Fingerprinting of Activated Sludge Systems Using PAGE Analysis of Total Protein Extractions for the Optimization of Biological Phosphorus Removal

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Report to the Water Research Commission
by the
Department of Microbiology and Plant Pathology
University of Pretoria

WRC Report No 776/1/98

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WRC Report No. 776/1/98
ISBN 1 86845 451 7

March 1998

Acknowledgements

The financial support of the Water Research Commission for the work and the support of the Steering Committee is acknowledge with gratitude. The members of the Steering Committee were: Dr N Mjoli (Chairman), Mr B Atkinson, Prof T E Cloete, Mr S Dill, Dr A Kühn, Prof M M Sibara, Dr I M Msibi, Prof M Wentzel, Miss M J U Wium (secretary).

Thanks are also due to:

* Prof M. Wentzel of the University of Capetown (UCT),
* Alison Thompson of East Rand Waterboard (ERWAT),
* Daspoort Water Treatment Plant for activated sludge samples and analysis provided.
* Prof P L Steyn for the use of the Gelcompar 4.0 programme.

EXECUTIVE SUMMARY

BACKGROUND:

The need for nutrient removal from wastewater is due to the worldwide problem of eutrophication. Eutrophication occurs when water bodies receive large volumes of water which contain excessive quantities of nutrients such as nitrates and more specifically phosphates. This leads to the growth of aquatic photosynthetic plants, notably algae. The large population of algae depletes oxygen in the water and the algae release toxins into the water. The decay of the algae following die-off results in the lowering of the dissolved oxygen content of the water and consequent death of other aquatic organisms. To prevent eutrophication, phosphate removal from effluents is necessary whether it is by chemical and/or biological means.

Until now it has not been possible to isolate a pure culture of bacteria that could be responsible for biological phosphorus removal. Due to the problems deriving from culture-dependent methods other methods were considered to determine the difference between P removing and non-P removing activated sludge systems. Therefore, there is a need for techniques that do not necessarily identify individual species, but that can differentiate bacterial communities in terms of their constituents. These methods alleviated the need for culturing and samples were analyzed in a more direct manner which prevent the selection of specific organisms. There is a need for better understanding the microbial community structure and function, in order to manage wastewater treatment systems to control bulking or to improve biological phosphate removal capacity.

Analysis of total proteins extracted from an environmental sample can be employed as a "fingerprint" to type the diversity in the sample, in a way similar to grouping of bacteria according to enzyme polymorphisms and immunological reactions. Such fingerprints may eventually be used to monitor the deterioration or enrichment of species diversity in microbial communities.

OBJECTIVES:

The objectives of this study were therefore to use the whole cell protein extraction and analysis of the proteins with the SDS-PAGE technique to study the microbial community structure in activated sludge in order to determine the differences between different activated sludge systems. This study will enable us to have a better understanding of activated sludge systems. It will also help to identify factors responsible for non performance, by comparing management systems, system parameters, chemical analysis and biomass with our bacterial community analysis findings.

The objectives were:

1. Monitoring a specific activated sludge plant over time (e.g. every week) using PAGE analysis of total cell protein extracts.
2. The comparison of different zones within the same system using PAGE analysis of total protein extracts.
3. Comparing different systems according to their design and phosphorus removal using PAGE analysis of total cell protein extracts.
4. The comparison of systems which remove phosphorus to those which do not remove phosphorus using PAGE analysis of total cell protein extracts.
5. Comparing the same system when removing phosphorus and when it's not removing phosphorus, using PAGE analysis.

SUMMARY OF RESULTS:

Whole cell protein extractions and SDS-PAGE

The results obtained with SDS-PAGE indicated that there were no differences amongst the bacterial community structures of the different activated sludge zones (anaerobic, anoxic and aerobic). The bacterial communities of phosphorus-, and nitrogen-removing laboratory scale systems indicated no difference amongst their protein profiles according to SDS-PAGE. System design and the type of waste treated by the plant did not result in altered protein patterns. This indicated that the microbial community structure of activated sludge plants were

closely related. Seasonal changes did not have an affect on the protein profiles of the bacterial community of the Daspoort activated sludge plant. The protein profiles of a phosphorus-removing and non-phosphorus-removing system indicated a high percentage of correlation indicating little variation in their bacterial communities.

Activated sludge systems were not dominated by one or a few specific bacterial species but consisted of a combination of different bacterial species which co-exist and function together in a complex community according to SDS-PAGE.

SDS-PAGE was a sensitive method to determine the similarities and differences between the protein profiles of the bacterial community structure of activated sludge samples. Resulting protein profiles, after SDS-PAGE were normalized and analyzed with the Gelcompar 4.0 programme. This programme calculated the % similarities and differences between each protein profile, with the Pearsons product moment correlation coefficient (r) between samples to construct a matrix. The samples were then clustered using the unweighted pair group method of arithmetic average (UPGMA) which resulted in a dendrogram.

Researchers tend to construct dendrograms consisting of only a few samples and then base the identification of a new genus or species on their findings. When samples are added to smaller dendrograms the dendrogram is more likely to vary. However, the larger the dendrogram, the more value can be attached to the results. When new samples are added, the groups will stay the same and only a small variation in the % correlation might appear. Each dendrogram must be evaluated on its own and not be compared with other dendrograms. These are the main reasons why no definite value of > 80 % for the same species and > 60 % for the same genus can be attached to a dendrogram. Percentage correlation between the samples must only be an indication of similarity.

One disadvantage of the SDS-PAGE method is that it needs to be standardized. Results between different laboratories may differ if standard methods are not followed. An exact value can not be attached to the % similarity or correlation of the resulting dendrogram after SDS-

PAGE. The % similarity can rather be used as an guideline. SDS-PAGE can therefore, not discriminate between the bacterial populations of the different activated sludge samples, it can only indicate samples with a high % similarity or not.

Immunochemical investigation

Antigen preparation from the anoxic and aerobic zones contained intact and lysed bacterial cells. Besides conventional immunization, subtractive immunization* using cyclophosphoamide was also used to focus the immune response on unique epitopes in the zones. *{Subtractive immunization, is a term referring to the homology to the process of "subtractive hybridization". To obtain messenger RNA's of an abundance, is used here to describe how antigen of low immunogenicity and absorbance can be made immunologically prominent by first immunizing with a crude immunogen containing the abundance of irrelevant antigens, followed by chemical paralysis of this immune response by cyclo-phosphamide treatment, and finally immunization of these animals with the complex antigen which then also contains the sought for antigenic determinant (Matthew & Patterson, 1983). This results in an immune response where the minor antigen acts prominently to elicit specific antibodies}. Neither strategy provided antibodies capable of distinguishing phenotypic diversity between the two zones, emphasizing the homogeneity of the microbial populations in the different zones of phosphate removing activated sludge systems. Nine stable hybridoma lines were established, all secreting IgM cross reactive to both antigen preparations but differing in the antigen specificity. Monoclonal antibody 7B9, putatively protein-directed, could clearly distinguish between the aerobic zones of the two activated sludge systems differing only in phosphate removal ability: immunoblot showed five discrete bands in the system successful at phosphate removal. The molecular weights of the bands appeared to be multiples of 18 kDa, indicating possible involvement of an 18 kDa proteinaceous monomer in phosphate uptake. A significant finding emerging from this study was based on the observation that antigenic differences were clearly detected between the aerobic zones of two activated sludge systems with differing phosphate removal ability. A monoclonal antibody was found that was capable of distinguishing between the two systems both by ELISA and immunoblot. Characterization of the antigen recognised suggested a protein nature.

Conclusions

* The main conclusion from SDS-PAGE is that the protein profiles do not differ significantly in any of the systems, suggesting that the bacterial community remains constant.

* Monoclonal antibodies can be used as diagnostic tools in phosphorus removing and non-removing activated sludge plants.

RECOMMENDATIONS FOR FURTHER RESEARCH:

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

Previous studies also indicated that biomass was related to phosphorus removal. The higher the "biomass" the better the P-removal. This suggested that the main difference between P-removing and non P-removing systems is biomass related and not due to the microbial community structure. The aims for future studies are therefore: i) To determine the relationship between biomass and P removing and non-P removing systems ii) To determine the P removal capacity of a system based on biomass iii) To determine the effect of

bioaugmentation on phosphorus removal in a conventional activated sludge system by adding biosupplements and/or anaerobic sludge in order to increase the biomass.

LIST OF PRODUCTS:

Degree:

PhD Microbiology - M M Ehlers

MSc Biochemistry - A Erasmus

BSc (Hons) Microbiology - F Molepo

Articles:

i) Direct extractions of proteins to monitor an activated sludge system on a weekly basis for 34 weeks using SDS-PAGE. Accepted for publication in *Water SA*.

ii) Protein profiles of phosphorus removing and nitrate removing activated sludge systems. Accepted for publication in *Systematic and Applied Microbiology*.

iii) Comparing the protein profiles of 21 different activated sludge systems after SDS-PAGE. Accepted for publication in *Water Research*.

iv) Antibody recognition of an 18 kDa proteinaceous monomer possibly involved in phosphate removal by activated sludge. Submitted for publications in *Water SA*.

Presentations:

Ehlers, M.M. and Cloete, T.E., 1997. Bacterial community structure of activated sludge determined by SDS-PAGE - Poster presentation. *American Society for Microbiology, May 5-7, 1997. Miami Beach, FL.*

Ehlers, M.M. and Cloete, T.E., 1997. Comparing the protein profiles of 21 different activated sludge systems after SDS-PAGE - Poster presentation. *Water Quality International 1998. Vancouver , B.C., Canada.*

Ehlers, M.M. and Cloete, T.E., 1997. Protein profiles of phosphorus removing and nitrate removing activated sludge systems - Poster presentation. *S.A. Society of Microbiology, July 5-8, 1998. Durban, RSA.*

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LIST OF ABBREVIATIONS:

Ac:	*Acinetobacter*
ATCC:	American Type Culture Collection
BSA:	bovine serum albumin
cfu:	colony forming unit
COD:	Chemical oxygen demand
DNA:	Deoxyribonucleic acids
DSM:	Deutsche Sammlung von Mikroorganismen und Zellkulturen
DVT:	Daveyton
EBPR:	Enhanced biological phosphate removal
ERWAT:	East Rand Waterboard
ESTR:	Ester Park Water Treatment Plant
g:	gram
h:	hour
HBFT:	Hartbeesfontein Water Treatment Plant
HDLB:	Heidelberg Water Treatment Plant
JPMS:	J.P.Marais Water Treatment Plant
l:	litre
LMG:	Laboratorium voor Microbiologie Gent Culture Collection, State University Gent, Belgium
mA:	milliampere
MDFN:	Modderfontein Water Treatment Plant
mg:	milligram
mmole:	millimole
mod:	module
M_r:	molecular mass
mS:	millisiemens
N:	Nitrogen
NYLS:	Nylstroom Water Treatment Plant
OLFT:	Olifantsfontein Water Treatment Plant
P:	Phosphorus
PBS:	Pphosphate buffered saline, pH 7.2

P-N:	Rynfield Water Treatment Plant - North
Prim:	Primary
P-S:	Rynfield Water Treatment Plant - South
POTG:	Potgietersrus Water Treatment Plant
RDLT:	Rondebult Water Treatment Plant
S.D.:	standard deviation
SDS-PAGE:	Sodium dodecyl sulphate polyacrylamide
Sec:	Secondary
TSKN:	Tsakane Water Treatment Plant
UPGMA:	Unweighted pair group method of arithmetic averages
UCT:	University of Capetown
VLKP:	Vlakplaats Water Treatment Plant
WTV:	Waterval Water Treatment Plant
ZKG:	Zeekoeigat Water Treatment Plant

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CHAPTER 1

INTRODUCTION

This project involves the combining of the theoretical aspects of biological phosphorus removal and the practical application of these tecniques. This project rounds of the work done in the previous two projects and takes the theory to practise. The treatment of wastewater by activated sludge systems is, in terms of metabolized matter, probably today's most important biotechnological process (Wagner, Amann, Lemmer & Schleifer, 1993). A lot of effort has been put into process engineering, whereas our current knowledge of microbial community structure-function correlations and consequently a microbiological understanding of the activated sludge process are still very limited. Diversity and dynamics of the microbial consortia in activated sludge have mostly been analyzed by culture-dependent methods (Wagner *et al.*, 1993). There is a large discrepancy between the total direct microscopic counts and viable plate counts (usually less than 1% of the former) for many ecosystems (Cloete & Steyn, 1987; Wagner *et al.*, 1993). Recoveries from activated sludge systems even with optimized media are only between 5 and 15% (Wagner *et al.*, 1993). Often the microscopically most prominent microorganisms like the filamentous bacteria in foaming activated sludge can not be found by standard cultivation procedures. This prompted efforts to gain more direct insights into the microbial community structure of activated sludge.

We expected to have a better understanding of biological phosphorus removal after the completion of this project, because our knowledge on the dynamics of the activated sludge microbial community, and therefore, our microbiological understanding of the activated sludge process is still very limited. The inefficiency of some activated sludge systems calls for a better understanding of how the biological phosphorus removal process relates to the microbial populations and their metabolism in the sludge.

There is interest to better understand the EBPR process, since it is not optimized and routinely fails (Bond *et al.*, 1995). To achieve this, a more complete knowledge of microbial phosphate metabolism in activated sludge is required. There is a need for techniques that do

not necessarily identify individual species, but that can differentiate bacterial communities in terms of their constituents (Lee & Fuhrman, 1990). This can be achieved by using a more direct method to analyze the bacterial community of activated sludge. Due to the problems deriving from culture-dependent methods several other methods were considered to determine the difference between P-removing and non-P-removing activated sludge systems. SDS-PAGE analyses of the total proteins and 16S rDNA analyses of activated sludge samples are methods that can be used. Expression of genes in microorganisms correlate with a variety of environmental stimuli, ranging from the presence of particular nutrients to changes in the physical-chemical conditions (Ogunseitan, 1993). Until now no direct method was developed to analyze the protein products of gene expressions of environmental samples (Ogunseitan, 1993).

The activated sludge process used to treat wastewater involves the biological degradation of organic material. The removal of nutrients such as nitrogen (N), and phosphorus (P) from wastewater can be obtained in activated sludge under specific conditions. The present design for P removal, termed enhanced biological phosphate removal (EBPR), requires the wastewater to pass through an initial anaerobic treatment and then an aerobic stage (Bond, Hugenholtz, Keller & Blackall, 1995). Empirical knowledge of the chemical transformations that occurs throughout the different stages of the treatment has provided insight into the biological mechanism of EBPR. Under favourable conditions, phosphate-removing sludge is observed to take several sludge ages to develop (Bond *et al.*, 1995).

Since Fuhs and Chen (1975) first implicated *Acinetobacter* spp. as having an important role in EBPR, most subsequent studies have focused on this bacterial genus. The reasons for this attention have not been entirely unjustified. Culture-dependent methods consistently indicated that *Acinetobacter* spp. were the numerically dominant members of EBPR systems. Furthermore, some, but not all, *Acinetobacter* strains isolated from activated sludge accumulated excessive amounts of polyphosphate in pure culture, suggesting their importance in the EBPR process (Bond *et al.*, 1995). However, researchers have difficulties reconciling the carbon and phosphorus transformations in pure cultures of *Acinetobacter* strains with the biochemical model for EBPR. In recent years, serious doubts have been raised as to the significance of *Acinetobacter* spp. in the EBPR process. The most compelling evidence for

this change of view has been the recent non-culture-dependent studies of phosphate-removing communities. In all cases, *Acinetobacter* spp. were found to represent only a small portion of the total EBPR microbial population. Instead, other bacterial groups such as the gram-positive bacteria and the beta subclass groups of the proteobacteria were numerically dominant. While the use of these non-culture-dependent methods reduces the significance of *Acinetobacter* spp. in EBPR processes, the resolution of the methods has not been sufficient to propose alternative EBPR candidate genera (Bond *et al.*, 1995).

Protein electrophoresis is a technique, yielding valuable information on the similarity or dissimilarity amongst bacterial cultures. This method could therefore, possibly also be used to determine the similarity or dissimilarity between different environmental samples containing microorganisms. SDS-PAGE of whole-cell soluble proteins, prepared under standard conditions, produced a complex banding pattern (called a protein electrophore gram or electrophoretic protein pattern), which is reproducible and can be considered as a "fingerprint" of the sample investigated (Kersters, 1990). The resulting protein profiles after SDS-PAGE could possibly lead to the better understanding of the diversity and dynamics of the microbial communities of phosphorus removing and non-phosphorus removing activated sludge systems, since this would indicate similarity or dissimilarity in those samples. This would indicate whether a difference existed in the microbial community structure between phosphorus removing, non-P-removing and N-removing systems.

The protein profiles of the different samples were scanned with a densitometer and analysed with the GelCompar computer program to determine the relatedness between the different zones and sludge systems. This gave an indication of the metabolic diversity amongst zones of the same system and amongst systems with different phosphate removing capabilities. The result furthermore indicate whether the metabolic pattern in different phosphorus removing systems was the same. Metabolic shifts should indicate either population shifts or a change in metabolic pattern of resident populations. Comparison of different activated sludge plant configurations should, furthermore, indicate whether the configuration of the plant has any effect on the metabolic pattern. It also indicate whether more than one biological phosphorus removal mechanism was involved.

Immunological laboratory methods have become powerful and indispensable research tools in biochemistry, molecular biology, microbiology, cell biology, and other related disciplines. Antibodies have been used to address problems that would have been difficult or impossible to approach by other methods. The main advantage of antibodies is that they can be raised against virtually any organic substance. Immunological techniques enjoy wide application due to their sensitivity and the specificity with which antibodies react with the target molecule and form an antigen-antibody complex, allowing detection, solation and quantification of these substances and/or organisms harbouring them (Drenckhahn *et al.*, 1993; Witzel, 1990).

The objectives of this study were therefore to use SDS-Page and immunological techniques, to study the microbial community structure in activated sludge in order to determine the differences between different activated sludge systems. This study will furthermore enable us to have a better understanding of activated sludge systems and to identify factors responsible for non performance, by comparing management systems, system parameters, chemical analysis and biomass with our bacterial community analysis findings.

The objectives for this study were therefore:

1. Monitoring a specific activated sludge plant over time (e.g. every week) using PAGE analysis of total cell protein extracts.
2. The comparison of different zones within the same system using PAGE analysis of total protein extracts.
3. Comparing different systems according to their design and phosphorus removal using PAGE analysis of total cell protein extracts.
4. The comparison of systems which remove phosphorus to those which do not remove phosphorus using PAGE analysis of total cell protein extracts.
5. Comparing the same system when removing phosphorus and when it's not removing phosphorus, using PAGE analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

In terms of metabolized matter the activated sludge process is probably today's most important biotechnological process (Wagner, Amann, Lemmer & Schleifer, 1993). Activated sludge is the biological treatment process for both domestic and industrial wastewater (Cloete & Muyima, 1997). The activated sludge process involves the biological degradation of organic material. Under specific conditions removal of nutrients such as nitrogen (N), and phosphorus (P) can be obtained in activated sludge.

