Diversity and bacterial numbers determine the ability of the community to resist stress (Atlas,

1984). When the number of species present and their abundance in the community remain constant over time the community is considered to be stable (Atlas, 1984). The more stable the community the more it would resist a decrease in both numbers and diversity. Due to stability, species diversity can be measured as the persistence of populations and the evenness of distribution of species abundance within the community. Fitness of a community is the ability of the community to withstand changes in the environment (Atlas, 1984). The relationship between diversity and stability is acceptable and useful for the examination of short and long-term effects of stress factors on the environment (Atlas, 1984).

Low species diversity characterizes areas where the intensity of one or more ecological factor approach extremes that could support life. Communities with low genetic diversity should be less able to withstand environmental stress, than those communities in which the genetic heterogeneity provided the means needed for dealing with the environmental modifications (Rashit and Bazin, 1987). Functional diversity could be examined as the number of different substrates that are used by the microbial community (Zak *et al.*, 1994).

Conditions that are different from the optimal growth requirements of bacterial cultures exert stress on the cells, where stress is any factor that influences a bacterial cell in a negative fashion. Bacteria react differently to various stress conditions (Brözel and Cloete, 1993).

Mature ecosystems are complex and the high species diversity results in numerous interspecies relationships. A low amount of energy is required to maintain the structure of a mature ecosystem (Atlas and Bartha, 1993). Diversity normally decreased when one or more populations attain high densities and high numbers. This results in successful competition and domination of a single population (Atlas and Bartha, 1993). Species that were tolerant to the stress factors would become

dominant, while certain species may not survive and this would lead to a decrease in diversity (Atlas, 1984). A stable community contained a high degree of information (species diversity) to recover or withstand environmental perturbations (chaos) (Atlas, 1984).

2.5 Koch's postulates for microbial ecology

The Koch's postulates (1884) have not been applied to the study of the ecology of micro-organisms. This is probably because it has not yet been possible to inoculate a system with a pure culture to investigate whether it would perform the same function after it has been isolated and studied in pure culture *in vivo*. Steyn and Cloete (1989) modified the original Koch's postulate (1884) for application in microbial ecology as follows:

1) A particular micro - organism 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.

These postulates rely on the isolation, culturability and identification of the micro-organisms, excluding the culturable 10% of micro-organisms in natural environments.

2.6 Methods for determining species diversity in natural environments

The environmental applications of microbiological, molecular and biochemical methods have experienced a stonier path than the implementation of the same methods in, for example, medicine. One of the major technical difficulties was the development of strategies to effectively and efficiently cope with the diversity and complexity of real environmental systems. Therefore, a need exist for non-culturing techniques to evaluate the diversity, equitability and abundance of micro - organisms in their natural environment.

2.6.1 Genotypic approaches to determining species diversity

Genotypic diversity measure the genetic potentials in microbial communities independent of the environmental conditions (Torsvik *et al.*, 1996). Genotypic methods are those that were directed towards DNA or RNA molecules (Van Damme *et al.*, 1996). The information in DNA from complex communities can be analyzed by two techniques providing an expression of the overall diversity (Torsvik *et al.*, 1996). Borneman and colleagues, (1996) state that genetic diversity was essential to life, since it permitted adaptation through the creation of new organisms by genetic transfer and mutations. The shift of analysis of isolates to total community analysis, creates a need for molecular techniques and especially nucleic acid analysis is been of importance (Griffiths *et al.*, 1997). Apart from the use of the ribosomal rRNA genes for molecular ecological studies, other functional genes such as the nitrogenase gene and the hydrogenase gene have been used to evaluate genetic diversity in an environment (Wawer and Muyzer, 1995; Zehr *et al.*,1995; Yap *et al.*, 1996).

Molecular methods provide tools for analysing the bacterial community, covering those bacteria that have not been cultured in the laboratory. Low resolution and

broad scale analysis of community DNA, like DNA-reassociation, allows assessment of the total genetical diversity of bacterial communities (Torsvik *et al.*, 1996). PCR - denaturing gradient gel electrophoresis (DGGE) analysis of rRNA genes gives somewhat higher resolution and provides information about changes in the gross community structure (Muyzer *et al.*, 1993). When DGGE analyses of rRNA genes are combined with hybridisation using phylogenetic probes or with sequencing, assessment of the phylogenetic affiliation of the numerically dominating members of a community can be obtained. Fluorescent *in situ* hybridisation (FISH) of bacterial cells with phylogenetic probes provides information about the overall taxon composition of bacterial communities or assemblies (Hahn *et al.*, 1992; Amann *et al.*, 1995). By cloning PCR-products from rRNA genes in whole community DNA, information about non-cultured bacteria is gained. This approach allows comparison of the structure of the cultivated fraction of a bacterial community with the total community with the total community. To discriminate at bacterial isolate and clone levels, DNA fingerprinting and sequencing have been applied (De Bruijn, 1992). These techniques are time-consuming, complex and the results are difficult to interpret (Amann *et al.*, 1995; Ehlers, 1998; Sakano and Kerkhof, 1998; Schwieger and Tebbe, 1998).

2.6.2 Phenotypic approaches to determining species diversity

Phenotypic diversity determinations include only the culturable (10%) species (Torsvik *et al.*, 1990). It involves the expression of genes under a given set of conditions (Torsvik *et al.*, 1996). Physiological diversity is based on cytological features (i.e. cell size) and can be determined by cell micromorphology and motility (O'Brien and Colwell, 1987). These methods were based on sampling, isolation for pure cultures and differentiation of phenotypic properties (Haldeman and Amy, 1993).

Morphological studies through colonial morphology and pigment production, as well as physiological tests such as growth temperature and heat or salt tolerance can be used (O'Brien and Colwell, 1987). Biochemical test such as formation of acetic acid from ethanol and acid production from carbohydrates, etc. are used to determine species diversity (O'Brien and Colwell, 1987). Gelatin hydrolysis, gluconate oxidation, haemolytic activity, hydrogen sulphide production, indole production, levan production, methyl red test, nitrate reduction, nitrite production, denitrification, oxidase activity, pectinase activity, urea hydrolysis and Voges-Proskauer reaction are used for determination of species diversity (O'Brien and Colwell, 1987). Nutritional testing for sources of carbon and/or nitrogen for growth and energy was also performed (O'Brien and Colwell, 1987). Antimicrobial susceptibility, phytopathogenicity or miscellaneous tests such as sensitivity, tolerance to dyes/heavy metals or growth on selective media are used in experiments (O'Brien and Colwell, 1987).

A limitation to the phenotypic approach is that bacterial strains have to be isolated from the biotype and pure cultures cultivated in laboratories before phenotypic testing can be performed (Torsvik *et al.*, 1990). Only a restricted part of the genetic information was revealed through phenotypic testing and it was not known if these organisms were representative of natural populations and their respective niches (Torsvik *et al.*, 1990).

2.6.3 Community based carbon source utilization patterns using the Biolog system

Garland and Mills, (1991) introduced the use of community-level carbon source utilization patterns for comparison of microbial communities from different habitats. The Biolog system (Biolog Inc., Hayward, USA.) is based on the different utilization of a large number of organic compounds by the test organisms.

Oxidation of the substrates is monitored by the colourimetrically determinable conversion of the redox dye tetrazolium violet into a vividly purple formazan (Biolog Inc., Hayward, USA). Metabolism of a substrate result in the formation of NADH and through an electron transport chain, in the irreversible reduction of the redox dye to the purple formazan. According to Zak and colleagues, (1994) the peak absorbance of the tetrazolium dye occurs at 590 nm.

Types of Biolog microplates

Standardized microplates allowed the simultaneous testing of 95 diagnostically significant carbon sources; with one reference well containing no carbon substrate (Bochner, 1989). Along with 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. The dried preparations in the individual wells are rehydrated by inoculation with the cell suspension (Biolog Inc., Hayward, USA).

Different microplate types are commercially available for characterization of Gram negative (GN Biolog plates) and Gram positive isolates (GP Biolog plates), where 62 of the substrates are common to both GN and GP plates, and each has 33 unique substrates (Kanopka *et al.*, 1998). In addition, empty plates (MT Biolog plates) are available that contain no carbon sources, but do contain the tetrazolium dye (Bochner, 1989; Garland and Mills, 1991; Guckert *et al.*, 1996). Thus, each Biolog microplate yields a specific pattern of activities representing the functional attributes of the inoculated sample with respect to a suite of substrates (Bochner, 1989).

Basic method for the Biolog System:

Community-level Biolog analysis is usually accomplished in four steps (Biolog Inc., Hayward, USA):

(a) process the sample to form a suspension of micro-organisms,

(b) inoculate the Biolog microplate(s) with aliquots (usually 150 μl) of the suspensions,

(c) incubate the plates for 4 - 24 h or more depending on sample, while monitoring colour development and

(d) analyze the results visually or automatically (590 mm).

The culturable fraction in the microplate could be higher due to:

a) co-operative effects between organisms, and

b) the inability of certain organisms to grow on solid media (Ullrich *et al.*, 1996).

The culturable fraction could be lower due to:

a) antagonistic interactions between organisms and

b) toxic effects of the redox dyes (Ullrich *et al.*, 1996).

Biolog system used for:

The Biolog system has already been used for characterization of naturally occurring bacteria and for classification of bacterial communities of different environments (Fredrickson *et al.*,1991; Garland and Mills, 1991; Verniere *et al.*, 1993; Winding, 1994; Zak *et al.*, 1994). The comparisons of Biolog results with other test systems, i.e. API and Biotype-100, have also been performed (Fredrickson *et al.*, 1991; Amy *et al.*, 1992; Klingler *et al.*, 1992; Verniere *et al.*, 1993). Community-level physiological profiles (CLPP) have been used to characterize microbial communities in freshwater, coastal and Lagoon areas, as well as soil, rhizosphere and bioreactors (Garland and Mills, 1991; Gorlenko and Kozhevin, 1994; Winding, 1994; Zak *et al.*, 1994; Bossio and Scow, 1995; Ellis *et al.*, 1995; Haack *et al.*, 1994; Lehman *et al.*, 1995; Vahjen *et al.*, 1995; Garland, 1996 (a); Garland, 1996 (b); Glimm *et*

al., 1997; Engelen *et al.*, 1998). Environments such as groundwater, phyllosphere, activated sludge and compost were also studied (Ellis *et al.*, 1995; Heuer *et al.*, 1995; Lehman *et al.*, 1995; Insam *et al.*, 1996; Victorio *et al.*, 1996). For rapidly assessing the dynamics of autochthonous microbial communities, the CLPP techniques have been used (Wünsche and Babel, 1995). Diversity can be calculated using univariate indices which do not capture all potential differences in the CLPP (Garland, 1997).

Victorio and colleagues (1996) showed that phenotypic fingerprinting using Biolog can be useful in distinguishing heterotrophic microbial communities within wastewater treatment systems. More diluted inocula result in protracted rates of colour development for both pure cultures and heterotrophic communities (Garland and Mills, 1991; Winding, 1994; Haack *et al.*, 1995; Kersters *et al.*, 1997).

According to Bochner (1989), this system allows the testing of carbon sources utilization's of bacteria not producing pH changes. This system works equally well with fermenting and non-fermenting bacteria (Bochner, 1989). However, false positive or negative results may be produced by oligotrophic bacteria (Bochner, 1989).

Unknown strains could be identified only if they belong to a species included in Biolog's data. Vahjen and colleagues (1995) defined an utilized substrate as any well in which the response was greater than the mean response of all positive wells. The threshold for a positive test could be defined as any positive value after background correction (Vahjen *et al.*, 1995). A higher positive value such as 0.25 absorbency units may eliminate weak false positive response (Garland, 1996(a); Garland, 1996(b)). An important advantage of the Biolog method is its ease of use and thus feasibility for use in large scale field studies.

Wünsche and Babel (1995), found that communities with cell densities ranging from 10^6 to 10^8 colony forming units (cfu).ml^{-1} use the same percentage of substrates after prolonged incubation. According to Wünsche and Babel (1995), the differentiation and grouping of natural communities by the number of utilized substrates seem to be possible using incubation periods of 48 h or more without taking into account the inoculum density. Kersters and colleagues, (1997) indicated that inoculum-dependent differences among the Biolog patterns for both pure cultures and heterotrophic microbial communities persist even after extended incubation periods. The reliable use of the Biolog system requires samples of approximately equivalent inoculum densities (Garland and Mills, 1991; Haack, 1994; Kersters *et al.*, 1997). Community comparisons would not be compromised by the different responses of inoculum density on the colour response data (Garland and Mills, 1991; Haack, 1994; Kersters *et al.*, 1997). Zak and colleagues (1994), found that two sites could exhibit identical substrate richness, evenness or diversity but still catabolize totally different substrates.

Previous studies have indicated that microbial communities produce habitat-specific and reproducible patterns of carbon source oxidation and therefore the method could be used to discern temporal and spatial differences among microbial communities from bulk soils, rhizospheres and subsurface cores (Smalla *et al.*, 1998). Bacterial growth occurred in the wells during the assay. Thus, the pattern of substrate used may only reflect the functional characteristics of organisms that are able to grow in the Biolog microplate wells under the assay conditions used. It remains unclear what fraction of the microbial population in a given community contributed to the observed pattern of carbon source oxidation. Studies on the effects of environmental perturbations or transgenic organisms on microbial communities should include determination of whether patterns of sole carbon source utilization were caused by a limited number of populations and whether these populations were numerically dominant in the community at the time of sampling or became dominant during the

course of the assay (Smalla *et al.*, 1998). It is unlikely that the carbon source utilization profiles obtained reflect the *in situ* function of the microbial communities used as inocula, because substantial bacterial growth occurs during the assays performed with Biolog microplates (Smalla *et al.*, 1998). If there was a significant change in the species composition or an alteration in the relative proportions of populations in the wells of the microtiter plate, the ability to extrapolate the findings of the assay to the microbial community being studied were compromised (Smalla *et al.*, 1998). The profile obtained does not necessarily reflect the functional potential of the numerically dominant members of the microbial community used as the inoculum (Smalla *et al.*, 1998).

Biolog for identification:

The Biolog metabolic pattern is a record of the carbon sources oxidized by a strain during respiration (Bochner 1989). To identify a strain, the breathprint (metabolic fingerprint) of the strain is established. Modification to the Biolog procedure that results in changes of the identification reflects the actual metabolic profile of the organism (Noble and Gow, 1998). Biolog identification software identifies bacteria by matching the profile off the test organism with the most likely match in a data bank of profiles (Noble and Gow, 1998). Using cluster analysis, overall similarities or dissimilarities between organisms that make up the set can be computed (Noble and Gow, 1998).

Theoretically, duplicates should yield identical results in the same laboratory, but differences often occur (Noble and Gow, 1998). Factors that might impair reproducibility of the results obtained from an experiment are 1) reading intervals, such as 24 h readings versus actual 22 h to 26 h readings and 2) variations in incubation temperature that occur because of fluctuations in temperature that occur from shelf to shelf within air-conducting incubators (D'Amato *et al.*, 1991).

Researchers have found variability in the GN microplate test results for the same cultures evaluated at different times (D'Amato *et al.*, 1991). Hartung and Cilerolo (1991) found that profiles in the Biolog assay were not reproducible. They showed that the profile varied in at least one carbon source between replicate assays of the same strain performed on different days and in duplicate readings of the same plate taken within 1 h (Hartung and Cilerolo, 1991). The manufacturers recommended an absorbency of at least 40% greater than the control well, to distinguish positive from negative for the oxidation of a particular substrate when the reaction was relatively weak (Hartung and Cilerolo, 1991; Verniere *et al.*, 1993).

According to Wünsche and Babel, (1995) the differentiation and grouping of natural communities by the number of utilized substrates seem to be possible using incubation periods of 48 h or more without taking into account the inoculum density. Kersters and colleagues, (1997) indicated that inoculum-dependent differences among the Biolog patterns for both pure cultures and heterotrophic microbial communities persist even after extended incubation periods. The reliable use of the Biolog system requires samples of approximately equivalent inoculum densities (Garland and Mills, 1991; Haack, 1994; Kersters *et al.*, 1997).

Hitzl and colleagues, (1997) proposed that the number of observed substrates was reduced from 95 to 31 or less, but at the same time measured more replicates of each substrate. A smaller number of discriminating substrates with an appropriate number of substrates (as necessary for a good separation) would potentially detect small differences among the substrate utilization patterns. According to Zak and colleagues, (1994) the appropriate observation period was determined by the rate at which colour develops, the time at which most of the substrates are used, a colour change occurs in the control well or by fungi growing in a well from the original inoculum.

Inoculum cell density:

Colour development in Biolog plates could be influenced by the inoculum cell density (Garland and Mills, 1991; Haack *et al.*, 1994; Garland, 1996(a); Garland, 1996(b)). The response in the wells is dependent on the growth of the organisms. The diversity of response is a function of both the richness and the evenness of response. A minimum number of metabolically active cells are required to produce an observable colour change (about 10^8 cfu. ml^{-1}) based on results from Haack *et al.*,1994. Colour development in each well reflected species activity and density, as well as the ability of the bacterial community to respond to particular substrates.

Garland (1997), states that differences in the overall rate of colour development (i.e. inoculum density) would produce variations in the diversity or pattern of colour development independent of any change in the types of organisms present. Garland and Mills (1991) as well as Haack and colleagues (1994) concluded that bacterial cell density in the inoculum affected the length of the incubation time (lag period) prior to colour development in the wells. If diluted inocula are used, colour will not develop until there were about 10^8 cfu.ml^{-1} in a well (Garland and Mills, 1991). If the inoculum contains $< 10^8$ cfu.ml^{-1}, colour will be a sigmoidal function of time (Van Elsas *et al.*, 1998).

