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

by

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Department of Microbiology and Plant Pathology

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

Signed: ____________ this 15 day of December 1999

Elke dag van jou lewe is werktyd : tyd om jou geleenthede te benut en jou ideale te verwesenlik; tyd om ander mense se hande te vat onderweg na die vervulling van jou drome; tyd om te groei tot die afgeronde mens wat jy graag wil wees

Johan Smit

Opgedra aan my ouers

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TABLE OF CONTENTS

Title page

Declaration

Dedication

Bedankings

List of Abbreviations

List of Tables

List of Figures

		p
SUMMARY		1-3
OPSOMMING		4-6
CHAPTER 1:	**INTRODUCTION**	7-13
CHAPTER 2:	**LITERATURE REVIEW**	
2.1	Introduction	14-15
2.2	Microbial population dynamics	15-18
2.3	Koch's postulates for microbial ecology	18-19
2.4	The Activated Sludge Process	19-20
2.4.1	Anaerobic zone	20-21
2.4.2	Anoxic zone	22
2.4.3	Aerobic zone	22-23
2.5	Phosphate removal in activated sludge systems	23-24
2.5.1	The role of biomass in phosphate removal	24-25
2.5.2	Enzyme activities in activated sludge	25-26
2.5.3	Phosphate removing bacteria	26-27

2.6 Microbial ecology of activated sludge 27-28

2.7 Methods for determining species diversity in natural environments 28-46

2.7.1 Genotypic approaches to determining species diversity 29-31

2.7.2 Phenotypic approaches to determining species diversity 31-32

2.7.3 Community based carbon source utilization patterns using the Biolog system 32-46

2.8 Summary 46-48

References

CHAPTER 3: BIOLOG FOR THE DETERMINATION OF DIVERSITY IN ACTIVATED SLUDGE SYSTEMS

3.1 Abstract 66

3.2 Introduction 66-70

3.3 Materials and Methods 70-71

3.4 Results and Discussion 72-98

References

CHAPTER 4: COMPARISON OF TWO KINDS OF BIOLOG MICROPLATES (GN AND ECO) IN THEIR ABILITY TO DETERMINE THE MICROBIAL COMMUNITY DYNAMICS IN ACTIVATED SLUDGE SYSTEMS

4.1 Abstract 104

4.2 Introduction 104-105

4.3 Materials and Methods 106-107

4.4 Results and Discussion 108-110

References

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UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

CHAPTER 5: CONCLUSIONS

LIST OF ABBREVIATIONS:

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

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VLKP: Vlakplaats Waste Water Treatment Plant

ZKG: Zeekoegat Waste Water Treatment Plant

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UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

LIST OF TABLES

p

TABLE 3.1: **Comparison of similarity amongst the dilutions of the anaerobic zone within the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein activated sludge systems samples inoculated into Biolog GN microplates** 86

TABLE 3.2: **Comparison of similarity amongst the dilutions of the anoxic zone within the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein activated sludge systems samples inoculated into Biolog GN microplates** 88

TABLE 3.3: **Comparison of similarity amongst the dilutions of the aerobic zone within the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein activated sludge systems samples inoculated into Biolog GN microplates** 90

TABLE 3.4: **Comparison of similarity amongst the dilutions of the anaerobic zone within the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein activated sludge systems samples inoculated into Biolog GP microplates** 92

TABLE 3.5: **Comparison of similarity amongst the dilutions of the anoxic zone within the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein activated sludge systems samples inoculated into Biolog GP microplates** 94

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

TABLE 3.6:	**Comparison of similarity amongst the dilutions of the aerobic zone within the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein activated sludge systems samples inoculated into Biolog GP microplates**	96
TABLE 3.7:	**Chemical analysis of Heidelberg, Vlakplaats, Tsakane and Hartebeesfontein activated sludge system at the time of sampling obtained from ERWAT**	97
TABLE 4.1:	**The twenty-five common carbon substrates within Biolog GN and ECO plates**	107

LIST OF FIGURES

p

Figure 3.1: Percentage C-sources utilized in Biolog GN and GP microplates after inoculation with activated sludge from the anaerobic zones at the Heidelberg (HDBL), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein (HBFT) water treatment plant 73

Figure 3.2: Dendrogram of the functional diversity of the microorganisms present in 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 74

Figure 3.3: Dendrogram of the functional diversity of the microorganisms present in 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 75

Figure 3.4: Dendrogram of the functional diversity of the microorganisms present in 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 76

Figure 3.5: Dendrogram of the functional diversity of the microorganisms in 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 77

Figure 3.6: Dendrogram of the functional diversity of the microorganisms present in the 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 78

Figure 3.7: Dendrogram of the functional diversity of the microorganisms present in 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 79

Figure 3.8: Dendrogram of the functional diversity of the microorganisms present in 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 80

Figure 3.9: Dendrogram of the functional diversity of the microorganisms present in 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 81

Figure 3.10: Dendrogram of the functional diversity of the microorganisms present in the Heidelberg (HDLB) water treatment plant afterinoculation 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 82

Figure 3.11: **Dendrogram of the functional diversity of the microorganisms present in 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** 83

Figure 3.12: **Dendrogram of the functional diversity of the microorganisms present in 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** 84

Figure 3.13: **Dendrogram of the functional diversity of the microorganisms present in 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 cannot be compared to each other** 85

Figure 3.14: **Dendrogram indicating the microbial community structure of the anaerobic zone (Anaer) of the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein (HBFT) waste water works samples inoculated into Biolog GN microplates** 85

Figure 3.15: **Dendrogram indicating the microbial community structure of the anoxic zone (Anox) of the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein (HBFT) waste water works samples inoculated into Biolog GN microplates** 87

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Figure 3.16: **Dendrogram indicating the microbial community structure of the aerobic zone (Aer) of the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein (HBFT) waste water works samples inoculated into Biolog GN microplates** 89

Figure 3.17: **Dendrogram indicating the microbial community structure of the anaerobic zone (Anaer) of the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein (HBFT) waste water works samples inoculated into Biolog GP microplates** 91

Figure 3.18: **Dendrogram indicating the microbial community structure of the anoxic zone (Anox) of the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein (HBFT) waste water works samples inoculated into Biolog GP microplates** 93

Figure 3.19: **Dendrogram indicating the microbial community structure of the aerobic zone (Aer) of the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein (HBFT) waste water works samples inoculated into Biolog GP microplates** 95

Figure 4.1: **Dendrogram indicating the similarity between carbon source utilization patterns of the twenty-five common carbon substrates within Biolog GN and ECO plates (TABLE 4.1)** 109

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SUMMARY

Diversity of micro-organism communities in activated sludge have been analyzed by culture -dependent methods, which exclude the majority of endogenous microbes due to the selective nature of the media. Molecular and biochemical techniques have been evaluated, but they are time - consuming, complex and the results are difficult to interpret. Methods such as community level carbon source utilization patterns (i.e. Biolog) are easy to use and detect different patterns, which could be related to diversity and function, in this and other studies.

Our aim was 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, therefore, lies in the interpretation of the results.

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. In this study the Biolog system was not considered as a culture - dependent method, but rather as a collection of metabolic tests (database) used for the purpose of generating a recognizable pattern for a specific community. Our hypothesis was that microbial community level carbon source utilization could be used to determine diversity and evenness in activated sludge systems.

In our study we used 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.

Our study of the different zones of the activated sludge systems tested indicated a high initial

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diversity, due to the high number of substrates utilized. In this study it was observed that phosphate removal could not be related to the functional diversity of the aerobic heterotrophic microbial community as determined on the basis of substrate utilization (Biolog).

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DIE BEPALING VAN MIKROBE SPESIE DIVERSITEIT EN EWEREDIGE VERSPREIDING IN GEAKTIVEERDE SLYK SISTEME DEUR GEBRUIK TE MAAK VAN VERSKILLENDE BIOLOG SISTEME

deur

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OPSOMMING

Die diversiteit van mikrobe gemeenskappe in geaktiveerde slyk is geanaliseer deur kwekings -afhanklike metodes, wat die meerderheid van die mikrobes uitskakel weens die selektiwiteit van die medium wat gebruik word. Molekulêre en biochemiese tegnieke is gebruik, maar die tegnieke is tydrowend, kompleks en die resultate is moeilik om te interpreteer. Metodes soos gemeenskap vlak koolstofbron benuttings patrone (bv. Biolog) is maklik om te gebruik en die verskillende patrone kan herlei word na diversiteit en funksie, in hierdie en ander studies.

Ons doel was nie om elke metaboliese reaksie van al die individue in die gemeenskap waar te neem nie, maar om die gemeenskaplike patroon vir 'n spesifieke gemeenskap te bepaal. Aangesien 1) 'n hoë spesies diversiteit sal lei tot 'n hoër relatiewe aantal substrate wat benut word, omdat daar meer moontlikhede is en 2) dat tydens verdunning, sommige organismes uit verdun sal word (wat 'n verlaging in spesies diversiteit veroorsaak) uit die gemeenskap, afhangend van hul getalle en die relatiewe bydra (miskien slegs een metaboliese reaksie in die sisteem), wat die aantal moontlikhede verlaag. Die gevolg van die verlaging van die moontlikhede tydens verdunning, behoort teoreties iets omtrent die gemeenskap struktuur te reflekteer. Die sleutel lê dus in die interpretasie van die resultate.