The need for nutrient removal from effluents (wastewater) is due to the worldwide problem of eutrophication. Eutrophication occurs when water bodies receive large volumes of water which contain excessive quantities of nutrients such as nitrates and more specifically phosphates (Slim, 1987; Toerien, Gerber, Lötter & Cloete, 1990). This leads to the growth of aquatic photosynthetic plants, notably algae. The large population of algae depletes oxygen in the water and the algae release toxins into the water. The decay of the algae following die-off results in the lowering of the dissolved oxygen content of the water and consequent death of other aquatic organisms (Gleisberg, 1992). To prevent eutrophication, phosphate removal from effluents is necessary whether it is by chemical and/or biological means (*Toerien et al.*, 1990).

Biological wastewater treatment systems rely on the interaction and metabolism of microorganisms. The present design for P removal, termed enhanced biological phosphate removal (EBPR), requires wastewater to pass through an initial anaerobic treatment and then an aerobic stage, during which P removal takes place (Bond, Hugenholtz, Keller & Blackall, 1995). Although a considerable amount of work has been done on system design and process engineering, the knowledge and understanding of the microbial community structure-function and consequently the microbiology behind the activated sludge process are limited. An understanding of the microbial community of a biological wastewater treatment system would

assist in improving system design and performance, since it is not optimized and routinely fails (Bond *et al.*, 1995; Cloete & Muyima, 1997).

Diversity and dynamics of the microbial consortia in activated sludge have mostly been analyzed by culture-dependent methods (Cloete & Steyn, 1987; Wagner *et al.*, 1993). There are problems using present culture methods with selective media to study the structure of bacterial communities in nature (Lee, Zo & Kim, 1996). The literature indicates that there is a discrepancy between the total direct microscopic counts and viable plate counts (usually less than 1% of the former) for ecosystems (Toerien *et al.*, 1990, Wagner *et al.*, 1993). Recoveries from activated sludge systems, even with optimized media, are only between 5% and 15% (Cloete & Steyn, 1987; Wagner *et al.*, 1993). Filamentous bacteria, which are often microscopically the most prominent microorganisms, in foaming activated sludge cannot be found by standard cultivation procedures (Wagner *et al.*, 1993). Culturing techniques have provided a misleading picture of bacterial community structure in activated sludge systems and in general the role of *Acinetobacter* species in activated sludge processes has been overrated (Cloete & Steyn, 1987; Wagner *et al.*, 1993).

Until now it has not been possible to isolate a pure culture of bacteria that could be responsible for biological phosphorus removal. Due to the problems deriving from culture-dependent methods, other methods were considered to determine the difference between P removing and non-P removing activated sludge systems. Therefore, there is a need for techniques that do not necessarily identify individual species, but that can differentiate bacterial communities in terms of their constituents (Lee & Fuhrman, 1990). These methods alleviate the need for culturing and samples are analyzed in a more direct manner which prevents the selection of specific organisms. The better we understand community structure and function, the better we can manage wastewater treatment systems to control bulking or to improve biological phosphate removal capacity (Cloete & Muyima, 1997).

2.2 Wastewater Treatment

Physical forces as well as chemical and biological processes drive the treatment of wastewater. Treatment methods that rely on physical forces are called unit operations.

These include screening, sedimentation, filtration and operations (Bitton, 1994). Treatment methods based on chemical and biological processes are called unit processes. Chemical processes include disinfection, adsorption and precipitation (Bitton, 1994). Biological unit processes involve microbial activity which is responsible for degradation of organic matter and removal of nutrients and includes, activated sludge, trickling filters and rotating biodisks (Metcalf & Eddy, 1991).

The activated sludge process has been developed for the removal of carbon, nitrogen and phosphate from wastewater. Activated sludge refers to a slurry of microorganisms that remove organics from wastewater, these organisms are themselves removed by sedimentation under aerobic conditions (Cloete & Muyima, 1997). The composition of the microbial community is determined by the type of wastewater (nutrient content) and a number of selective pressures such as mean cell retention time (MCRT), aerobiosis, anaerobiosis, temperature and other extrinsic factors (Cloete & Muyima, 1997). The wastewater composition has significant influence on the process; for example the easily degradable carbon sources are the key to the efficiency of the biological phosphorus removal process (Henze, 1996).

Activated sludge treatment removes from the wastewater the biodegradable organics which are degraded by the bacteria in an aerated basin. In this process biomass growt occurs. The trapped solids and biomass is carried over with the influent into a clarifier where solids are allowed to settle and concentrate and are then removed. Also, part of the unsettelable unbiodegradable suspended solids and other constituents can be adsorbed on, or entrapped by the activated sludge floc (Cloete & Muyima, 1997). Part of the microbes and other solids are retained in the system as an activated sludge and recycled to the aeration basin (return sludge) to maintain a high concentration of bacteria, the rest is drawn as waste (Eckenfelder, Patoczka, Watkin, 1985; Toerien *et al.*, 1990).

Wastewater treatment comprises the following four steps:

i) Preliminary treatment: The objective of this operation is to remove debris and coarse materials that may clog equipment in the plant.

ii) Primary treatment: Treatment is brought about by physical processes (unit operations)

such as screening and sedimentation.

iii) Secondary treatment: Biological (e.g. activated sludge trickling filters, oxidation ponds) and chemical (e.g. disinfection) unit processes are used to treat wastewater-removal of nutrients and generally occurs during secondary treatment of wastewater.

iv) Tertiary or advanced treatment: Unit operations and chemical unit processes are used to further remove BOD, nutrients, pathogens, parasites and sometimes toxic substances (Bitton, 1994).

2.2.1 Composition of Wastewater

Domestic wastewater is mainly composed of proteins (40% - 60%), carbohydrates (25% - 50%), fats and oils (10%), urea derived from urine, and large numbers of trace organics which include pesticides, surfactants, phenols and priority pollutants. The latter category comprises nonmetals (As, Se), metals (e.g. Cd, Hg, Pb), benzene compounds (e.g. benzene, ethylbenzene) and chlorinated compounds (e.g. chlorobenzene, lindane, tetrachloroethane, trichloroethane) (Metcalf & Eddy, 1991). The bulk of organic matter in domestic wastewater is biodegradable and consists mainly of carbohydrates, amino acids, peptides and proteins, volatile acids and fatty acids and their esters (Painter & Viney, 1959).

In domestic wastewater organic matter is found as dissolved organic carbon (DOC) and particulate organic carbon (POC). POC represents approximately 60% of organic carbon and some of it is of sufficient size to be removed by sedimentation.

In fixed-film processes, DOC is directly absorbed by the biofilm, whereas POC is adsorbed to the biofilm surface to be subsequently hydrolysed by microbial action. Four main tests are used for determination of organic matter in wastewater:

i) **Biochemical oxygen demand (BOD)**

BOD is the amount of dissolved oxygen (DO) consumed by microorganisms for the biochemical oxidation of organic (carbonaceous BOD) and inorganic matter (autotrophic or nitrogenous BOD) (Bitton, 1994). As a standard, the test is run for 5 days, BOD_5.

ii) **Nitrogenous oxygen demand (NOD)**

Autotrophic bacteria such as nitrifying bacteria also require oxygen to oxidize NH_4^+ to nitrate. The oxygen demand exerted by nitrifiers is called autotrophic BOD or nitrogenous oxygen demand (Bitton, 1994).

iii) **Chemical oxygen demand (COD)**

COD is the amount of oxygen necessary to oxidize the organic carbon completely to CO_2 and H_2O and ammonia (Sawyer & McCarthy, 1978). In general 1 g of carbohydrate or 1 g of protein is approximately equivalent to 1 g of COD (Bitton, 1994).

iv) **Total organic carbon (TOC)**

TOC represents the total organic carbon in a given sample and is independent of the oxidation and energy state of the organic matter (Bitton, 1994).

Trace organics are detected and measured by means of sophisticated instruments such as gas chromatography and mass spectroscopy (Bitton, 1994).

2.3 The Activated Sludge Process

The dominating technology for phosphorus removal is chemical precipitation. Biological phosphorus removal receives increased interest due to the low sludge production, the fertilizer value of the sludge and the use of the wastewater components as "chemical" for the process (Henze, 1996). Chemical precipitation is used as a polishing process in combination with biological phosphorus removal. The chemicals primarily used are ferrous sulphate, ferric chloride and aluminum sulphate (Henze, 1996). The general tendency in recent years has been a decline in pre-and postprecipitation, and an increase in biological phosphorus removal and simultaneous precipitation, the latter is also cheaper (Henze, 1996).

The flocculation of microorganisms is the basic operational unit of activated sludge. The exact nature of the flocculation material is not well known, but it seems bacterial orientated. Flocculant growth is responsible for the removal of colloidal and ionic matter from

wastewater by means of adsorption and agglomeration in the aeration tank (Cloete & Muyima, 1997). Good flocculant growth plays an important role in the sedimentation tank for rapid and efficient separation of sludge from the treated effluent. The adsorption capacity of the floc depends on the suitable cell surface available. The cells have to metabolize the adsorbed material first before further material can be adsorbed. The removal process depends on continuous re-inoculation with recycled settled sludge. Only floc-forming organisms that settle rapidly in the sedimentation tank are selected to be retained. Thus, the process is microbially self-regulating with the required selected flocs recirculated (Cloete & Muyima, 1997). To understand the activated sludge process better, a description of the different stages is necessary.

2.3.1 Anaerobic Zone

The principal function of the anaerobic zone is to establish a facultatively anaerobic microbial community as indicated by the fermentation pattern. During anaerobiosis this bacterial community produce compounds such as ethanol, acetate and succinate, which serve as carbon sources for phosphate-accumulating bacteria (Cloete & Muyima, 1997).

In the anaerobic zone dissolved oxygen and oxidized nitrogen (nitrate or nitrite) are absent, and are not input in any significant concentrations (Barnard, 1976; Buchan, 1984). Sludge from the clarifier flows together with the influent wastewater into this zone. 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 & Muyima, 1997). The release of a certain quantity of phosphate from the biomass into the solution indicates that the bacteria have been suitably conditioned (Pitman, 1984). The retention time (about 1 h) of the influent wastewater is of extreme importance. Nitrates and dissolved oxygen discharge into the zone must be zero or as near to zero as possible at all times (Cloete & Muyima, 1997). The presence of nitrate in an anaerobic zone interferes with the phosphate removing capability of the activated sludge during aerobiosis (Barnard, 1976; Nicholls & Osborn, 1979; Marais, Loewenthal & Siebritz, 1983). The reason 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 & Muyima, 1997). However, the use of unsettled influent and the presence of sludge from the sludge treatment in the primary clarifiers, probably producing lower fatty acids, had a positive effect on the phosphate removal (Mulder & Rensink, 1987). The degree of nitrate feedback that can be tolerated depends on the strength of the sewage feed to the anaerobic zone and its readily biodegradable COD concentration and the desired P removal (Pitman, 1984).

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

Phosphate release or uptake can be induced by certain carbon sources. The additions of acetate to the medium and the lowering of the pH as well as phosphate starvation under anaerobic conditions, resulted in phosphate release (Fuhs & Chen, 1975; Barnard, 1976; Buchan, 1983). Nicholls and Osborn (1979), confirmed that bacteria relieved from stress conditions rapidly take up phosphate in an overplus reaction immediately on entering the aerobic zone, where stress conditions are relieved and phosphate together with an abundant source of energy is available. Phosphate seemed to be released from the acid-soluble fraction of cells and to a minimal degree from the RNA and DNA (Cloete & Muyima, 1997).

2.3.2 Primary Anoxic Zone

Anoxic refers to the presence of nitrates and the absence of dissolved oxygen (Buchan, 1984; Pitman, 1984; Streichan, Golecki & Schon, 1990). The primary anoxic zone is the main denitrification reactor in the activated sludge process. It is fed by effluent from the anaerobic zone and by mixed liquor recycled from the aerobic zone (Cloete & Muyima, 1997). The

absence of oxygen and the availibility of nitrate and nitrite leads to the enrichment of denitrifying bacteria, which reduce nitrate or nitrite to molecular nitrogen. Soluble and enmeshed particulate biodegradable matter are partly removed in the primary anoxic zone. It was found that phosphate release were induced in the anoxic zone in the presence of lower fatty acids or their salts such as acetate, formate and propionate (Gerber, Mostert, Winter & De Villiers, 1986). Phosphate release was not only affected by the substrate but also by the dosage of the substrate concentrations of soluble, readily biodegradable carbon substrates (Cloete & Muyima, 1997). The limited phosphate uptake in the anoxic zone can be due to the reduction of nitrate to nitrogen gas by some phosphate-accumulating bacteria such as *Acinetobacter*.

2.3.3 Primary Aerobic Zone

The main function of the primary aerobic zone is to oxidize organic material in the sewage, to oxidize ammonia to nitrite and then to nitrate (by chemoautotrophs), and 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 feed sewage (Cloete & Muyima, 1997). Ammonia is oxidized to nitrite by *Nitrosomonas, Nitrosospira* and *Nitrosolobus* spp. Nitrite is oxidized to nitrate by *Nitrobacter, Nitrospira* and *Nitrococcus* spp. (Buchan, 1984). The principal operational determinant of the efficiency of phosphate removal seems to be the aeration rate, which should be sufficient to promote the rapid uptake of released and feed phosphate. The aeration rate should ensure the oxidation of the carbon compounds and ammonia and to suppress the growth of filamentous microorganisms that produce poorly settling sludge (Pitman, 1984). High rate aeration resulted in maximum uptake of phosphate within 2 h (Levin & Shapiro, 1965; Shapiro, 1967, Carberry & Tenny, 1973). Wentzel and colleagues (1985) indicated that the excessive phosphate uptake in the aerobic stage was associated directly with the degree of phosphate release during the previous anaerobic phase, therefore, more phosphate released leads to more phosphate uptake. There is a linear relation between phosphate release and uptake (Wentzel, Dold, Ekama & Marais, 1985).

Other parameters also play a role in enhanced phosphate removal such as: the presence of readily biodegradable compounds, especially volatile fatty acids, produced by fermentative

bacteria from organic compounds in the influent. These volatile fatty acids are removed during the anaerobic phase and polymerized at the expense of energy obtained from the breakdown of polyphosphate (Cloete & Muyima, 1997). Poly-ß-hydroxybutyric acid (PHB) plays an important role in the mechanism of phosphate uptake and release (Nicholls & Osborn, 1979; Deinema, Habets, Scholten, Turkstra & Webers, 1980; Lawson & Tonhazy, 1980; Comeau, Hall, Hancock & Oldham, 1986). The presence of PHB in bio-polyphosphate bacteria help them to grow and rebuild their own polyphosphate by taking up soluble phosphate from the solution (Comeau *et al.*, 1986). PHB and polyphosphate will therefore play a mutually interdependent role to assist aerobic bacteria to survive through an anaerobic period (Nicholls & Osborn, 1979).

2.3.4 Secondary Anoxic Zone

The function of this zone is the removal of excess nitrates which were not removed in the primary anoxic zone. Denitrification in this zone is very slow and therefore, the quantity of nitrate removed is small. Due to the low COD the retention time in this anoxic zone is relatively long (Cloete & Muyima, 1997).

2.3.5 Secondary Aerobic Zone and Clarifier

The main function of the secondary aerobic zone is to increase the dissolved oxygen to a level between 2 and 4 $mg.l^{-1}$ in the mixed liquor before it enters the clarifier (Barnard, 1976), and to refine the final effluent by the removal of additional phosphate and the oxidation of residual ammonia (Cloete & Muyima, 1997). Mixed liquor must be aerated for at least 1 h before it passes into the clarifier to promote phosphate uptake and maintain good aeration conditions (Cloete & Muyima, 1997). Excess aeration should be prevented as it would result in the conversion of organically bond nitrogen to nitrate and cause the slow aerobic release of phosphate from the solids (Keay, 1984; Pitman, 1984). Ammonia nitrification as well as phosphate removal, which has not been completed, will continue in the secondary aerobic zone (Buchan, 1984).

The function of the clarifier is to produce a clear effluent free of suspended solids, and a

thickened sludge for recycling to the inlet of the process. The quality of the underflow sludge should be such that nitrate is not recycled to the anaerobic zone (Ekama, Marais & Siebritz, 1984).

2.4 Activated Sludge Systems

Activated sludge technologies are available, most of which are basically identical, using combined sludge process with nitrification-denitrification included (Henze, 1996). The main stream processes dominate over the side stream processes (Henze, 1996). The removal capacity of the process can be limited by the wrong design or operation of the treatment plant (Henze, 1996).

The most widespread main stream processes are the: A/O, BARDENPHO, UCT, PHOREDOX, BIODENIPHO and ISAH/Johannesburg. In the side stream process, the release of the stored phosphorus occurs in the sludge recycle stream, from which it is removed chemically, as in the PHOSTRIP process or biologically as in the OWASA process (Henze, 1996).

2.4.1 Phoredox and Bardenpho Activated Sludge Processes

The basic process for the simultaneous biological removal of phosphate and nitrogen was proposed by Barnard in 1976 and is known as the Phoredox process (phosphorus reduction oxidation) in South Africa and the Bardenpho or modified Bardenpho in the United States (Cloete & Muyima, 1997). The Phoredox activated sludge processes are a five-stage process designed to remove nitrogen as well as phosphate, while the Bardenpho is a four-stage process designed for nitrogen removal (Cloete & Muyima, 1997). The two systems consist of primary anoxic and aerated as well as secondary anoxic and aerated basins, followed by a clarifier. The incorporation of an anaerobic zone in the Phoredox activated sludge process results in the release of phosphate which is a basic requirement for successful phosphate uptake. The anaerobic zone encourages the production of low molecular mass organics to promote the growth of phosphate removing bacteria. After phosphate has been released from the biomass in the anaerobic zone, phosphate is reincorporated into the biomass in the first

aerobic zone, together with part of all the influent phosphate (Gerber *et al.*, 1986). Barnard (1976) found that the application of this process in South Africa has lead to the removal of phosphate in the final effluent of activated sludge plants between 0.2 - 0.8 $mg.l^{-1}$, together with the removal of 80% - 90% of the nitrogen (Cloete & Muyima, 1997). The primary and second anoxic zone, are responsible for denitrification. The final aerobic zone stimulates the release of nitrogen gas and improves the settleability of the sludge (Streichan *et al.*, 1990; Van Strakenburg, Rensink & Rijs, 1993).

2.4.2 A/O Activated Sludge Process

The A/O (aerobic/oxic) process is used for high-loaded activated sludge systems. Return sludge is mixed with incoming wastewater. The mixed liquor passes through an anaerobic zone and then through an aerobic zone (no anoxic). Phosphorus accumulates in the sludge and is removed by the surplus sludge (Cloete & Muyima, 1997).

2.4.3 UCT Activated Sludge Process

The UCT (University of Cape Town) process is a modified Phoredox process which includes three internal recirculations which prevents nitrate recycling to the anaerobic zone. Returned sludge is recycled into the anoxic zone instead of the anaerobic zone. The other two recirculations are located between the aerobic and anoxic zones and between the anoxic and anaerobic zones (Van Strakenburg *et al.*, 1993).