For most environmental samples, the cell density in the original inoculum will be far below 10^8 cfu.ml^{-1} (Van Elsas *et al.*, 1998). Aquatic samples contain about 10^5 - 10^6 cfu.ml^{-1} (Van Elsas *et al.*, 1998). Soil suspensions prepared at 1:10 (w/w) dilution in water contain 10^5 - 10^7 cfu.ml^{-1} and up to 10^9 cfu.ml^{-1} (Van Elsas *et al.*, 1998). Zak and colleagues (1994), used dilutions of 10^{-4}, which meant that their inocula probably contained 10^2 - 10^4 cfu.ml^{-1}.

Inoculum densities should be kept as high as possible ($> 10^5$ cfu.ml^{-1}) to ensure that the communities inoculated into the individual wells are the same (Van Elsas, 1998). If the total cell density is 10^4 ml^{-1}, a species that comprises 1% of the population will have 15 individuals (on average) per 150 ml aliquot (Van Elsas *et al.*, 1998). If the cells are randomly distributed in the suspension, the probability that a well receives no individuals of the species is $< 10^6$ (Van Elsas *et al.*, 1998).

Insam and colleagues (1996), determined the cell density microscopically after staining with aniline blue and set the inoculum density at 10^5 cfu.ml^{-1}. While Verschuere and colleagues (1997), used microbial ATP-measurements to quantify the amount of active microbial biomass. Guckert and colleagues (1996), used the optical density of the supernatant of an activated sludge sample. Colour development may be dominated by those species that are either present in relatively high numbers initially or have high maximum growth rates. This was based on microscopic examination of coloured wells and on the observed correlation of AWCD with inoculum density rather than activity. The number of stained cells at a given incubation is a function of the number of cells in the original inoculum able to use the substrate as a sole carbon source and the growth of these cells during incubation. The immediate development of colour in certain response wells suggests that the inoculum contains a large fraction of micro-organisms able to utilize the substrate (Kersters *et al.*, 1997). The Biolog substrate oxidation responses and AWCDs often exhibited a lag phase, an exponential phase and a stationary phase (Kersters *et al.*, 1997). The lag phase in colour development in other wells indicates that a longer period of growth was necessary to produce a sufficient density of stained cells, reflecting a smaller percentage of micro-organisms in the inoculum able to utilize the substrate as a sole carbon source (Kersters *et al.*, 1997).

A sample with a high species diversity would produce a different pattern of colour response data than a sample of identical composition but lower species diversity for

similar incubation times (Kersters *et al.*, 1997). Several methods may be used to overcome this influence of inoculum density on colour response data: i) dilution or concentration of samples to achieve equivalent inoculum densities, ii) use of multiple readings over a time course of incubation, or transformation of the data to account for different inoculum densities (Kersters *et al.*, 1997).

Absorbency within Biolog microplate wells:

The absorbance value of the control well was subtracted from the well absorption, yielding a single difference value (SDV) and the average well colour development (AWCD) for the plates were then calculated by summing all these SDV values and dividing by 95 (Garland and Mills, 1991; Kersters *et al.*, 1997). This approach is effective at reducing effects of different rates of overall colour formation when variation in AWCD is 30 - 50% (Garland, 1996(a); Garland, 1996(b)). All well reactions were then normalized to the AWCD for each microplate to account for different inoculum densities (Garland, 1996(a); Garland, (b)). Hitzl and colleagues (1997), used the minimum value of all wells instead of the water (control) well as reference control to avoid negative values while calculating the AWCD.

Limiting the effect of differences in the colour development can be reached by semi-continuous monitoring of colour development in plates so that samples of equivalent AWCD (Garland, 1997). This approach allows for effects caused by differences in inoculum density and effects due to different types of activities of organisms to be separately quantified (Garland, 1997).

The interval between readings would depend on the rate of colour development in the microplates, which is a function of inoculum density and incubation temperature (Garland, 1997). According to Garland (1997) samples of moderate density (i.e. 10^5 - 10^6 cfu.ml^{-1}) incubated at room temperature may need an automated plate reader

with a 2 h interval, while for samples of lower density incubated at cooler temperatures daily readings may be sufficient. The specific point in AWCD used for analysis did not appear to influence classification of samples, but did affect which carbon sources were responsible for the differences between the samples (Garland, 1997). The point in AWCD is unimportant for purposes of classification, but it is important if a complete idea of differences in carbon source utilization was desired (Garland, 1997). Comparison of the integral of the colour development in each well after an extended incubation period may be more effective than comparison of absorbance values at a single setpoint in AWCD. The integral could capture differences in colour production that occurred both early and late in the incubation period (Guckert, 1997).

Kersters and colleagues (1997), reported that the reproducibility of the control well colour development (CWCD) was poor for heterotrophic microbial communities. The CWCD was included in formulas for the assignment of positive or negative responses for the 95 substrates of a microplate and for calculating the average well colour development (AWCD) (Garland and Mills, 1991; Haack *et al.*, 1994; Wünsche and Babel, 1995; Garland, 1996(a); Garland, 1996(b)). Kersters and colleagues (1997) indicated that if the interpretation and transformation of the 95 raw colour response data rely on the CWCD. It should be taken into account that the reproducibility of the CWCD was poor (Kersters *et al.*, 1997).

The manufacturer (Biolog Inc., Hayward, USA) suggested that colour in the substance blank well could be due to cell lysis or utilization of endogenous or extracellular polymers. Kersters and colleagues (1997) as well as Garland and Mills (1991) stated that colour development in the control well appeared to be the result of carbon present in the inoculum since dilution of environmental samples before inoculation reduced colour production in the control well.

The profiles of the communities in the wells of Biolog GN plates with each inoculum had several prominent bands, suggesting that more than one population contributed to the carbon source oxidation (Smalla *et al.*, 1998). There were differences among the profiles of the various wells analyzed, since the relative proportions of the numerically dominant populations in activated sludge were altered during incubation in the wells of the Biolog plates (Smalla *et al.*, 1998). Whether a bacterial population becomes numerically dominant is dependent on numerous factors, including the ability of the organisms to oxidize a carbon source at the concentration provided and their competitiveness under the cultivation conditions used (Smalla *et al.*, 1998). Various factors determine the competitiveness of the populations, including their nutritional requirements and their generation times under the prevailing conditions, as well as antagonistic and synergistic interactions among the populations (Smalla *et al.*, 1998).

Bacterial populations becoming dominant in various wells of the Biolog microplates, are versatile with respect to the ability to use different carbon sources and were competitive under the conditions of the assay (Smalla *et al.*, 1998). Biolog microplate patterns generated by activated sludge communities may resemble the density of the inoculum and the catabolic potential of the numerically dominant bacteria in activated sludge (Smalla *et al.*, 1998). The carbon source utilization patterns of Biolog microplates may be habitat specific. The pattern of a community does not necessarily reflect the functional potential of the community at the time of inoculation (Smalla *et al.*, 1998). The use of carbon source utilization profiles to characterize microbial communities remained a valuable tool for comparison of microbial communities, although it had some limitations (Smalla *et al.*, 1998).

Metabolic patterns within Biolog microplates:

In this regard the following should be considered:

i) 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

ii) Dominance would be indicated by the same number of carbon sources utilized upon dilution of the sample

iii) The number of the organisms would change and not its carbon source utilization profile, as long as the inoculum density and incubation time were sufficient

iv) Equitability would be indicated by the same number of carbon sources utilized upon dilution of the sample, because the organisms were diluted at the same ratio, as long as the inoculum density and incubation time were sufficient

v) 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 species would be diluted out and their contribution to carbon source utilization would disappear (Smalla *et al.*, 1998).

The theoretical model for functional diversity using Biolog:

By inoculating Biolog microplates with microbial samples, carbon source utilization profiles (Fig.1) are obtained.

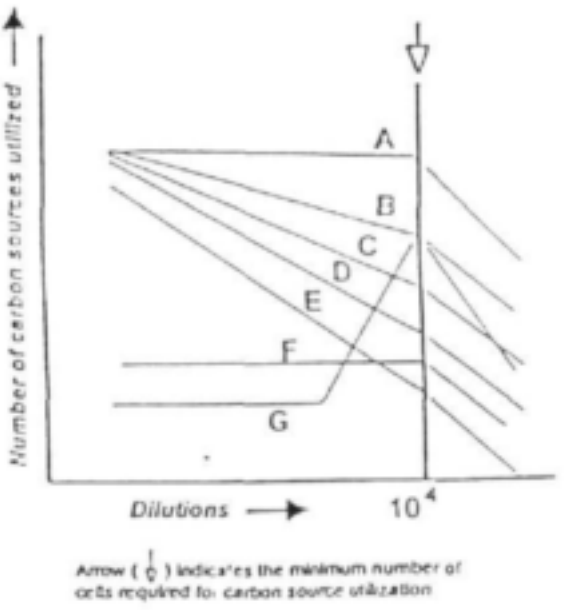


Figure 1: **Predicted carbon source utilization patterns of diluted bacterial samples using the Biolog system (Included from Korf, 1998).**

In Fig. 1, the theoretically pattern "A" indicates evenness, because the contribution of each species in the community remains the same upon dilution, because all the species present are diluted in the same ratio. However, theoretically pattern "A" may also indicate dominance. Absolute evenness is however highly unlikely to occur in natural microbial communities, therefore, pattern "A", would rather be indicative of dominance and not evenness.

In Fig. 1 theoretically pattern "B" would indicate an uneven distribution of species according to the above hypothesis. Accordingly a decline in evenness is represented

in the following order "A" > "B" > "C" > "D" > "E". Hence, the larger the gradient of the lines, the more uneven the distribution of the species. Pattern "F" reflects dominance but with a low percentage of carbon source utilization. Pattern "F", however, does not reflect a high species diversity and evenness, due to the low number of carbon sources utilized in contrast to pattern "A" which is a straight line.

Pattern "G" indicates a microbial community where the dominant organism is suppressed by non-fermentative organisms in that community. When the competitive organisms have been diluted out, the dominant organism would start to utilize carbon sources and this would lead to the increase in the number of carbon sources utilized upon dilution as indicated by pattern "G" (Korf, 1998).

Biolog summary:

There is a need for techniques that do not necessarily identify individual species, but with a reasonable chance of detecting patterns, which could be related to diversity and evenness. The Biolog system unlike traditional culture - dependent methods, which are generally selective for the component of the community that has to be cultured, can reflect the activities of a broad range of bacteria (Zak *et al.*, 1994). The Biolog system is therefore not considered as a culture -dependent method in this study, but rather as a collection of metabolic tests (database) used for the purpose of generating a recognizable pattern for a specific community.

In this study, inoculating Biolog GN-, GP- and ECO (ecological) - microplates, with different dilutions of microbial communities were determined. The resulting carbon source profiles were compared to indicate diversity and evenness in activated sludge systems.

2.7 Summary:

It was suggested by Bosch, (1992) that total biomass rather than individual species or specific populations may be important in terms of phosphate removal. Momba, (1995) indicated that a cell has a limit to the amount of phosphate that can be accumulated per cell irrespective of nutrient availability. Enzyme activities are influenced by the biomass concentration and inactivation rates for different enzymes are different within different zones of activated sludge systems (Goel *et al.*, 1998).

Species diversity could be expressed as species richness and evenness, which is an index expressing the ratio between the number of species and the number of individuals in a community (Atlas, 1984). Stability in a community is dependent on a high species diversity to overcome stress situations to shift back towards their original state of equilibrium (Atlas, 1984). Diversity and dynamics of microbial communities in activated sludge have mostly been analyzed by culture-dependent methods, which can exclude the majority of endogenous microbes due to the selective nature of the media (Wagner *et al.*, 1993).

Molecular and biochemical techniques are available for identification and phylogenetic characterization of micro-organisms without cultivation. These techniques are time-consuming, complex and difficult to interpret and therefore new methods are needed (Schwieger and Tebbe, 1998). Methods that are easy to use and detect different patterns which can be related to diversity and function, i.e. carbon utilization patterns are currently examined in this and other studies.

Community-level carbon source utilization patterns (i.e. Biolog), have recently been introduced for classifying microbial communities on the basis of heterotrophic metabolism (Garland and Mills, 1991). An "even" community would have a greater index of diversity than one in which the community was dominated by one or a few

species in which the diversity would be less (Pielou, 1975). Such a classification system might allow microbial ecologists to compare microbial communities from different environments without isolation and identification of community members (Haack *et al.*, 1994). By this study the latter would be investigated and partially explained for activated sludge systems.

CHAPTER 3

MATERIALS AND METHODS

Biolog plates. Biolog GN, GP and ECO microplates (Biolog Inc., Hayward, CA) were used in this study.

Bacterial strains. Studies were performed using the following pure cultures: *Serratia marcescens* (DSM 30121), *Staphylococcus aureus* (ATCC 196E), *Pasteurella multocida* (LMG 2851), *Alcaligenes eutrophus* (DSM 531), *Acinetobacter calcoaceticus* (DSM 588), *Rhodococcus equi* (LMG 5358) and *Micrococcus luteus* (DSM 20030). The LMG culture (*Pasteurella multocida)* was obtained from the LMG culture collection (Gent, Belgium) and DSM cultures (*Serratia marcescens*, *Alcaligenes eutrophus*, *Acinetobacter calcoaceticus*, *Micrococcus luteus*) from Germany and the ATCC culture (*Staphylococcus aureus*) from the USA. The percentage carbon sources utilized by the cultures when reaching the recommended cell density (10^8 cell.ml^{-1}) (Biolog Inc., Hayward, CA.) is summarized in Table 1. Profiles of diversity, evenness (ME) and unevenness (MU) were obtained by inoculating GN Biolog plates with different mixtures of pure cultures (Table 2 and 3).

Table 1: Percentage of carbon sources utilized in Biolog GN microplates by 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

Table 2: Mixtures of pure cultures used to determine the effect of evenness

Mixtures of pure cultures*	*S. aureus* (cfu.ml^{-1})	*P. multocida* (cfu.ml^{-1})	*A. eutrophus* (cfu.ml^{-1})	*A. calcoaceticus* (cfu.ml^{-1})	*M. luteus* (cfu.ml^{-1})	*R. equi* (cfu.ml^{-1})
ME^{0}	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}

* Mixtures of pure cultures used as inoculum for GN Biolog microwell plates.

Table 3: Mixtures of pure cultures used to determine the effect of unevenly distributed micro-organisms

Mixtures of pure cultures*	*S. aureus* (cfu.ml^{-1})	*P. multocida* (cfu.ml^{-1})	*A. eutrophus* (cfu.ml^{-1})	*A. calcoaceticus* (cfu.ml^{-1})	*M. luteus* (cfu.ml^{-1})	*R. equi* (cfu.ml^{-1})
MU^{0}	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^{-2}	10^{2}	10^{1}	10^{2}	10^{3}	10^{1}	10^{2}

* Mixtures of pure cultures used as inoculum for GN Biolog microwell plates.

Plate counts. Viable counts were determined by the spread plate procedure. Samples were serially diluted in 0.85% NaCl and 100 ml 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 24 h (*S.marcescens, P.multocida, A.eutrophus, A. calcoaceticus*) and 48 h (*S.aureus,*

R.equi and *M.luteus*).

Sample sources and inoculum preparation. Mixed liquor grab samples (1 l) were drawn from the aerobic, anoxic and anaerobic zones of the following activated sludge plants: Heidelberg (3 stage), Vlakplaats (3 stage), Tsakane (3 stage), Hartebeesfontein (2 stage) and aerobic zone samples were drawn from Daspoort (3 stage), Centurion (3 stage), Baviaanspoort (3 stage), Rooiwal (3 stage) and Zeekoegat Gauteng, South Africa, East Rand Water Care Company (ERWAT). Hot water spring samples were collected from Warmbad (South Africa). All samples were collected in sterile screw-capped plastic bottles and samples were analyzed within 12 h after collection. Chemical analysis for the anaerobic, anoxic and aerobic zones of Heidelberg, Vlakplaats, Tsakane and Hartebeesfontein systems were obtained from ERWAT (Table 11).

Inoculation and incubation of Biolog microplates. Pure cultures inoculum for microplates were obtained by growing pure cultures on Nutrient agar and *P.multocida* on BHI agar. *S. marcescens, P. multocida, A. eutrophus* and *A. calcoaceticus* were collected after 24 h and *S. aureus, R. equi* and *M. luteus* after 48 h growth. Pure cultures were suspended in sterile saline (0.85% NaCl). Biolog GN microplate wells were inoculated with 150 μl of the cell suspensions (Engelen *et al.*, 1998). Microplates were incubated aerobic in the dark at 28°C without agitation. Colour formation in the individual cells of the microtiter plates was measured at 620 nm with an Anthos reader 2001 (Anthos Labtec Instruments). Readings of the microplates were made in duplicate after 24 h, 48 h and 72 h of incubation. Readings were also made at 590 nm to compare it with readings at 620 nm and plates were also visually studied. According to our results better absorbency readings were obtained at 620 nm.

The hot water spring samples were pipetted directly into the Biolog plates. The microbial suspension was diluted with sterile saline and used as inocula for Biolog GN microwell plates. Biolog GN microplate wells were inoculated with 150 μl of the cell suspensions (Kersters *et al.*, 1997). Microplates were incubated aerobically in the dark at 37°C (hot water springs) without agitation.

Biolog GN and GP microplates were inoculated in duplicate with undiluted and diluted activated sludge samples from Heidelberg, Vlakplaats, Tsakane and Hartebeesfontein waste water treatment plant (Gauteng, South Africa). Biolog GN and ECO plates were inoculated in duplicate with undiluted and diluted activated sludge from Baviaanspoort, Daspoort, Centurion, Rooiwal and Zeekoegat waste water treatment plants (Gauteng, South Africa). All the microbial activated sludge suspensions were diluted with sterile saline (0.85 % NaCl) and used as inoculum for Biolog GN, GP and ECO microwell plates. Biolog GN, GP and ECO microplate wells were inoculated with 150 μl of the activated sludge suspensions (Zak *et al.*, 1994). Microplates were incubated aerobically in the dark at 21°C without agitation. Colour formation in the individual cells of the microtiter plates was measured with an Anthos reader 2001 (Anthos Labtec Instruments) at 620 nm. Readings of the microplates were made in duplicate after 24 h, 48 h and 72 h of incubation and microwells were also visually studied.