Die Biolog sisteem anders as tradisionele kwekings - tegnieke, wat normaalweg selektief is vir die komponent van die gemeenskap wat gekweek moet word, kan die aktiwiteite van 'n wye reeks van bakterieë aangedui word. In die studie was die Biolog sisteem nie beskou as 'n kwekings - afhanklike tegniek nie, maar eerder as 'n versameling van metaboliese toetse (databasis) wat gebruik word vir die daarstelling van 'n waarneembare patroon vir 'n spesifieke gemeenskap. Ons hipotese was dat mikrobe gemeenskapvlakkoolstofbronbenutting gebruik kan word om die diversiteit en eweredige verspreiding van organismes in geaktiveerde slyk mee te bestudeer.

In ons studie waar geaktiveerde slyksisteme verteenwoordigend was van 'n omgewing met 'n hoë spesies diversiteit en onewe verspreiding van spesies, het ons resultate aangetoon dat met verdunning sommige van die substrate nie meer benut word nie, weens die verlies van sekere spesies.

CHAPTER 1

INTRODUCTION

The activated sludge treatment system uses undefined populations of microorganisms to treat a variety of wastewaters. The conditions in a reactor are manipulated to promote the presence and activity of desirable organisms, such as ammonia oxidisers and to discourage the growth of undesirable organisms such as the bacteria associated with foaming. The design and maintenance of such systems is, in effect, the engineering of the microbial populations and their activities.

Unfortunately, it is difficult to engineer something that can not be measured. Consequently, the explicit engineering of microbial populations has been impeded by the crude, labour - intensive and inadequate nature of the methods available to quantify and identify bacteria : suspended solids measurements, morphological examination and culturing of the bacteria (Bitton, 1994). The technological limitations of the available methods have undoubtedly contributed to the difficulties surrounding key areas such as the control of foaming, the organisms responsible for phosphorus removal and the estimation of growth rates for nitrifiers.

The lack of effective methods for studying the distribution of microbial communities in their natrural environments has therefore limited the understanding of the activated sludge process (Griffiths *et al.*, 1997). Methods based on describing the distribution of individual micro-organisms are time-consuming and biassed due to their reliance on culturing techniques

(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.

Garland and Mills (1991) used an approach, which involved direct inoculation of environmental samples into Biolog microplates and use of the resulting response to describe differences in microbial communities. Standardized Biolog microplates allow the simultaneous testing of 95 diagnostically significant carbon sources; with one reference well containing no carbon source (Biolog Inc., Hayward, CA). The Biolog 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. The key therefore lies in the interpretation of the results.

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 *et al.*, 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).

Our aim was 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.

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 was any differences in the effectiveness of these techniques for studying activated sludge system microbial community structure dynamics.

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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).

Microorganisms (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 microorganisms (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). Microorganisms 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).

2.2 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) (Hurlbert, 1971). According to Odum (1971) climax 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 can serve as an indicator of stress conditions (Atlas *et al.*, 1991).

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).

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). Studies of bacterial species diversity differ in the: method of isolation; mode of characterization; clustering methods used for grouping or identification; the 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 characterized areas where the intensity of one or more ecological factor approached extremes that could support life (Atlas, 1984). 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 were used by the microbial community (Zak *et al.*, 1994).

Conditions that were 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 reacted differently to various stress conditions (Brözel and Cloete, 1993).

Mature ecosystems are complex and the high species diversity results in numerous interspecies relationships (Atlas and Bartha, 1993). 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).

2.3 Koch's postulates for microbial ecology

The Koch's postulates (1884) have not been applied to the study of the ecology of microorganisms. This is probably because it has not yet been possible to inoculate a system with a pure culture to 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 for application in microbial ecology as follows:

1) A particular microorganism must always be associated with a certain phenomenon;
2) the same function(s) observed in the natural habitat must also be performed in pure culture;
3) the organism must be enumerated in the natural habitat, isolated and studied in pure

culture;

4) the function must be quantified (activity per cell determined); and

5) the product of the number of individual cells and activity per cell should account for the magnitude of the phenomenon observed in the natural habitat.

2.4 The Activated Sludge Process

The term "activated sludge" refers to an aerobic slurry of microorganisms which can be removed from the process through sedimentation and returned, in quantifiable amounts, to the wastewater stream (Grady and Lim, 1980). The activated sludge system has been developed primarily for Carbon, Nitrogen and Phosphate removal. Barnard (1976) proposed the basic process for simultaneous biological removal of phosphates and nitrogen, known as the Bardenpho or Modified Bardenpho process. The Bardenpho activated sludge process consists of four stages designed mainly for removing of nitrogen, while the five stage systems remove nitrogen and phosphate (Barnard, 1976). Activated sludge systems modified for enhanced phosphate removal during effluent treatment are employed in at least eight countries around the world i.e. Australia, Brazil, Canada, France, New Zealand, South Africa, United States and Zimbabwe (Toerien *et al.*, 1990).

The primary objective of the activated sludge process is the removal of soluble biodegradable compounds. The processes involved in nutrient removal must not only be technically possible, but practical and must be able to meet the effluent criteria in an economical manner (Grady and Lim, 1980).

Some problems with the activated sludge plants:

1) this process is a direct result of its controllability;

2) it requires relatively sophisticated operation in order to achieve the desired results; and

3) the decision to install such a system requires considerable commitment on the part of an industry or municipality (Grady and Lim, 1980).

A high rate of microbial growth is achieved in the presence of nutrients and oxygen (Gray, 1989). Microbial metabolism of the organic matter present results in the production of oxidised end-products such as carbon dioxide, nitrates, sulphates and phosphates, as well as the biosynthesis of new microbial biomass (Gray, 1989; Horan, 1990; Bitton, 1994; Muyima *et al.*, 1997).

Since its inception in the early 20th century, the activated sludge process has been widely adopted and further developed to cope with increased effluent loads and contaminants. The greatest feature of the process has proven to be its flexibility and its ability to deal with the ever increasing stress placed upon it such as biological nutrient removal (BNR) operations. There exist different types of activated sludge systems, the most basic type will be discussed.

2.4.1 Anaerobic Zone

The anaerobic zone's main function is to establish a facultatively anaerobic microbial community. This bacterial community produce compounds such as ethanol, acetate and succinate, which serves as carbon sources for phosphate-accumulating bacteria during

anaerobiosis (Cloete and Muyima, 1997).

The anaerobic zone is essential for the removal of phosphate, because the bacteria in the activated sludge passing through this zone are preconditioned to take up excess phosphate under aerobic conditions (Cloete and Muyima, 1997). The release of a certain quantity of phosphate from the biomass into the solution indicates that the bacteria have been suitably conditioned for phosphate uptake within the aerobic zone (Pitman, 1984). The presence of nitrate in an anaerobic zone interferes with the phosphate removing capability of the activated sludge during aerobiosis (Barnard, 1976; Marais *et al.*, 1983). This may be due to competition for substrate between phosphate-accumulating and denitrifying organisms. In the presence of nitrate, the redox potential is too high to produce fatty acids for the release of phosphate (Cloete and Muyima, 1997).

The microorganisms found in the anaerobic zone are normally from soil and water environments and are capable of fermentation (species of *Aeromonas, Citrobacter, Klebsiella, Pasteurella, Proteus* and *Serratia*) (Fuhs and Chen, 1975; Buchan, 1984). These microorganisms accumulate and produce organic compounds such as lactic acid, succinic acid, propionic acid, butyric acid, acetic acid and ethanol during fermentation (Fuhs and Chen, 1975; Buchan, 1984). These organic compounds serve as electron donor and acceptor, but cannot be utilized under anaerobic conditions. Therefore it seems as though the anaerobic zone provides substances for the reproduction and growth of aerobic phosphate-accumulating bacteria (Fuhs and Chen, 1975; Buchan, 1984).

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2.4.2 Anoxic Zone

Anoxic refers to the presence of nitrates and the absence of dissolved oxygen (Buchan, 1984; Pitman, 1984; Streichan *et al.*, 1990). The primary anoxic zone is the principal denitrification reactor in the activated sludge process. The primary anoxic zone is fed by effluent from the anaerobic zone and by mixed liquor recycled from the aerobic zone (Cloete and Muyima, 1997). The presence of nitrate and nitrite in the absence of oxygen leads to the enrichment of denitrifying microorganisms, which reduce nitrate or nitrite to molecular nitrogen. It was found that phosphate release were induced in the anoxic zone in the presence of low fatty acids or their salts such as acetate, formate and propionate (Gerber *et al.*, 1986). Phosphate release was not only affected by the presence of the substrate but also by the substrate concentrations of soluble, readily biodegradable carbon substrates (Cloete and Muyima, 1997).

2.4.3 Aerobic Zone

The main function of the primary aerobic zone is to oxidize organic material in the sewage and to oxidize ammonia to nitrite and then to nitrate (by chemoautotrophs) (Cloete and Muyima, 1997). The oxidizetion needs to take place in order to provide an environment in which the biomass can take up all the phosphate released in the anaerobic zone, plus all the phosphate that enters the process in the sewage (Cloete and Muyima, 1997). Ammonia is oxidized to nitrite by *Nitrosomonas, Nitrosospira* and *Nitrosolobus* spp. (Atlas and Bartha, 1993). Nitrite is oxidized to nitrate by *Nitrobacter, Nitrospira* and *Nitrococcus* spp. (Buchan, 1984; Atlas and Bartha, 1993). Wentzel and colleagues (1985) indicated that when more phosphate is released

in the anaerobic zone, more phosphate gets taken up in the aerobic zone.