2.4.4 Biodenipho Activated Sludge Process

The Biodenipho (biological denitrification phosphorus removal) consists of an alternating supply of two coupled aeration tanks with anoxic and aerobic phases. An anaerobic zone precedes this zone to promote the growth of phosphorus-accumulating bacteria. The wastewater and return sludge flow into the anaerobic zone which consists of three compartments to promote the plug-flow character. Optimum nitrification and denitrification occurs due to the alternating wastewater supply. Recirculation of nitrified water to the anoxic zone is excluded because both processes take place in the same tank (Van Strakenburg

et al., 1993).

2.4.5 BB Activated Sludge Process

The BB process is characterized by the activated sludge undergoing anaerobic, anoxic and aerobic phases over time as a result of alternating aeration. The mixed liquor settles to the bottom of the first aeration tank during the anaerobic/anoxic phase. Phosphate release is not inhibited by the nitrate rich supernatant in the first aeration tank. Nitrification and denitrification take place in the first and second aeration tanks. Phosphate-accumulating bacteria take up phosphate during periods of aeration in the first and second aeration tanks (Van Strakenburg *et al.*, 1993).

2.4.6 The Modified Oxidation Ditch Process

In a carousel or oxidation ditch process the activated sludge mixed liquor flows continuously around a loo-type channel. The activated sludge is aerated by an aeration system. With low oxygenation it is possible to create an aerobic zone capable of nitrification, immediately downstream of the aerator and an anoxic zone some distance upstream of the aerator. The discharging of the influent at the upstream limit of the anoxic zone favours the use of the wastewater carbon source for denitrification. An optimum combination of nitrogen and phosphorus removal is due to the position of the anaerobic zone in the front, where the return sludge meets the influent. The following modifications must be considered for biological phosphorus removal: the first two sections serve as the anaerobic zone, the recirculation flow must go from last to the third section, and the section for sedimentation serve as an anoxic zone (Van Strakenburg *et al.*, 1993).

2.4.7 Phostrip Activated Sludge Process

The Phostrip activated sludge process is a combined biological and chemical process for the removal of phosphorus from wastewater. This side stream process relies on the ability of the sludge microorganisms to release phosphorus in a more concentrated form in a small anaerobic phosphate stripper tank. Recycled sludge stripped of phosphorus is introduced to

an influent feed in an activated sludge reactor. Aerated mixed liquor enters a settler, where activated solids are separated and the supernatant is discharged as effluent. The activated sludge discharge to an anaerobic stripper tank, which is a clarifier, where the phosphorus is released from the organisms into the supernatant. The phosphate stripped sludge is returned to the head of the activated sludge reactor, while the phosphate enriched secondary supernatant is treated separately with lime to precipitate phosphorus (Van Strakenburg *et al.*, 1993).

2.4.8 Biofilm Reactors, Anaerobic Digesters and Lagoon Systems

These different systems for wastewater treatment will be discussed in short. Biofilm technology has developed rapidly during the past two decades and is widely used for wastewater treatment. A biofilm refers to a complex structure of cells and cellular product, such as extracelluar polymers, attached to a solid surface or substratum (Cloete & Muyima, 1997). Biofilm growth results in the digestion and metabolization of organic matter, oxygen, trace elements, required for biological activity, from the liquid phase with which it is in contact (Cloete & Muyima, 1997). Biofilm reactors include the following: trickling filters, rotating biological contactors, submerged biofilters, fluidized-bed reactor and airlift reactors (Cloete & Muyima, 1997).

Anaerobic digesters are the oldest means of wastewater treatment. The primary use of the anaerobic digester is the stabilization of suspended organic matter (Grady & Lim, 1980). Anaerobic digestion refers to a biological engineering process by which a feedstock is converted into a range of simpler compounds including methane, produced by microorganisms in the absence of oxygen (Hughes, 1979). From an energy consumption view point this method of wastewater treatment seems to be the most economical (Cloete & Muyima, 1997).

Lagoon systems provide a low-cost sewage treatment system for small communities. Five types of systems are used for wastewater treatment: aerobic, facultative, anaerobic, aerated and maturation lagoons. The biological diversity of a lagoon system is higher than any other biological wastewater treatment process. Therefore, the lagoon system is a very stable

process (Cloete & Muyima, 1997).

2.5 Nutrient Requirements in the Activated Sludge Process

Nitrogen and phosphorus are the most important nutrients in the activated sludge process. Phosphorus is directly involved in biosynthesis, whereas nitrogen is involved also in the energy transfer system of microorganisms. The lack of these nutrients might effect the growth of the activated sludge/biofilm microorganisms. It can also result in the selection of undesirable filamentous microorganisms (Cloete & Muyima, 1997). High concentrations of nitrogen and phosphate in effluent from the activated sludge process contributes to the problem of eutrophication in receiving water (Cloete & Muyima, 1997).

2.5.1 Nitrogen Removal

Nitrogen is found in wastewater in the reduced form of organic nitrogen and ammonia nitrogen (amino acids, proteins, nitrogen heterocyclic compounds) (Bitton, 1994; Cloete & Muyima, 1997). In biological treated effluents, approximately 90% of the nitrogen is in the form of ammonia and NO_3 (Bitton, 1994). In domestic sewage in RSA the average total nitrogen concentration is approximately 60 $mg.l^{-1}$ - 120 $mg.l^{-1}$ (60 $mg.l^{-1}$ - 120 $mg.l^{-1}$ for RSA). Approximately 15% of the total nitrogen, mainly solid-associated organic nitrogen is removed from domestic wastewater during primary treatment. Conventional biological treatment removes approximately another 10% - 20% of the nitrogen that is associated with cell biomass, which settles in the sedimentation tank (Bitton, 1994). During carbonaceous oxidation of wastewater, organic nitrogen is converted to ammonia nitrogen (ammonification).

2.5.1.1 Nitrification

Nitrification is the process where ammonia, the reduced form of nitrogen is oxidized by autotrophic nitrifying bacteria to nitrite and nitrate. Ammonia is oxidized to nitrite by *Nitrosomonas*, *Nitrosospira* and *Nitrosolobus* spp (Buchan, 1984). Nitrite is oxidized to

nitrate by *Nitrobacter, Nitrospira* and *Nitrococcus* spp. (Buchan, 1984). Nitrification is a chemolithotrophic process. Microorganisms utilize energy generated from the oxidation of inorganic compounds (Cloete & Muyima, 1997). Nitrification is possible at a broad range of pH values, but the optimum is between pH 7.5 - 8. Nitrification is affected by temperature as well as the presence of heavy metals in the ionic form which inhibits the process. A substantial increase in oxygen supply (25% - 35%) is required when nitrification is included (Ekama & Marais, 1984).

The process of nitrification can be described by the following chemical equations:

$$NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + H_2O + 2\ H^+ + 250\ kJ$$

$$NO_2^- + 0.5\ O_2 \rightarrow NO_3^- + 75\ kJ$$

The cost of nitrification and the concentration of nitrate being discharged in the effluent can be decreased when the facility for denitrification is incorporated into the system, because of reduced oxygen requirements and alkalinity consumption (Cloete & Muyima, 1997).

2.5.1.2 Denitrification

When reduced nitrogen is incorporated into newly synthesized biomass the process is termed assimilative nitrate reduction (Cloete & Muyima, 1997). When nitrate nitrogen is reduced to elementary nitrogen and serves as an electron acceptor, the process is known as denitrification (Cloete & Muyima, 1997). A wide range of heterotrophic bacteria can accomplish denitrification under anoxic conditions. Denitrification can take place at temperatures as low as 5°C. The pH range for optimum denitrification is between pH 7.0 - 7.5 (Cloete & Muyima, 1997).

Denitrification can be described by the following equation:

$$NO_3^- + 6\ H^+ + 5\ e^- \rightarrow 0.5\ N_2 + 3\ H_2O$$

The heterotrophic reduction of nitrite and nitrate to molecular nitrogen leads to a substantial elimination of nitrogen from wastewater (Cloete & Muyima, 1997).

2.5.2 Phosphorus Removal in Activated Sludge

Phosphorus (P) is a necessary macronutrient for all living cells. It is an important cellular structural component of adenosine triphosphate (ATP), nuclei acids (DNA and RNA), proteins and phospholipids in cell membranes (Toerien *et al.*, 1990; Bitton, 1994).

Common forms of phosphorus in wastewater are orthophosphate (PO_4^{3-}) (50% - 70% of phosphorus), polyphosphates, and phosphorus tied to organic compounds. Orthophosphate comprises approximately 90% of phosphorus in biologically treated effluents (Bitton, 1994).

The bulk (40% - 60%) of phosphorus in wastewater comes from detergents. The average concentration of total phosphorus (inorganic and organic forms) in wastewater is in the range of 10 - 20 $mg.l^{-1}$ (Bitton, 1994). In the Republic of South Africa the Water Act of 1956 as amended in 1980 limits the concentration of soluble orthophosphate in effluent discharged to certain catchment areas to 1 $mg.l^{-1}$ (Slim, 1987).

Phosphorus is a limiting nutrient in regard to algal growth in lakes. Excess phosphorus in natural water is responsible for eutrophication. Eutrophication leads to significant changes in water quality and lowers the value of surface waters for fishing as well as industrial and recreational uses. By reducing the phosphorus input to receiving water eutrophication can be controlled (Cloete & Muyima, 1997).

Phosphorus is present in wastewater in both the inorganic and organic forms. Phosphorus present in wastewater is often converted to orthophosphate. In this more assimilable form, phosphorus enters the biochemical reaction system (Cloete & Muyima, 1997). The removal of phosphates can be achieved by chemical and/or biological means. Phosphorus removal by chemical precipitation is expensive and increases the salt concentration in effluents. This

is not the ideal solution and may aggravate an already serious mineralisation problem (Slim, 1987). Enhanced phosphate removal by biological means is thus the method of choice in activated sludge systems.

Several biological and chemical mechanisms are responsible for phosphorus removal in wastewater treatment plants:

i) Chemical precipitation, which is controlled by pH and cations such as Ca, Fe and Al.

ii) Phosphorus assimilation by wastewater microorganisms.

iii) Polyphosphate accumulation by microorganisms.

iv) Microorganism-mediated enhanced chemical precipitation (Bitton, 1994).

2.5.2.1 Microbiology of the Phosphorus Cycle

i) Mineralization

Organic phosphorus compounds (e.g. phytin, inositol phosphates, nuclei acids, phospholipids) are mineralized to orthophosphate by a wide range of microorganisms that include bacteria (e.g. *Bacillus subtilis*, *Arthrobacter*), actinomycetes (e.g. *Streptomyces*), and fungi (e.g. *Aspergillus, Penicillium*). Phosphatases are the enzymes responsible for degradation of phosphorus compounds. Enzyme activity can be altered by the presence of different substrate concentrations or product accumulation. Catabolic enzymes are activated by ADP or AMP and inhibited by ATP (Toerien *et al.*, 1990; Bitton, 1994).

ii) Assimilation

Microorganisms assimilate phosphorus, which enters into the composition of several macromolecules in the cell. Some microorganisms have the ability to store phosphorus as polyphosphates in special volutin granules. Volutin granules containing polyphosphate (polyP) have been demonstrated in bacteria by a number of researchers (Toerien *et al.*, 1990; Bitton, 1994).

iii) Precipitation of phosphorus compounds

The presence of Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+} as well as the pH of the aquatic environment control the solubility of orthophosphate. Insoluble compounds such as hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), vivianite $Fe_3(PO_4).8H_2O$ or variscite $AlPO_4.2H_2O$

are formed when precipitation occurs (Bitton, 1994).

iv) **Microbial solubilization of insoluble forms of phosphorus**

Through their metabolic activity, microorganisms help in the solubilization of phosphorus compounds. The metabolic processes of solubilization involves enzymes, production of organic and inorganic acids by microorganisms (e.g. succinic acid, oxalic acid, nitric and sulphuric acid), production of CO_2, which lowers the pH, production of H_2S, which may react with ironphosphate and liberate orthophosphate and the production of chelators that can complex Ca, Fe or Al (Bitton, 1994).

Primary treatment of wastewater removes only 5% - 15% of phosphorus (that associated with particulate organic matter) and conventional biological treatment does not remove a substantial amount of phosphorus (approximately 10% - 25%) (Bitton, 1994). Most of the retained phosphorus is transformed to sludge. Additional phosphorus removal can be achieved chemically by adding iron and aluminum salts or lime to wastewater. Commercially available aluminum and iron salts are alum, ferric chloride, ferric sulphate, ferrous sulphate and waste pickle liquor from the steel industry. These are generally added in excess due to the competitive formation of metal hydroxides. Lime is less frequently used for phosphorus removal because of increased production of sludge as well as the operation and maintenance problems associated with its use.

Aluminum reacts with phosphorus to form aluminum phosphate:

$$Al^{3+} + PO_4^{3-} \rightarrow AlPO_4$$

Ferric chloride reacts with phosphorus to form ferric phosphate:

$$FeCl_3 + PO_4^{3-} \rightarrow FePO_4 + 3\ Cl^-$$

Other treatments for removal of phosphorus include adsorption to activated alumina, ion exchange, electrochemical methods and deep-bed filtration (Bitton, 1994).

2.5.2.2 Enhanced Biological Phosphorus Removal (EBPR)

In addition to chemical precipitation, phosphorus can also be removed by biological means. Phosphorus removal by activated sludge was first observed by Srinath and his colleagues, (1959). Shapiro and his collaborators (1967) demonstrated the role of microorganisms in the uptake and release of phosphorus in the activated sludge system (Shapiro, 1967; Shapiro, Levin & Zea, 1967). Prokaryotes and eucaryotes can store phosphorus as polyphosphates in intracellular volutin granules. Enhanced biological removal of phosphorus is based on the enrichment of activated sludge with bacteria capable of accumulating orthophosphate in excess of the normal metabolic requirements in the cell (Cloete & Muyima, 1997).

EBPR was promoted if a sizable portion of the influent organic carbon was in the form that was readily biodegradable (e.g. acetate) and present at the initial anaerobic stage of treatment (Bond *et al.*, 1995). After phosphate has been from the biomass in an anaerobic stage, phosphate is reincorporated in the biomass during aeration, together with part or all of the influent phosphorus (Fig. 1) (Toerien *et al.*, 1990; Van Veen, Abee, Kortstee, Konings & Zehnder, 1993). The primary purpose of the stored polyP in most bacteria is that it serves as a phosphorus source for periods of phosphorus starvation (Wanner, 1994).

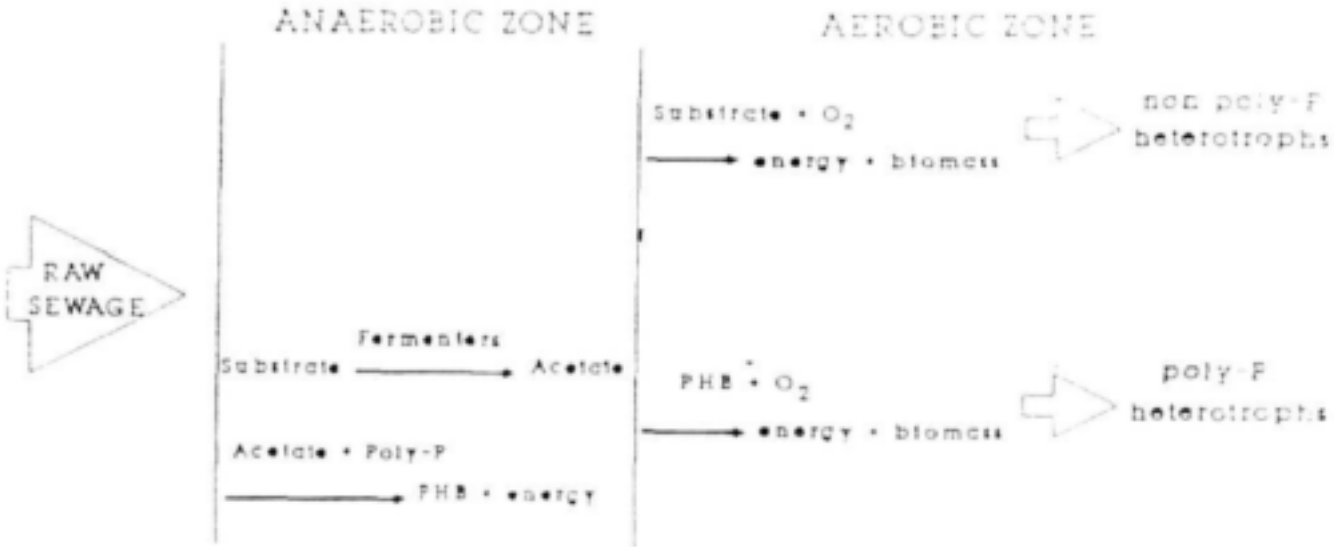


Figure 1: Acetate formation and subsequent storage of PHB by PolyP bacteria

(Meganck & Faup, 1988)

2.5.2.3 Enzymes Involved in EBPR

Two enzymes are involved in the degradation of polyphosphate i) Polyphosphates, ATP: polyphosphate phosphotransferase (Polyphosphate kinase), Polyphosphate glycokinase and ii) Polyphosphate: AMP-phosphotransferases (Towner, Bergogne-Bèrèzin & Fewson, 1991; Van Veen *et al.*, 1993). The latter enzyme enables (in combination with adenylate kinase) the organism to conserve the energy from the phosphate bonds in polyP and so use the accumulated polymer as a source of ATP when energy cannot be obtained otherwise (Van Veen *et al.*, 1993). The synthesis of poly-ß-hydroxybutyrate during the anaerobic phase allows its use in the subsequent aerobic phase to provide oxidizable substrate for phosphate uptake and polyphosphate synthesis and cell synthesis (Towner *et al.*, 1991). The production of volatile fatty acids during the anaerobic phase provides the conditions of excess carbon and energy sources required in the aerobic phase for the accumulation of energy reserves, with the result that both poly-ß-hydroxybutyrate and polyphosphate accumulate (Towner *et al.*, 1991). Phosphorus uptake occurs against a concentration gradient and depends on the presence of an oxidizable energy source which is inhibited by respiratory chain inhibitors and uncouplers of the oxidative phosphorylation (Van Veen *et al.*, 1993).