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.

Data handling. Carbon source utilization patterns were obtained by determining the percentage carbon sources utilized after a 72 h incubation period. The number of carbon sources utilized was divided by 95 and expressed as a percentage value, which represented carbon source profiles/utilization. Data from each well (within

the Biolog GN and GP microplates) and data from each of the 25 common substrates (Table 4) in the experiment using both *GN* and *ECO* plates were directly stored on a computer in the Bionum version 1.1 for Windows (Applied Maths, Kortrijk, Belgium) using a distance-based approach to calculate the pairwise distances between all the dilutions/activated sludge systems investigated, meaning the negative ("0 ") and positive ("1") of all bands for a particular dilution/activated sludge system were used to calculate the similarity of the dilution/activated sludge systems to one another. The Simple matching coefficient (S_{SM}) used for determining the similarity between samples (data sets) was used for construction of a distance (similarity) matrix by using the GelCompar 4.0 program (Applied Maths, Kortrijk, Belgium). The program clustered the samples using the unweighted pair group method of arithmetic averages (UPGMA).

Table 4: The twenty-five common carbon substrates within Biolog GN and ECO plates.

Polymers	
	α-cyclodextrin
	glycogen
	Tween 40
	Tween 80
Carbohydrates	
	i-erythritol
	D-galactonic acid lactone
	N-acetyl-D-glucosamine
	glucose-1-phosphate
	β-methyl-D-glucoside
	D,L-α-glycerol phosphate
	α-D-lactose
	D-mannitol
	methyl pyruvate**
Carboxylic acids	
	g-hydroxybutyric acid
	α-ketobutyric acid
	D-galacturonic acid
	D-glucosaminic acid
	itaconic acid
Amino acids	
	L-asparagine
	glycyl-L-glutamic acid
	L-phenylalanine
	L-serine
	L-threonine
Amines	
	phenyl ethylamine
	putrescine

** Methyl pyruvate within GN plates and methyl pyruvate ester within ECO plates.

CHAPTER 4

RESULTS AND DISCUSSIONS

Colour development in each well of the GN, GP and ECO Biolog microplates reflected the ability of the bacterial community to utilize that specific carbon source. According to Insam and colleagues (1996) 10^4 cfu.ml^{-1} were required as an initial inoculum density for Biolog microplate wells. Our results indicated that incubation periods of 24 h and 48 h were not sufficient for complete colour development in the Biolog microplate wells with an initial inoculum density of *ca.* 10^4 cfu.ml^{-1}. Our results indicated complete colour development after 72 h of incubation. All our results were therefore based on a 72 h incubation period. An initial inoculum density of 10^4 cfu.ml^{-1} was used in this study in compliance with the recommendations of Biolog.

In this study we set out to indicate that metabolic diversity (substrate utilization) as determined with the Biolog system could be used to learn more about species diversity and evenness in natural habitats. Mixtures of pure cultures to create artificial communities and samples from natural environments were used in this study.

The first artificial community contained only *S. marcescens* in order to simulate dominance, as one might expect in an extreme habitat. Dilution of *S.marcescens* did not result in a decline ($< 10\%$ difference) in the number of substrates utilized (Fig 2). This indicated, as postulated, that the presence of one organism (dominance) in a community would result in the same number of carbon sources utilized upon dilution, given that the initial inoculum density and incubation time were sufficient. This was furthermore substantiated by the dendrogram, which was constructed from these results (Fig. 3). A similarity of more than 98% was observed amongst these

dilutions.

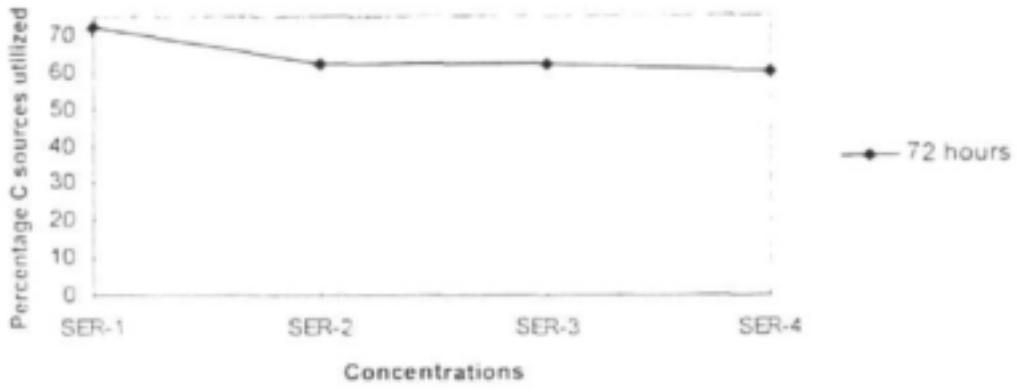


Figure 2: Percentage of carbon sources utilized by *Serratia marcescens* using concentrations SER-1, SER-2, SER-3 and SER-4, (representative of dominance)

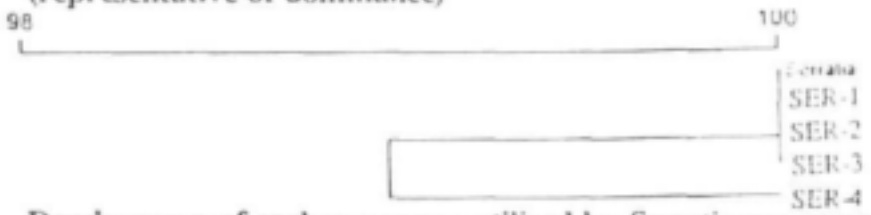


Figure 3: Dendrogram of carbon sources utilized by *Serratia marcescens* using concentrations SER-1, SER-2, SER-3 and SER-4, (representative of dominance). The relationship are determined by using the Simple matching coefficient (S_{SM}) values, which were grouped by the unweighted average pair group method, using GelCompar 4.0

The second artificial community was constructed to reflect evenly distributed species (Table 2). Upon dilution, no decline in the number of substrates utilized by the evenly distributed mixtures of pure bacterial cultures (Table 2) was observed after 72 h incubation (Fig. 4). This indicated, that "absolute" evenness would also result in the same number of substrates utilized upon dilution, given that the inoculum density ($>10^4$ cfu.ml^{-1}) and incubation time (72 h) were sufficient. This was substantiated by the dendrogram, which was constructed from these results, indicating that there was a similarity of more than 99% amongst the four dilutions (Fig. 5). However, it is unlikely that one would find this sort of evenness in nature. This pattern would rather indicate dominance than "absolute" evenness.

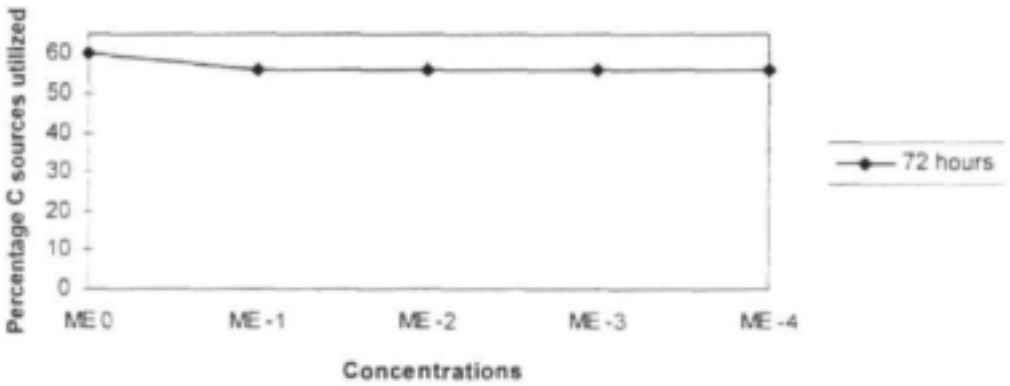


Figure 4: **Percentage of carbon sources utilized by the mixture of pure cultures (ME0, ME-1, ME-2, ME-3 and ME-4), (representative of evenness) (Table 2)**

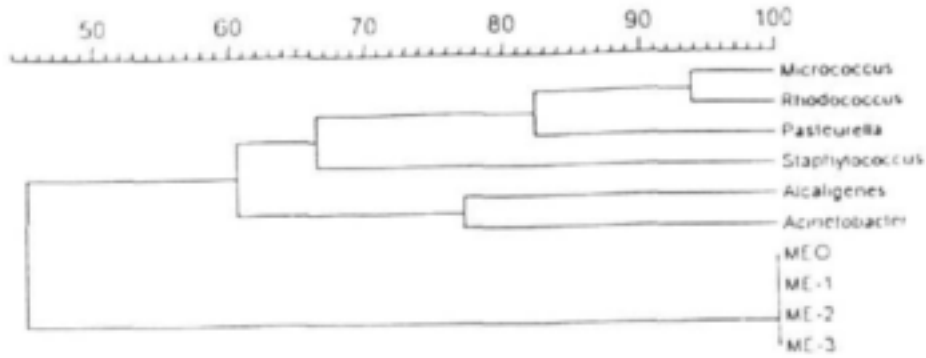


Figure 5: **Dendrogram of carbon sources utilized by the mixture of pure cultures (ME0, ME-1, ME-2, ME-3 and ME-4) (representative of evenness) (Table 2). The relationship are determined by using the Simple matching coefficient (S_{SM}) values, which were grouped by the unweighted average pair group method, using GelCompar 4.0**

The third artificial community was constructed to reflect an uneven distribution of the species in the community (Table 3). In the sample where the mixture of the organisms was representative of an uneven distribution and abundance of species (Table 3), a decline in the number of substrates utilized was observed after dilution (Fig. 6). This was expected, because some of the species were diluted out and their contribution to substrate utilization was lost. The undiluted sample (MU0) and the 10^{-1} dilution (MU-1) were 91% similar (Fig. 7). This was expected, as postulated, since a relatively large number of species were present initially in adequate quantities for colour development in the Biolog microplates (Table 3). The 10^{-2} (MU-2) dilution was 51% similar to the undiluted sample (MU0) and 10^{-1} dilution (MU-1). This reflected unevenness, as was expected, since the different species in the mixture were not represented in even numbers (Table 3). The 10^{-3} (MU-3) dilution was 77% similar to the 10^{-2} (MU-2) dilution, confirming the latter conclusion.

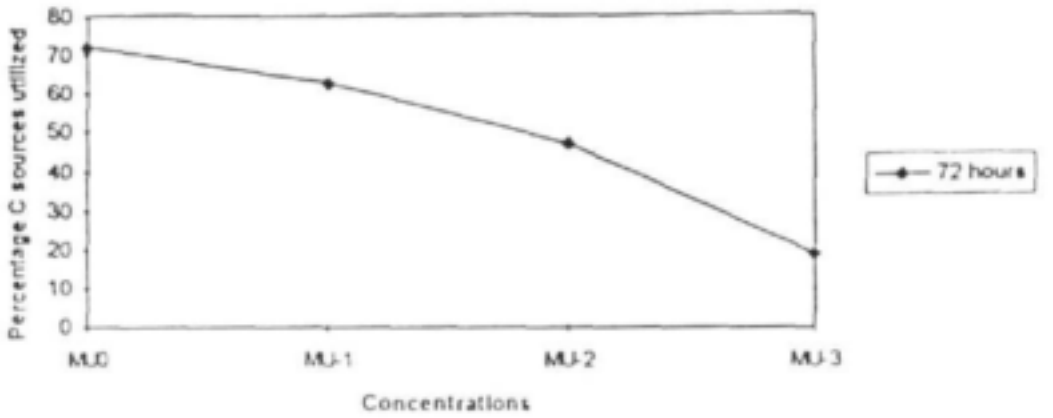


Figure 6: Percentage of carbon sources utilized by the mixture of pure cultures representative of an uneven distribution of micro-organisms (MU0, MU-1, MU-2 and MU-3) (Table 3)

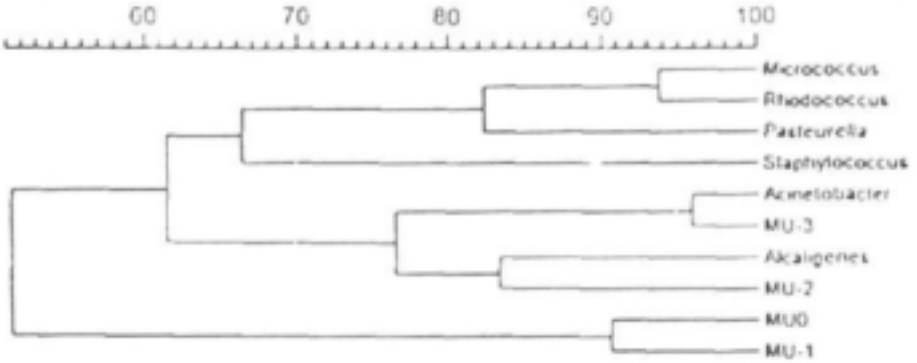


Figure 7: Dendrogram of carbon sources utilized by the mixture of pure cultures (MU0, MU-1, MU-2 and MU-3) representative of an uneven distribution of micro - organisms (Table 3). The relationship are determined by using the Simple matching coefficient (S_{SM}) values, which were grouped by the unweighted average pair group method, using GelCompar 4.0

The first natural environment investigated was a hot water spring (HWS) representative of an extreme environment. Upon dilution, the percentage substrates utilized, stayed consistent for the hot water spring samples (Fig. 8). This reflected dominance as previously suggested (Fig. 2). The hot water spring (HWS) data was represented in a dendrogram (Fig. 9) and all the dilutions were more than 98% related to one another. Due to the extreme environment, dominance was expected and indicated. This confirmed our hypothesis with regards to dominance.

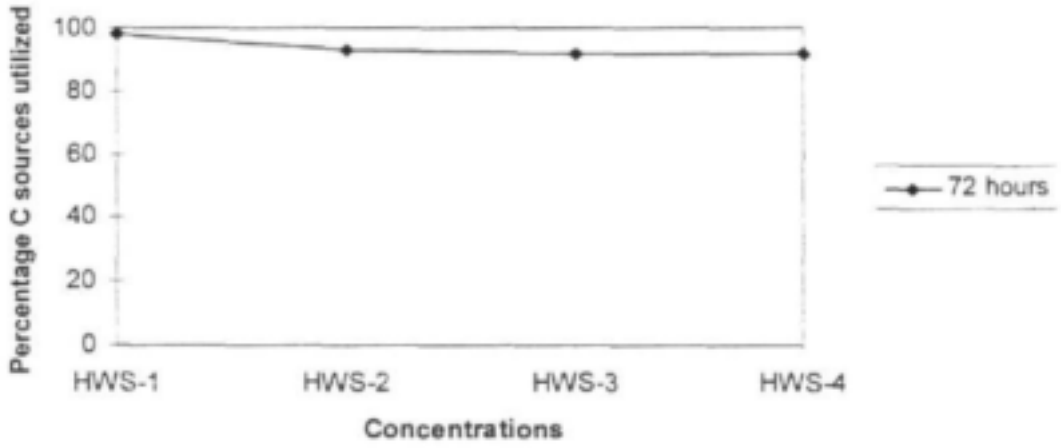


Figure 8: **Percentage carbon sources utilized in Biolog GN microplates of hot water springs (HWS) at different initial cells densities. HWS-1, HWS-2, HWS-3, HWS-4**

Figure 9: **Dendrogram of carbon sources utilized in Biolog GN microplates of hot water springs (HWS), HWS-1, HWS-2, HWS-3, HWS-4. The relationship are determined by using the Simple matching coefficient (S_{SM}) values, which were grouped by the unweighted average pair group method, using GelCompar 4.0**

The second natural environment investigated was an activated sludge system, representative of a high species diversity and uneven distribution of species. Upon dilution, the percentage of substrates utilized from the anaerobic zone of Heidelberg (GN and GP), Vlakplaats (GN and GP) and Hartebeesfontein (GN) water treatment plant, decreased upon dilution (Fig. 10). This indicated a pattern representing unevenness. Tsakane (GN and GP) and Hartebeesfontein (GP) indicated a more even distribution (10^{-1} to 10^{-3} dilutions). The high number of substrates utilized at the lower dilutions (10^{-1} and 10^{-2}) indicated a high initial microbial diversity in the community, but not necessarily evenness of each species. Evenness of each species would be reflected only upon further dilution. The steeper the gradient, the more uneven the distribution of species. It was concluded that the lower the similarity amongst dilutions, the lower the evenness and *vice versa*.