The presence of readily biodegradable compounds, especially volatile fatty acids, produced by fermentative bacteria from organic compounds in the influent also play a role in enhanced phosphate removal (Cloete and Muyima, 1997). These volatile fatty acids are removed during the anaerobic phase and polymerized at the expense of energy obtained from the breakdown of polyphosphate (Cloete and Muyima, 1997). Poly-ß-hydroxybutyric acid (PHB) plays an important role in the mechanism of phosphate uptake and release (Nicholls and Osborn, 1979; Comeau *et al.*, 1986).

2.5 Phosphate removal in activated sludge

Phosphorus is present in wastewater in both the inorganic and organic forms (Toerien *et al.*, 1990). 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 standard (< 1 mg ortho phosphate.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 required (Toerien *et al.*, 1990; Bitton, 1994).

Biological phosphorus removal can be performed by a rather broad group of microorganisms, the Phosphate Accumulating Organisms (PAO's) (Henze, 1996). The mechanism of enhanced phosphorus removal in activated sludge systems must therefore, depend on a group of

organisms which in nature are favoured by fluctuating conditions of aerobiosis - anaerobiosis (Toerien *et al.*, 1990).

2.5.1 The role of biomass in phosphate removal

Under favourable conditions, phosphate removing sludge is observed to take several sludge ages to develop (Bond *et al.*, 1995). This suggests that phosphorus removing bacterial community may need to be established and may not occur merely as a result of conditioning of the existing population (Henze, 1996).

Bacteria grown in low nutrient environments, accumulated 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; Henze, 1996). Under conditions of increased nutrient (PO_4^{3-}) availability, total phosphate removal increased, possibly due to the increase in biomass (Bosch, 1992; Momba, 1995; Muyima, 1995). Wentzel and colleagues (1985) indicated that when more phosphate is released in the anaerobic zone, more phosphate gets taken up in the aerobic zone. This suggested that total biomass may be more important in terms of phosphate removal, than individual species or specific populations.

The heterotrophic biomass mediates the biodegradation process of the chemical oxygen demand (COD) removal and denitrification (Ubisi *et al.*, 1997). Thus, the rates of these processes are directly related to the heterotrophic active biomass present and the specific rates should be

expressed in terms of this parameter to allow a meaningful comparison of the rates measured in different systems (Dold *et al.*, 1980; Ubisi *et al.*, 1997). However, the heterotrophic active biomass parameter has been hypothetical and has not been measured directly, primarily due to the lack of suitable simple measurement techniques.

2.5.2 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 system; 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 are qual to 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 α-glucosidase is higher under aerobic incubation as compared to anaerobic incubation (Goel *et al.*, 1998). Protease activities seem to be marginally affected by the electron acceptor conditions (Goel *et al.*, 1998). Protease activities were observed to be stable (Goel *et al.*, 1998). 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 α-glucosidase activities were approximately calculated to be 46 h and 1 h, respectively (Goel *et al.*, 1998).

2.5.3 Phosphate removing bacteria

In enhanced biological phosphate removal (EBPR), groups of polyphosphate - accumulating bacteria are enriched in the activated sludge recycling of the sludge in anaerobic and aerobic zones. The polyphosphate - accumulating bacteria are able to take up the fermentation products viz., acetic, lactic, succinic, propionic and butyric acids, and store them as intracellular carbon and energy reserves ie., poly-β-hydroxyalkonoates (PHA's) (Satoh *et al.*, 1992; Lilley *et al.*, 1997). As a result of internal carbohydrate accumulation, the Polyphosphate - accumulating bacteria release phosphate into the sludge and the ortho-P concentration in the anaerobic zone increases (Wentzel, 1992). In the aerobic zone the polyphosphate - accumulating bacteria utilise the internally stored carbon for growth which increases their biomass in the sludge (Wentzel, 1992). Phosphate removal, together with an increase in the amount of polyphosphate

- accumulating bacteria in the system, leads to a "total" removal of phosphate from the activated sludge system (Wentzel, 1992). Soluble phosphate, accumulated as biological poly-phosphate in the solid phase, is then removed from the system with the waste sludge.

Uptake of phosphate in the aerobic zone is directly related to the amount of phosphate released in the anaerobic zone. The amount of phosphate, which can be removed by bio-phosphate activity is directly coupled to the amount of volatile fatty acid (VFA) that the polyphosphate accumulating bacteria remove in the anaerobic tank (Henze, 1996).

Although the process of EBPR is technically well understood, it remains difficult to achieve consistent and reproducible removal rates at full-scale due to the lack of understanding of the biochemical and microbiological processes (Satoh *et al.*, 1996; Wang and Park, 1998). Without understanding the correlation between the polyphosphate - accumulating bacteria community structure within activated sludge systems and the activated sludge system's performance, reproducable and effective biological phosphorus removal will remain difficult to design.

2.6 Microbial ecology of activated sludge

According to Grady and Lim (1980), the expression "activated sludge" refers to a slurry of microorganisms that removes organic compounds from wastewater and these microorganisms were 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 phagothrophic protozoa and microscopic metazoa (Cloete and Muyima, 1997).

The microbial population of activated sludge consists of 95% decomposers, pre - dominantly bacteria (Cloete and Muyima, 1997). The bacteria indicated that the function of microfauna in the removal of organic pollution and nutrients was not significant (Cloete and Muyima, 1997). In studies conducted on activated sludge water, attention had been directed toward the aerobic heterotrophs, since they were 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.

2.7 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 efficiently cope with the diversity and complexity of real environmental systems. Therefore,

a need exists for non - culturing techniques to evaluate the diversity, equitability and abundance of microorganisms in their natural environment.

2.7.1 Genotypic approaches to determining species diversity

Genotypic diversity measured 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), stated 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, created a need for molecular techniques and especially nucleic acid analysis has 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, such as DNA - reassociation, allows assessment of the total genetic 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 (Muyzer *et al.*, 1993). 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. 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).

Analysis of phospholipid fatty acids has proven to be a powerful tool for structural analysis of microbial communities (Buyer and Drinkwater, 1997). Phospholipids are extracted and purified from environmental sample and then fatty acids are liberated by saponification and esterified. The resulting fatty acid methyl esters are identified by gas chromotography. The method does not require growth in culture, so it is non-selective (Buyer and Drinkwater, 1997). Certain taxonomic groups may be identified by specific phospholipid fatty acids, although as further taxa are tested by this method we may find that fatty acids believed to belong to only one group may also occur in another group (Buyer and Drinkwater, 1997). The phospholipid fatty acid analysis is much more technically demanding, which has tended to restrict its use

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(Buyer and Drinkwater, 1997).

2.7.2 Phenotypic approaches to determining species diversity

Phenotypic diversity determinations included only the culturable (10%) species (Torsvik *et al.*, 1990). It involved the expression of genes under a given set of conditions (Torsvik *et al.*, 1996). Physiological diversity was based on cytological features (i.e. cell size) and which can be determined by cell micromorphology and motility (O'Brien & 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 tests 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 was that bacterial strains had to be isolated from the biotype and pure cultures cultivated in laboratories before phenotypic testing could 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).

2.7.3 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.

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 were 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), Gram positive isolates (GP Biolog plates), where 62 of the substrates are common to GN and GP plates, and each has 33 unique substrates (Konopka *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). Biolog (Biolog Inc., Hayward, CA) also have special purpose microplates i.e. ecoligical (ECO) microplates containing three identical sets of 31 carbon sources and are used to monitor and study changes in microbial communities. 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).

In all previous environmental studies employing Biolog microplates, the Gram - negative (GN) plates have been used. Because these plates were designed to identify bacterial isolates, it is not yet clear whether they optimally characterize heterotrophic communities. In response to the scientific community's interactions with Biolog, Inc. (Insam, 1997), the company now produces "ecology" (ECO) plates, which contain a greatly reduced number of substrates compared to the GN plates (31 vs. 95), but provide within-plate replication of those substrates. Choi and Dobbs (1999) have compared the GN and ECO plates to determine whether the two kinds of plates were equally effective in distinguishing among heterotrophic bacterial communities from a variety of aquatic environments. Inter-plate differences in utilization of the common substrates are attributed to differences in the nutrient regime experienced by the inoculated bacteria. The Biolog GN and ECO plates demonstrated an equal capacity to discriminate among the heterotrophic expressions of the microbial communities (Choi and

Dobbs, 1999).

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 and/or automatically (590 nm).

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

a) cooperative 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).

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 and 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; Heuer and Smalla, 1997). Community - level physiological profiles (CLPP) have been used to characterize microbial communities in freshwater, activated sludge, coastal and Lagoon areas, sediments as well as soil, rhizosphere, bioreactors and gnotobiotic mixtures (Fredrickson *et al.*, 1991; Garland and Mills, 1991; Gorlenko and Kozhevin, 1994; Haack *et al.*, 1994; Winding, 1994; Zak *et al.*, 1994; Bossio and Scow, 1995; Ellis *et al.*, 1995; Lehman *et al.*, 1995; Vahjen *et al.*, 1995; Garland, 1996 (a); Garland, 1996 (b); Guckert *et al.*, 1996; 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; Heuer and Smalla, 1997). 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 has been useful in distinguishing heterotrophic microbial communities within wastewater treatment systems. More diluted inocula resulted 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 allowed 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 (Biolog, Inc, Hayward, CA). 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 was its ease and 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} used 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 seemed 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 persisted 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 substrates used may only reflect the functional characteristics of organisms that were 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).