2.5.2.4 Modes for EBPR

Two modes of biological phosphate uptake have been identified: The first mode is termed polyphosphate "overplus" observed by Harold (1964). Polyphosphate overplus designates the accumulation of polyphosphate upon the addition of orthophosphate to phosphate starved organisms. This phenomenon consists of a rapid uptake of inorganic-phosphate by the phosphate-starved bacteria on transfer to a phosphate-rich media containing a carbon and energy source. Some of the inorganic phosphate taken up is converted to polyphosphate which can be seen as volutin granules within the cell. After a short period of growth this polyphosphate is degraded and utilised to supply phosphate for synthesis of nucleic acid by the growing bacteria (Towner *et al.*, 1991). The overplus phenomenon is not likely to occur in wastewater treatment however, since phosphate starvation does not occur in any step of

the treatment (Fuhs & Chen, 1975). Levin and Shapiro (1965) introduced the term "luxury uptake" to describe the ability of activated sludge to remove more phosphate than is required for growth. Fuhs and Chen, (1975) termed these bacteria accumulating polyphosphate, polyP bacteria. Since then, two approaches have been taken to explain the mechanisms of enhanced biological phosphorus removal in wastewater treatment plants: microorganism-mediated chemical precipitation and microorganism-mediated enhanced uptake of phosphorus (Bitton, 1994).

i) **Microorganism-mediated chemical precipitation**

According to this approach, precipitation of phosphate and its subsequent removal from wastewater is mediated by microbial activity in the aeration tank of the activated sludge process. At the head of a plug-flow aeration tank microbial activity leads to low pH, which solubilizes phosphate compounds. At the end of the tank, a biologically mediated increase in pH leads to phosphate precipitation and incorporation into sludge. Biologically mediated phosphate precipitation also occurs inside denitrifying biofilms. Since denitrification produces alkalinity, denitrifier activity leads to an increase in pH and subsequently precipitation of calcium/phosphate in biofilms. Precipitation of phosphorus can also be induced by the increase in phosphate concentration that results from release of phosphorus from the polyphosphate pool under anaerobic conditions.

ii) **Microorganism-mediated enhanced uptake of phosphorus**

Enhanced removal of phosphorus is the result of microbial action in the activated sludge process. Several mechanisms have been proposed to explain the enhanced uptake of phosphorus by microorganisms in wastewater. Phosphorus is accumulated intracellularly in polyphosphate granules (e.g. volutin granules), which can be easily observed under bright-field or phase contrast microscopy. Polyphosphates serve as energy and phosphorus sources in microorganisms.

An enzyme, polyphosphate kinase, catalyses polyphosphate biosynthesis in the presence of Mg^{2+} ions by transferring the terminal phosphorylgroup from ATP to the polyphosphate chain (Bitton, 1994).

Polyphosphate degradation is driven by several enzymes according to the following reactions (Bitton, 1994):

polyphosphate AMP phosphotransferase

$$Polyphosphate_n + AMP \rightarrow (polyphosphate)_{n-1} + ADP$$

adenylate kinase

$$2\ ADP \leftrightarrow ATP + AMP$$

2.5.2.5 Conditions for EBPR

Biological phosphorus removal is characterized by complex interactions between different intracellular components (poly-ß-hydroxybutyrate; PHB, poly-phosphates; polyP and possibly glycogen) and is induced under alternating anaerobic and aerobic or anoxic conditions (Peterson, Temmink, Henze and Isaacs, 1996). The stability of the process depends highly on a maintenance of these intercellular interactions which inevitably is connected to extracelluar factors. Readily biodegradable organic substrates are stored intracellularly as PHB under anaerobic conditions by polyphosphate accumulating organisms (PAO's) (Peterson *et al.*, 1996). The energy to this anaerobic process is derived from hydrolysis of polyP, the result being a release of phosphate. Glycogen is possibly degraded anaerobically to produce reduction equivalents which are required for the PHB synthesis. Under the carbon-limited aerobic or anoxic conditions PHB is utilised for growth, glycogen synthesis and uptake of phosphate for storage as polyP (Bitton, 1994).

Polyphosphatases are hydrolytic enzymes that are involved in polyphosphate degradation. In some bacteria, the hydrolysis of polyphosphates is driven by polyphosphate glucokinase and polyphosphate fructokinase, resulting in the phosphorylation of glucose and fructose, respectively. Polyphosphate accumulating aerobic bacteria such as *Acinetobacter* take up phosphorus under aerobic conditions, accumulate it as polyphosphate granules and release

it under anaerobic conditions (Bitton, 1994). For example *Acinetobacter calcoaceticus* takes up phosphorus under aerobic conditions at a rate of 0.4 - 0.5 mmole.g^{-1} dry cells per hour and releases it under anaerobic conditions at a rate of 0.015 mmole.g^{-1} dry cells per hour (Bitton, 1994). Magnesium plays an important role in polyphosphates and is taken up and released simultaneously with phosphate. Other studies have shown that in addition to Mg^{2+}, K^{+} and and to a much lesser extent Ca^{2+} are also co-transported with phosphate (Comeau *et al.*, 1986).

2.5.2.6 Microorganisms Involved in EBPR

There is controversy over whether *Acinetobacter* is the predominant microorganism involved in enhanced phosphorus uptake. Cloete and Steyn (1987) found no correlation between the number of *Acinetobacter* and the extent of phosphorus removal. The use of respiratory quinone profiles to characterize the bacterial population structure of the anaerobic-aerobic activated sludge system showed that *Acinetobacter* species were not important in the system (Hiraishi, Masamune & Kitamura, 1989). However, it was shown that *Acinetobacter*, as detected by the biomarker diaminopropane, was the dominant organism only in wastewater treatment plants with low organic loading (Auling, Pilz, Busse, Karrasch, Streichan & Schon, 1991).

Many bacteria have been reported to accumulate polyphosphate in excess of the normal cell requirement which is around 1% - 3% of the cell dry weight (Bitton, 1994). The following PAO's have been reported: *Acinetobacter* sp., *Aeromonas hydrophila*, *Pseudomonas* sp., *Moraxella* sp., *Enterobacter* sp., *Xanthobacter* sp., *Camomonas-Pseudomonas* group, *Zoogloea ramigera*, some filamentous bacteria, coryneform bacteria and some gram positive bacteria (Bitton, 1994; Cloete & Muyima, 1997). Transmission electron microscopy indicated that the type of phosphate-accumulating bacteria depends on the wastewater composition and on the process used for phosphorus removal (Streichan *et al.*, 1990).

2.5.3 Phosphorus Release under Anaerobic Conditions

Under anaerobic conditions bacteria use energy derived from polyphosphate to take up carbon

substrates, which are stored as PHB reserves and to regulate the pH gradient across the cytoplasmic membrane (Bitton, 1994). This phenomenon leads to the release of inorganic phosphorus. Simple organic substrates such as acetate are taken up by microorganisms and stored intracellularly as PHB, which subsequently are used as a carbon source during the aerobic phase (Toerien *et al.*, 1990). Acetate is converted to acetyl-CoA and the reaction is driven by energy supplied by the hydrolysis of accumulated intracellular polyphosphates. NADH which provides the reduction power for PHB synthesis is derived from the consumption of intracellular carbohydrate (Arun, Mino & Matsuo, 1988). Some investigators have suggested that the anaerobic zone serves as a fermentation milieu in which microorganisms such as *Aeromonas* produce volatile fatty acids such as acetate which are taken up by PAO's and stored as PHB (Brodisch & Joyner, 1983; Meganck, Malnou, Le Flohic, Faup & Rovel, 1984)

To maximize carbon storage under anaerobic conditions, Comeau and colleagues (1987) suggested increasing the addition of simple carbon sources (e.g. septic wastewater, fermented primary sludge supernatant, acetate salts) and minimizing the addition of electron acceptors such as O_2 and NO_3. Readily biodegradable short-chain carbon substrates (butyric and isobutyric acids, valeric and isovaleric acids, ethanol, acetic acid, methanol, sodium acetate) can enhance removal of phosphorus (Jones, Tadwalkar & Hsu, 1987; Abu-Ghararah & Randall, 1990).

2.5.4 Enhanced Uptake under Aerobic Conditions

Under aerobic conditions the energy derived from the metabolism of stored (e.g. PHB) or external carbon sources in the presence of O_2 or NO_3 is used for the accumulation of polyphosphates inside the cells (Bitton, 1994). Under these conditions, inorganic phosphorus is taken up by the cells and stored as polyphosphates. Toxicants such as 2.4-dinitrophenol and H_2S have an adverse effect on phosphorus uptake under aerobic conditions (Comeau, Rabinowitz, Hall & Oldham, 1987). Methods have been developed for distinguishing intracellular polyphosphate from extracelluar precipitated orthophosphate. The use of these methods has indicated that polyphosphate is the predominant form of bioaccumulated phosphorus in activated sludge (Bitton, 1994).

2.6 Activated Sludge Microbial Population Dynamics

The term "activated sludge population dynamics" can be understood as a branch of water science and technology dealing with the phenomena that covers the relationships between individual members of a complex microbial consortium traditionally called activated sludge. Studies concerning the structure and function of bacterial communities are crucial to understand the dynamics and stability of natural ecosystems. The limited methodology available makes it difficult to study species composition and diversity of whole bacterial communities. Therefore, the knowledge of microbial community structure and function and their correlation with plant performance is limited (Wallner, Erhart & Amann, 1995). It should be emphasized that the activated sludge system is invariable carbon limited, this is the factor that dominates the competitive interactions between heterotrophic bacteria.

2.6.1 Microbial Ecology of Activated Sludge

Activated sludge can be defined as an artificial living ecosystem under the continuous influence of abiotic and biotic factors. Due to strong competition between the different organisms only the best adapted organisms will win. Activated sludge consists of different species which co-exist in aggregates and function together.

The activated sludge microorganisms can be divided into two major groups:

i) decomposers, are responsible for biochemical degradation of polluting substances in wastewater. This group represents bacteria, fungi and colourless cyanophyta.

ii) consumers, utilize bacterial and other microbial cells as substrates. This group of activated sludge microfauna, consists of phagothrophic protozoa and microscopic metazoa (Cloete & Muyima, 1997).

Decomposers represent 95% of the microbial population of activated sludge, especially by bacteria. This indicated that the role of microfauna in the removal of organic pollution and nutrients are only marginal (Cloete & Muyima, 1997).

Bacteria specialized to specific substrates can be concentrated in activated sludge after proper adaptation. Oxic organotrophic microorganisms are able to degrade complex organic substrates by exo-and endoenzymes (Cloete & Muyima, 1997). Fermentative bacteria are responsible for the conversion of organic compounds to volatile fatty acids which are essential for EBPR (Cloete & Muyima, 1997). A wide range of bacteria are able to use nitrate nitrogen as the final acceptor in biochemical reactions. These organisms are called anoxic organotrophic microorganisms or the denitrifiers. Nitrifying bacteria are responsible for nitrification in the aerobic phase of the activated sludge process. The PAO's are able to remove phosphate from wastewater by the EBPR mechanism (Cloete & Muyima, 1997). Sulphur bacteria also play an important role in the activated sludge process because they can cause bulking problems (Cloete & Muyima, 1997).

2.6.1.1 r- and K-Strategists

Microorganisms, like higher plants and animals, have evolved strategies that enable them to successfully survive and maintain themselves within communities. One artificial scheme for viewing these strategies classifies organisms along a r-K gradient (Andrews & Harris, 1985; Atlas & Bartha, 1993). K refers to carrying capacity and r to the maximal intrinsic rate of natural increase. In practice, most natural microbial environments will show characteristic oscillations between extreme r-and extreme K-conditions (Andrews & Harris, 1985).

Biological factors that play a role is that organisms strive to maximize their fitness for survival through r- or K-strategies. In uncrowded environments, r-strategists can attain maximum specific growth rates; in crowded environments, K-strategists can attain maximum population densities (Toerien *et al.*, 1990).

Although in comparison to macroorganisms, all microorganisms might appear to be r-strategists and have been viewed in this manner, compared to each other, some microorganisms can be considered K-strategists. The main bacteria fraction counted under the microscope is the K-selected population, composed of a large number of different bacteria, having a lower growth rate than the colony-forming bacteria (Torsvik, Goksøyr & Daae, 1990b). K-strategists, which reproduce more slowly, tend to be successful in

resource-limited situations. A K-environmental condition exists when the environment/population relationship is such that the specific rate of increase of the population is close to or at zero and the population density is correspondingly close to or at the carry capacity K (Andrews & Harris, 1985). The non-platable K-selected population must consist of a large number of different, highly specialized bacteria.

A r-strategist microorganism would be one that through rapid growth rates, takes over and dominates situations in which resources are temporarily abundant (Atlas & Bartha, 1993). The r-strategists rely upon high reproductive rates for continued survival within the community, whereas the K-strategists depend upon physiological adaptations to the environmental resources of carrying capacity of the environment. Although r- strategists have high reproductive rates, they have few other competitive adaptations, they tend to prevail in situations that are not resource-limited, that is where nutrients are not severely limiting, and in which high reproduction rates outweigh the advantages of other competitive adaptations (Atlas & Bartha, 1993).

A r-condition may arise temporarily because of an increase in the food supply and/or a catastrophic decline in population density. Theoretically bacteria growing on plates are r-selected bacterial populations having fairly high growth rates and growing on high nutrient concentrations (Torsvik *et al.*, 1990b). Populations of r-strategists are subject to extreme fluctuations. When resources became scarce and conditions unfavourable, their populations experience rapid reduction. Populations of K-strategists tend to be more stable and permanent members of the community. They tend to prevail in uncrowded communities and devote a large portion of their resources to reproduction (Andrews & Harris, 1985; Atlas & Bartha, 1993).

Factors in enhanced P-removal activated sludge systems tending to favour K-strategists:

i) high-density population achieved by physically returning microorganisms to the systems after gravity settling in the secondary settling tank.
ii) long sludge ages (15 - 20 days) in systems.
iii) low nutrient concentrations

Factors that favour r-strategists include:

i) alternating anaerobic and aerobic conditions which stress aerobic and anaerobic organisms, respectively.

ii) high nutrient concentrations at the point of entrance of influent and,

iii) the presence of predators such as protozoa and rotifers (Toerien *et al.*, 1990).

It can be expected that both r- and K-strategists will occur in enhanced nutrient removal activated sludge systems and that keen competition between these organisms partly determine the microbial population composition in these systems (Toerien *et al.*, 1990).

TABLE 1: Summary of the properties of r- and K-strategists compiled from Andrews and Harris, (1985).

	r-strategists	K-strategists
A Properties		
1 Numbers:	Low - uncrowded	High - crowded
2 Nutrient uptake:	High acquisition rate	High affinity rate
3 Growth rate:	High growth rate	Low growth rate
4 Resources:	Used for reproduction	Used for maintenance
5 Nutrients in surrounding:	Fluctuate (temporarily abundant)	Constant (low)
6 Abilities:	Specialists (do one thing well)	Generalists (do many things indifferently)
7 Stress resistance:	Resistant spores	High sensitivity of spores to germinate
8 Temperature requirements:	Grow at higher temperatures (tropical)	Grow at cooler temperatures (polar)
B Abilities		
1 Constancy:	Extreme fluctuations	Stable community numbers
2 Competitive adaptations:	Few (occur in situations that are not resource limited)	Many (compete for nutrients in limited nutrient surroundings)
3 Survival within community:	High reproductive rate. Take over by rapid growth rate where resources are temporarily abundant	Physiological adaptation to carrying capacity of environment
4 Fitness: (Proportion of genes left in the gene pool)	Fit in uncrowded selection	Fit in crowded environment
5 Term refers to:	Maximum specific growth rate minus minimum specific death rate	Density of individuals that a given environment can support at the population equilibrium

2.6.2 Microbiology of Activated Sludge

The microbial community structure of activated sludge consists of a complex consortium of individual members. The following organisms have been observed as part of the microbial community of activated sludge: bacteria, protozoa, fungi and filamentous microorganisms. The role of algae and fungi are considered not so important, whereas protozoa, filamentous organisms and bacteria actively participate in the biological treatment of the activated sludge system (Cloete & Muyima, 1997). Protozoa are responsible for "grooming" the zoogloeal mass by grazing on it, and are consumed by other organisms present in the system. Ciliates such as *Virticella, Opercularias* and *Epistylis* play an important role in the activated sludge system (Shapiro, 1967). Certain amoebae prey on bacteria cells (Buchan, 1983).

The nitrifying bacteria were originally soil microbes. In an aquatic environment the following genera of nitrifiers are reported: *Nitrosomonas, Nitrosococcus, Nitrosospira* and *Nitrosocystis* for the oixidation of ammonia. *Nitrobacter, Nitrospina* and *Nitrococcus* for the final oxidation of nitrite to nitrate (Cloete & Muyima, 1997). The role of the filamentous microorganisms are important as they promote the formation of bacterial flocs. The sedimentation ability of the sludge depends on floc formation (Jenkins, Richard & Daigger, 1986). However, when the filamentous population exceeds a certain level (e.g. 10^7 $\mu m.ml^{-1}$ or 10^4 $m.g^{-1}$) settling and compaction problems occur such as: low settling velocities and excessive volume of the settled sludge which leads to a dilution of the mixed liquor and causes operation problems. Filamentous organisms are directly responsible for bulking (due to the proliferation of these organisms) and foaming (absence of these organisms) which results in separation problems and that the final effluent is contaminated with bacteria-rich sludge (Jenkins *et al.*, 1986; Blackall & Hugenholtz, 1993). The most frequently found filamentous organisms are: *Microthrix parvicella, Halicsomenobacter hydrossis, type 0092, type 1701, Norcardia amarae, Norcardia pinensis, Sphaerotilus natans, Nostocoida limicola type 0961, 021N, Thiothrix, 0041 0675* and *Rhodococcus* spp. (Jenkins *et al.*, 1986; Blackall & Hugenholtz, 1993; Duchene, 1993; Eikelboom, 1993; Wanner, 1994). The excessive growth of *Norcardia erythropolis* and *Microthrix parvicella* leads to scum formation, offensive odours and when discharged with the final effluent, to a significant increase in the COD and suspended solids content.

The microbial community of activated sludge contains of heterotrophic bacteria. Researchers found the following bacterial species in activated sludge: *Acinetobacter, Moraxella, Flavobacterium, Pseudomonas, Vibrio, Achromobacter, Alcaligenes, Enterobacter, Serratia, Proteus, Aeromonas, Proteobacter, Xanthobacter, Aerobacter, Klebsiella, Bordetella, Comamonas, Zoogloea, Citrobacter, Shigella, Pasteurella, Yersinia* spp, *Escherichia intermedium* and *Bacillus cereus* (Dias & Bhat, 1964; Harold, 1966; Shapiro, 1967; Fuhs & Chen, 1975; Deinema *et al.*, 1980; Shoda, Oshumi & Udaka, 1980; Brodisch & Joyner, 1983; Gersberg & Allen, 1984; Lötter, 1985; Lötter & Murphy, 1985; Suresh, Warburg, Timmerman, Weels, Cocia, Roberts & Halvorson, 1985; Venter, Lötter, De Haas & MacDonald, 1989; Streichen *et al.*, 1990; Auling *et al.*, 1991; Kavanaugh & Randall, 1994).

Activated sludge is therefore a diverse ecosystem which consists of different bacterial species which all function together to maintain a stable bacterial community.

2.6.3 Polyphosphate Accumulating Organisms (PAO's)

Biological phosphorus removal can be performed by a rather broad group of microorganisms, the Phosphate Accumulating Organisms (PAO's) (Henze, 1996). The microorganisms responsible for biological phosphorus removal are still partly unknown with respect to taxonomy, but we know that they are present in all biological wastewater treatment processes, although not showing any biological phosphorus removal activity until being activated (Henze, 1996). It take time(days) to activate them and further grow them (Bond *et al.*, 1995; Henze, 1996). Their growth rate is approximately 2 d^{-1} at 20°C, which is twice that of nitrifying bacteria (Henze, 1996).