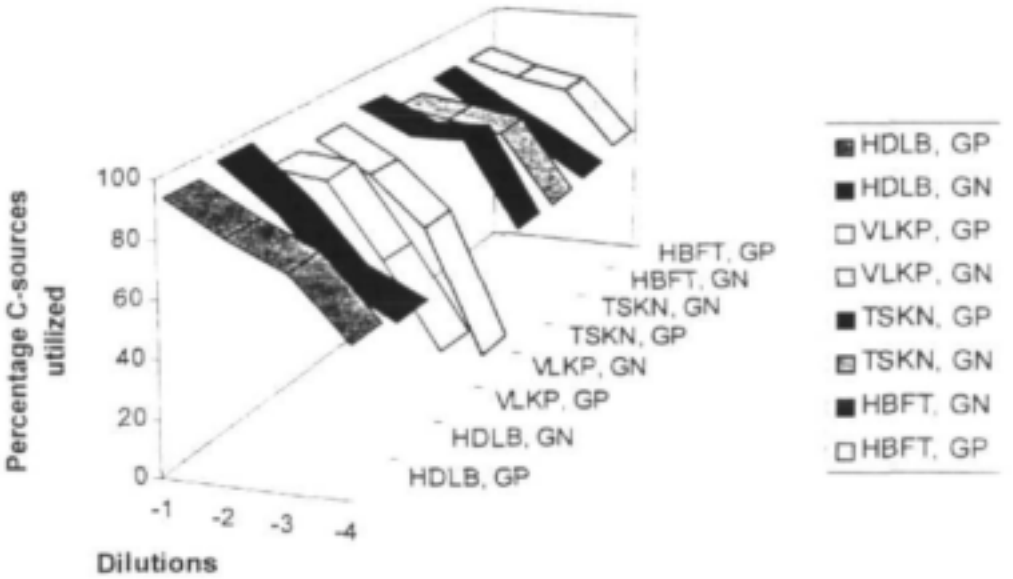


Figure 10: **Percentage C-sources utilized in Biolog GN and GP microplates after inoculation with activated sludge from the anaerobic zones at the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein (HBFT) waste water treatment plant**

Using Dendrograms to illustrate unevenness within the Heidelberg, Vlakplaats, Tsakane and Hartebeesfontein Systems:

Fig.11 gives an example of how the Biolog data for a specific sample (Vlakplaats, anaerobic zone, Biolog GN) could be more accurately interpreted, without considering the impact of oxygen on the community. The 10^{-1} and 10^{-2} dilutions were 92% similar, indicating a high initial diversity. The 10^{-3} dilution was 78%

similar to the 10^{-1} and 10^{-2} dilutions. This indicated a decrease in the species diversity and an uneven distribution of the species. Upon further dilution (10^{-4}), the similarity decreased to 33% indicating a further loss of species. This indicated a difference from the previous dilution (78% to 33%), due to a high initial diversity but low evenness of species present within this community. The lower the similarity amongst the dilutions, the more uneven the distribution of the species represented. The latter was reflected in the decrease of substrates utilized, due to a substantial loss of the contribution to substrate utilization by the species, which are lost upon dilution.

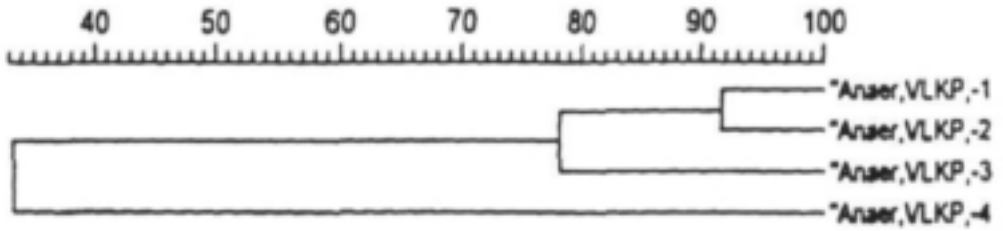


Figure 11: **Dendrogram of the Vlakplaats (VLKP) water treatment plant after inoculation of a Biolog GN microplate with activated sludge from the anaerobic (Anaer) zone, (indicating an uneven distribution of species) . The relationship are determined by using the Simple matching coefficient (S_{SM}) values, which were grouped by the unweighted average pair group method, using GelCompar 4.0**

In order to prove that the Biolog system could be used to generate an useful database without taking all possibilities into consideration, the Biolog GN and GP systems were compared

Heidelberg:

Anaerobic:

Two groups were formed one containing the 10^{-1} to 10^{-3} dilutions of the Biolog GP microplates 83%, indicating a high similarity between these dilutions. The other group contained the 10^{-1} and 10^{-2} dilutions of the Biolog GN microplates, with an 87% similarity. These two groups were 72% similar.

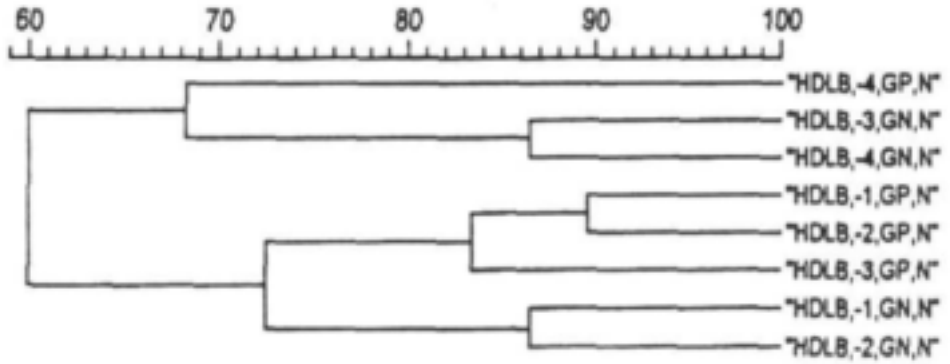


Figure 12: **Dendrogram of the Heidelberg (HDLB) water treatment plant after inoculation of a Biolog GN and GP microplate with activated sludge from the anaerobic (Anaer) zone, (indicating that GN and GP plates cannot be compared to each other). The relationship are determined by using the Simple matching coefficient (S_{SM}) values, which were grouped by the unweighted average pair group method, using GelCompar 4.0**

Vlakplaats:

Anaerobic:

The 10^{-1} and 10^{-2} dilutions from the Biolog GN microplates were 92% similar. The Biolog GP microplates 10^{-1} and 10^{-2} dilutions were 95% similar. This indicated a high initial diversity. The GN and GP Biolog microplates formed separate groups, being 68% similar. The 10^{-4} dilutions of both GN and GP microplates were 71% similar, indicating a difference in the community remaining within this dilution. This should not be the case and therefore GN and GP microplate data should not be compared.

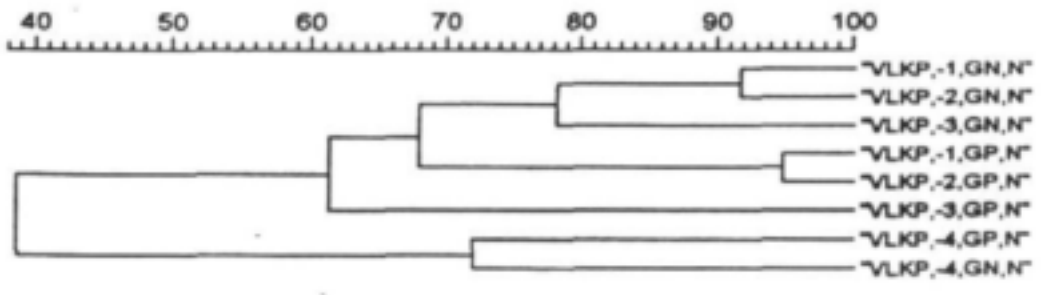


Figure 13: **Dendrogram of the Vlakplaats (VLKP) water treatment plant after inoculation of a Biolog GN and GP microplate with activated sludge from the anaerobic (Anaer) zone, (indicating that GN and GP plates cannot be compared to each other). The relationship are determined by using the Simple matching coefficient (S_{SM}) values, which were grouped by the unweighted average pair group method, using GelCompar 4.0**

Tsakane:

Anaerobic:

Two groups were formed, one contained the 10^{-1} to 10^{-3} dilutions of the Biolog GN

microplates, being 83% similar. The other group contained the 10^{-1} to 10^{-3} dilutions of the same sample within Biolog GP microplates, with a 91% similarity. The two groups were 68% similar. Indicating a difference between the GN and GP data, therefore it is best not to compare the GN and GP microplate data with each other.

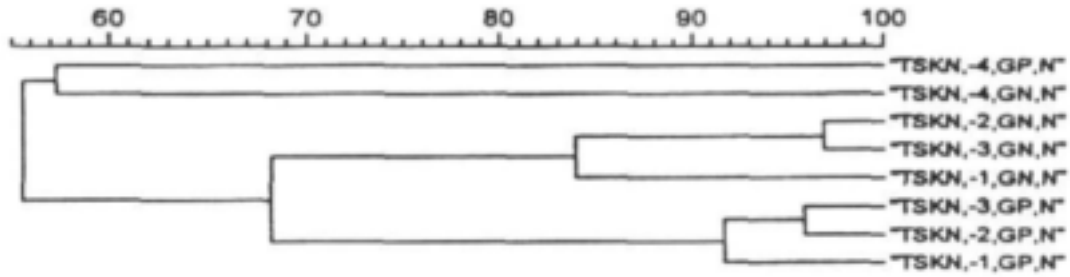


Figure 14: **Dendrogram of the Tsakane (TSKN) water treatment plant after inoculation of a Biolog GN and GP microplate with activated sludge from the anaerobic (Anaer) zone, (indicating that GN and GP plates cannot be compared to each other). The relationship are determined by using the Simple matching coefficient (S_{SM}) values, which were grouped by the unweighted average pair group method, using GelCompar 4.0**

Hartebeesfontein:

Anaerobic:

The 10^{-1} to 10^{-4} dilutions of the Biolog GN microplates were 78% similar. The 10^{-1} to 10^{-4} dilutions of the Biolog GP microplates were 73% similar. The Biolog GN and GP microplates were less than 60% similar, indicating that GN and GP microplate data should not be compared to each other.

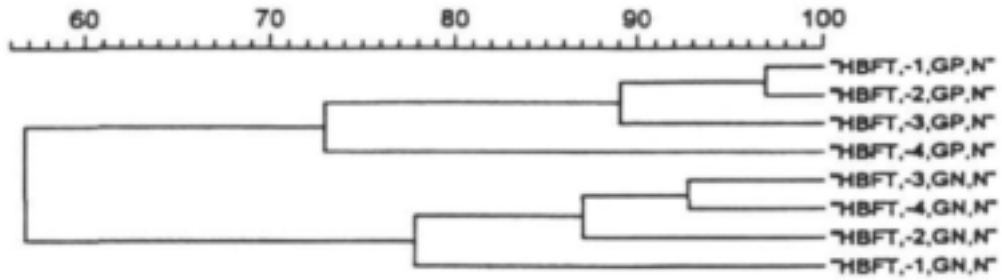


Figure 15: **Dendrogram of the Hartebeesfontein (HBFT) water treatment plant after inoculation of a Biolog GN and GP microplate with activated sludge from the anaerobic (Anaer) zone, (indicating that GN and GP plates cannot be compared to each other). The relationship are determined by using the Simple matching coefficient (S_{SM}) values, which were grouped by the unweighted average pair group method, using GelCompar 4.0**

Heidelberg:

Anoxic:

The 10^{-1} and 10^{-2} dilutions of the Biolog GN microplate data were 93% similar, indicating a high initial diversity. The 10^{-2} and 10^{-3} dilutions were 69% similar. The 10^{-3} and 10^{-4} dilutions were less than 40% similar. The 10^{-1} and 10^{-2} dilutions were more than 99% similar. The GN and GP Biolog microplates formed separate groups, being 60% similar. The 10^{-4} dilutions of both the GN and GP microplates were 98% similar.

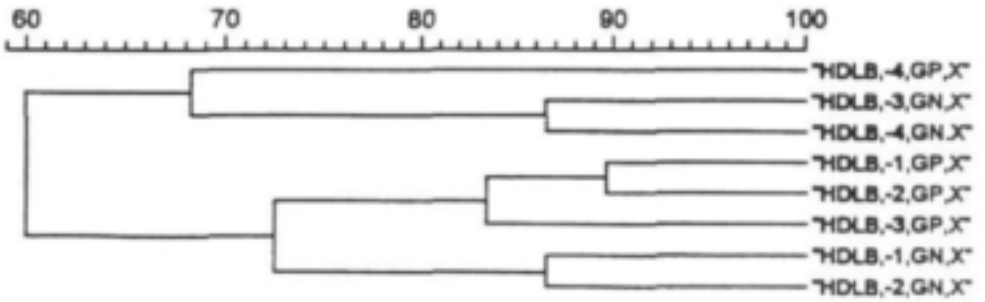


Figure 16: **Dendrogram of the Heidelberg (HDLB) water treatment plant after inoculation of a Biolog GN and GP microplate with activated sludge from the anoxic (Anox) zone, (indicating that GN and GP plates cannot be compared to each other). The relationship are determined by using the Simple matching coefficient (S_{SM}) values, which were grouped by the unweighted average pair group method, using GelCompar 4.0**

Vlakplaats:

Anoxic:

The 10^{-1} and 10^{-2} dilutions of the Biolog GN microplates were 94% similar, indicating a high initial diversity. The Biolog GP microplates 10^{-1} and 10^{-2} dilutions were 89% similar. This indicated a high initial diversity. The GN and GP Biolog microplates formed separate groups, being 75% similar. The 10^{-4} dilutions of both GN and GP microplates were 93% similar.

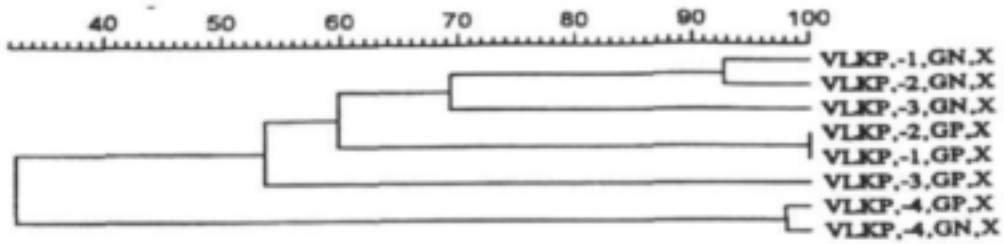


Figure 17: **Dendrogram of the Vlakplaats (VLKP) water treatment plant after inoculation of a Biolog GN and GP microplate with activated sludge from the anoxic (Anox) zone, (indicating that GN and GP plates cannot be compared to each other). The relationship are determined by using the Simple matching coefficient (S_{SM}) values, which were grouped by the unweighted average pair group method, using GelCompar 4.0**

Tsakane:

Anoxic:

The 10^{-1} dilutions of both the GN and GP microplates were 96% similar. The 10^{-2} dilutions of both the GN and GP microplates were 78% similar. Indicating a high similarity between these dilutions as expected. The 10^{-3} and 10^{-4} dilutions of both the GN and GP microplates were less than 60% similar, indicating differences between the GN and GP microplate data. This should not be the case and therefore Biolog GN and GP data should not be compared.

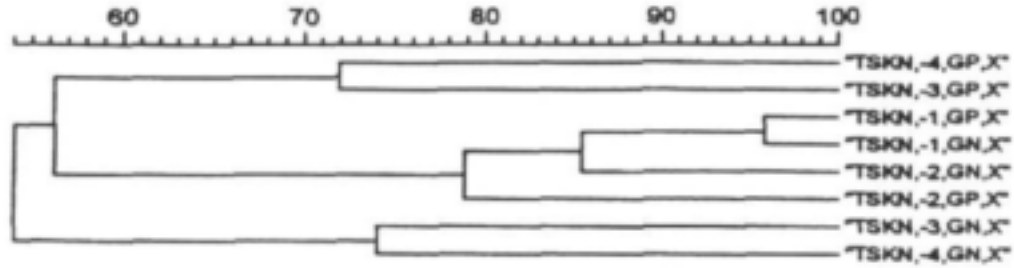


Figure 18: **Dendrogram of the Tsakane (TSKN) water treatment plant after inoculation of a Biolog GN and GP microplate with activated sludge from the anoxic (Anox) zone, (indicating that GN and GP plates cannot be compared to each other). The relationship are determined by using the Simple matching coefficient (S_{SM}) values, which were grouped by the unweighted average pair group method, using GelCompar 4.0**

Heidelberg:

Aerobic:

The 10^{-1} and 10^{-2} dilutions of the Biolog GN microplates were 88% similar, indicating a high initial diversity. The 10^{-1} dilution of the Biolog GP microplates were 78% similar to the latter group. The 10^{-4} dilutions of both GN and GP microplates were more than 99% similar, indicating a similar community remaining within the sample upon dilution.

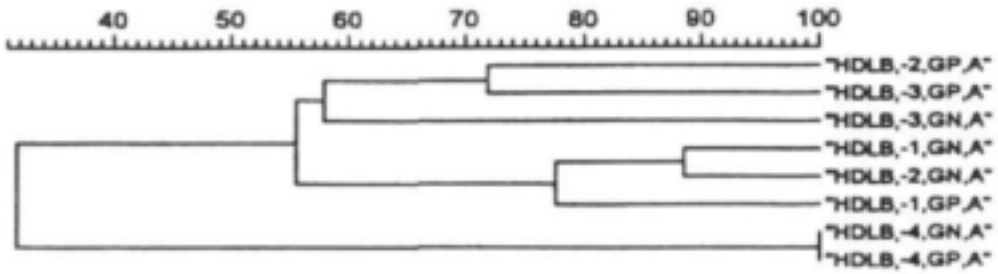


Figure 19: **Dendrogram of the Heidelberg (HDLB) water treatment plant after inoculation of a Biolog GN and GP microplate with activated sludge from the aerobic (Aer) zone, (indicating that GN and GP plates cannot be compared to each other). The relationship are determined by using the Simple matching coefficient (S_{SM}) values, which were grouped by the unweighted average pair group method, using GelCompar 4.0**

Vlakplaats:

Aerobic:

The 10^{-1} and 10^{-2} dilutions of the Biolog GN microplates were 89% similar, indicating a high initial diversity. The Biolog GP microplates 10^{-1} and 10^{-2} dilutions were 91% similar. This indicated a high initial diversity. The GN and GP Biolog microplates groups were 66% similar, indicating that Biolog GN and GP data should not be compared.