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 fingerprints) 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 a) reading intervals, such as 24 h readings versus actual 22 h to 26 h readings and b) 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 manufacturer's 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 seemed 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 persisted 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 be 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.

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 were 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), stated 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 μl 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 $cfu.ml^{-1}$. 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 were 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 average well colour density (AWCD) with inoculum density rather than activity. The number of stained cells at a given incubation was 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 suggested that the inoculum contained 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 indicated 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 iii) transformation of the data to account for different inoculum densities (Kersters *et al.*,

1997).

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 was 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, 1996(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 allowed 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 was unimportant for purposes of classification, but it was 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, 1996).

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 was 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, were versatile with respect to the ability to use different carbon sources and 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 did 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).

The following must 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; and

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).

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, equitability, abundance and function. In this study, inoculating Biolog GN - , GP - and ECO - microwell plates, with different dilutions of microbial communities were determined and the resulting carbon source profiles were compared to indicate diversity and evenness in activated sludge systems.

Previous studies done by Korf (1998) using a sample where *Serratia marcescens* was used in order to simulate dominance, as might be expected in an extreme environment, 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, Korf (1998) 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.

Korf (1998) indicated that 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 was an extreme environment (Korf, 1998).

2.8 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). In this study the latter would be investigated and partially explained for activated sludge systems.

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CHAPTER 3

BIOLOG FOR THE DETERMINATION OF MICROBIAL DIVERSITY IN ACTIVATED SLUDGE SYSTEMS.

3.1 ABSTRACT

In this study, different carbon source profiles were generated by inoculating Biolog GN and GP microwell plates, with different dilutions of microbial communities from a number of activated sludge systems. This led to the successful generation of patterns reflecting diversity and evenness in the different systems. The high number of substrates utilized at the lower dilutions (10^{-1} and 10^{-2}) indicated a high functional diversity in the community, but not necessarily evenness of the functionality. Functional evenness of each species was reflected upon further dilution. Our results indicated differences in the functional diversity of the microbial communities amongst some of the activated sludge systems studied. These differences were not specifically related to phosphate removing and non phosphate removing systems.

3.2 INTRODUCTION

The majority of microorganisms in their natural habitat cannot be cultured and remain unidentified (Cloete *et al.*, 1992; Haldeman and Amy, 1993; Wagner *et al.*, 1993). This has led to a lack of knowledge of 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 (Pace *et al.*, 1986; 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). The techniques are also qualitative and not quantitative (Muyima *et al.*, 1997). 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.

In search of these techniques, microbial communities can be considered as systems containing information (data), which is related primarily to microbial diversity. The higher the diversity, the more information. Current attempts, such as molecular techniques, have tried to achieve the translation of this information into meaningful data (Ehlers, 1995). Molecular techniques rely on information in a microbial community, extracted as molecules, related to the genome of the different members of the community (Torsvik *et al.*, 1990). This would indicate genetic complexity, which could again be related to microbial diversity in the system. A more simplistic and effective method is however required to achieve this translation and interpretation of data.

In any given system one or more species exists 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 to functional diversity. The hypothesis being that the more substrates utilized the higher the diversity due to the collective

action of individual species.

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 micro - organisms 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 was therefore 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. 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

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Biolog method can reflect the activities of a broad range of bacteria (Zak *et al.*, 1994). We therefore do not consider the Biolog system as a culture - dependent method, but rather as a battery of metabolic tests to use for generating a database for a specific community as discussed.

Therefore, this technique could be useful for understanding the biological phosphorus removal phenomenon in activated sludge systems. Previous work, has indicated that phosphorus removal was not as a result of a specific microbial community composition, but rather related to the total biomass in the aerobic zone of a system (Mills and Wasel, 1980; Ehlers, 1998). Our hypothesis was that microbial community level carbon source utilization could be used to determine functional diversity and evenness in activated sludge systems.

3.3 MATERIALS AND METHODS

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

Sample sources and inoculum preparation. Mixed liquor grab samples (1l) were drawn from the aerobic, anoxic and anaerobic zones of the following activated sludge plants : Heidelberg (3 stage); Vlakplaats (3 stage); Tsakane (3 stage); and Hartebeesfontein (2 stage), Gauteng, South Africa (ERWAT). Chemical analysis for the anaerobic, anoxic and aerobic zones of Heidelberg, Vlakplaats, Tsakane and Hartebeesfontein systems were obtained from East Rand Waterboard (ERWAT) (TABLE 3.1).

Inoculation and incubation of Biolog microplates. Biolog GN and GP microplates were inoculated with undiluted and diluted activated sludge samples from Heidelberg, Vlakplaats, Tsakane and Hartebeesfontein waste water works. All the microbial activated sludge suspensions were diluted with sterile saline (0.85 % NaCl) and used as inoculum for Biolog GN and GP microwell plates. Biolog GN and GP microplate wells were inoculated with 150 μl of the activated sludge suspensions. Microplates were incubated in the dark at 21 ^{0}C without agitation. Colour formation in the individual cells of the microtiter plates was measured with an Anthos reader 2001 (Anthos Labtec Instruments) at 590 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.

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 was directly stored on a computer in the Bionum version 1.1 for Windows (Applied Maths, Kortrijk, Belgium) as "0" (negative) and "1" (positive) to be calculated according to the Simple matching coefficient (S_{SM}) used for determining the correlation between samples (data sets) in the Gelcompare 4.0 program. Analyses and dendrogram constructions were done with the GelCompar 4.0 program (Applied Maths, Kortrijk, Belgium). The program clustered the samples using the unweighted pair group method of arithmetic averages (UPGMA).

3.4 RESULTS AND DISCUSSIONS

Colour development in each well of the GN and GP Biolog microplates reflected the ability of the bacterial community to utilize that specific carbon source. According to Insam *et al.* (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 functional diversity and evenness in activated sludge systems. The activated sludge system was representative of an environment with 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 plants decreased (Fig. 3.1).

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

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of species. It was concluded that the lower the similarity amongst dilutions, the lower the evenness and *vice versa*.

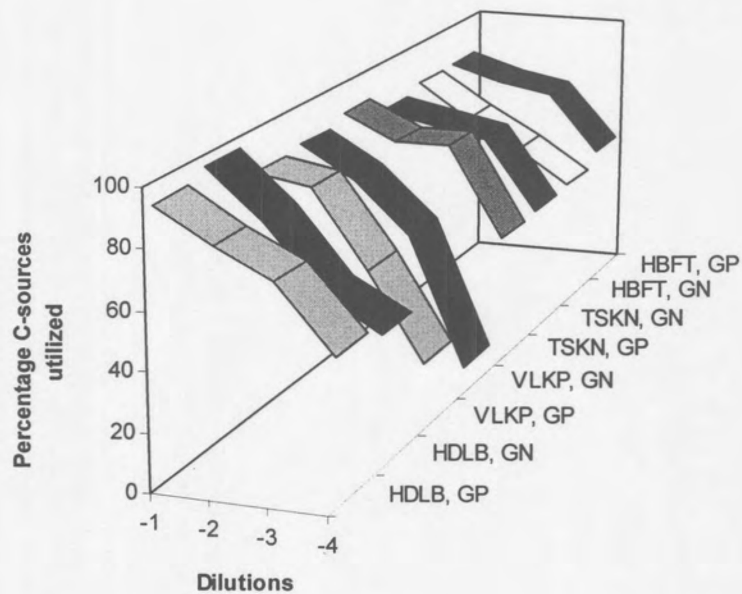

Figure 3.1: Percentage C-sources utilized in Biolog GN and GP microplates after inoculation with activated sludge from the anaerobic zones at the Heidelberg (HDBL), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein (HBFT) water treatment plant.

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

Fig. 3.2 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.

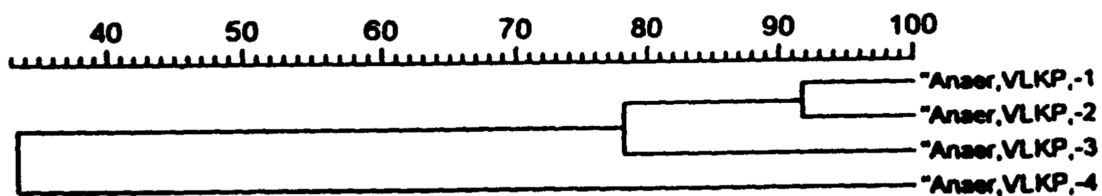

Figure 3.2: Dendrogram of the functional diversity of the microorganisms present in the Vlakplaats (VLKP) water treatment plant after inoculation of a Biolog GN microplate with activated sludge from the anaerobic (Anaer) zone, indicating an uneven distribution of species.

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.