Since Fuhs and Chen (1975) first implicated *Acinetobacter* spp. as having an important role in EBPR, subsequent studies have focused on this bacterial genus. It has been thought that bacteria of the gamma subclass of the proteobacteria, such as *Acinetobacter* species, dominated phosphorus removing activated sludge populations. In a sludge that was conditioned to remove phosphate by the addition of acetate, bacteria from the genus *Acinetobacter* were reported to make up to 90% of the cultivated heterotrophs (Bond *et al.*, 1995). Culture-dependent methods consistently indicated that *Acinetobacter* spp. were the

numerically dominant members of EBPR systems. *Acinetobacter* strains isolated from activated sludge accumulated excessive amounts of polyphosphate in pure culture, suggesting their importance in the EBPR process (Bond *et al.*, 1995). Other members of the gamma subclass such as aeromonads, vibrio's and coliforms were also reported to dominate an activated sludge community as determined by plate culturing onto various media (Bond *et al.*, 1995).

By contrast, non-culture-dependent methods such as quinone profiles and fluorescent in situ hybridization probes indicated that *Acinetobacter* spp. were present in small proportions in activated sludge (ca. 3% to 6%) (Kavanaugh & Randall, 1994). Wagner *et al.*, (1993) found that members of the gamma proteobacterial subclass were selected for by cultivation with nutrient media, explaining the results of Kavanaugh and Randall (1994). Bond *et al.*, (1995) also indicated that *Acinetobacter* spp. made up only a small portion of the community (ca. 2%). Experimental studies, therefore, indicated that *Acinetobacter* spp. were not the dominant, nor the only organism responsible for enhanced biological removal of phosphate (Cloete & Muyima, 1997). Researchers have difficulties reconciling the carbon and phosphorus transformations in pure cultures of *Acinetobacter* strains with the biochemical model for EBPR. Doubts have been raised as to the significance of *Acinetobacter* spp. in the EBPR process. Compelling evidence for this change of view has been the recent non-culture-dependent studies of phosphorus removing communities. In all cases, *Acinetobacter* spp. were found to represent only a small portion of the total EBPR microbial population, instead, other bacterial groups such as the gram-positive bacteria and the beta subclass groups of the proteobacteria were numerically dominant (Bond *et al.*, 1995). These non-culturable-dependent methods diminishes the significance of *Acinetobacter* spp. in EBPR processes, however, the resolution of the methods has not been sufficient to propose alternative EBPR candidate genera (Bond *et al.*, 1995). The presence of other bacteria was also recorded: *Pseudomonas, Klebsiella, Moraxella, Aeromonas, Escherichia coli, Flavobacteria, Bordetella, Citrobacter, Shigella, Pasteurella* and *Yersinia*. The presence of gram positive organisms like *Bacillus* and *Staphylococcus* were also recorded (Cloete, Bosch & Mienni, 1992; Bond *et al.*, 1995).

These data suggested that culturing techniques have provided a misleading picture of bacterial

community structure in activated sludge systems and in general the role of *Acinetobacter* spp. in activated sludge processes has been overrated. The larger percentages of bacteria found by Bond and colleagues (1995) were from the proteobacterial subclass (ca. 28%). Bacteria from this subclass have been observed by fluorescent in situ hybridization to be dominant in activated sludge communities (Manz, Wagner, Amann & Schleifer, 1994; Wagner *et al.*, 1993; Wagner, Erhart, Manz, Amann, Lemmer, Wedi & Schleifer, 1994).

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. Their selective advantages requires (1) their presence (2) alternating aerobiosis-anaerobiosis, (3) degradable organic matter which can be fermented by acidogenic bacteria, and (4) the presence of a sufficient quantity of P to allow uptake of fatty acids during anaerobiosis and phosphorus uptake during aerobiosis (Toerien *et al.*, 1990). A certain quantity of phosphate is removed for normal metabolic requirements by all the microorganisms in activated sludge. Menar and Jenkins (1970) pointed out that this removal can only account for a maximum of 20% - 30% of the phosphorus present and not the enhanced removal rates (> 90%) observed. However the precise mechanism of enhanced phosphorus removal in activated sludge systems has not yet been clarified.

Under favourable conditions, phosphate removing sludge is observed to take several sludge ages to develop (Bond *et al.*, 1995). This suggests that the phosphorus removing bacterial community may need to be established and may not occur merely as a result of conditioning of the existing population. The ability of the PAO's to store polyphosphate is dependent on the availability of low molecular organics, mainly fatty acids, under anaerobic conditions. The amount of phosphorus that can be removed biologically is directly coupled to the amount of substrate that the PAO's can take up in the anaerobic tank. The substrate will be available for the process either from the raw wastewater or from fermentation in the anaerobic tank (Henze, 1996). In the anaerobic tank stored phosphorus is released to the bulk liquid. The PAO's need alternating anaerobic-aerobic conditions in order to build-up their internal energy storage components, organic polymers and polyphosphates (Fig. 2). These energy pools allow the organisms to utilize the energy in the organic substrate for polyphosphate build-up. The details in the metabolism are still not agreed upon, but polyalkanoates and glycogen are

involved (Henze, 1996).

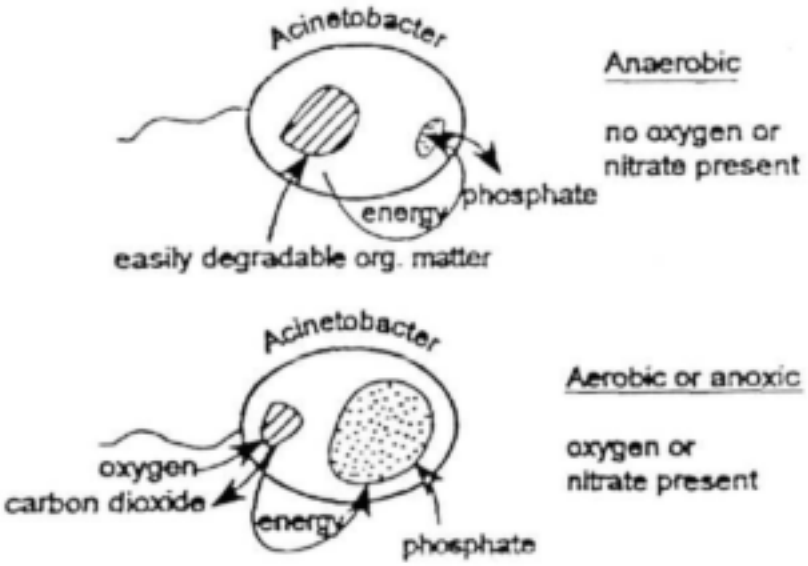


Figure 2: **The alternation of energy storage compounds in phosphate storing organisms such as *Acinetobacter* (Henze, Gujer, Mino, Matsuo, Wentzel, and Marais, 1995)**

2.6.4 Possible Metabolism of PAO's

Polyphosphate accumulating organisms (PAO's) which are responsible for biological phosphate removal and glycogen accumulating organisms (GAO's) which appear when phosphate removal deteriorates are the two important microbiological populations in EBPR processes (Mino, Liu, Satoh & Matsuo, 1996).

The anaerobic-aerobic activated sludge process for EBPR is characterized by the introduction of an anaerobic zone at the influent end of the conventional activated sludge process. It is an established process but its phosphate removal mechanisms are not fully understood from the microbiological and biochemical point of views (Mino *et al.*, 1996). When the anaerobic zone is introduced, a biological selective pressure is created by the condition that the

activated sludge biomass come into contact with organic substrates under anaerobic conditions. Microorganisms that have the capability to take up organic substrates anaerobically can exclusively grow under such a selective pressure. Two microbial populations known to have such a capability, polyphosphate accumulating organisms (PAO's) responsible for EBPR and glycogen accumulating non-polyP organisms (GAO's) which appear when phosphate removal deteriorates (Mino *et al.*, 1996).

PAO's take up organic substrates in the anaerobic phase, accompanied by degradation of polyP and subsequent release of orthophosphates (Pi), consumption of intracellularly stored glycogen, and accumulation of intracellular polyhydroxyalkanoates (PHA). In the subsequent aerobic phase, the polyphosphate and glycogen consumed in the anaerobic phase are recovered with the consumption of the previously stored PHA as well as "new" stored polyP and glycogen. GAO's also take up organic substrates under anaerobic conditions, accompanied by consumption of stored glycogen and accumulation of PHA. The glycogen level is recovered in the subsequent aerobic phase along with the consumption of the accumulated PHA. GAO's metabolism apparently resembles that of PAO's, except that there is no involvement of polyP metabolism (Fig. 3).

Under "normal conditions", PAO's get dominant in EBPR processes and good phosphorus removal efficiency is achieved. If GAO's happen to dominate, phosphorus removal deteriorates. The anaerobic metabolism between PAO's and GAO's are very similar in the sense that glycolysis enables the redox balance control in the cell and that the redox balance regulates the whole metabolism. The major difference between them is the source of energy: PAO's generate energy by degrading stored polyP, whereas GAO's degrade glycogen through glycolysis to generate energy. The latter metabolism is much more complicated than the former, which seems to be a disadvantage for GAO's. PAO's enriched sludges take up acetate more and faster than GAO enriched sludges (Mino *et al.*, 1996). However, the causes for the change from PAO to GAO dominant behaviour are not understood. Indeed, the microbiology of enhanced P-removal activated sludge systems has received some attention but is far from completely understood.

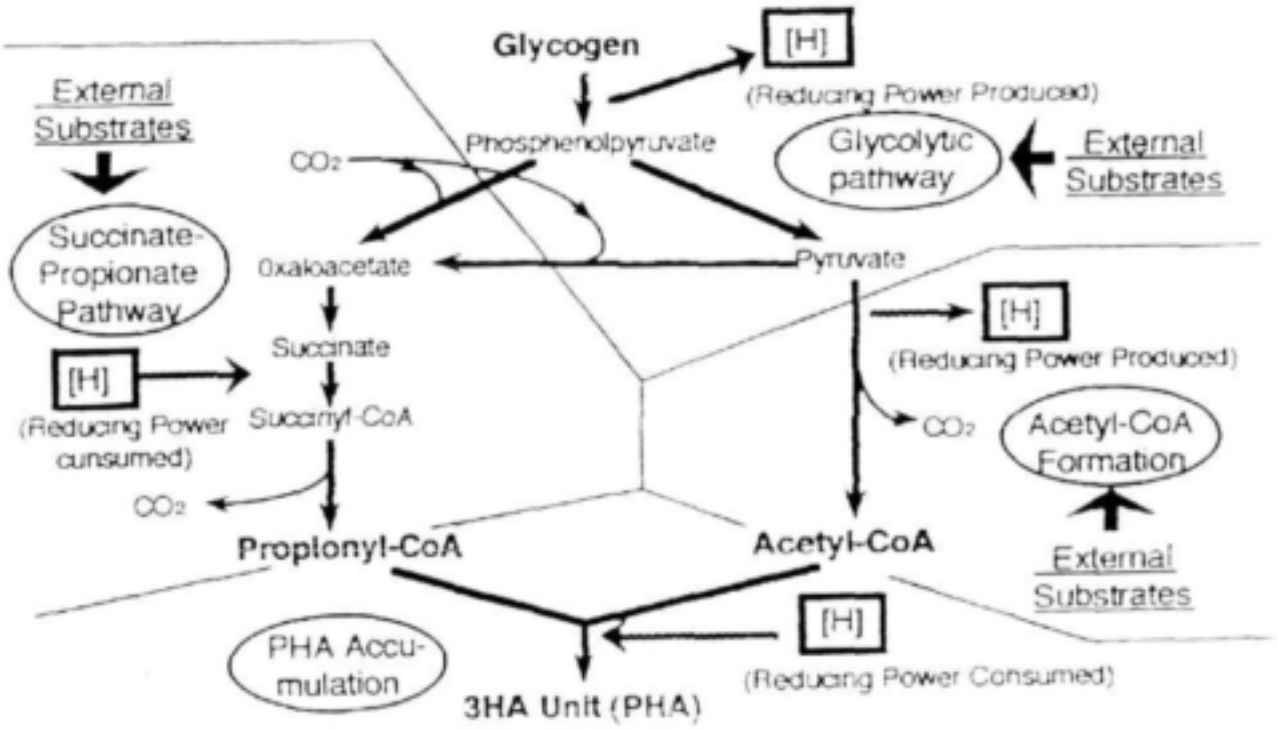


Figure 3: **Key pathways of anaerobic substrate uptake and its conversion to PHA by PAO's and GAO's (Mino *et al.*, 1996).**

2.7 Methods to determine the Bacterial Community Structure of Activated Sludge

Until now it has not been possible to isolate a pure culture of bacteria that could be responsible for biological phosphorus removal. There are many problems using present culture methods with selective media to study the structure of bacterial communities in nature (Lee *et al.*, 1996). Cloete and Steyn (1987) found that less than 10% of microscopic cell counts of activated sludges could be accounted for in agar plate enumerations. Due to the problems deriving from culture-dependent methods, several other methods were considered to determine the bacterial community structure of activated sludge. These methods alleviate the need for culturing and sludge samples are directly analyzed.

Genotypic information is derived from the nucleic acids (DNA and RNA) present in the cell, whereas phenotypic information is derived from proteins and their functions, different

chemotaxonomic markers and a wide range of other expressed features (Van Damme, Pot, Gillis, De Vos, Kersters & Swings, 1996). Expression of genes in many microorganisms correlates with a variety of environmental stimuli ranging from the presence of particular nutrients to changes in physical-chemical conditions (Ogunseitan, 1993).

2.7.1 SDS-PAGE

In chemosystematic analysis bacterial cells are chemically characterised. Growth conditions can effect the biochemical and physiological tests used in numerical taxonomy considerably. The determination of DNA base composition, protein profiles, fatty acid profiles and phospholipid analysis have become established in groups of bacteria where morphological characteristics have failed to provide classification (Schleifer & Stackebrandt, 1983; O'Brien & Colwell, 1987; Haldeman & Amy, 1993). The lack of rapid methods to detect the degree of relatedness between bacteria at the genomic level led to the introduction of methods of comparing gene products. One of the fastest and easiest is the electrophoretic separation of whole-cell proteins (Schleifer & Stackebrandt, 1983). SDS-PAGE of whole-cell proteins are a valuable tool and has extensively been used in the classification of bacteria (Kersters, 1990).

Comparative electrophoresis of proteins should be equivalent to DNA:DNA hybridization, since bacteria are being compared on the translational products of most of the cell's chromosome. It is to be expected, therefore, that it will be useful at the species level, and of little value when comparing distantly related bacteria. In a number of cases it has been well proved that PAGE of whole-cell proteins discriminates at much the same level as DNA:DNA hybridization. The two methods give highly congruent results (Priest & Austen, 1993).

The main advantages of numerical analysis of electrophoretic patterns of large numbers of bacteria are: i) rapid grouping, ii) allocation of unknown microorganisms to a group and its possible identification, iii) storage of large numbers of patterns in data banks for reference, iv) a quick decision on whether two colony types in a culture are due to variation or contamination, v) information on epidemiology spreading of animal and plant pathogens, and

vi) DNA:DNA homology determinations of large numbers of strains can be reduced to hybridization of DNA's from the typical representative of each group, previously established by gel electrophoresis (Kerstes & De Ley, 1975).

Several techniques have been developed for detecting and analysing the abundance of genes, and mRNA transcripts in natural microbial communities. No direct methods were developed until now for analysing the protein products of genes expresses from environmental samples (Ogunseitan, 1993).

The extraction of proteins directly from environmental samples is desirable for multiple reasons: i) Analysis of proteins extracted from environmental samples may help characterize the response of microbial communities to stressful conditions such as contamination with toxic chemicals, starvation, heat or oxygen levels ii) Analysis of total proteins extracted from an environmental sample can be employed as a "fingerprint" to type the diversity in the sample, in a way similar to grouping of bacteria according to enzyme polymorphisms and immunological reactions. Such fingerprints may eventually be used to monitor the deterioration or enrichment of species diversity in microbial communities iii) The abundance of proteins to which specific antibodies are available can be directly measured in total proteins extracted from complex ecosystems and used as an index for monitoring the progress of a biocatalytic reaction in situ (Ogunseitan, 1993).

The bacterial genome contains information involved in the production of some 2000 proteins, which function either enzymatically or structurally (Jackman, 1987). Electrophoresis of the total cellular proteins in polyacrylamide gels (PAGE) provides a partial separation in which individual bands represent several proteins (Kersters, 1990 in Clement, Rudolph & Sands, 1990). This complex pattern represents a "fingerprint" of a specific strain or sample that can be used for comparative purposes (Priest & Austin, 1993; Van Damme *et al.*, 1996). Previously this technique has been used for comparative taxonomical studies. Oguseitan (1993) developed techniques for the extraction of whole cell proteins from activated sludge and soil and suggested that SDS PAGE can be a useful tool in comparative studies of environmental samples. SDS forms a SDS-peptide complex, thereby masking the normal electric charge of the molecule, while the heat denatures the protein so that peptide chains

with no secondary structure are obtained, thus allowing only size to play a role in separation. It was necessary to develop a method to realise the bacterial cells from the flocs. Activated sludge samples are first homogenized with glass beads, followed by several washing and centrifuging steps to separate the bacterial cells from the flocs. The standard methodology for pure culture whole cell protein extractions were then used for the extraction of proteins from the activated sludge samples. The bacterial cells are broken, usually by physical means, and the lysate applied directly to the gel. One or two reference proteins are included and, after electrophoresis, the gel is stained with, for example, Coomassie brilliant blue. A densitometer trace of the stained gel provides the quantitative data for the sample. Analysis of the densitometer traces uses traditional numerical methods. Similarities are calculated between each sample using a suitable similarity coefficient, the Pearson product moment correlation coefficient (r). The resultant matrix is clustered using the average linkage algorithm (also known as unweighted pair group method of arithmetic averages, UPGMA) to provide a sorted similarity matrix or dendrogram. Computer programs such as the Gelcompar 4.0 can be used to analyze the results.

Polyacrylamide gels have the property of possessing a pore size of the same order as biological macromolecules (Jackman, 1987). The pore size can be varied using gels of different concentration (range 3% - 30%) to allow separation of a variety of particles, including whole ribosomes, DNA fragments and proteins down to a molecular weight of 5000 (Jackman, 1987).

2.7.2 Molecular Methods

Exploitation of molecular biology techniques has led to the development of new methods such as extraction of nucleic acids from soils or sediments, to study the dominant, non-culturable bacteria (Leff, Dana, McArthur & Shimkets, 1995). Less than 1% of the bacteria visible microscopically can be cultured (Leff *et al.*, 1995). Analysis of directly extracted DNA has the potential to detect specific genes of cryptic microorganisms, or monitor changes in the genotypic diversity of microbial communities (Steffan, Goksøyr, Bej & Atlas, 1988; Somerville, Knight, Straube & Colwell, 1989; Muyzer, De Waal & Uitterlinden, 1993; Tebbe & Vahjen, 1993; Fox, 1994; Tiedje, 1994; Leff *et al.*, 1995). Microbial ecologists

have begun to apply genome based techniques to the study of microorganisms in natural systems obviating the need for cell culture (Torsvik, Salte, Sørheim & Goksøyr, 1990a; Torsvik *et al.*, 1990b). Extraction and purification of DNA from environmental samples may be more tedious than colony hybridization, but more representable of the species diversity (Ogram & Sayler, 1988).