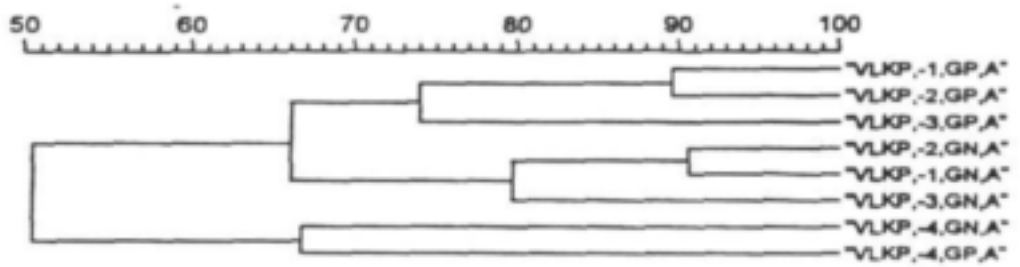


Figure 20: **Dendrogram of the Vlakplaats (VLKP) water treatment plant after inoculation of a Biolog GN and GP microplate with activated sludge from the aerobic (Aer) zone, (indicating that GN and GP plates cannot be compared to each other). The relationship are determined by using the Simple matching coefficient (S_{SM}) values, which were grouped by the unweighted average pair group method, using GelCompar 4.0**

Tsakane:

Aerobic:

The 10^{-1} and 10^{-2} dilutions of the Biolog GN microplates were 90% similar, indicating a high initial diversity. The Biolog GP microplates 10^{-1} and 10^{-2} dilutions were 89% similar. The rest of the dilutions (10^{-3} and 10^{-4}) of both GN and GP microplates were less than 63% similar, indicating that Biolog GN and GP data should not be compared.

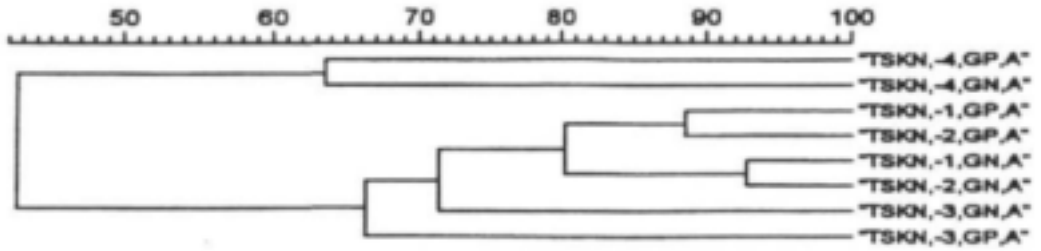


Figure 21: **Dendrogram of the Tsakane (TSKN) water treatment plant after inoculation of a Biolog GN and GP microplate with activated sludge from the aerobic (Aer) zone, (indicating that GN and GP plates cannot be compared to each other). The relationship are determined by using the Simple matching coefficient (S_{SM}) values, which were grouped by the unweighted average pair group method, using GelCompar 4.0**

Hartebeesfontein:

Aerobic:

The 10^{-1} and 10^{-2} dilutions of the Biolog GN microplates were 89% similar, indicating a high initial diversity. The Biolog GP microplates 10^{-1} and 10^{-2} dilutions were 90% similar. This indicated a high initial species diversity. These two groups were 63% similar. The rest of the dilutions (10^{-3} and 10^{-4}) of both GN and GP microplates formed separate groups, being less than 65% similar. This indicated a significant difference between the GN and GP profiles.

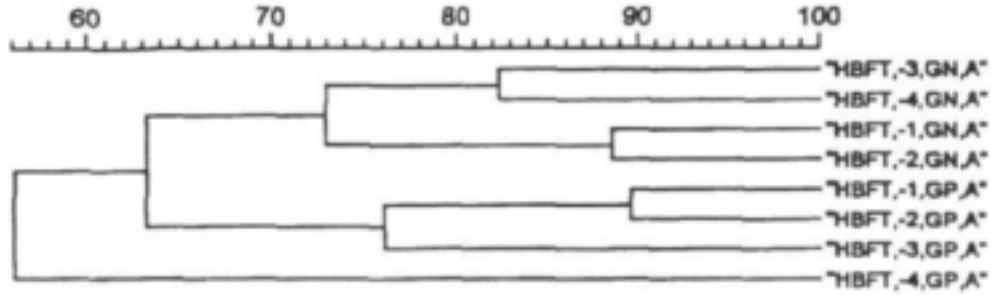


Figure 22: Dendrogram of the Hartebeesfontein (HBFT) water treatment plant after inoculation of a Biolog GN and GP microplate with activated sludge from the aerobic (Aer) zone, (indicating that GN and GP plates can not be compared to each other). The relationship are determined by using the Simple matching coefficient (S_{SM}) values, which were grouped by the unweighted average pair group method, using GelCompar 4.0

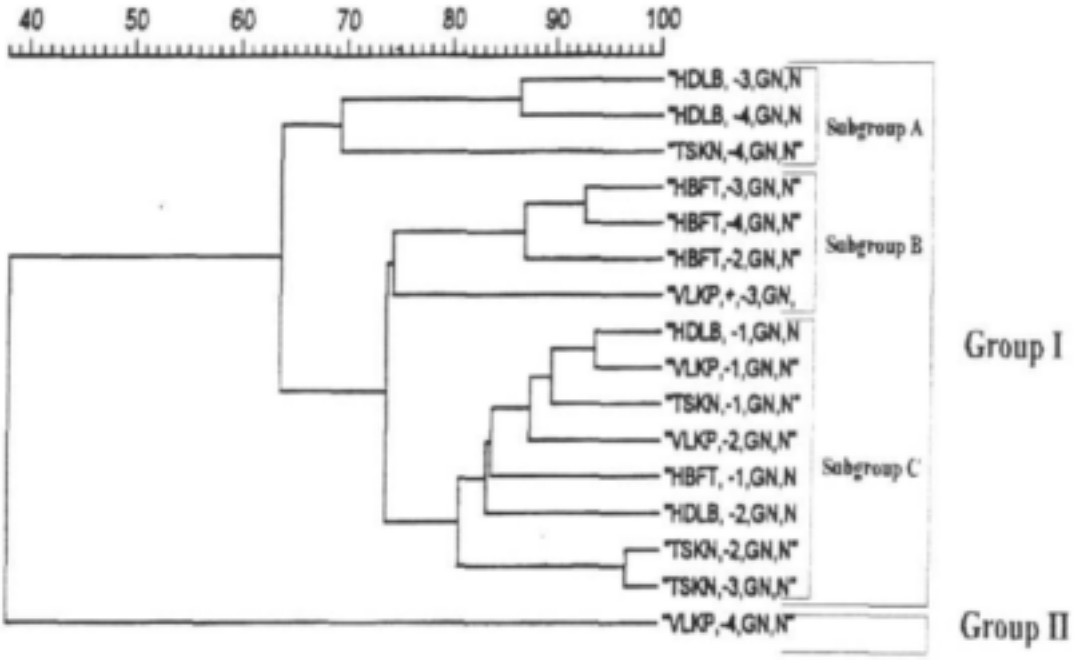


Figure 23: **Dendrogram indicating the microbial community structure of the activated sludge from the anaerobic zone (Anaer) of the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein (HBFT) wastewater works inoculated into Biolog GN microplates. The relationship are determined by using the Simple matching coefficient (S_{SM}) values, which were grouped by the unweighted average pair group method, using GelCompar *4.0***

The dendrogram in Fig. 23 can be divided into two Groups. Group I and Group II were less than 40% similar. Group I could be divided into three subgroups with a 64% similarity. Subgroup A consists of the 10^{-3} and 10^{-4} dilutions of Heidelberg, which were 86% similar and the 10^{-4} dilution of Tsakane, which were 69% similar to the rest of the subgroup. Subgroup B contained the 10^{-2} to 10^{-4} dilutions of Hartebeesfontein, which were 87% similar and the 10^{-3} dilution of Vlakplaats with a 75% similarity to the rest of the subgroup. Subgroup C contained the 10^{-1} and 10^{-2} dilutions of Heidelberg, Vlakplaats and Tsakane being 81% similar. This indicated

that there were no differences amongst these dilutions. Group II contained the 10^{-4} dilution of the Vlakplaats system.

Table 5: Comparison of similarity amongst the dilutions of the activated sludge from the anaerobic zone within the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein (HBFT) activated sludge systems inoculated into Biolog GN microplates at the time of sampling

Dilutions		HDLB	VLKP	TSKN	HBFT
10^{-1}	HDLB	100%	95%	65%	85%
	VLKP	95%	100%	65%	85%
	TSKN	65%	65%	100%	65%
	HBFT	85%	85%	65%	100%
10^{-2}	HDLB	100%	84%	81%	74%
	VLKP	84%	100%	81%	75%
	TSKN	81%	81%	100%	75%
	HBFT	74%	75%	75%	100%
10^{-3}	HDLB	100%	64%	64%	64%
	VLKP	64%	100%	74%	75%
	TSKN	64%	74%	100%	74%
	HBFT	64%	75%	74%	100%
10^{-4}	HDLB	100%	38%	64%	64%
	VLKP	38%	100%	38%	38%
	TSKN	64%	38%	100%	74%
	HBFT	64%	38%	74%	100%

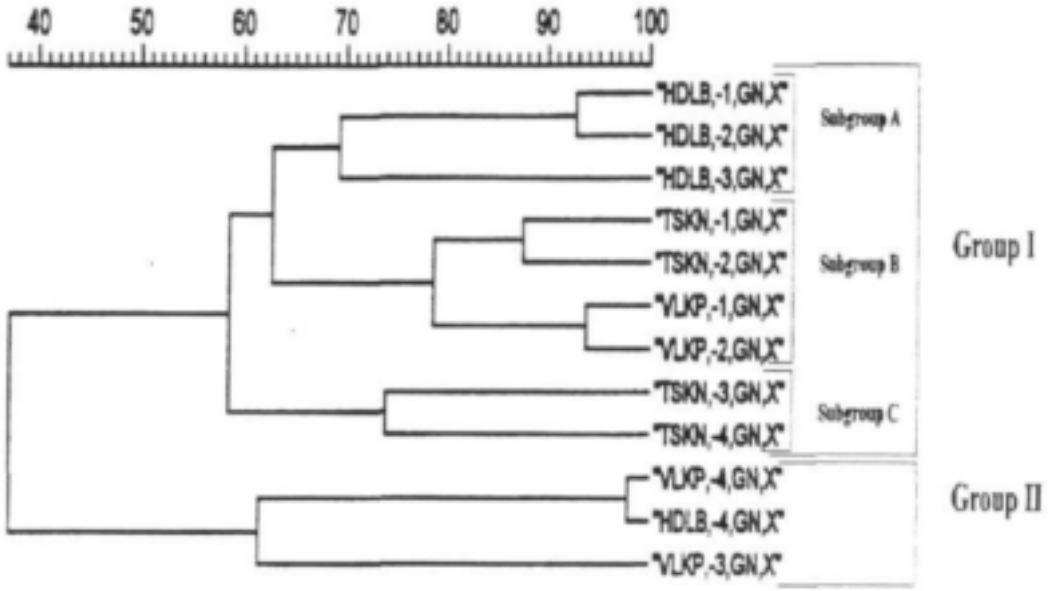


Figure 24: **Dendrogram indicating the microbial community structure of the activated sludge from the anoxic zone (Anox) of the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein (HBFT) wastewater works inoculated into Biolog GN microplates. The relationship are determined by using the Simple matching coefficient (S_{SM}) values, which were grouped by the unweighted average pair group method, using GelCompar 4.0**

The dendrogram in Fig. 24 can be divided two Groups, which were less than 40% similar. Within Group I there were three subgroups which were 59% similar. Subgroup A contained the 10^{-1} and 10^{-2} dilutions of Heidelberg with a 93% similarity and the 10^{-3} dilution of Heidelberg being 69% similar to the rest of the subgroup. Subgroup B contained the 10^{-1} and 10^{-2} dilutions of Tsakane and the 10^{-1} and 10^{-2} dilutions of Vlakplaats with a 79% similarity. Subgroup C contained the 10^{-3} and 10^{-4} dilutions of Tsakane with a 73% similarity.

Group II contained the 10^{-3} dilution of Vlakplaats (62% similar to the rest of Group) and both the 10^{-4} dilutions of the Vlakplaats (VLKP) and Heidelberg (HDLB)

activated sludge systems with a 98% similarity, which indicated that these two dilutions were closely related.

Table 6: **Comparison of similarity between the dilutions of the activated sludge from the anoxic zone within the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein activated sludge system inoculated into Biolog GN microplates at the time of sampling**

Dilutions		HDLB	VLKP	TSKN	HBFT
10^{-1}	HDLB	100%	63%	63%	NA
	VLKP	63%	100%	79%	NA
	TSKN	63%	79%	100%	NA
	HBFT	NA	NA	NA	NA
10^{-2}	HDLB	100%	63%	63%	NA
	VLKP	63%	100%	79%	NA
	TSKN	63%	79%	100%	NA
	HBFT	NA	NA	NA	NA
10^{-3}	HDLB	100%	37%	59%	NA
	VLKP	37%	100%	37%	NA
	TSKN	59%	37%	100%	NA
	HBFT	NA	NA	NA	NA
10^{-4}	HDLB	100%	98%	38%	NA
	VLKP	98%	100%	38%	NA
	TSKN	38%	38%	100%	NA
	HBFT	NA	NA	NA	NA

NA = Hartebeesfontein (HBFT) does not have an anoxic zone.

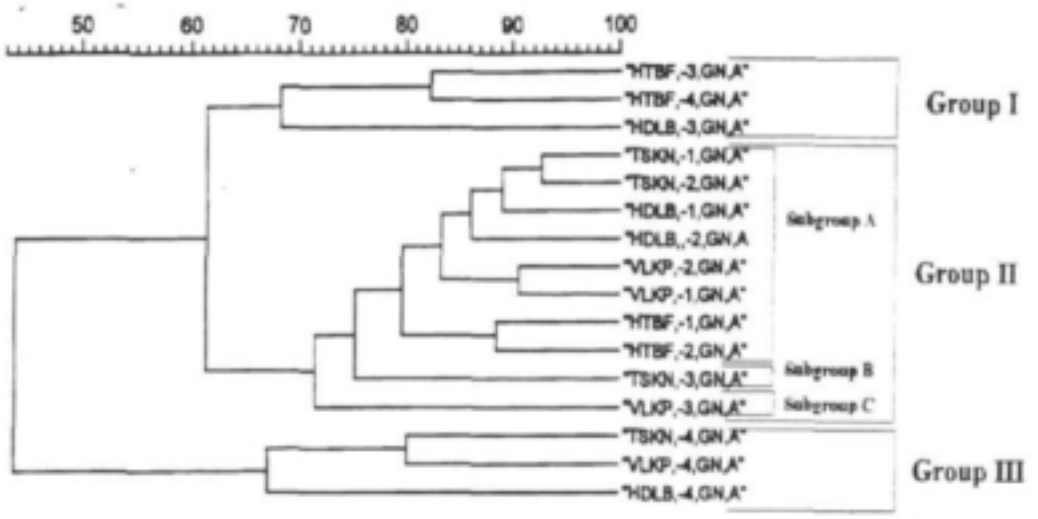


Figure 25: **Dendrogram indicating the microbial community structure of the activated sludge from the aerobic zone (Aer) of the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein (HBFT) waste water works inoculated into Biolog GN microplates. The relationship are determined by using the Simple matching coefficient (S_{SM}) values, which were grouped by the unweighted average pair group method, using GelCompar 4.0**

The dendrogram in Fig. 25, could be divided into three Groups. Groups I, II and III were less than 50% similar. Group I contained the 10^{-3} and 10^{-4} dilutions of the Hartebeesfontein system, with an 82% similarity and the 10^{-3} dilution of the Heidelberg system being 68% similar to the rest of the Group.

Group II can be divided into three subgroups: subgroup A contained all the 10^{-1} and 10^{-2} dilutions of the four activated sludge systems with a 80% similarity. Subgroup B contained the 10^{-3} dilution of Tsakane, which were 75% similar to subgroup A. Subgroup C contained the 10^{-3} dilution of Vlakplaats being 72% similar to subgroup A and B.

Group III contained the 10^{-4} dilutions of Vlakplaats and Tsakane, which were 82% similar to each other and the 10^{-4} dilution of Heidelberg, which were 67% similar to the latter.

Table 7: Comparison of similarity between the dilutions of the activated sludge from the aerobic zone within the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein activated sludge system inoculated into Biolog GN microplates at the time of sampling

Dilutions		HDLB	VLKP	TSKN	HBFT
10^{-1}	HDLB	100%	84%	89%	79%
	VLKP	84%	100%	84%	79%
	TSKN	89%	84%	100%	79%
	HBFT	79%	79%	79%	100%
10^{-2}	HDLB	100%	84%	84%	79%
	VLKP	84%	100%	83%	79%
	TSKN	84%	83%	100%	79%
	HBFT	79%	79%	79%	100%
10^{-3}	HDLB	100%	61%	61%	68%
	VLKP	61%	100%	71%	61%
	TSKN	61%	71%	100%	61%
	HBFT	68%	61%	61%	100%
10^{-4}	HDLB	100%	68%	68%	43%
	VLKP	68%	100%	80%	43%
	TSKN	68%	80%	100%	43%
	HBFT	43%	43%	43%	100%

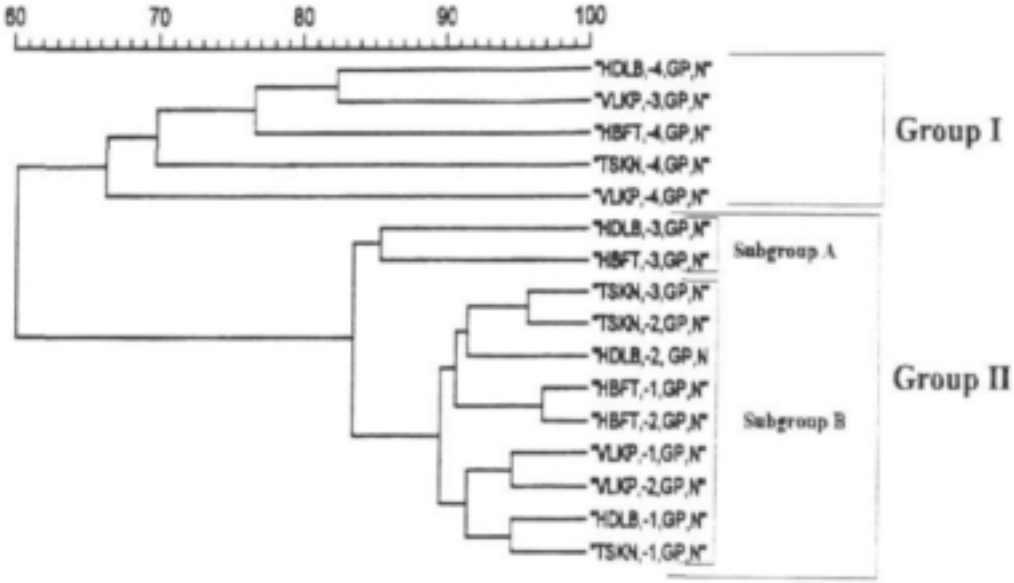


Figure 26: **Dendrogram indicating the microbial community structure of the activated sludge from the anaerobic zone (Anaer) of the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein (HBFT) waste water works inoculated into Biolog GP microplates. The relationship are determined by using the Simple matching coefficient (S_{SM}) values, which were grouped by the unweighted average pair group method, using GelCompar 4.0**

The dendrogram in Fig. 26 could be divided into two Groups. Group I and Group II were 60% similar. Group I could be divided into a subgroup containing the 10^{-4} dilution of Heidelberg and the 10^{-3} dilution of Vlakplaats being 82% similar. Group II could be divided into two subgroups that were 84% similar. Subgroup A contained the 10^{-3} dilution of Heidelberg and the 10^{-3} dilution of Hartebeesfontein being 85% similar. Subgroup B contained all the 10^{-1} and 10^{-2} dilutions of the four activated sludge systems with an 89% similarity, the 10^{-3} dilution of Tsakane was contained within this group with a 96% similarity to the 10^{-2} dilution of Tsakane.