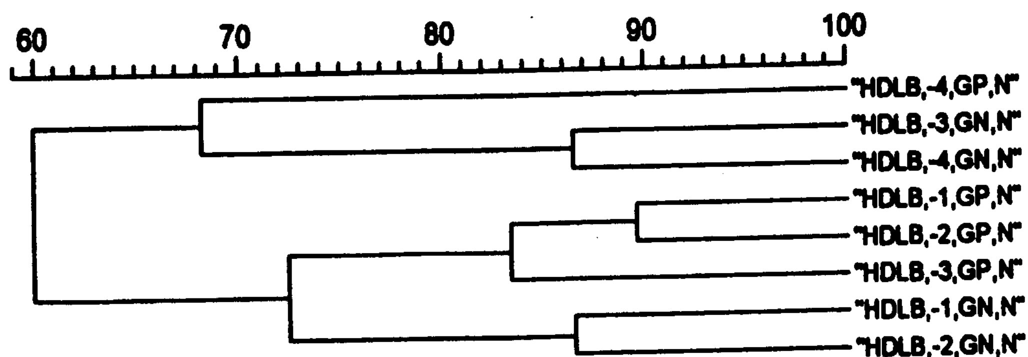

Figure 3.3: Dendrogram of the functional diversity of the microorganisms present in the Heidelberg (HDLB) water treatment plant after inoculation of a Biolog GN and GP microplate with activated sludge from the anaerobic (Anaer) zone, indicating that GN and GP plates cannot be compared to each other.

This indicated a significant difference between the GN and GP profiles. 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.

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.

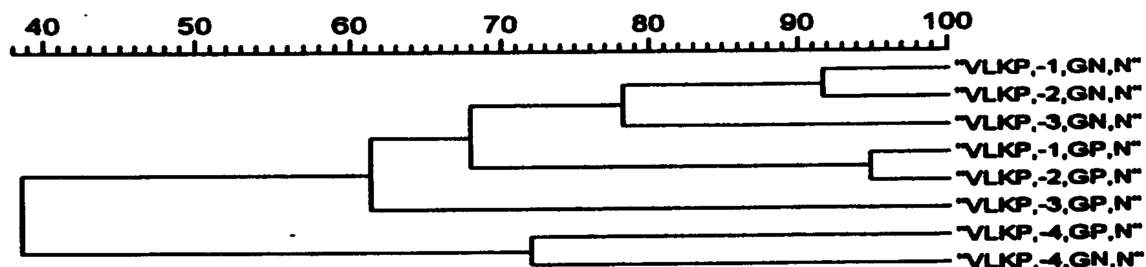

Figure 3.4: Dendrogram of the functional diversity of the microorganisms present in the Vlakplaats (VLKP) water treatment plant after inoculation of a Biolog GN and GP microplate with activated sludge from the anaerobic (Anaer) zone, indicating that GN and GP plates cannot be compared to each other.

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.

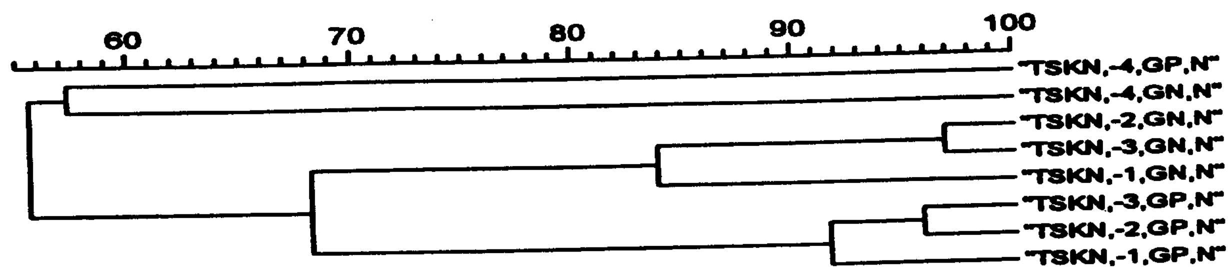

Figure 3.5: Dendrogram of the functional diversity of the microrganisms in the Tsakane (TSKN) water treatment plant after inoculation of a Biolog GN and GP microplate with activated sludge from the anaerobic (Anaer) zone, indicating that GN and GP plates cannot be compared to each other.

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.

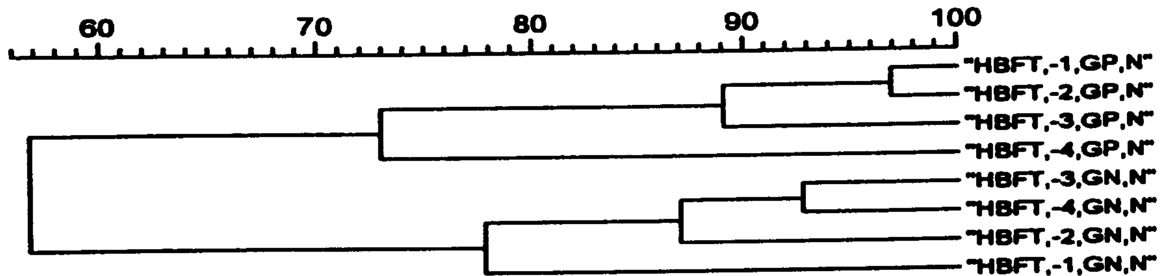

Figure 3.6: Dendrogram of the functional diversity of the microorganisms present in the Hartebeesfontein (HBFT) water treatment plant after inoculation of a Biolog GN and GP microplate with activated sludge from the anaerobic (Anaer) zone, indicating that GN and GP plates cannot be compared to each other.

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.

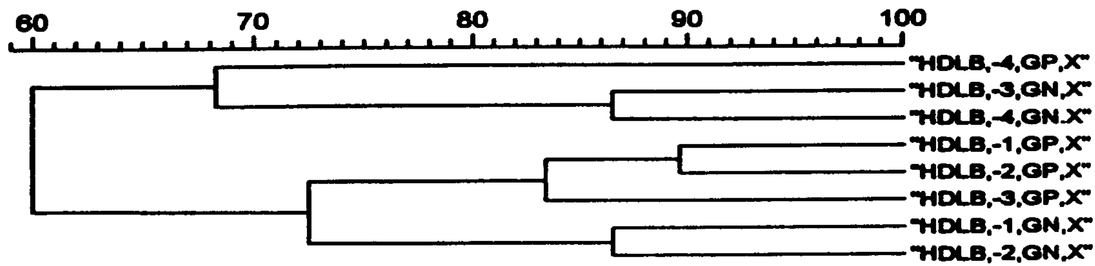

Figure 3.7: Dendrogram of the functional diversity of the microorganisms present in the Heidelberg (HDLB) water treatment plant after inoculation of a Biolog GN and GP microplate with activated sludge from the anoxic (Anox) zone, indicating that GN and GP plates cannot be compared to each other.

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.

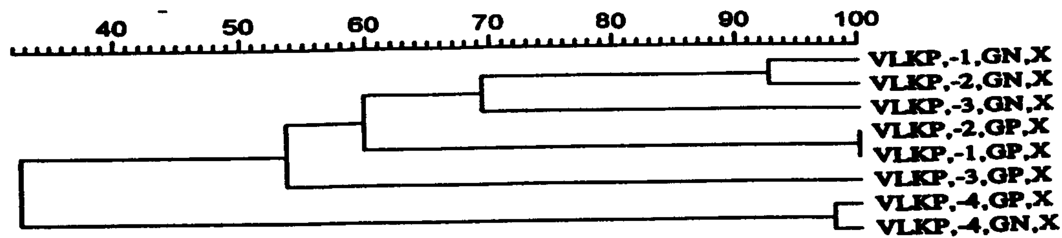

Figure 3.8: Dendrogram of the functional diversity of the microorganisms present in the Vlakplaats (VLKP) water treatment plant after inoculation of a Biolog GN and GP microplate with activated sludge from the anoxic (Anox) zone, indicating that GN and GP plates cannot be compared to each other

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.

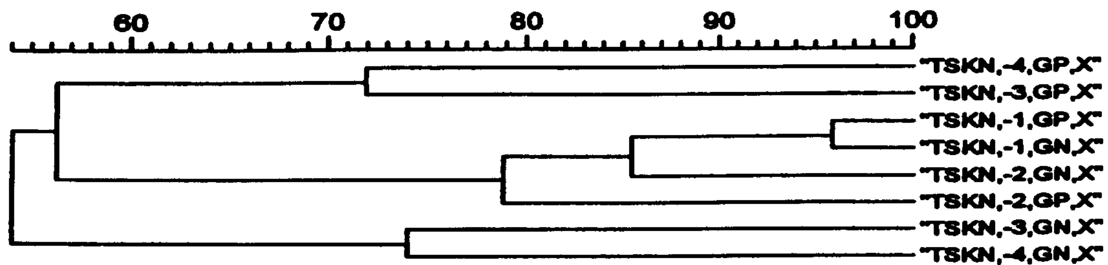

Figure 3.9: Dendrogram of the functional diversity of the microorganisms present in the Tsakane (TSKN) water treatment plant after inoculation of a Biolog GN and GP microplate with activated sludge from the anoxic (Anox) zone, indicating that GN and GP plates cannot be compared to each other

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.