Genotypic methods are those that are directed towards DNA or RNA molecules (Van Damme *et al.*, 1996). Various molecular methods have been used to attempt to determine the species composition of bacterial populations without enrichment culture. Many of these attempts involve cloning and sequencing of the 16S rRNA (Lee *et al.*, 1996).

There are practical problems in using molecular biological methods for ecological studies, because large numbers of samples must be analyzed. Sequencing methods are inadequate because they require a lot of time and effort (Lee *et al.*, 1996).

Microbial populations can be analyzed by using gene probes directed to 5S, 16S and 23S r RNA (Pace, Stahl, Lane & Olsen, 1986; Ogram & Sayler, 1988; Lee & Fuhrman, 1990; Stahl, 1993). Sequence determination approaches are based on analysis of material and are tedious, time consuming and cannot be applied readily to large numbers of strains (Schleifer & Stackebrandt, 1983). These methods can be seen as part of chemosystematic analysis because all the genetic features of the organisms are revealed (Torsvik *et al.*, 1990a). These methods are restricted to the systems on which the initial work was performed as the probes only detect those isolates for which they are made (Ogram & Sayler, 1988). The unculturable species of the community will remain undetected.

The non-culture-dependent molecular approach of cloning and sequencing 16S rDNA from environmental samples has previously been used to determine microbial community structure in soil, ground water and marine habitats (Bond *et al.*, 1995). The 16S-like rRNA has been the common target for determinative hybridization probes. Some regions of the rRNA have remained essentially unchanged, these can be used as targets for universal probes. Universal probes have been used to measure total rRNA abundance in the environment and to assess differences in cellular rRNA content (Amann, Binder, Olson, Chrisholm, Devereux & Stahl,

1990). Chromosomal DNA and RNA are the only chemical components unaffected by growth conditions (Priest & Austen, 1993). Phylogenetic studies on prokaryotes are based on the comparison of homologous genetic sequences, (which are "living fossil records"), in the genetic material of species (Schleifer & Stackebrandt, 1983). Methods like DNA - DNA hybridization are used to determine the genetic homology between organisms (Palleroni, 1994). DNA-DNA hybridization is an indirect parameter of the sequence similarity between two entire genomes. It is, however, debatable whether data which were obtained with short oligonucleotides and experimentally induced mispairing can be extrapolated to entire genomes. At present it remains impossible to convert a percent DNA-binding or DNA-DNA hybridization value into a percentage of whole-genome sequence similarity (Van Damme *et al.*, 1996).

Bond and his colleagues (1995) determined the bacterial community structure of phosphate-removing and non-phosphate removing activated sludge systems. They sequenced the 16S rRNA clone libraries of two aerobic activated sludge samples. After total DNA extractions of the samples the DNA were amplified with PCR by using two universal primers. The amplifications were cloned into a vector and sequenced. A total of 189 clones were sequenced and compared with the sequences of known bacterial cultures. The frequency between the clones and known bacterial sequences were given in percentage similarity. Their results indicated that the *Rhodocyclus* group were more dominant in the sample which removed phosphorus (Bond *et al.*, 1995).

The aim of the study by Bond and his colleagues (1995) was to apply this approach to determine the community structure of activated sludge samples obtained from two different laboratory-scale sequencing batch reactors which differed in phosphate-removing capability (Bond *et al.*, 1995). The bacterial community structures from the two reactors were compared in order to identify differences which may indicate groups or genera important in the EBPR process. This technique seems to have great potential in studies concerning the determination of the organisms responsible for phosphorus removal in activated sludge systems.

2.7.3 Monoclonal and Polyclonal Antibodies

Serotyping is based on the presence of variability in the antigenic constituents of the cells. Structural components such as capsules, cell envelopes, flagella or fimbriae and intercellular molecules and secretion products such as enzymes and toxins have all been used in serological studies. Antigens may be proteins or carbohydrates and may be thermostable or thermolabile (Van Damme *et al.*, 1996).

Bacteria can be identified by their serological properties. Structures on the cell wall of bacteria can induce an immune response in the bodies of mammals. With this method it is possible to generate antibodies against these antigenic structures i.e. proteins, flagella and lipopolysaccharides. The bacteria can be identified through serological reactions, for example the enzyme-linked immunosorbent assay (ELISA) (Priest & Austen, 1993). Antigenic diversity does not correlate with diversity as determined by other methods (Priest & Austen, 1993).

The immunofluorescence approach has successfully been used to identify bacteria in complex environments, e.g. low numbers of *Sphaerotilus natans* in thick flocs of activated sludge (Wagner *et al.*, 1993). The limitation of the immunofluorescence is the production of antibodies which requires a pure culture of the organisms of interest. Fluorescent rRNA-targeted oligonucleotide probes are an alternative to fluorescent antibodies in the identification of bacteria. A combination of polymerase chain reaction-assisted direct retrieval of rRNA sequences and fluorescent in situ probing enabled the specific detection and identification of uncultured bacteria (Wagner *et al.*, 1993; Wallner *et al.*, 1995). Wagner and colleagues (1993) used group specific oligonucleotide probes for in situ analysis of microbial community structure in activated sludge. However, immunofluorescence studies have severe limitations i.e., the specificity of antibodies is generally restricted to the species or subspecies level (Wagner *et al.*, 1994).

There is a need for techniques that do not necessarily identify individual species, but that can differentiate bacterial communities in terms of their constituents (Lee & Fuhrman, 1990). In this study SDS-PAGE was used in different experiments to determine the sensitivity and

application of this method in the determination of the bacterial community structure of activated sludge. However, it is suggested that future research on environmental samples should include the use of both SDS-PAGE and 16S rRNA techniques in comparative studies.

2.8 Summary

In terms of metabolized matter the activated sludge process is probably today's most important biotechnological process (Wagner *et al.*, 1993). Activated sludge is the biological treatment process for both domestic and industrial wastewater (Cloete & Muyima, 1997). The activated sludge process involves the biological degradation of organic material. Under specific conditions nutrients such as nitrogen (N), and phosphorus (P) removal can be obtained in activated sludge. The need for nutrient removal from effluents (wastewater) is due to the worldwide problem of eutrophication.

Many technologies are used for phosphorus removal from wastewater. The dominating technology is simultaneous chemical precipitation. Biological phosphorus removal received interest due to the low sludge production and the fertilizer value of the sludge (Henze, 1996). Substantial savings are achieved through biological rather than chemical P removal (Toerien *et al.*, 1990).

Optimization of enhanced phosphorus removal processes depends on a complete understanding of the ecophysiology of polyP organisms. These organisms in the activated sludge process are selected (or are adapted) by i) alternating anaerobic-aerobic conditions receiving their influent organic load in the anaerobic zone and ii) the physical and chemical properties of the sludge flocs containing the bacteria and which are settled out in sedimentation tanks (clarifiers) before being returned to the influent end of the plant (Toerien *et al.*, 1990).

Diversity and dynamics of the microbial consortia in activated sludge have mostly been analyzed by culture-dependent methods (Cloete & Steyn, 1987; Wagner *et al.*, 1993). Culturing techniques have provided a misleading picture of bacterial community structure in activated sludge systems and in general the role of *Acinetobacter* species in activated sludge

processes has been overrated (Cloete & Steyn, 1987; Wagner *et al.*, 1993). Therefore, there is a need for techniques that do not necessarily identify individual species, but that can differentiate bacterial communities in terms of their constituents (Lee & Fuhrman, 1990). These methods alleviate the need for culturing and samples are analyzed in a more direct manner which prevents the selection of specific organisms. These methods include SDS-PAGE, molecular techniques and monoclonal and polyclonal antibodies.

A thorough knowledge of the bacterial populations responsible for a functioning activated sludge process can only originate from the combination of different approaches. Changes in community composition have been followed not on the population (species) level but on a rougher scale. Population shifts could serve as early indicators from up coming malfunctions (e.g. filamentous bacteria as indicators for sludge bulking) so that corrective measurements could be made in time. Keeping in mind the biases caused by cultivation, future studies should rely on in situ identification of individual cells with immuno- or nucleic acid probes (Wagner *et al.*, 1993).

The better we understand community structure and function, the better we can manage wastewater treatment systems to control bulking or to improve biological phosphate removal capacity (Cloete & Muyima, 1997).

CHAPTER 3

MATERIALS AND METHODS

3.1 *Organism used in the Sensitivity study:*

Acinetobacter calcoaceticus ATCC 23055^T was used as pure culture.

3.1.1 *Samples used in the Sensitivity study:*

Aerobic activated sludge was collected from the Daspoort activated sludge plant (3-stage, Bardenpho).

3.1.2 *Experimental work for the Sensitivity study:*

1. After the cells were broken with glass beads, *Acinetobacter calcoaceticus* ATCC 23055^T (1.2×10^8 colony forming units. ml^{-1}) was mixed in the following ratios with aerobic activated sludge (1.3×10^6 cfu. ml^{-1}): 1:1; 2:1; 10:1; 1:2 and 1:10 (Table 1).

2. The change in the microbial community during the different stages of aerobic, anaerobic and aerobic conditions were monitored for a 48 h period. A 500 ml aerobic sludge sample was used. 100 ml aerobic sludge was prepared for SDS-PAGE. The remaining volume of sludge was incubated anaerobically for 24 h, after which another 100 ml sample was prepared for SDS-PAGE. The remaining volume of activated sludge was again incubated aerobically (with air bubbling through) for 24 h.

3.2 *Organisms used in the comparison studies:*

Fifty eight LMG and ATCC bacterial strains (Table 2) were used as reference cultures (Strains from the collection of the Department of Microbiology and Plant Pathology).

3.3 *Samples:*

The activated sludge samples (grab samples) were obtained from:

i) Daspoort Water treatment Plant (3 stage Bardenpho): collected on a weekly basis from

the anaerobic, anoxic and aerobic zones for a period of 34 weeks (Table 3). Average values for the following chemical analysis at Daspoort were: $COD_{(raw)}$ (408 $mg.l^{-1}$); Ammonia (14.61 $mg.l^{-1}$) and nitrate (0.39 $mg.l^{-1}$). The Daspoort system treats both domestic and industrial water.

ii) Six laboratory-scale systems at UCT: 3 UCT-type N-and P-removing (ML, MS, MT) and 3 modified Ludzack Etlinger (MLE) type N-Removing (HM, PW, MU). At least four samples of each system were monthly obtained (Table 4).

iii) Twenty one other activated sludge systems: Nineteen from Gauteng province and two from the Northern province were collected (Table 5 and Table 6).

3.4 *Sample preparation*

The following method for the separation of the bacterial cells from the activated sludge flocs was developed during this study:

Protein extractions were carried out using of different centrifuging and buffer washing steps. 100 ml activated sludge samples and 70 g glass beads where homogenized for 10 min. The supernatant was centrifuged for 15 min at 1000 rpm in a Hermle 360 K centrifuge. The supernatants were pelleted by centrifuging for 15 min at 7000 rpm. Pellets were resuspended in 2 ml 40 mM Tris pH 7.4. 1 ml of Percoll (Merck) was added to each sample, mixed and centrifuged for 10 min at 12 000 rpm in the eppendorf rotor of the Hermle 360 K centrifuge. The percoll band was extracted from each sample with a syringe. Samples were washed 3 times with 0.2 M Tris pH 7: 0.8% NaCl and centrifuged each time for 10 min at 12 000 rpm to remove the percoll.

3.5 *Polyacrylamide gel electrophoresis of proteins*

3.5.1 *Extraction of Proteins from activated sludge samples*

The whole cell protein extractions for SDS-PAGE were performed as described by Dagutat (1990). Samples were washed 3 times in 0.2 M phosphate buffer (pH 6.88) and centrifuged for 8 min at 12 000 rpm. 75 μl of sample treatment buffer (STB) [0.5 M Tris-HCl pH 6.8, 5% (v/v) 2-B-mercaptoethanol (BDH), 10% (v/v) glycerol (Merck), and 2% (m/v) SDS (Univar)], was added to each pellet and boiled for 5 min at 94 °C. Cell pellets were kept on ice and cells were disrupted by sonication using an Cole-Parmer Ultrasonic Homogenizer

(Series 4710) at 50% maximum output (40 watt) for up to 45 s using 15 s pulses. The second volume of 75 µl sample buffer was added and mixed. Cell debris were removed by centrifuging at 12 000 rpm for 8 min. The clear supernatant was stored at -20 °C until required.

3.5.2 *Standard conditions for SDS-PAGE*

SDS-PAGE were performed by the method described by Laemmli (1970), modified according to Kiredjian, Holmes, Kersters, Guilvout & De Ley (1986). Proteins were separated on gels (1.5 mm thick and 125 mm long) run in a Hoefer SE600 dual cooled vertical slab unit. The separation gel (12 %, 1.5 M Tris-HCl pH 8.66, conductivity 16.5 mS) and stacking gel (5% 0.5 M Tris-HCl pH 6.6, conductivity 28.1 mS) were prepared from monomer solution containing 29.2% (m/v) acrylamide (BDH Electran) and 0.8% (m/v) N^1-N^1-bismethylene acrylamide (BDH Electran). Electrophoresis was performed at a constant current of 30 mA through the stacking gel, and at 60 mA through the separation gel at 10°C. After electrophoresis, gels were stained for 1 h in a Coomassie Blue solution [12.5% (v/v) Coomassie Blue stock solution, 50% (v/v) methanol (UniVar) and 10% (v/v) acetic acid (UniVar) prepared from a 2% (m/v) Coomassie Brilliant Blue R (Unilab) stock solution. After staining, gels were destained overnight in a solution containing 25% (v/v) methanol (UniVar) and 10% (v/v) acetic acid (UniVar).

3.5.3 *Analysis of protein patterns*

Gels with the protein profiles were analyzed with a Hoefer GS300 densitometer. Data obtained were directly stored on a computer and analyzed with the GelCompar 3:1 programme (Applied Maths, Kortrijk, Belgium). The programme calculated the Pearson product moment correlation coefficient (r) between the samples, and clustered the samples using the unweighted pair group method of arithmetic averages (UPGMA). *Psychrobacter immobilis* LMG 1125 was used as reference pattern on each gel. Reproducibility of electrophoresis was determined by comparing these tracks with a *Psychrobacter immobilis* protein profile selected in the GelCompar 3.1 programme as standard. A relationship of $>90\%$ between gels was presumed acceptable for reproducible gels.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 To determine the sensitivity of the SDS-PAGE technique and its applicability to activated sludge analysis.

Figure 1 indicates that two groups could be distinguished in the dendrogram subdivided at the 86% correlation. Group A included the pure *Acinetobacter calcoaceticus* ATCC 23055^T culture and the following four mixtures: 1:10; 1:1; 2:1 and 1:2. Group A showed a correlation of > 86%. Activated sludge samples deliberately spiked with *Acinetobacter calcoaceticus* ATCC 23055^T at different ratios (Table 1) showed a correlation (> 80%) with the pure *Acinetobacter calcoaceticus* ATCC 23055^T strain and a lower (< 60%) correlation with the unspiked activated sludge.

TABLE 1: The percentage correlation between different mixtures of a pure *Acinetobacter calcoaceticus* ATCC 23055^T strain and activated sludge samples after SDS-PAGE

SAMPLES	% Correlation With *Acinetobacter calcoaceticus* ATCC23055^T	% Correlation With Activated Sludge
Acinetobacter calcoaceticus ATCC 23055^T	100	59
Aerobic Activated sludge sample	59	100
"1:1""	88	59
"2:1""	86	59
"10:1""	59	78
"1:2""	88	59
"1:10""	96	59
"1:1"" After 24 h	75	59

" Activated sludge
"" *Acinetobacter calcoaceticus* ATCC 23055^T

Unspiked activated sludge showed a 59% correlation with the pure culture of *Acinetobacter calcoaceticus* ATCC 23055^T. This suggested that the activated sludge microbial community contained *Acinetobacter* organisms and/or organisms closely related to *Acinetobacter*. The question now is, whether one can conclude from this that at least 59% of this particular sludge consisted of *Acinetobacter* species.

The high correlation (> 80%) between the spiked activated sludge samples and the *Acinetobacter* culture does indicate that *Acinetobacter calcoaceticus* ATCC 23055^T was pre-dominant (at least 59% representation) in this particular activated sludge and that 80% above, gives the combined effect of the spiked *Acinetobacter* and those occurring naturally. This is substantiated by the 59% minimum correlation between *Acinetobacter* and the unspiked activated sludge and the other ratios as in Table 1.

This suggested, that SDS-PAGE analysis could possibly be used to determine whether *Acinetobacter* pre-dominated in a particular activated sludge. Hence, pure cultures of *Acinetobacter* were included in the next study where samples from different activated sludge systems were used.

A 1:1 spiked activated sludge sample kept aerobically for 24 h indicated a decrease in correlation from 86% to 75% with the pure culture and a 59% correlation with the unspiked activated sludge. This suggested that the microbial community was changing towards a situation where *Acinetobacter* would eventually not pre-dominate and that the equilibrium condition represented by the unspiked activated sludge sample would eventually be reached. This indicated the sensitivity of the SDS-PAGE method, indicating that predominance of *Acinetobacter* could be indicated in mixtures of activated sludge, even over time.

The dendrogram of the second experiment (Fig. 2) indicated a > 94% correlation between the original aerobic sample and the same sample when it was incubated for 24 h under anaerobic conditions. When the same sample was again incubated for another 24 h under aerobic conditions the sample indicated a > 89% correlation. These results indicated that the bacterial community stayed the same during the different aerobic and anaerobic conditions, therefore indicating that the same bacteria probably were present throughout the

activated sludge process. This indicates a stable microbial community structure.

The results indicated that SDS-PAGE was a sensitive tool which can be used in bacterial community studies of activated sludge. Protein electrophoresis was therefore considered a useful technique to determine the bacterial community structure of activated sludge and to compare different sludge samples, such as those which remove phosphorus to those which do not remove phosphorus.