Table 8: Comparison of similarity amongst the dilutions of the activated sludge from the anaerobic zone within the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein activated sludge system inoculated into Biolog GP microplates at the time of sampling

Dilutions		HDLB	VLKP	TSKN	HBFT
10^{-1}	HDLB	100%	92%	95%	89%
	VLKP	92%	100%	92%	89%
	TSKN	95%	92%	100%	89%
	HBFT	89%	89%	89%	100%
10^{-2}	HDLB	100%	89%	92%	91%
	VLKP	89%	100%	89%	89%
	TSKN	92%	89%	100%	91%
	HBFT	91%	89%	91%	100%
10^{-3}	HDLB	100%	60%	83%	85%
	VLKP	60%	100%	60%	60%
	TSKN	84%	60%	100%	83%
	HBFT	85%	60%	83%	100%
10^{-4}	HDLB	100%	66%	70%	77%
	VLKP	66%	100%	66%	66%
	TSKN	70%	66%	100%	70%
	HBFT	77%	66%	70%	100%

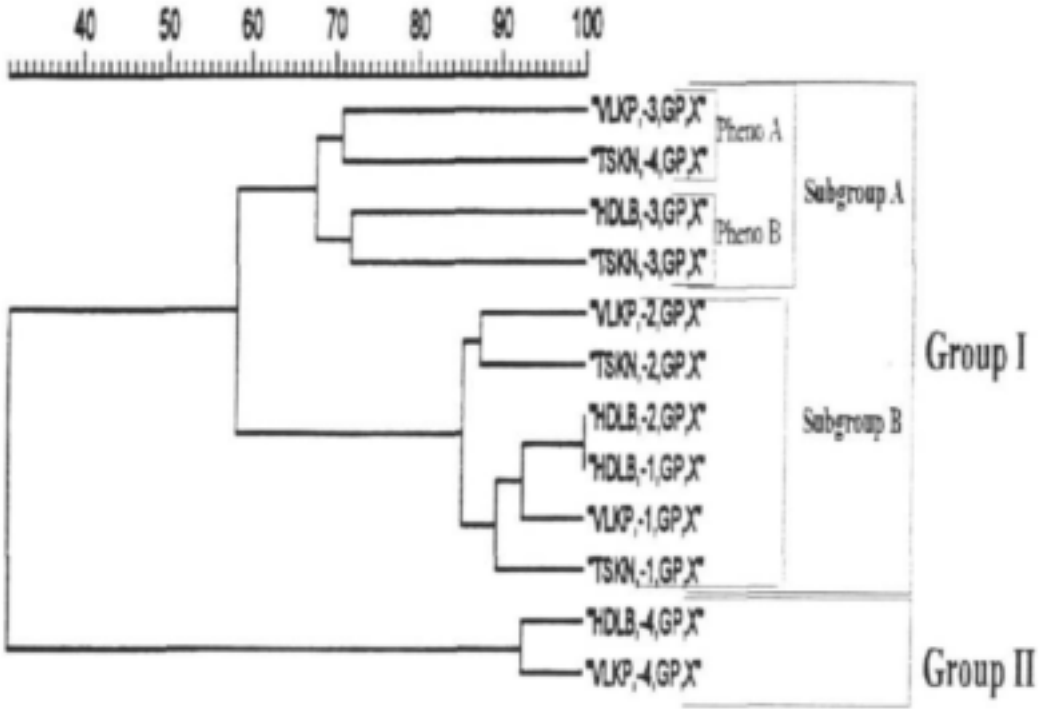


Figure 27: **Dendrogram indicating the microbial community structure of the activated sludge from the anoxic zone (Anox) of the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein (HBFT) waste water works inoculated into Biolog GP microplates. The relationship are determined by using the Simple matching coefficient (S_{SM}) values, which were grouped by the unweighted average pair group method, using GelCompar 4.0**

The dendrogram in Fig. 27 could be divided into two Groups with a 31% similarity. Group I could be divided into two subgroups with a 58% similarity. Subgroup A contained two pheno with an 68% similarity. Pheno A contained the 10^{-3} dilution of Vlakplaats and the 10^{-4} dilution of Tsakane being 71% similar. Pheno B contained the 10^{-3} dilutions of Heidelberg and Tsakane being 72% similar. Subgroup B contained all the 10^{-1} and 10^{-2} dilutions of the Heidelberg, Vlakplaats and Tsakane systems with a 86% similarity.

Group II contained the 10^{-4} dilutions of both Heidelberg and Vlakplaats with a 93% similarity.

Table 9: Comparison of similarity between the dilutions of the activated sludge from the anoxic zone within the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein activated sludge system inoculated into Biolog GP microplates at the time of sampling

Dilutions		HDLB	VLKP	TSKN	HBFT
10^{-1}	HDLB	100%	93%	89%	NA
	VLKP	93%	100%	89%	NA
	TSKN	89%	89%	100%	NA
	HBFT	NA	NA	NA	NA
10^{-2}	HDLB	100%	86%	86%	NA
	VLKP	86%	100%	88%	NA
	TSKN	86%	88%	100%	NA
	HBFT	NA	NA	NA	NA
10^{-3}	HDLB	100%	68%	72%	NA
	VLKP	68%	100%	68%	NA
	TSKN	72%	68%	100%	NA
	HBFT	NA	NA	NA	NA
10^{-4}	HDLB	100%	93%	31%	NA
	VLKP	93%	100%	31%	NA
	TSKN	31%	31%	100%	NA
	HBFT	NA	NA	NA	NA

NA = Hartebeesfontein (HBFT) does not have an anoxic zone.

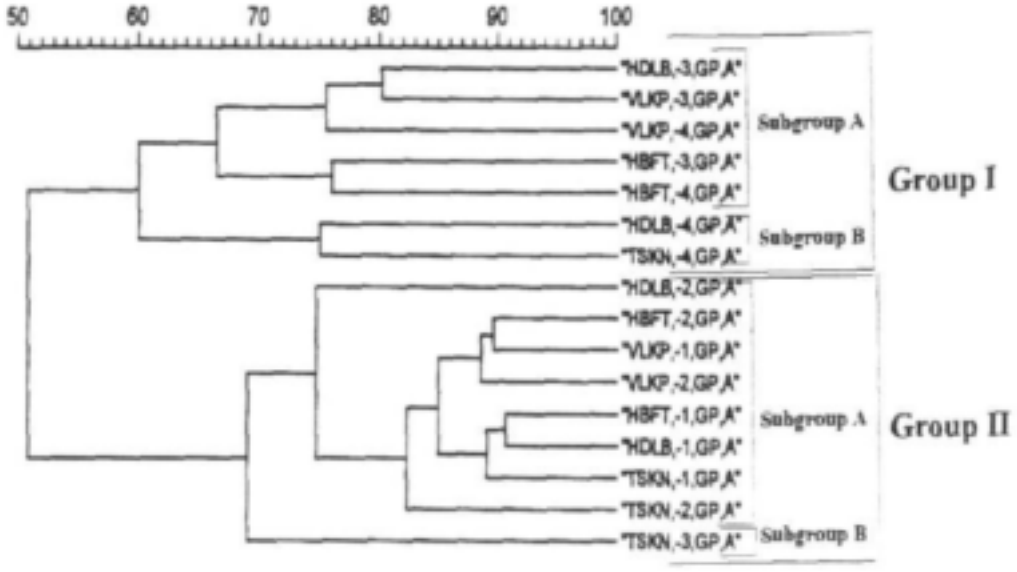


Figure 28: **Dendrogram indicating the microbial community structure of the activated sludge from the aerobic zone (Aer) of the Heidelberg (HDLB),Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein (HBFT) waste water works inoculated into Biolog GP microplates. The relationship are determined by using the Simple matching coefficient (S_{SM}) values, which were grouped by the unweighted average pair group method, using GelCompar 4.0**

The dendrogram in Fig. 28 could be divided into two Groups being 51% similar. Group I could be divided into two subgroups with a 60% similarity. Subgroup A contained the 10^{-3} dilutions of Heidelberg, Vlakplaats and Hartebeesfontein and the 10^{-4} dilutions of Vlakplaats and Hartebeesfontein with a 66% similarity. Subgroup B contained the 10^{-4} dilutions of Heidelberg and Tsakane with a 76% similarity.

Group II could be divided into two groups with a 69% similarity. Subgroup A contained all the 10^{-1} and 10^{-2} dilutions of the four activated sludge systems with a 75% similarity. Subgroup B contained the 10^{-3} dilution of Tsakane.

Table 10: **Comparison of similarity between the dilutions of the activated sludge from the aerobic zone within the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein activated sludge system inoculated into Biolog GP microplates at the time of sampling**

Dilutions		HDLB	VLKP	TSKN	HBFT
10^{-1}	HDLB	100%	85%	89%	91%
	VLKP	85%	100%	85%	85%
	TSKN	89%	85%	100%	89%
	HBFT	91%	85%	89%	100%
10^{-2}	HDLB	100%	75%	75%	75%
	VLKP	75%	100%	82%	88%
	TSKN	75%	82%	100%	82%
	HBFT	75%	88%	82%	100%
10^{-3}	HDLB	100%	80%	50%	67%
	VLKP	80%	100%	50%	67%
	TSKN	50%	50%	100%	50%
	HBFT	67%	67%	50%	100%
10^{-4}	HDLB	100%	60%	75%	60%
	VLKP	60%	100%	60%	67%
	TSKN	75%	60%	100%	60%
	HBFT	60%	67%	60%	100%

Comparison of the similarity between carbon source utilization patterns of the twenty-five common carbon substrates within Biolog GN and ECO plates

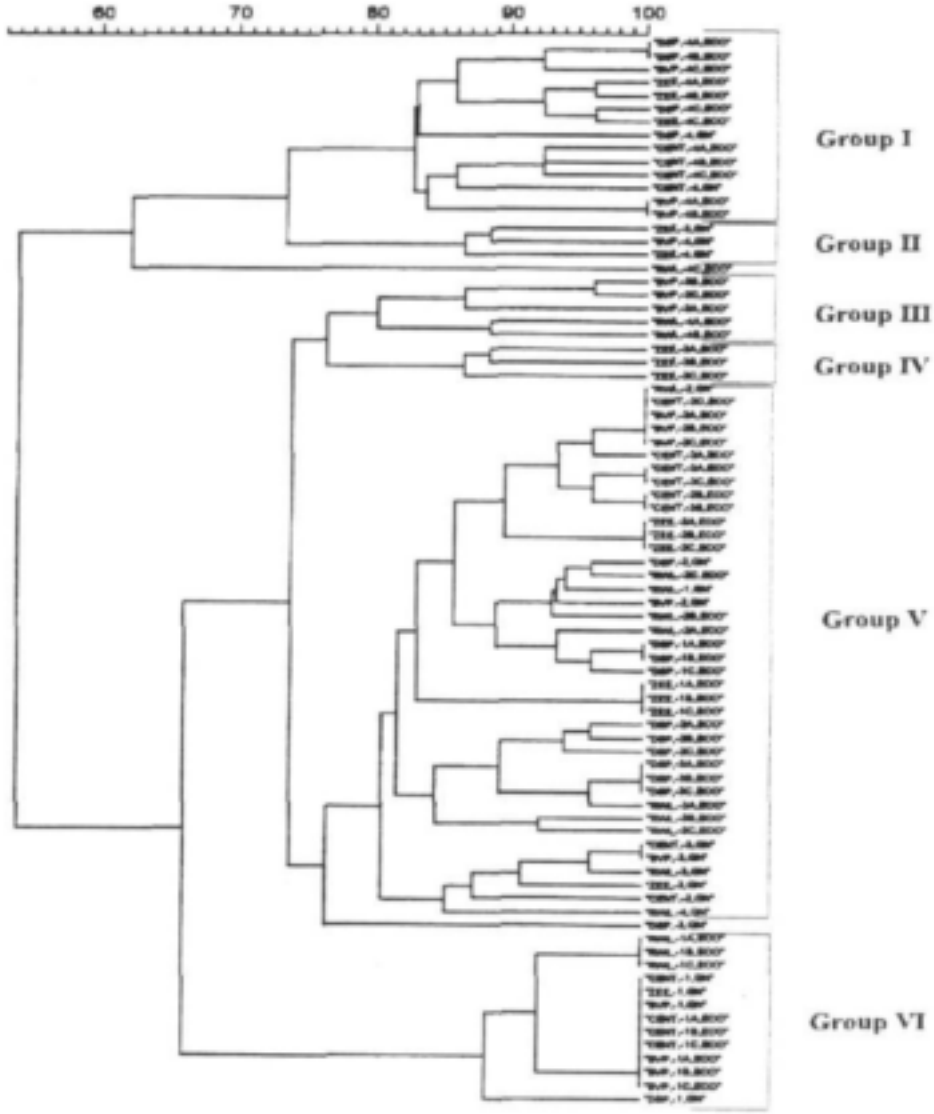


Figure 29 : Dendrogram indicating the similarity between carbon source utilization patterns of the twenty-five common carbon substrates within Biolog GN and ECO plates (Table 4). The relationship are determined by using the Simple matching coefficient (S_{SM}) values, which were grouped by the unweighted average pair group method, using GelCompar 4.0

The Dendrogram in Fig. 29, gives the comparison of the 25 common substrates within the Biolog GN and ECO plates of Baviaanspoort, Centurion, Daspoort, Rooiwal and Zeekoegat (Fig. 29). This data could be divided into six Groups, at an 80 % similarity level. The following samples were present in Group I the 10^{-4} dilutions of Daspoort (A, B and C of the ECOplates); Daspoort (GN); Baviaanspoort (A, B and C of ECO plates); Zeekoegat (A, B and C of ECOplates); Centurion (A, B and C of the ECO plates) and Centurion (GN).

Group II contained the 10^{-3} dilution of Zeekoegat (GN) and the 10^{-4} dilutions of Baviaanspoort (GN); Zeekoegat (GN) and Rooiwal (C of the ECO plates).

Group III contained the 10^{-3} dilutions of Baviaanspoort (A, B and C of the ECO plates) and the 10^{-4} dilution of Rooiwal (A and B of the ECO plates).

Group IV contained the 10^{-3} dilutions of Zeekoegat (A, B and C ECO plates).

Group V contained the following samples the 10^{-1} dilutions of Rooiwal (GN); Daspoort (A, B and C of the ECO plates); Zeekoegat (A, B and C of the ECO plates); the 10^{-2} dilutions of Rooiwal (GN); Rooiwal (A, B and C of the ECO plates); Centurion (A, B and C of the ECO plates); Centurion (GN); Baviaanspoort (A, B and C of the ECO plates); Baviaanspoort (GN); Zeekoegat (A, B and C of the ECO plates); Zeekoegat (GN); Daspoort (GN); Daspoort (A, B and C of the ECO plates); the 10^{-3} dilutions of Centurion (A, B and C of the ECO plates); Centurion (GN); Daspoort (A, B and C of the ECO plates); Rooiwal (A, B and C of the ECO plates); Baviaanspoort (GN); Rooiwal (GN) and the 10^{-4} dilution of Rooiwal (GN).

Group VI contained the 10^{-1} dilutions of Rooiwal (A, B and C of the ECO plates); Centurion (GN); Zeekoegat (GN); Baviaanspoort (GN); Centurion (A, B and C of the ECO plates); Baviaanspoort (A, B and C of the ECO plates) and Daspoort (GN).

Table 11: Chemical analysis of the Heidelberg, Vlakplaats, Tsakane and Hartebeesfontein activated sludge system at the time of sampling obtained from ERWAT

System	Design	Waste type	pH	COD $mg.l^{-1}$	NH_3/N $mgN.l^{-1}$	NO_3/NO_2 $mg.l^{-1}$	PO_4/P $mgP.l^{-1}$	MLSS $mg.l^{-1}$
Heidelberg		Industrial						
Anaerobic	2-stage	and	7.4	102	12.7	0.1	8.7	ND
Anoxic	2-culture	Domestic	7.5	98	0.4	14.8	0.3	ND
Aerobic			7.5	10	0.2	14.2	0.1	5862
Vlakplaats		Industrial						
Anaerobic	3-stage	and	7.4	ND	14.7	1.4	9.9	ND
Anoxic	Bardenpho	Domestic	7.4	ND	8.6	1.5	7.9	ND
Aerobic			7.4	ND	1.4	6.1	5.2	3370
Tsakane								
Anaerobic	3-stage	Domestic	6.9	77	17.0	ND	9.7	ND
Anoxic	Bardenpho		7.1	44	18.8	ND	7.9	ND
Aerobic			7.1	83	10.6	6.2	0.1	4166
Hartebeesfontein		Industrial						
Anaerobic	3-stage	and	ND	ND	19.7	0.4	2.7	9620
Aerobic	Bardenpho	Domestic	ND	ND	19.6	1.5	4.3	2710

ND = Not determined.