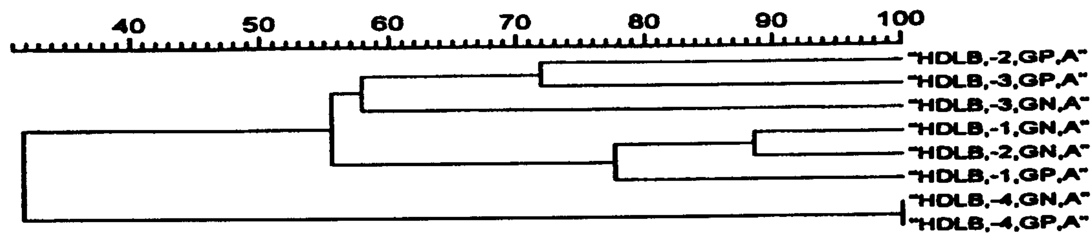

Figure 3.10: Dendrogram of the functional diversity of the microorganisms present in the Heidelberg (HDLB) water treatment plant after inoculation of a Biolog GN and GP microplate with activated sludge from the aerobic (Aer) zone, indicating that GN and GP plates cannot be compared to each other

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.

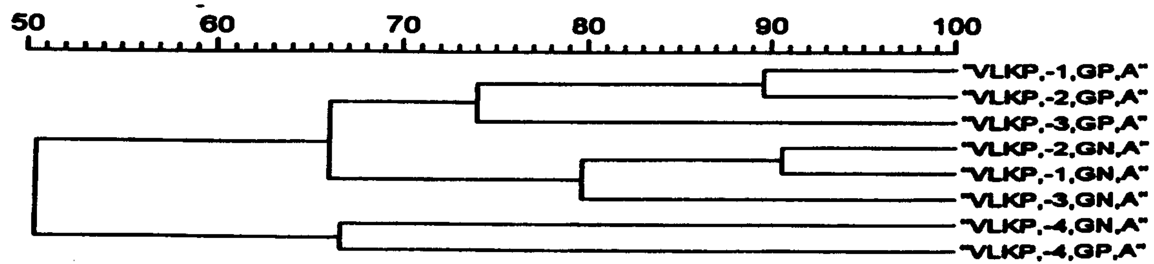

Figure 3.11: Dendrogram of the functional diversity of the microorganisms present in the Vlakplaats (VLKP) water treatment plant after inoculation of a Biolog GN and GP microplate with activated sludge from the aerobic (Aer) zone, indicating that GN and GP plates cannot be compared to each other

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.

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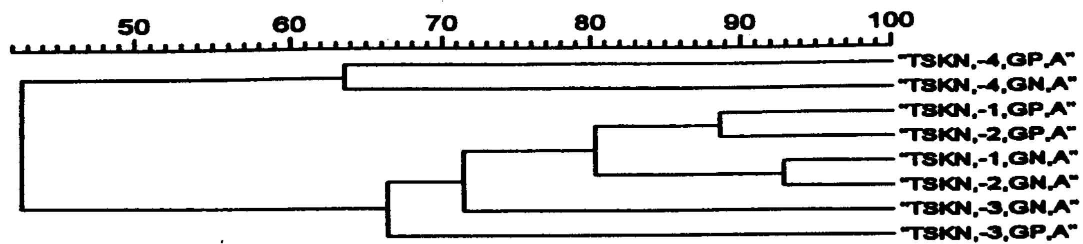

Figure 3.12: Dendrogram of the functional diversity of the microorganisms present in the Tsakane (TSKN) water treatment plant after inoculation of a Biolog GN and GP microplate with activated sludge from the aerobic (Aer) zone, indicating that GN and GP plates cannot be compared to each other

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.

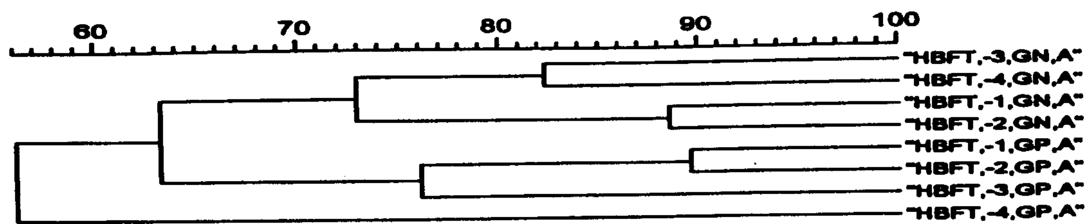

Figure 3.13: Dendrogram of the functional diversity of the microorganisms present in the Hartebeesfontein (HBFT) water treatment plant after inoculation of a Biolog GN and GP microplate with activated sludge from the aerobic (Aer) zone, indicating that GN and GP plates can not be compared to each other

Comparison amongst different dilutions within different activated sludge systems

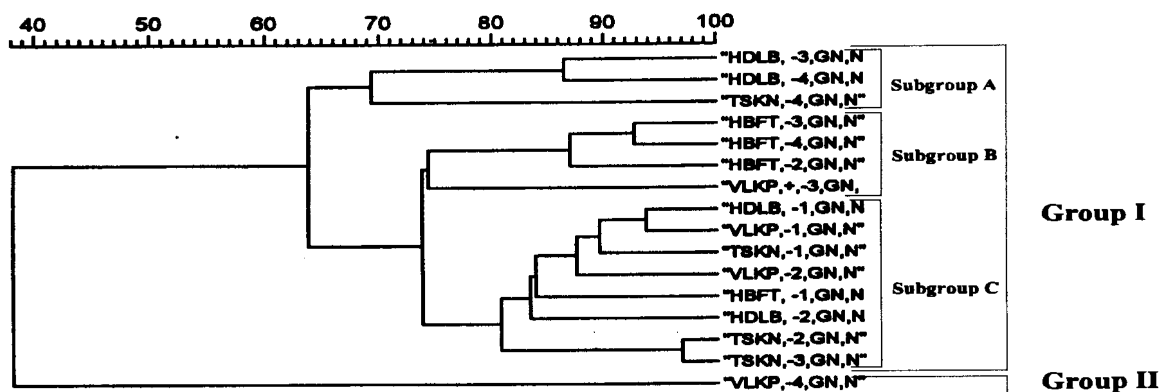

Figure 3.14: Dendrogram indicating the microbial community structure of the activated sludge from the anaerobic zone (Anaer) of the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein (HBFT) waste water works samples inoculated into Biolog GN microplates

The dendrogram in Fig. 3.14 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 3.1: Comparison of similarity amongst the dilutions of the anaerobic zone within the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein (HBFT) activated sludge systems inoculated into Biolog GN microplates

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%

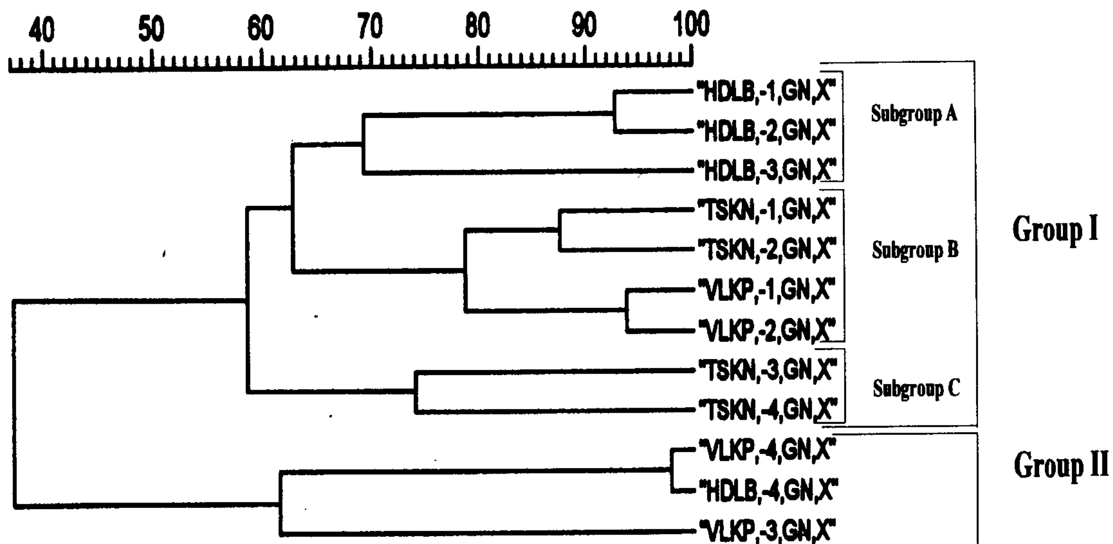

Figure 3.15: Dendrogram indicating the microbial community structure of the activated sludge from the anoxic zone (Anox) of the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein (HBFT) waste water works samples inoculated into Biolog GN microplates

The dendrogram in Fig. 3.15 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 3.2: Comparison of similarity between the dilutions of the anoxic zone within the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein activated sludge system samples inoculated into Biolog GN microplates

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.