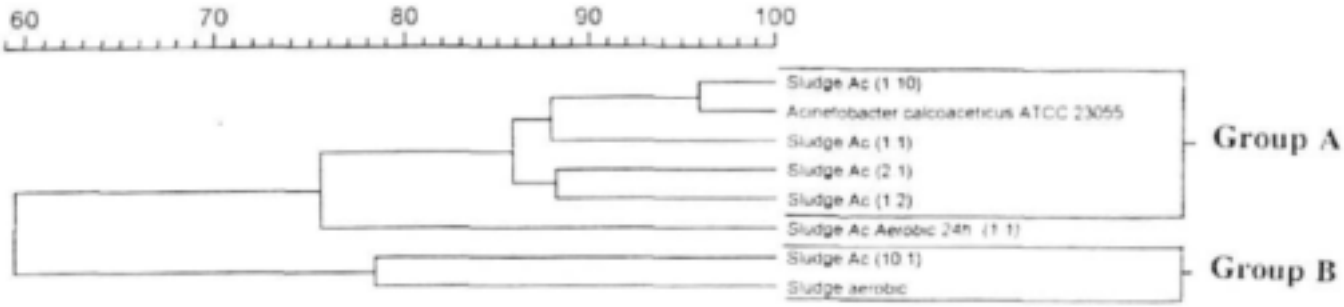


Figure 1: **Dendrogram of the electrophoretic patterns of mixtures of *Acinetobacter calcoaceticus* ATCC 23055 and activated sludge samples based on UPGMA analysis of the correlation coefficients (r) of the protein patterns.**

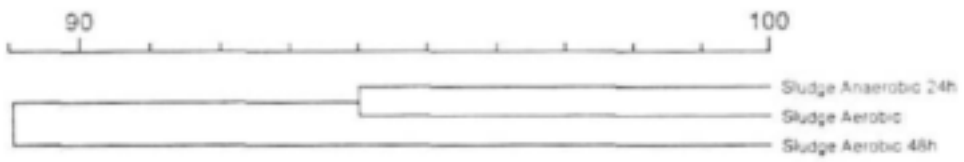


Figure 2: **Dendrogram of the electrophoretic patterns of an activated sludge samples incubated anaerobically and aerobically for 48 h, based on UPGMA analysis of the correlation coefficients (r) of the protein patterns.**

4.2 Determining whether the activated sludge community was pre-dominated by specific organisms.

Pure bacterial cultures (Table 2) previously isolated or associated with activated sludge were used as reference strains to determine if any dominated within a specific activated sludge sample or system. This would be indicated when a dominant organism showed a high percentage correlation with the protein profiles of sludge samples in a specific group or subgroup.

In Figure 3, 58 pure bacterial cultures known to be present in activated sludge were compared with 240 activated sludge samples representing the anaerobic, anoxic and aerobic zones. The activated sludge samples included samples of the Daspoort water treatment plant collected on a weekly basis for 34 weeks, samples of 6 activated sludge systems operated at UCT and 19 activated sludge systems in the Gauteng province and two from the Northern Province. The protein fingerprints of the pure bacterial cultures were used as references to determine if the sludge samples clustered with a specific pure culture. This would have indicated pre-dominance.

The dendrogram (Fig.3) was divided into two sections. Section I with a > 48% correlation indicated that 216 of the activated sludge samples clustered together. This Section was divided in to 7 Groups. Group A, with 82% correlation included 51 samples representing the sludge samples collected from Rynfield, Vlakplaats, Olifantsfontein, Nylstroom, Potgietersrus, Heidelberg, Modderfontein, JP Marais, Daspoort and UCT plants.

Group B, with 80% similarity represented 50 activated sludge samples from the following plants: Rynfield North and South, Nylstroom, Olifantsfontein, Esterpark, Daspoort and UCT. Group C at 78% correlation, consisted of 6 which included 5 Daspoort and one Vlakplaats sample. In Group D, correlating at 78%, 22 of the 36 samples were from the Daspoort activated sludge plant. Group E, with a 70% similarity consisted of 25 sludge samples, 17 samples represented the UCT plants. Group F, correlating at 67%. This group consisted of 44 samples, which included samples from Daspoort, Tsakane, Daveyton, Waterval North and South, Rondebult, Rynfield North, Hartbeesfontein and UCT. In Group

G, correlating at 70%, included 4 Hartbeesfontein samples. The pure cultures *Bacillus cereus* and *Staphylococcus aureus* were the only two pure cultures that clustered together with the sludge samples in Section I.

Section II with a 31% similarity represented the pure bacterial cultures. Three groups were distinguished. Group A, correlating at 55%, included 30 *Acinetobacter* species. Group B, with 60% similarity consisted 3 pure cultures and 6 sludge samples. In Group C, with 25% correlation several loose groups of pure cultures as well as a few activated sludge samples with no close relationship to the other samples were observed. Hence, these samples were not discussed further.

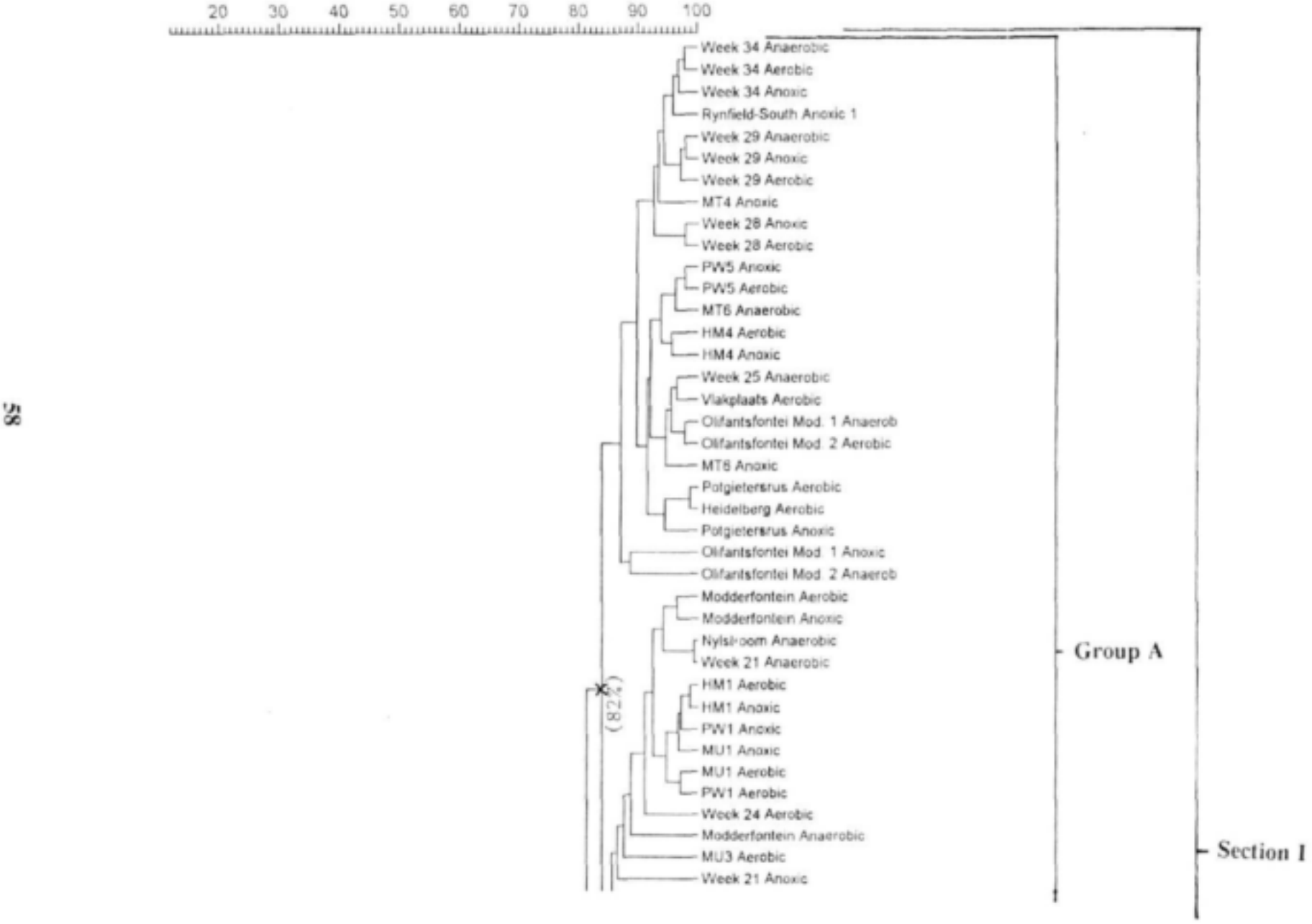
The resulting activated sludge protein profiles, as indicated in this dendrogram (Fig.3), indicated that a specific activated sludge system is not dominated by a specific organism because no correlation was found between protein bands of pure cultures compared to any of the activated sludge subgroups. The only exception was, two gram positive organisms *Bacillus cereus* and *Staphylococcus aureus* which clustered together with the activated sludge samples in Section I. This is interesting because literature usually indicated gram negative organisms like *Acinetobacter* species to be dominant in activated sludge, when culture-dependant methods were used (Bond *et al.*, 1995). However, a survey by Bux and Kasan (1994) indicated that the culturable fraction of a number of activated sludge plants was dominated by *Bacillus* species.

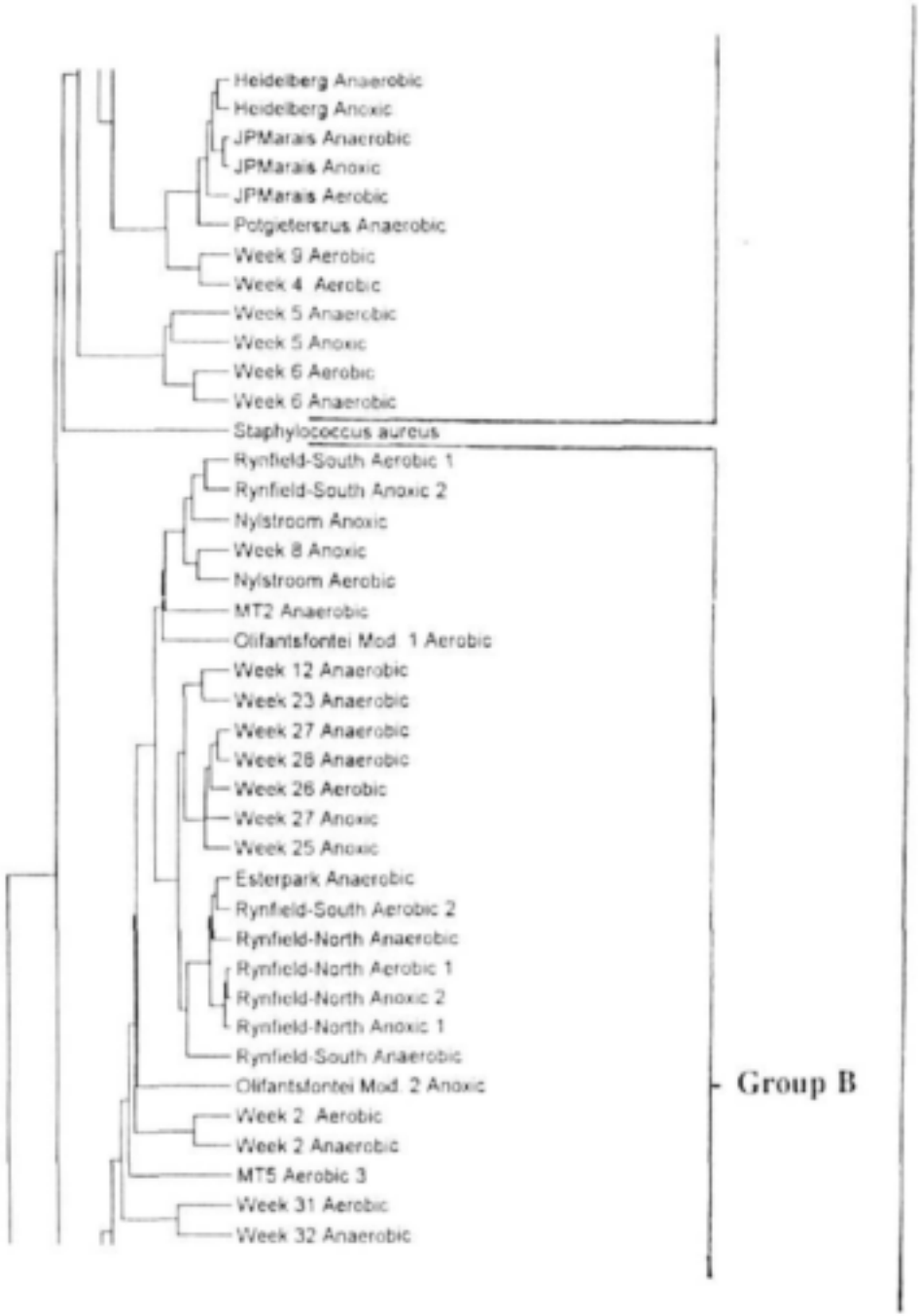
The results indicated that the activated sludge bacteria community consisted of a diverse and dynamic community of different organisms that interact and co-exist on a specific level. There was a tendency amongst samples of the same systems to cluster together, at a higher level, although not significantly different from other plants. The conclusion that was made considering the low correlation amongst the protein profiles of 58 pure bacterial cultures associated with activated sludge and activated sludge samples was that no specific organism pre-dominated. Hence, it was clear that activated sludge consists of a diverse bacterial community.

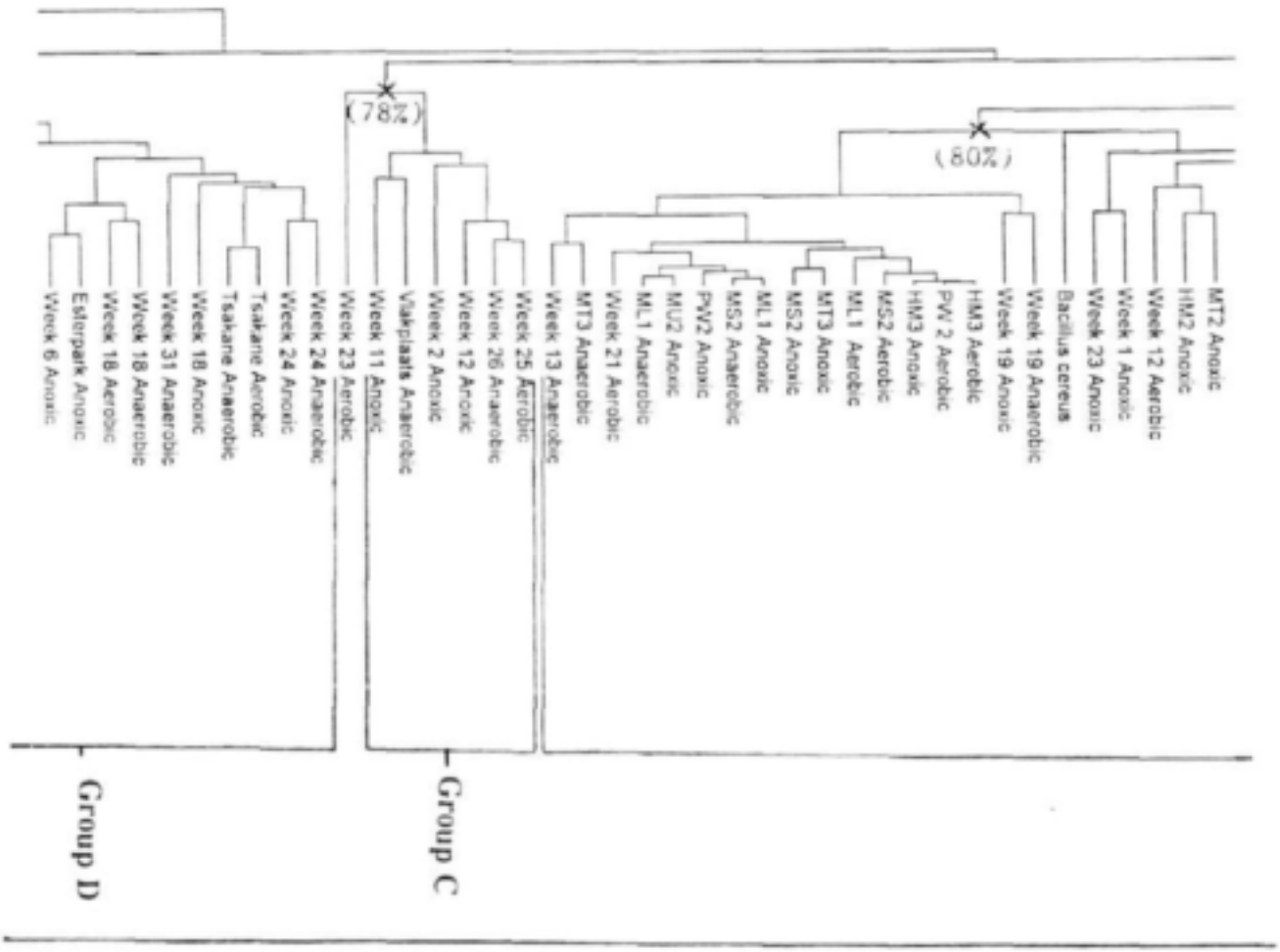
TABLE 2: List of 58 bacterial strains used as reference strains.

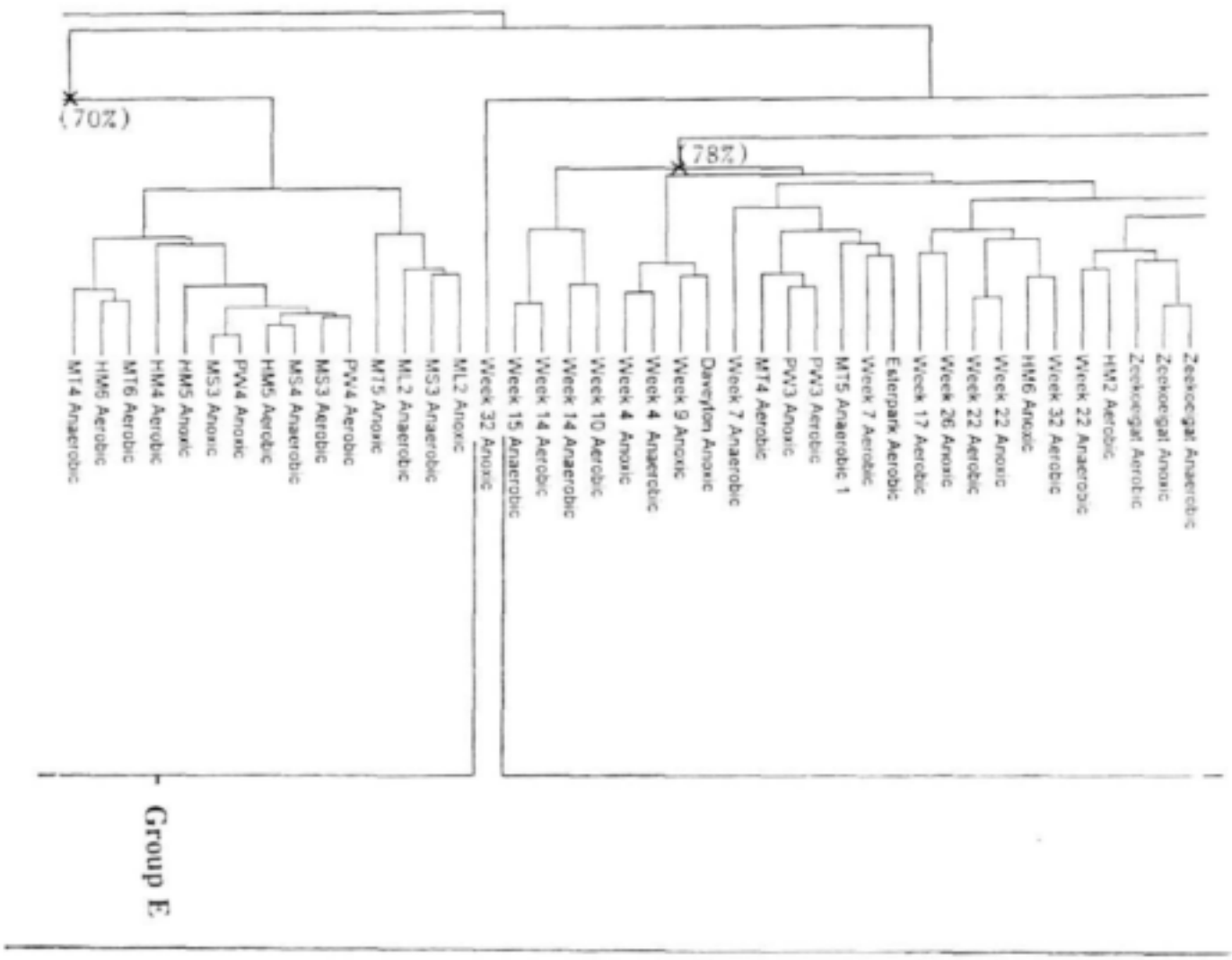
Bacterial strains	Bacterial strains	Bacterial strains
**Acinetobacter baumannii* AC, B1, ϕ5, Ao8; M:3,4,9,16,21,26,27,29; AS:98,99,100,101,103, 104,105; DSM 1139; ATCC 19606^T	*Camomonas testosteroni* A20 slime and non-slime forming	*Pseudomonas aeruginosa*
**A. calcoaceticus* ATCC 17912^T; ATCC 23055^T	*Cytophaga heparina* LMG 10345, LMG 10348	*Rhodocyclus purpureus* LMG 7759^T
**A. junii* AS:33,60,64,78,79, 87,89,96; ATCC 17908^T	*Entrobacter cloacea*	*Serratia marcescens*
**A. lwoffii* AS: 92,93,97; ATCC 21130^T	*Escherichia coli*	*Shigella dysenteriae*
**A. johnsonnii* ATCC 17906^T	*Klebsiella pneumonia*	*Staphylococcus aureus*
Alcaligenes DSM 531	*Micrococcus luteus*	*Vitrocella stercoraria* LMG 7756^T
Bacillus cereus	*Micrococcus roseus*	*Yersinia* sp
Brevibacterium lactofermentum ATCC 13869	*Proteus vulgaris*	*Zooglea ramigera* LMG 17136^T

* *Acinetobacter* strains identified with API 20E (M. Bosch, M.Sc. Microbiology, thesis, 1992) were obtained from the culture collection of the Department of Microbiology and Plant Pathology at the University of Pretoria.