The results in Table 11, indicate that effective COD removal, effective nitrification and a sufficient MLSS concentration in the aerobic zone were required for effective PO_4^{3-} removal. This was in agreement with previous studies (Buchan, 1980; Bosch, 1992; Schwieger and Tebbe, 1998). This suggested that the biomass concentration was more important for effective PO_4^{3-} removal, than the specific microbial community structure, as was previously indicated (Mills and Wassel, 1980; Kersters *et al.*, 1997).

Biolog GN and GP microplate comparisons:

All the above dendrograms (Fig. 12 to Fig. 22) indicating the comparison between Biolog GN and GP microplate data showed differences between GN and GP microplate data. It is therefore important, not to compare the GN and GP profiles,

since this would show a difference in community structure, which is not related to the true community structure, but rather to the substrates used in the GN and GP plates. However, considered in isolation (GN or GP), the same conclusions could be made for a specific community in terms of dominance and evenness. This indicated, that a significantly different set of substrates (given that a sufficient number of substrates are incorporated) could be utilized and yet the data generated substantiates the hypothesis, that upon dilution some members of the community will be lost and this would be reflected in the metabolic pattern (database) which is generated.

Phosphate removal:

Our hypothesis was that differences in microbial community structure in activated sludge systems might exist, but that this had no bearing on the effectivity with which these systems removed phosphate. If PO_4^{3-} removal was related to the microbial community composition, a high correlation of the Biolog patterns would be expected amongst PO_4^{3-} removing systems. However, this was not the case in our study. All the different zones of systems tested indicated a high initial diversity (10^{-1} to 10^{-2} dilutions), due to the high number of substrates utilized. No specific patterns could, however be identified for PO_4^{3-} removing systems, indicating that PO_4^{3-} removal was not related to the functionality of the aerobic heterotrophic microbial community which was determined using the Biolog system This agrees with previous studies (Kersters *et al.*, 1997).

Biolog GN and ECO plate comparisons:

In this study, the Biolog community-level metabolic carbon source utilization patterns were evaluated to determine whether the GN- and ECO-plates were equally effective in distinguishing microbial metabolic patterns from a number of activated sludge systems and to relate this to the microbial diversity and evenness within the activated

sludge systems.

The metabolic activity patterns obtained from the different activated sludge systems all indicated patterns resembling an uneven distribution of species upon dilution (Fig. 29). This is due to the fact that some of the species were lost upon dilution and therefore their contribution to substrate utilization were lost. Both of the Biolog microplates used indicated a high initial diversity (10^{-1} and 10^{-2} dilutions), due to the high number of substrates utilized. Inter-plate differences in utilization of the common substrates (Table 4) are attributed to differences in the formulation of the low-nutrient media within the plates (Fig. 29). The GN and ECO plates were equally effective in distinguishing microbial metabolic patterns for a number of activated sludge systems and to relate these patterns to the microbial diversity and evenness within the activated sludge systems. This indicated that irrespective of the type of Biolog plate used the hypothesis that upon dilution some members of the community will be lost and this would be reflected in the metabolic pattern which is generated, will be true.

Limitations:

Although the results indicate the usefulness of this technique, some limitations may make it impossible to draw a valid conclusion. One such a scenario, would be where the diversity in a system is so high, that irrespective of dilution, all the substrates remain to be utilized. However, whether this type of community exists, is arguable. Therefore, should a loss of substrate utilization occur, upon dilution, this technique definitely reflects the extent of evenness in a microbial community, as was illustrated for activated sludge in our case. As proved by this and other studies, the use of substrate utilization profiles to characterize microbial communities have clear limitations, but this rapid technique remains a valuable tool for comparison of microbial communities, provided the data are cautiously interpreted (Smalla *et al.*,

1998). Future research on using Biolog for community-level carbon source utilization patterns should focus on a range of diverse environments including, for example hot water springs, rivers, etc. in order to further validate the method.

CHAPTER 5

CONCLUSIONS

In this study it was indicated that:

- The Biolog system could be used to determine functional (species) diversity and evenness

- Metabolic profiles in the activated sludge reflected a pattern resembling unevenness, indicated by some of the substrates no longer being utilized after the loss of some of the species upon dilution

- When using the Biolog system, no specific patterns could be identified for phosphate removing and non-phosphate removing systems, indicating that phosphate removal could not be related to the functionality of the aerobic heterotrophic microbial community which was determined using the Biolog system

- Different Biolog systems (GN, GP, ECO plates) could be used and yet the data generated substantiates the hypothesis, that upon dilution some members of the community will be lost and this would be reflected in the metabolic pattern (database) which is generated

- Some limitations might make it impossible to draw a valid conclusion. One such a scenario, would be where the diversity in a system is so high, that irrespective of dilution, all the substrates remain to be utilized. However, whether this type of community exists, is arguable. Therefore, should a loss of substrate utilization occur upon dilution, this technique will reflect the extent of evenness in a

microbial community, as was illustrated for activated sludge in this case

- As proven by this and other studies, the use of substrate utilization profiles to characterize microbial communities have clear limitations, but this rapid technique remains a valuable tool for the comparison of microbial communities, provided the data are cautiously interpreted

REFERENCES

Amann, R. I., Ludwig, W. Schleifer, K.H. 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59**: 143-169.

Amy, P. S., Haldeman, D. L., Ringelberg, D., Hall, D. H. and Russell, C. 1992. Comparison of identification systems for classification of bacteria isolated from water and endolithic habitats within the deep subsurface. *Appl. Environ. Microbiol.* **58**:3367- 3373.

Atlas, R.M. 1984. Diversity of microbial communities. *Advanced Microbial Ecology* 7: 1-47.

Atlas, R.M., Howrowitz, A., Kirchevsky, M. and Bej, A. K. 1991. Response of microbialpopulations to environmental disturbance. *Micro. Ecol.* 222: 249-256.

Atlas, R.M., Colwell, R. R., Pramer, D., Tiedje, J.M., Vidaver, A. K. and Wodzinski, R.J.1992. Microbial ecology. *ASM News.* 58: 4-7.

Atlas, R.M. and Bartha, R. 1993. *Microbial Ecology. Fundamentals and applications.* 3rd ed. The Benjamin/Cummings Publishing Company, Inc. New York.

Barnard, J.L. 1976. A review of biological phosphorus removal in activated sludge process. *Water. S.A.* **2**: 136-144.

Bitton, G. 1994. *Wastewater microbiology.* John Wiley and Son, Inc. New York. p 59-303.

Bochner, B. R. 1989. Breathprints at the microbial level. *Am. Soc. Microbiol. News.* **55:** 536-539.

Borneman, J., Skroch, P.W., O'Sullivan, K.M., Palus, J. A., Rumjanek, N.G., Jansen, J. L., Nienhuis and Triplett, E.W. 1996. Molecular microbial diversity of an agricultural soil in Wisconsin. *Appl. Eviron. Microbiol.* **62:** 1935-1943.

Bosch, M. 1992. *Phosphorus uptake kinetics of Acinetobacter in activated mixed liquor*. MSc.thesis, Department Microbiology and Plant Pathology, University of Pretoria, Pretoria,South Africa.

Bossio, D. A. and Scow, K. M. 1995. Impact of carbon and flooding on the metabolic diversity of microbial communities in soils. *Appl. Environ. Microbiol.* **11**: 4043-4050.

Buchan, L. 1980. *The location and nature of accumulated phosphorus in activated sludge*. D.Sc. thesis, Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, South Africa.

Cloete, T.E. and Muyima, N.Y.U. 1997. Microbial community analysis: The key to the design of biological wastewater treatment systems. *International Association on Water quality*, Cambridge, Great Britain.

D'Amato, R.F., Bottone, E.J. and Amsterdam, D. 1991. Substrate profile systems for the identification of bacteria and yeasts using rapid and automated approaches. In: *Manual of clinical microbiology*. 5th ed. Balows, A; W.J. Haulser, Jr.; K. Herrmann, H. D. Isenberg, and H.J. Shadomy. American Society for Microbiology, Washington, DC p 128-136.

De Bruijn, F.J. 1992. Use of repetitive (repetitive extragenic element and enterobacterial repetitive intergenic consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. *Appl. Environ. Microbiol.* **58:** 2180-2187.

Ehlers, M. M. 1998. *Bacterial community structure of activated sludge determined with SDS-PAGE*. Ph.D. Thesis, Department of Microbiology and Plant Pathology,University of Pretoria, Pretoria, South Africa.

Ellis, R.J., Thompson, I.P. and Bailey, M.J. 1995. Metabolic profiling as a means of characterizing plant-associated microbial communities. *FEMS Microbiol. Ecol.* **16**: 9-18.

Engelen, B., Meinken, K., Von Wintzingerode, F., Heuer, H., Malkomes, H.-P. and Backhaus, H. 1998. Monitoring impact of a pesticide treatment on

bacterial soil communities by metabolic and genetic fingerprinting in addition to conventional testing procedures. *Appl. Environ. Microbiol.* **64:** 2814-2821.

Frederickson, J.K., Balkwill, D. L., Zachara, J.M., Li, S. W., Brockman, F.W. and Simmons, M. A. 1991. Physiological diversity and distributions of heterotrophic bacteria in deep cretaceous sediments of the Atlantic coastal plain. *Appl. Environ. Microbiol.* **57:** 402-411.

Frostegard, A., Tunlid, A. and Baath, E. 1993. Phospholipid fatty acid composition,biomass and activity of microbial communities from two soil types experimentally exposed to different heavy metals. *Appl. Environ. Microbiol.* **59:** 3605-3617.

Garland, J.L. and Mills, A. L. 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. *Appl. Environ. Microbiol.* **57:** 2351-2359.

Garland, J.L. 1996(a). Analytical approaches to the characterization of samples of microbial communities using patterns of potential C source utilization. *Soil. Biol. Biochem.* **28**: 213-221.

Garland, J.L. 1996(b). Patterns of potential C source utilization by rhizosphere communities.*Soil. Boil. Biochem.* **28**: 223-230.

Garland, J.L. 1997. Analysis and interpretation of community-level physiological profiles in microbial ecology. *FEMS Microbiol. Ecol.* **24**: 289-300.

Glimm, E., Heuer, H., Engelen, B., Smalla, K. and Backhaus, H. 1997. Statistical comparisons of community catabolic profiles. *J. Microbiol. Methods.* **30**: 71-80.

Goel, R., Mino T., Satoh H. and Matsuo, T. 1998. Enzyme activities under anaerobic and aerobic conditions in activated sludge sequencing batch reactors. *Water Res.* **32**:2081-2088.

Gorlenko, M. V. and Kozhevin, P.A. 1994. Differentiation of soil microbial communities by multisubstrate testing. *Microbiol.* **63:** 158-161.

Grady, C. P. L. and Lim, H. C. 1980. Activated sludge. In: *PN Cheremisinoff(ed)*

Biological wastewater treatment theory and applications. Marcel Dekker (Inc.) New York.

Griffiths, B. S., Ritz, K. and Wheatly, R. E. 1997. Relationship between functional diversity and genetic diversity in complex microbial communities. In: H. Insam and Ranger, A. (ed.). *Microbial communities. Functional versus structural approaches*. Springer Verslag, Berlin, Heidelberg. New York, p 1-9.

Guckert, J.B., Carr, G.J., Johnson, T. D., Hamm, B.G., Davidson, D. H. and Kumagai, Y. 1996. Community analysis by Biolog: curve integration for statistical analysis of activated sludge microbial habitats. *J. Microbiol. Methods*. **27:** 183-197.

Haack, S. K., Garchow, H., Klug, M.J. and Forney, L.J. 1994. Analysis of factors affecting the accuracy, reproducibility, and interpretation of microbial community carbon source utilization patterns. *Appl. Environ. Microbiol*. **61:** 1458-1468.

Haldeman, D. L. and Amy, P. S. 1993. Diversity within a colony morphotype: implications for ecological research. *Appl. Environ. Microbiol*. **59**: 933-935.

Hahn, D., Amann, R.I., Ludwig, W., Akkermans, A.D.L., Schleifer, K.H. 1992. Detection of microorganisms in soil after *in situ* hybridization with rRNA-targeted, fluoresently labelled oligonucleotides. *J. Gen. Microbiol*. **138:** 879-887.

Hartung, J. S. and Cilerolo, E. L. 1991. Variation among strains of *Xanthomonas campertris* causing citrus bacterial spot. *Plant Diseases* **75:** 622-626.

Heuer, H., Hartung, K., Engelen, B. and Smalla, K. 1995. Studies on microbial communities associated with potato plants by Biolog and TGGE patterns. *Med. Fac. Landbouww. Univ. Gent*. **60**: 2639-2645.

Heuer, H. and Smalla, K. 1997. Evaluation of community - level catabolic profiling using Biolog GN microplates to study community changes in potato phyllosphere. *J. Microbiol. Methods*. **30**: 49 - 61.

Hitzl, W., Ranger, A., Sharma, S. and Insam, H. 1997. Separation power of the 95 substrates of the Biolog system determined in various soils. *FEMS Microbiol. Ecol.* **22**: 167-174.

Insam, H., Amor, K., Renner, M. and Crepaz, C. 1996. Changes in functional abilities of the microbial community during composting of manure. *Microb. Ecol.* **31**: 77-87.

Insam, H. and Ranger, A. 1997. *Microbial communities: functional versus structural approaches.* Springer, Heidelberg, Germany.

Kaneko, T. and Atlas, R.M. 1977. Diversity of bacterial populations in the Beaufort sea. *Nature.* **270:** 596-599.

Kersters, I., Van Vooren, L., Verschuere, L., Vauterin, L., Wouters, A., Mergaert, J. Swings, J. and Verstraete, W. 1997. Utility of the Biolog system for the characterization of heterotrophic microbial communities. *System. Appl. Microbiol.* **20**: 439-447.

Klinger, J.M., Stowe, R. P., Obenhuber, D. C., Groves, T. O., Mishra, S. K. and Pierson, D. L. 1992. Evaluation of the Biolog automated microbial identification system. *Appl. Environ. Microbiol.* **58**: 2089-2092.

Konopka, A., Oliver, L. and Turco, R.F. (Jr.). 1998. The use of carbon source utilization patterns in environmental and ecological microbiology. *Microbial Ecology* **35**: 103-115.

Korf, C. 1998. *The Biolog system as a method to determine microbial species diversity in natural habitats.* M. Sc. Thesis, Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, South Africa.

Lee, S. and Fuhrman, J.A. 1990. DNA hybridisation to compare species compositions of natural bacterioplankton assemblages. *Appl. Environ. Microbiol.* **56**: 739-746.

Lehman, R.M., Colwell, F. S., Ringelberg, D. B. and White, D. C. 1995. Combined microbial community-level analysis for quality assurance of

terrestrial subsurface cores. *J. Microbiol. Methods.* **22:** 263-281.

Lovejoy, T. 1993. Why should we care about biodiversity? *ASM News*. **59**: 322-323.

Mallory, L. M. and Saylor, G. S. 1984. Application of FAME(fatty acid methyl ester) analysis in the numerical taxonomic determination of bacterial guild structure. *Micro. Ecol.* **10**: 283-296.

Mills, A. L. and Wasel, R. A. 1980. Aspects of diversity measurement for microbial communities. *Appl. Environ. Microbiol.* **40:** 578-586.

Momba, M.N.B. 1995. *Phosphate removal in activated sludge and its relationship to biomass*. M.Sc. thesis, Department Microbiology and Plant Pathology, University of Pretoria, Pretoria, South Africa.

Moyer, C. L., Dobbs, F.C. and Karl, D. M. 1994. Estimation of diversity and community structure through restriction fragment length polymorphism distribution analysis of bacterial 16S rRNA genes from a microbial mat at an active, hydrothermal vent system, Loihi Seamount, Hawaii. *Appl. Environ. Microbiol.* **60**: 871-879.

Muyima, N.Y.O. 1995. *Enhanced biological phosphate removal by immobilized Acinetobacter and activated sludge microbial populations*. Ph.D. Thesis, University of Pretoria, Pretoria, South Africa.

Muyima N. Y. O., Momba M. N. B. and Cloete T. E. 1997. Biological methods for the treatment of wastewaters. In: *Microbial Community Analysis: The key to the design of biological wastewater treatment systems.* (eds. Cloete T. E. and Muyima N. Y. O). International Association on Water Quality. Cambridge, Great Britain.

Muyzer, G., De Waal, E. C. and Uitterlinden, A.G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of

polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* **59**: 695-700.

Noble, L.D. and Gow, J.A. 1998. The effect of suspending solution supplemented with marine cations on the oxidation of Biolog GN microplate substrates by Vibrionaceae bacteria. *Can. J. Microbiol.* **44:** 251-258.

O'Brien, M. and Colwell, R. 1987. Characterization test for numerical taxonomy studies. *Methods in microbiology*. **19**: 69-103.

Odum, P. E. 1971. *Fundamentals of ecology*. Saunders, Philadelphia.

Palleroni, N.J. 1994. Some reflections on bacterial diversity. *ASM News*. **60**: 537-540.

Parkasam, T.B.S. and Dondero, N.C. 1967. Aerobic heterotrophic populations of sewage and activated sludge. *Appl. Microbiol.* **15:** 461-467.

Pielou, E. C. 1975. *Ecological diversity*. John Wiley and Sons, interscience, New York.