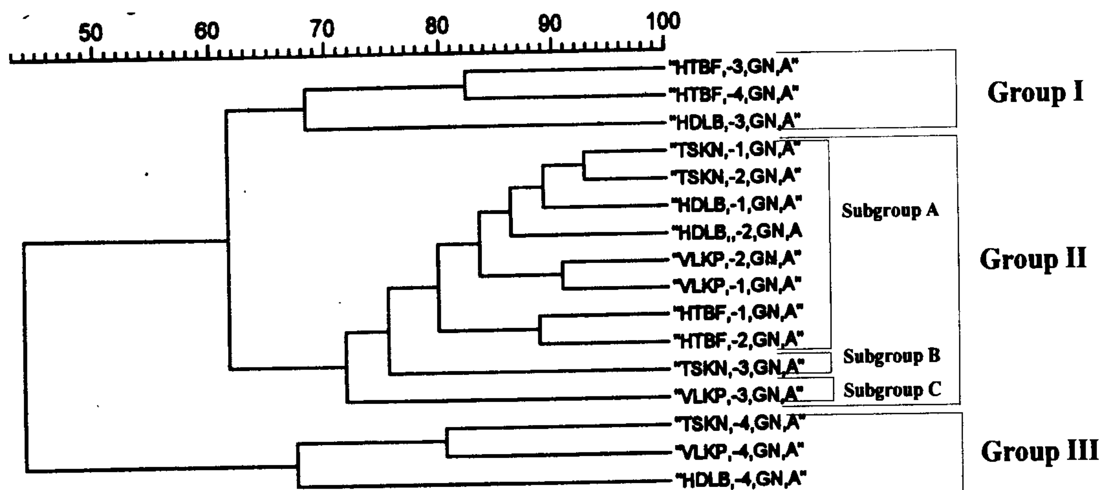

Figure 3.16: Dendrogram indicating the microbial community structure of the activated sludge from the aerobic zone (Aer) of the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein (HBFT) waste water works samples inoculated into Biolog GN microplates

The dendrogram in Fig. 3.16, 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 3.3: Comparison of similarity between the dilutions of the aerobic zone within the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein activated sludge system samples inoculated into Biolog GN microplates

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%

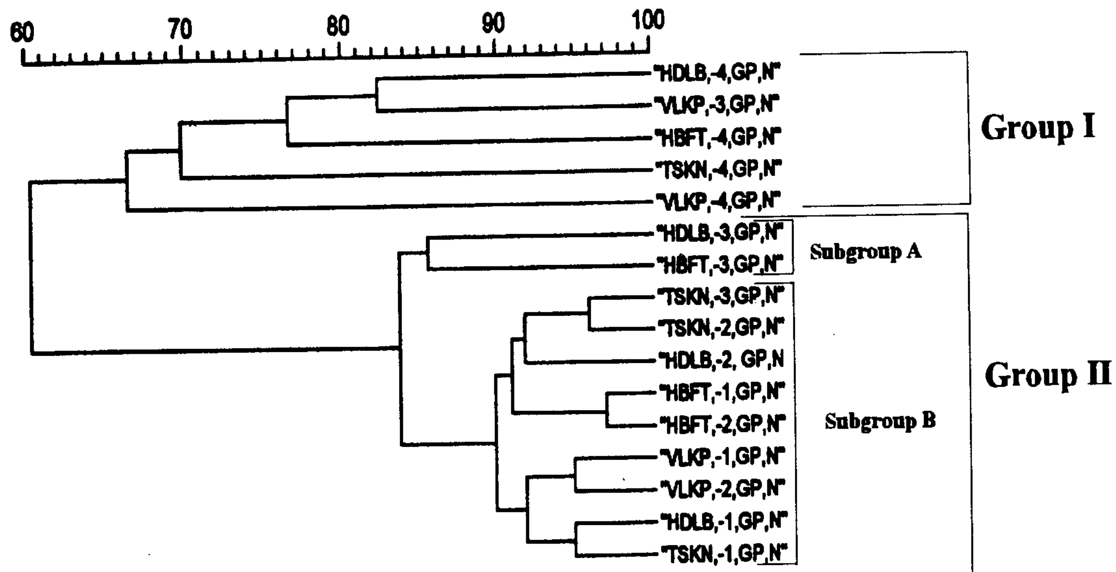

Figure 3.17: Dendrogram indicating the microbial community structure of the activated sludge from the anaerobic zone (Anaer) of the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein (HBFT) waste water works samples inoculated into Biolog GP microplates

The dendrogram in Fig. 3.17 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 3.4: Comparison of similarity amongst the dilutions of the anaerobic zone within the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein activated sludge system samples inoculated into Biolog GP microplates

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%

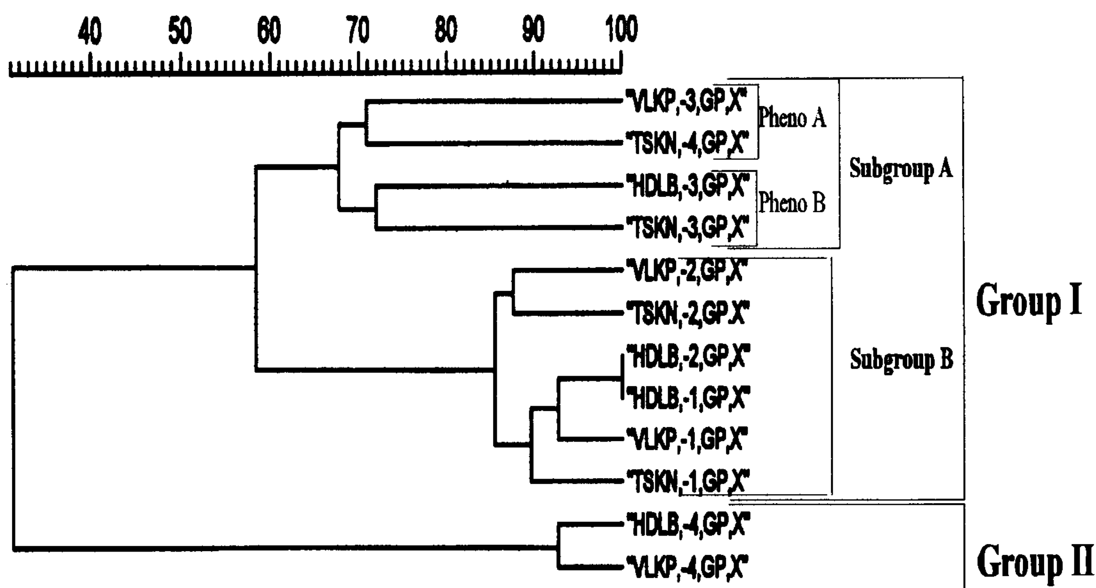

Figure 3.18: Dendrogram indicating the microbial community structure of the activated sludge from the anoxic zone (Anox) of the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein (HBFT) waste water works samples inoculated into Biolog GP microplates

The dendrogram in Fig. 3.18 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 3.5: 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 samples inoculated into Biolog GP microplates

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.

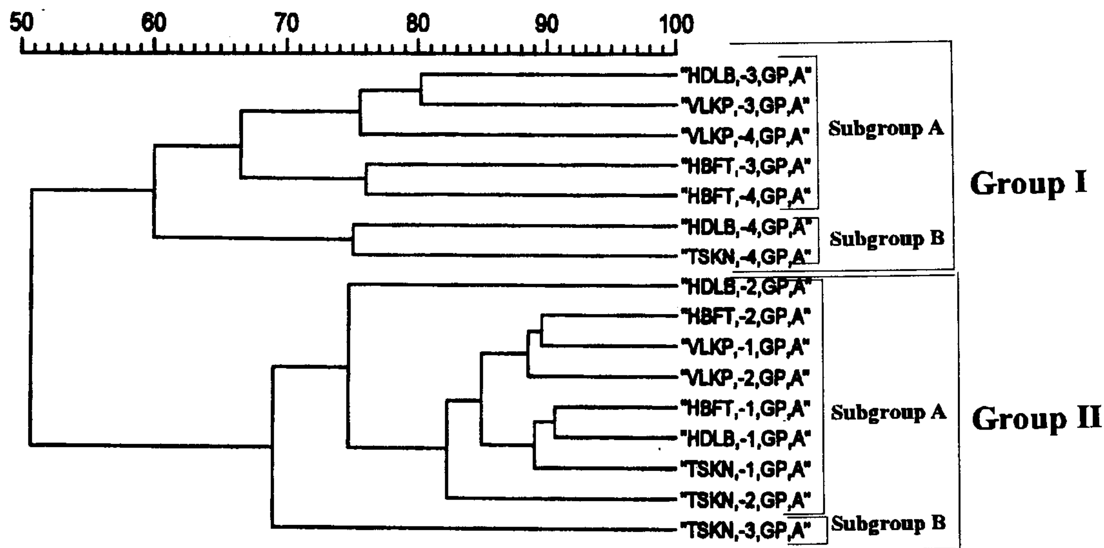

Figure 3.19: Dendrogram indicating the microbial community structure of the activated sludge from the aerobic zone (Aer) of the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein (HBFT) waste water works samples inoculated into Biolog GP microplates

The dendrogram in Fig. 3.19 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 3.6: Comparison of similarity between the dilutions of the aerobic zone within the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein activated sludge system samples inoculated into Biolog GP microplates

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%

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 could not be 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).

Table 3.7: 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	Badenpho	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	Badenpho		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	N	ND	19.7	0.4	2.7	9620
Aerobic	Badenpho	Domestic	D	ND	19.6	1.5	4.3	2710
			N					
			D					

ND = Not determined.

The results in Table 3.7, 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 functionality of the aerobic heterotrophic microbial community which as determined using the Biolog system, as was previously indicated (Mills and Wassel, 1980; Kersters *et al.*, 1997).

Our results support the notion that biomass concentration is more important in PO_4^{3-} removal (Bosch, 1992; Momba, 1995; Muyima, 1995), than community composition determined using the Biolog system. In terms of PO_4^{3-} removal, research should focus on the role of biomass concentration and its relationship to PO_4^{3-} removal and methods to determine the biomass accurately. 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,

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etc. in order to further validate the method. Biolog measures the functionality and not necessarily the community structure, to determine the community structure alternative techniques such as PLFA analysis could be used.

The dendrograms (Fig. 3.3 to Fig. 3.13) indicate 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, GN or GP) 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.

Although these 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).

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CHAPTER 4

COMPARISON OF TWO KINDS OF BIOLOG MICROPLATES (GN AND ECO) IN THEIR ABILITY TO DETERMINE THE MICROBIAL COMMUNITY DYNAMICS IN ACTIVATED SLUDGE SYSTEMS.

4.1 ABSTRACT

Different carbon source profiles were generated by inoculating Biolog GN (95 different substrates) and ECO (31 substrates in triplicate) microwell plates, with different dilutions of microbial communities from a number of activated sludge systems. This led to the generation of metabolic patterns reflecting the degree of diversity and evenness within the different activated sludge systems. The high number of substrates utilized at the lower dilutions (10^{-1} and 10^{-2}) indicated a high microbial diversity in the community, but not necessarily evenness amongst the microbial communities. Evenness of the individual microbial communities was indicated upon further dilution. Our results indicated that the two types of microplates had an equal capacity to detect metabolic patterns for activated sludge microbial communities. Therefore, we concluded that either GN or ECO plates could be used to detect diversity and evenness within activated sludge systems.

4.2 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 microorganisms 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 microorganisms, 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

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the molecular techniques.

Individual level approaches for analyzing microbial communities rely on (a) isolation of microorganisms, (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 microorganisms (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, but rather as a collection of metabolic tests (database) used to generate a recognizable pattern for a specific microbial community. In most previous environmental studies Biolog GN (Gram-negative) microplates have been used. As previously observed by Choi and Dobbs (1999) it is not yet clear whether these plates optimally characterize heterotrophic communities, since they were designed to identify bacterial isolates. For studies of environmental communities Biolog, Inc. now produces ECO ("ecology") plates, which contains a reduced number of substrates compared to the GN plates (31 vs. 95), but provide a replication of those substrates within a single plate (Choi and Dobbs, 1999). To our knowledge, the GN and ECO plates have not been compared for activated sludge water environments. Our aim, therefore was to determine whether the two kinds of 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.

4.3 MATERIALS AND METHODS

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

Sample sources and inoculum preparation. Mixed liquor grab samples (1 L) were drawn from the aerobic zones of the following activated sludge plants: Centurion; Zeekoegat; Baviaanspoort; Rooiwal; and Daspoort, Pretoria, Gauteng, South Africa. All samples were collected in sterile screw-capped bottles (1 L) and samples were analyzed within 12 h after collection.

Inoculation and incubation of Biolog microplates. Biolog GN and ECO plates were inoculated with undiluted and diluted activated sludge samples from Centurion, Zeekoegat, Baviaanspoort, Rooiwal and Daspoort activated sludge systems. All the microbial activated sludge suspensions were diluted with sterile saline (0.85% NaCl) (Korf, 1998) and used as inoculum for Biolog GN and ECO plates. Biolog GN and Eco plate wells were inoculated with 150 μl of the activated sludge suspensions (Kersters *et al.*, 1997; Korf, 1998). Microplates were incubated in the dark at 21°C without agitation (Korf, 1998). Colour formation in the individual cells of the microtiter plates was measured with an Anthos reader 2001 (Anthos Labtec Instruments) at 590 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.

Data handling. Carbon source utilization patterns were obtained by determining the percentage carbon sources utilized after a 72 h-incubation period (Korf, 1998). Data from each of the 25 common substrates (Table 1) in both types of microplates was directly stored on a computer in the Bionum version 1.1 for Windows (Applied Maths, Kortrijk, Belgium) as "0" (negative) and "1" (positive) to be calculated according to the Simple matching coefficient (S_{SM}) used for determining the correlation between samples (data sets) in the Gelcompare 4.0 program. Analyses and a dendrogram construction were done with the GelCompar 4.0 programme (Applied Maths, Kortrijk, Belgium). The programme clustered the samples using the unweighted pair group method of arithmetic averages (UPGMA).

Table 4.1: The twenty-five common carbon substrates within Biolog GN and ECO plates.

Category	Substrate
Polymers	
	α-cyclodextrin
	glycogen
	Tween 40
	Tween 80
Carbohydrates	
	i-erythritol
	D-galactonic acid lactone
	N-acetyl-D-glucosamine
	glucose-1-phosphate
	ß-methyl-D-glucoside
	D,L-α-glycerol phosphate
	α-D-lactose
	D-mannitol
	methyl pyruvate**
Carboxylic acids	
	γ-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

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

RESULTS AND DISCUSSION

The Dendrogram in Fig. 4.1, gives the comparison of the utilization of the 25 common substrates within the Biolog GN and ECO plates of Baviaanspoort, Centurion, Daspoort, Rooiwal and Zeekoegat (Fig. 4.1). 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).

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

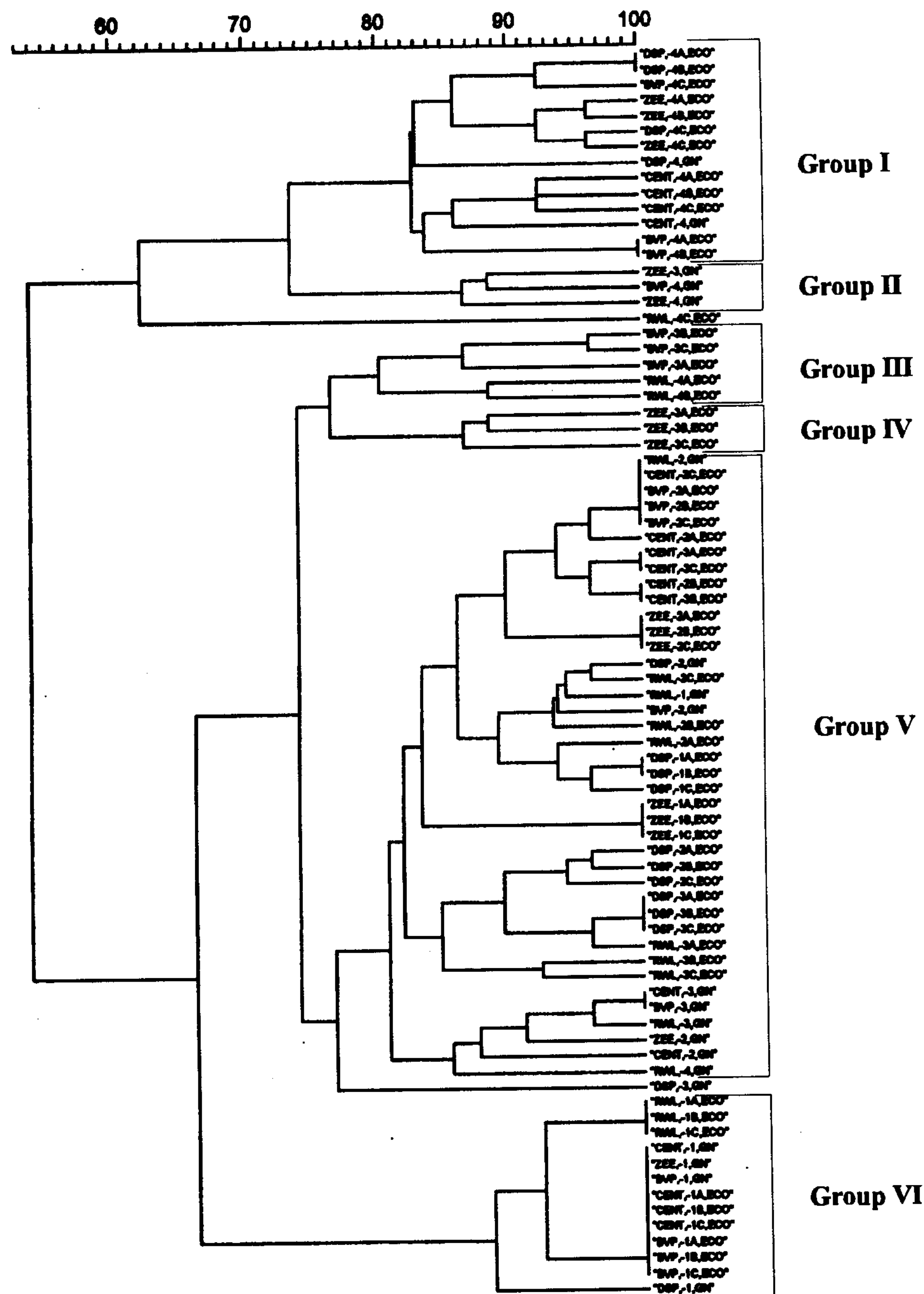

Figure 4.1 : **Dendrogram indicating the similarity between carbon source utilization patterns of the twenty-five common carbon substrates within Biolog GN and ECO plates (Table 4.1)**

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 functional diversity and evenness of the aerobic heterotrophic microbial community 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. 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 4.1) are attributed to differences in the formulation of the low-nutrient media within the plates. 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.

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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 functionality 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 was not community structure specific

- 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