Sakano, Y. and Kerkhof, L. 1998. Assessment of changes in microbial community structure during operation of an ammonia biofilter with molecular tools. *Appl. Environ. Microbiol.* **64:** 4877 - 4882.

Schmidt, T.M., DeLong, E.F. and Pace, N. R. 1991. Analysis of a marine picoplakton community by 16S rRNA gene cloning and sequencing. *J. Bacteriol.* **173:** 4371-4378.

Schwieger, F. and Tebbe, C.C. 1998. A new approach to utilize PCR single-strand conformation polymorphism for 16S rRNA gene-based microbial community

analysis. *Appl. Environ. Microbiol.* **64**: 4870 - 4876.

Smalla, K., Wachtendorf, U., Krögerrecklenfort, E. and Heuer, H. 1996. Which members of the bacterial population of the potato phylosphere and rhizosphere contribute most to the BIOLOG community pattern? In: *Abstracts of the symposium on bacterial genetics and ecology* BAGECO 5, May. Nafplion, Greece. p. 93.

Smalla, K., Wachtendorf, U., Heuer, H., Liu, W. and Forney, L. 1998. Analysis of BIOLOG GN substrate utilization patterns by microbial communities. *Appl. Environ. Microbiol.* **64**: 1220-1225.

Sneath, P.H.A. and Sokal, R.R. 1973. *Numerical taxonomy - the principles and practices of numerical classification.* Freeman, W.H. and Co., San Francisco.

Steyn, P. L. and Cloete, T.E. 1989. The adaptation of Koch's postulates to microbial ecology *S.A. J. Science.* **85:** 360-362.

Tiedje, J.M. 1994. Microbial diversity: Of value to whom? *ASM News.* **60:** 524-525.

Toerien, D.F., Gerber, A., Lötter, L.H. and Cloete, T.E. 1990. Enhanced biological phosphorus removal in activated sludge systems. *Adv. Microb. Ecol.* **11:** 173-230.

Torsvik, V., Goksoyr, J. and Daae, F. L. 1990. High diversity of DNA in soil bacteria. *Appl. Eviron. Microbiol.* **56:** 782-787.

Torsvik, V., Sfrheim, R. and Gfksoyr, J. 1996. Total bacterial diversity in soil and sediment communities-a review. *J. Ind. Microbiol.* **17:** 170-178.

Tunlid, A. and White, D.C. 1992. Biochemical analysis of biomass, community structure, nutritional status and metabolic activity of microbial communities in soil. *Soil Biochem.* **7**: 229-262.

Ullrich, S., Karrasch, B., Hoppe, H. G., Jeskulke, K. and Mehrens, M. 1996. Toxic effects on bacterial metabolism of the redox dye 5-cyano-2,3-ditolyl tetrazolium chloride.*Appl. Environ. Microbiol.* **62**: 4587-4593.

Vahjen, W., Munch, J.C. and Tebbe, C.C. 1995. Carbon source utilization of soil extracted micro-organisms supplemented with genetically engineered and non-engineered *Corynebacterium glutamicum* and a recombinant peptide at the community level. *FEMS Microbiol. Ecol.* **18:** 317-328.

Van Damme, P., Pot, B., Gillis, M., De Vos, P., Kersters, K. and Swings, J. 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. *Micro. Rev.* **60:** 407-438.

Van Elsas, J.D., Duarte, G.F., Rosado, A. S. and Smalla, K. 1998. Microbiological and molecular biological methods for monitoring microbial inoculants and their effects in soil environment. *J. Microbiol. Methods.* **32:** 133-154.

Verniere C., Pruvost, O., Civerolo, E. L., Gambin, O., Jaquemon-Collet, J. and Luisetti, J. 1993. Evaluation of the Biolog substrate utilization system to identify and assess metabolic variation among strains of *Xanthomonas campestris* pv. *citri*. *Appl. Environ. Microbiol.* **59**: 243-249.

Victorio, L., Gilbride, K. A., Allen, D. G. and Liss, S.N. 1996. Phenotypic fingerprinting of microbial communities in wastewater treatment systems. *Wat. Res.* **30:** 1077-1086.

Wagner, M., Amann, R., Lemmer, H. and Schleiffer, K.-H. 1993. Probing activated sludge with oligonucleotides specific for proteobacteria: inadequacy of culture-dependent methods for describing microbial community structure. *Appl. Environ. Microbiol.* **59:** 1520 - 1525.

Watve, M. G. and Gangal, R.M. 1996. Problems in measuring bacterial diversity and a possible solution. *Appl. Environ. Microbiol.* **62:** 4299-4301.

Wawer, C and Muyzer, G. 1995. Genetic diversity of *Desulfovibrio* spp. in environmental samples analyzed by denaturing gradient gel electrophoresis of [NiFe] hydrogenase gene fragments. *Appl. Environ. Microbiol.* **61:** 2203-

2210.

Winding, A. 1994. Fingerprinting bacterial soil communities using Biolog microtiter plates. In: Ritz, K., Dighton, K.E. and Giller, K.E. (eds.). *Beyond the biomass : compositional and functional analysis of soil microbial communities*. Chichester,Wiley.

Wünsche, L. and Babel, W. 1995. The suitability of the Biolog automated microbial identification system for assessing the taxonomical composition of terrestrial bacterial communities. *Microbiol. Res.* **151:** 133-143.

Yap, W.H., Li, X., Soong, T.W. and Davies, J.E. 1996. Genetic diversity of soil micro-organisms assessed by analysis of hsp 70 (dna K) sequences. *J. Ind. Microbiol.* **17:** 79-184.

Zak, J.C., Willig, M. R., Moorhead, D. L. and Wildman, H. G. 1994. Functional diversity of microbial communities: a quantitative approach. *Soil. Biol. Biochem.* **26:** 1101-1108.

Zehr, J. P., Mellon, M., Braun, S., Litaker, W., Steppe, T. and Paerl, H.W.1995. Diversity of heterotrophic nitrogen fixation genes in a marine cyanobacterial mat.*Appl. Environ. Microbiol.* **61:** 2527-2532.

APPENDIX

Eco MicroPlate

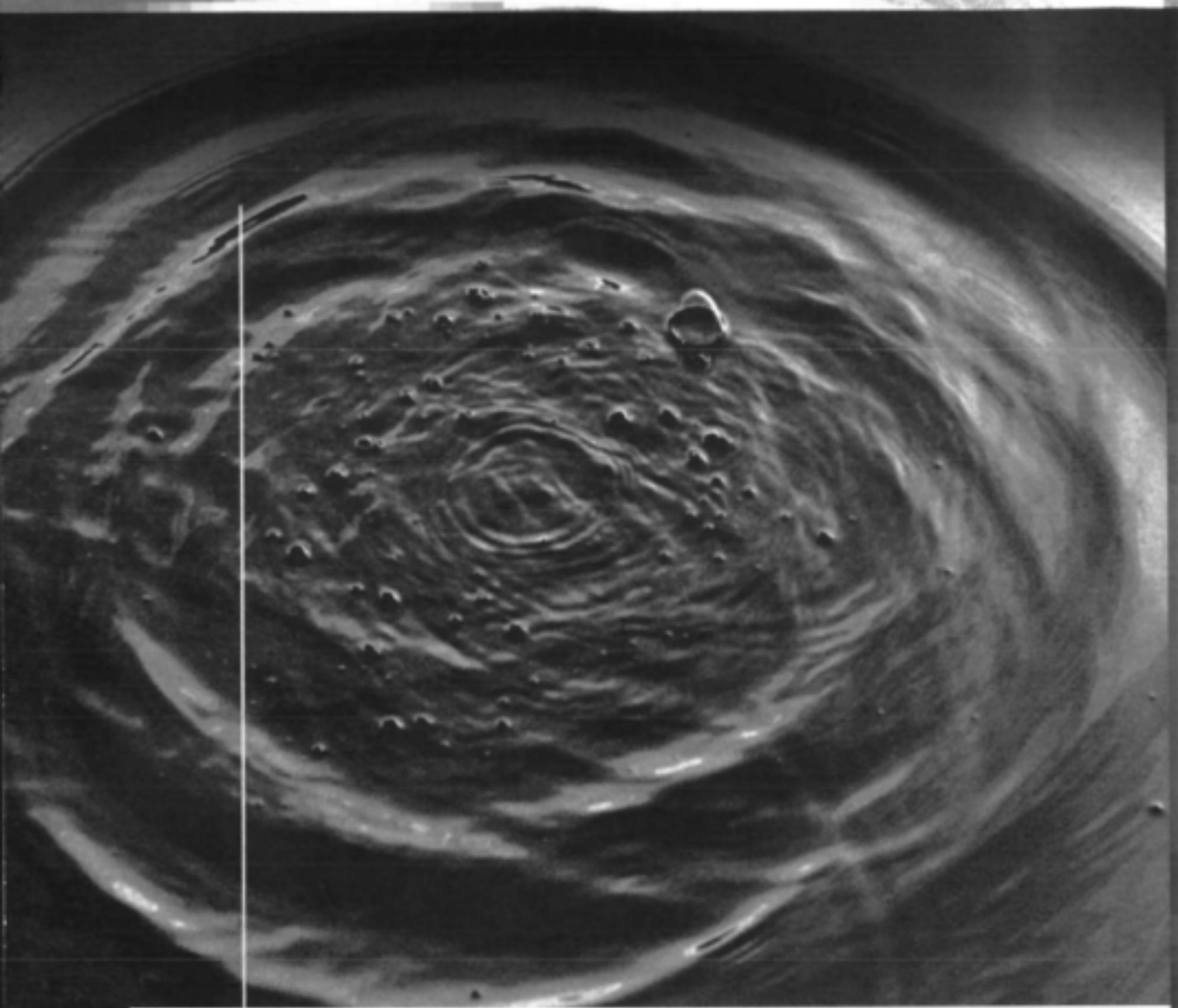
Water	b-Methyl-D-Glucoside	D-Galactonic Acid g-Lactone	L-Arginine	Water	b-Methyl-D-Glucoside	D-Galactonic Acid g-Lactone	L-Arginine	Water	b-Methyl-D-Glucoside	D-Galactonic Acid g-Lactone	L-Arginine
Pyruvic Acid Methyl Ester	D-Xylose	D-Galacturonic Acid	L-Asparagine	Pyruvic Acid Methyl Ester	D-Xylose	D-Galacturonic Acid	L-Asparagine	Pyruvic Acid Methyl Ester	D-Xylose	D-Galacturonic Acid	L-Asparagine
Tween 40	i-Erythritol	2-Hydroxy Benzoic Acid	L-Phenylalanine	Tween 40	i-Erythritol	2-Hydroxy Benzoic Acid	L-Phenylalanine	Tween 40	i-Erythritol	2-Hydroxy Benzoic Acid	L-Phenylalanine
Tween 80	D-Mannitol	4-Hydroxy Benzoic Acid	L-Serine	Tween 80	D-Mannitol	4-Hydroxy Benzoic Acid	L-Serine	Tween 80	D-Mannitol	4-Hydroxy Benzoic Acid	L-Serine
a-Cyclodextrin	N-Acetyl-D-Glucosamine	g-Hydroxybutyric Acid	L-Threonine	a-Cyclodextrin	N-Acetyl-D-Glucosamine	g-Hydroxybutyric Acid	L-Threonine	a-Cyclodextrin	N-Acetyl-D-Glucosamine	g-Hydroxybutyric Acid	L-Threonine
Glycogen	D-Glucosaminic Acid	Itaconic Acid	Glycyl-L-Glutamic Acid	Glycogen	D-Glucosaminic Acid	Itaconic Acid	Glycyl-L-Glutamic Acid	Glycogen	D-Glucosaminic Acid	Itaconic Acid	Glycyl-L-Glutamic Acid
D-Cellobiose	Glucose-1-Phosphate	a-Keto Butyric Acid	Phenylethyl-amine	D-Cellobiose	Glucose-1-Phosphate	a-Keto Butyric Acid	Phenylethyl-amine	D-Cellobiose	Glucose-1-Phosphate	a-Keto Butyric Acid	Phenylethyl-amine
a-D-Lactose	D,L-a-Glycerol Phosphate	D-Malic Acid	Putrescine	a-D-Lactose	D,L-a-Glycerol Phosphate	D-Malic Acid	Putrescine	a-D-Lactose	D,L-a-Glycerol Phosphate	D-Malic Acid	Putrescine

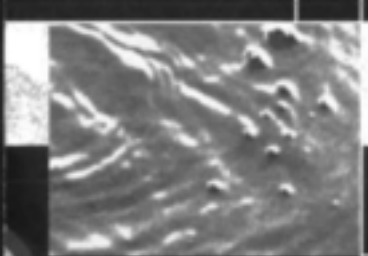
GN MicroPlate™

A1 water	A2 α- cyclodextrin	A3 dextrin	A4 glycogen	A5 tween 40	A6 tween 80	A7 N-acetyl-D- galactosamine	A8 N-acetyl-D- glucosamine	A9 adonitol	A10 L- arabinose	A11 D- arabitol	A12 cellobiose
B1 i- erythritol	B2 D- fructose	B3 L- fucose	B4 D- galactose	B5 gentiobiose	B6 α-D- glucose	B7 m- inositol	B8 α-D- lactose	B9 lactulose	B10 maltose	B11 D- mannitol	B12 D- mannose
C1 D- melibiose	C2 β-methyl D-glucoside	C3 D- psicose	C4 D- raffinose	C5 L- rhamnose	C6 D- sorbitol	C7 sucrose	C8 D- trehalose	C9 turanose	C10 xylitol	C11 methyl pyruvate	C12 mono-methyl succinate
D1 acetic acid	D2 cis- aconitic acid	D3 citric acid	D4 formic acid	D5 D- galactonic acid lactone	D6 D- galacturonic acid	D7 D- gluconic acid	D8 D- glucosaminic acid	D9 D- glucuronic acid	D10 α- hydroxybutyric acid	D11 β- hydroxybutyric acid	D12 γ- hydroxybutyric acid
E1 p-hydroxy phenylacetic acid	E2 itaconic acid	E3 α-keto butyric acid	E4 α-keto glutaric acid	E5 α-keto valeric acid	E6 D,L- lactic acid	E7 malonic acid	E8 propionic acid	E9 quinic acid	E10 D- saccharic acid	E11 sebacic acid	E12 succinic acid
F1 bromo succinic acid	F2 succinamic acid	F3 glucuronamide	F4 alaninamide	F5 D- alanine	F6 L- alanine	F7 L-alanyl glycine	F8 L- asparagine	F9 L- aspartic acid	F10 L- glutamic acid	F11 glycyl-L- aspartic acid	F12 glycyl-L- glutamic acid
G1 L- histidine	G2 hydroxy-L- proline	G3 L- leucine	G4 L- ornithine	G5 L- phenylalanine	G6 L- proline	G7 L- pyroglutamic acid	G8 D- serine	G9 L- serine	G10 L- threonine	G11 D,L- carnitine	G12 γ-amino butyric acid
H1 urocanic acid	H2 inosine	H3 uridine	H4 thymidine	H5 phenyl ethylamine	H6 putrescine	H7 2-amino ethanol	H8 2,3- butanediol	H9 2-methyl	H10 D,L-α- glycerol phosphate	H11 glucose-1- phosphate	H12 glucose-6- phosphate

GP MicroPlate™

A1 water	A2 α- cyclodextrin	A3 β- cyclodextrin	A4 dextrin	A5 glycogen	A6 inulin	A7 mannan	A8 tween 40	A9 tween 80	A10 N-acetyl-D- glucosamine	A11 N-acetyl-D- mannosamine	A12 amygdalin
B1 L- arabinose	B2 D- arabitol	B3 arbutin	B4 cellobiose	B5 D- fructose	B6 L- fucose	B7 D- galactose	B8 D- galacturonic acid	B9 gentiobiose	B10 D- gluconic acid	B11 α-D- glucose	B12 m- inositol
C1 α-D- lactose	C2 lactulose	C3 maltose	C4 maltotriose	C5 D- mannitol	C6 D- mannose	C7 D- melezitose	C8 D- melibiose	C9 α-methyl D-galactoside	C10 β-methyl D-galactoside	C11 3-methyl glucose	C12 α-methyl D-glucoside
D1 β-methyl D-glucoside	D2 α-methyl D-mannoside	D3 palatinose	D4 D- psicose	D5 D- raffinose	D6 L- rhamnose	D7 D- ribose	D8 salicin	D9 sedoheptulosan	D10 D- sorbitol	D11 stachyose	D12 sucrose
E1 D- tagatose	E2 D- trehalose	E3 turanose	E4 xylitol	E5 D- xylose	E6 acetic acid	E7 α- hydroxybutyric acid	E8 β- hydroxybutyric acid	E9 γ- hydroxybutyric acid	E10 p- hydroxyphenyl acetic acid	E11 α-keto glutaric acid	E12 α-keto valeric acid
F1 lactamide	F2 D- lactic acid methyl ester	F3 L- lactic acid	F4 D- malic acid	F5 L- malic acid	F6 methyl pyruvate	F7 mono-methyl succinate	F8 propionic acid	F9 pyruvic acid	F10 succinamic acid	F11 succinic acid	F12 N-acetyl L-glutamic acid
G1 alaninamide	G2 D- alanine	G3 L- alanine	G4 L-alanyl-glycine	G5 L- asparagine	G6 L- glutamic acid	G7 glycyl-L- glutamic acid	G8 L- pyroglutamic acid	G9 L- serine	G10 putrescine	G11 2,3- butanediol	G12 glycerol
H1 adenosine	H2 2'- deoxy- adenosine	H3 inosine	H4 thymidine	H5 uridine	H6 adenosine-5'- monophosphate	H7 thymidine-5'- monophosphate	H8 uridine-5'- monophosphate	H9 fructose-6- phosphate	H10 glucose-1- phosphate	H11 glucose-6- phosphate	H12 D,L-α- glycerol phosphate





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