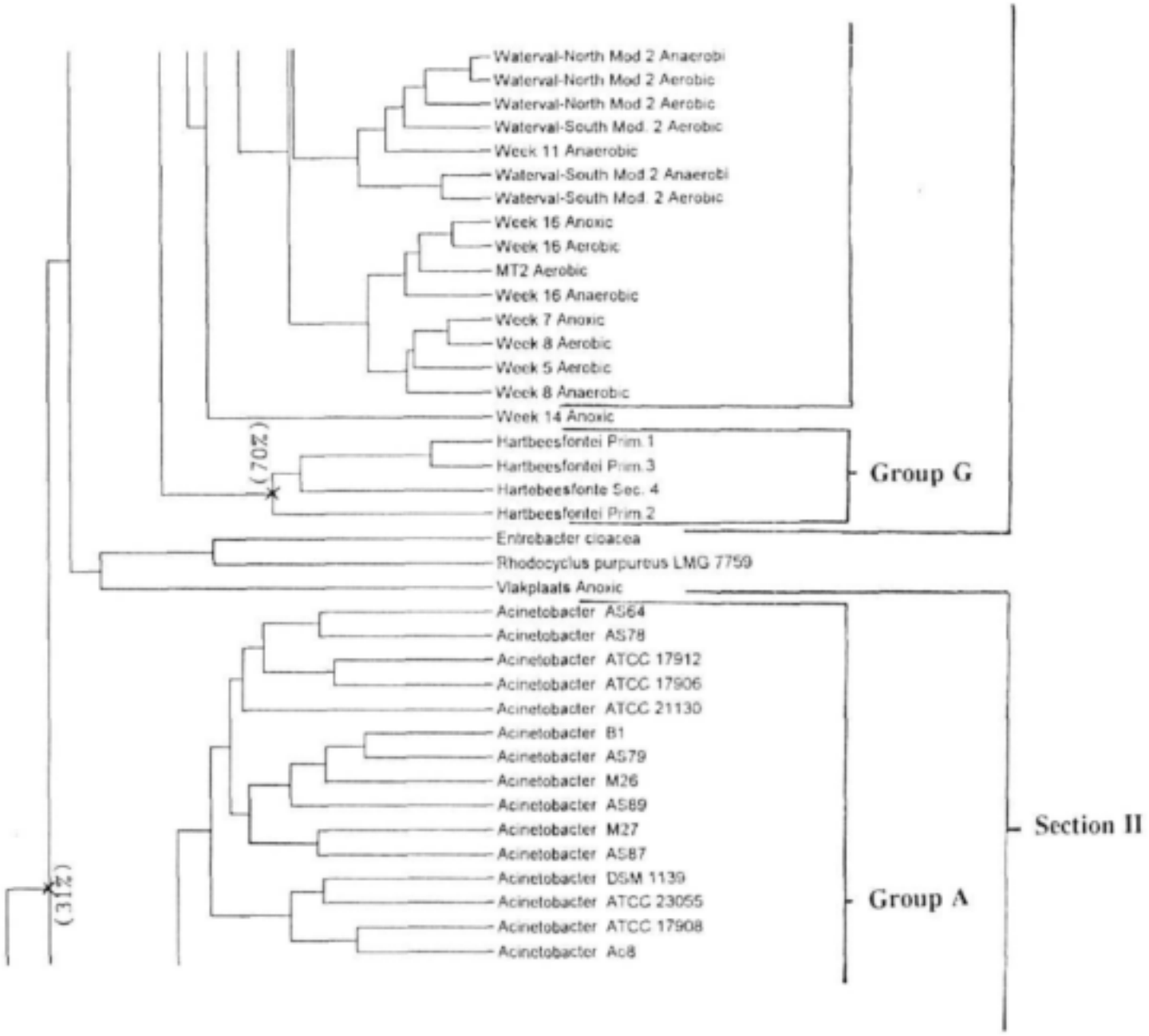

20
30
40
50
60
70
80
90
100
Week 34 Anaerobic
Week 34 Aerobic
Week 34 Anoxic
Rynfield-South Anoxic 1
Week 29 Anaerobic
Week 29 Anoxic
Week 29 Aerobic
MT4 Anoxic
Week 28 Anoxic
Week 28 Aerobic
PW5 Anoxic
PW5 Aerobic
MT6 Anaerobic
HM4 Aerobic
HM4 Anoxic
Week 25 Anaerobic
Vlakplaats Aerobic
Olifantsfontei Mod. 1 Anaerob
Olifantsfontei Mod. 2 Aerobic
MT8 Anoxic
Potgietersrus Aerobic
Heidelberg Aerobic
Potgietersrus Anoxic
Olifantsfontei Mod. 1 Anoxic
Olifantsfontei Mod. 2 Anaerob
Modderfontein Aerobic
Modderfontein Anoxic
Nylsvloom Anaerobic
Week 21 Anaerobic
HM1 Aerobic
HM1 Anoxic
PW1 Anoxic
MU1 Anoxic
MU1 Aerobic
PW1 Aerobic
Week 24 Aerobic
Modderfontein Anaerobic
MU3 Aerobic
Week 21 Anoxic
(82%)
Group A
Section I


Heidelberg Anaerobic
Heidelberg Anoxic
JPMarais Anaerobic
JPMarais Anoxic
JPMarais Aerobic
Potgietersrus Anaerobic
Week 9 Aerobic
Week 4 Aerobic
Week 5 Anaerobic
Week 5 Anoxic
Week 6 Aerobic
Week 6 Anaerobic
Staphylococcus aureus
Rynfield-South Aerobic 1
Rynfield-South Anoxic 2
Nylstroom Anoxic
Week 8 Anoxic
Nylstroom Aerobic
MT2 Anaerobic
Olifantsfontei Mod 1 Aerobic
Week 12 Anaerobic
Week 23 Anaerobic
Week 27 Anaerobic
Week 28 Anaerobic
Week 26 Aerobic
Week 27 Anoxic
Week 25 Anoxic
Esterpark Anaerobic
Rynfield-South Aerobic 2
Rynfield-North Anaerobic
Rynfield-North Aerobic 1
Rynfield-North Anoxic 2
Rynfield-North Anoxic 1
Rynfield-South Anaerobic
Olifantsfontei Mod 2 Anoxic
Week 2 Aerobic
Week 2 Anaerobic
MT5 Aerobic 3
Week 31 Aerobic
Week 32 Anaerobic
Group B


(78%)
(80%)
Week 6 Anoxic
Esterpark Anoxic
Week 18 Aerobic
Week 18 Anaerobic
Week 31 Anaerobic
Week 18 Anoxic
Tsakane Anaerobic
Week 24 Anoxic
Week 24 Anaerobic
Week 23 Aerobic
Week 11 Anoxic
Vlakplaats Anaerobic
Week 2 Anoxic
Week 12 Anoxic
Week 26 Anaerobic
Week 25 Aerobic
Week 13 Anaerobic
MT3 Anaerobic
Week 21 Aerobic
ML1 Anaerobic
MU2 Anoxic
PW2 Anoxic
MS2 Anaerobic
ML1 Anoxic
MS2 Anoxic
MT3 Anoxic
ML1 Aerobic
MS2 Aerobic
HM3 Anoxic
PW 2 Aerobic
HM3 Aerobic
Week 19 Anoxic
Week 19 Anaerobic
Bacillus cereus
Week 23 Anoxic
Week 1 Anoxic
Week 12 Aerobic
HM2 Anoxic
MT2 Anoxic
Group D
Group C


(70%)
(78%)
MT4 Anaerobic
MT6 Aerobic
HM6 Aerobic
HM4 Aerobic
HM5 Anoxic
MS3 Anoxic
PW4 Anoxic
HM5 Aerobic
MS4 Anaerobic
MS3 Aerobic
PW4 Aerobic
MT5 Anoxic
ML2 Anaerobic
MS3 Anaerobic
ML2 Anoxic
Week 32 Anoxic
Week 15 Anaerobic
Week 14 Aerobic
Week 14 Anaerobic
Week 10 Aerobic
Week 4 Anoxic
Week 4 Anaerobic
Week 9 Anoxic
Davelyton Anoxic
Week 7 Anaerobic
MT4 Aerobic
PW3 Anoxic
PW3 Aerobic
MT5 Anaerobic 1
Week 7 Aerobic
Esterpark Aerobic
Week 17 Aerobic
Week 26 Anoxic
Week 22 Aerobic
Week 22 Anoxic
HM6 Anoxic
Week 32 Aerobic
Week 22 Anaerobic
HM2 Aerobic
Zeekoegat Aerobic
Zeekoegat Anoxic
Zeekoegat Anaerobic
Group E

Week 3 Aerobic
Week 3 Anaerobic
Week 13 Aerobic
MU4 Aerobic
MU4 Anoxic
Week 27 Aerobic
Week 2 Aerobic
Week 3 Anoxic
Hartbeesfontei Sec. 1
Week 1 Aerobic
Week 17 Anaerobic
Tsakane Anoxic
Daveyton Aerobic
Daveyton Anaerobic
MT1 Anoxic
Week 20 Anoxic
Waterval-North Mod.1 Aerobic
Waterval-North Mod.1 Anaerobi
Waterval-South Mod.1 Aerobic
Waterval-South Mod.1 Anaerobi
Waterval-North Mod.1 Aerobic
Waterval-South Mod.1 Aerobic
Rondebult Aerobic
Week 11 Aerobic
Rynfield-North Aerobic 2
Week 30 Anaerobic
Week 33 Aerobic
Week 30 Anoxic
Week 30 Aerobic
Week 33 Anoxic
MU3 Anoxic
Hartbeesfontei Prim.4
Hartbeesfontei Sec. 3
Hartbeesfontei Sec. 2
Week 10 Anaerobic
MS4 Aerobic
Week 10 Anoxic
MU5 Anoxic
MU5 Aerobic
(48%)
(67%)
Group F


Waterval-North Mod 2 Anaerobi
Waterval-North Mod 2 Aerobic
Waterval-North Mod 2 Aerobic
Waterval-South Mod. 2 Aerobic
Week 11 Anaerobic
Waterval-South Mod 2 Anaerobi
Waterval-South Mod. 2 Aerobic
Week 16 Anoxic
Week 16 Aerobic
MT2 Aerobic
Week 16 Anaerobic
Week 7 Anoxic
Week 8 Aerobic
Week 5 Aerobic
Week 8 Anaerobic
Week 14 Anoxic
Hartbeesfontei Prim.1
Hartbeesfontei Prim.3
Hartebeesfonte Sec. 4
Hartbeesfontei Prim.2
(70%)
Group G
Enterobacter cloacea
Rhodocyclus purpureus LMG 7759
Vlakplaats Anoxic
Acinetobacter AS64
Acinetobacter AS78
Acinetobacter ATCC 17912
Acinetobacter ATCC 17906
Acinetobacter ATCC 21130
Acinetobacter B1
Acinetobacter AS79
Acinetobacter M26
Acinetobacter AS89
Acinetobacter M27
Acinetobacter AS87
Acinetobacter DSM 1139
Acinetobacter ATCC 23055
Acinetobacter ATCC 17908
Acinetobacter Ac8
(31%)
Group A
Section II

(55%)
(60%)
(25%)
Acinetobacter AS105
Acinetobacter AS101
Acinetobacter ATCC 19606
Acinetobacter 05
Acinetobacter M3
Acinetobacter M29
Acinetobacter M9
Acinetobacter AS99
Acinetobacter M4
Acinetobacter M21
Acinetobacter M16
Acinetobacter AS104
Acinetobacter AS100
Acinetobacter AS98
Acinetobacter AS103
Alcaligenes DSM 531
Acinetobacter AS97
Acinetobacter As96
MS1 Anaerobic
MT1 Aerobic
MS1 Anoxic
MT1 Anaerobic
Week 9 Anaerobic
MS1 Aerobic
Group B
Camomonas testosteroni A20 (Slime)
Camomonas testosteroni A20 (Non-Slime
MS4 Anoxic
Proteus vulgaris
Acinetobacter AS33
Week 15 Aerobic
Acinetobacter AS92
Week 15 Anoxic
Acinetobacter AS60
Acinetobacter AC
Week 1 Anaerobic
Pseudomonas aeroguriosa
Acinetobacter As93
Week 33 Anaerobic
Week 17 Anoxic
Week 31 Anoxic
Escherichia coli
Group C

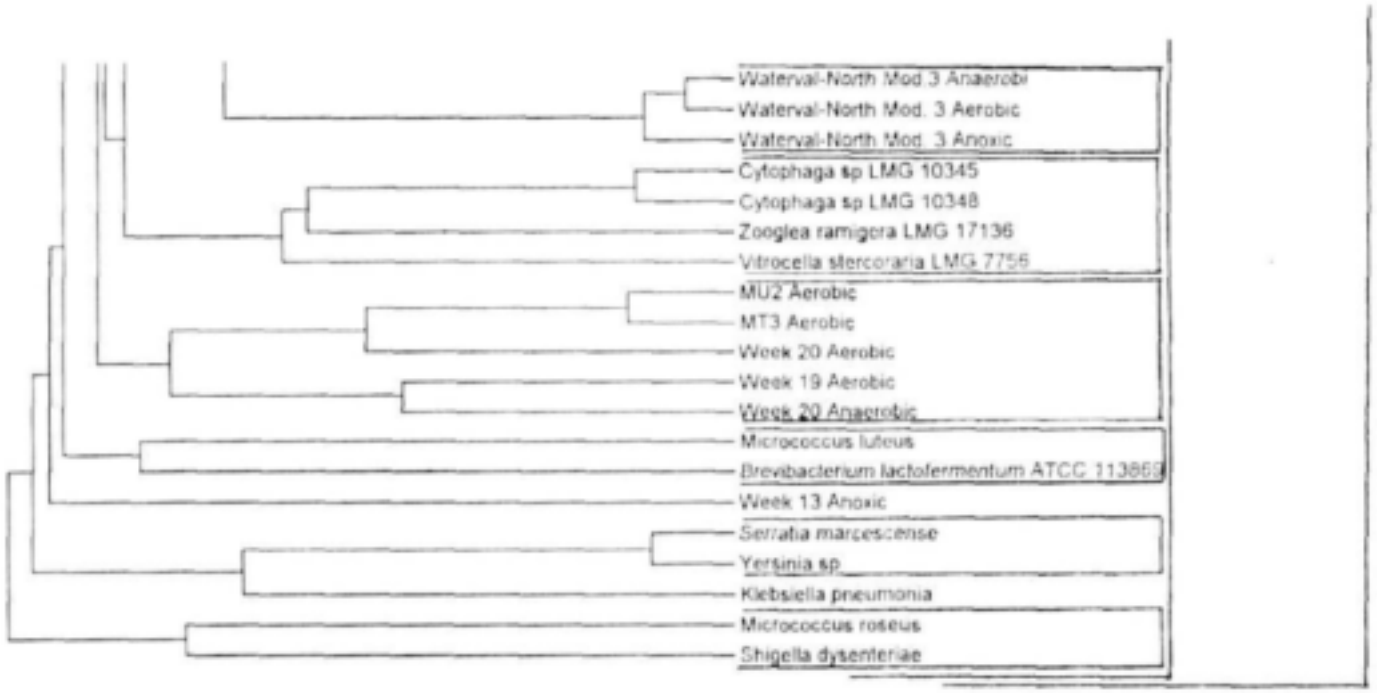


FIGURE 3: **Dendrogram of the electrophoretic patterns of 58 pure bacterial cultures compared with 240 activated sludge samples of the anaerobic, anoxic and aerobic zones, based on UPGMA analysis of the correlation coefficients (r) of the protein patterns.**

4.3 Determination of the effect of seasonal changes on the microbial community of an activated sludge system.

The dendrogram of the protein profiles for the three zones of the Daspoort activated sludge plant as compiled over 34 weeks was grouped into 6 sections (Fig. 4). Each of these sections was divided into smaller groups and subgroups. Section I, representing 76 of the 102 samples, showed a 63% correlation. Samples taken throughout the sampling period were represented in this section indicating no definite community structure changes. These samples represented winter and summer conditions. The results hence indicated that the community composition remained similar irrespective of the season.

Section I was divided into group A representing 57 samples with a 69% correlation. This group was further sub-divided into subgroup 1 which consisted of 13 samples with a 87% correlation. This subgroup also represented different sampling weeks and zones. Subgroup 2, consisting of only 3 samples, representing all three zones, showed a 86% correlation. Subgroup 3 consisted of 5 samples with a 86% correlation. Subgroup 4 consisted of 10 samples with a 92% correlation indicating a high percentage relatedness. This subgroup included samples taken during weeks 25, 28, 29 and 34 of experimentation, which coincided with summer temperatures, except for one sample (anoxic zone) taken during the 8 th week. The three different zones of weeks 29 and 34 were all present in this subgroup indicating almost identical protein profiles for the different zones, indicating a stable bacterial community structure in the activated sludge plant. Subgroup 5 showed 84% correlation and consisted of 5 samples. Subgroup 6 consisted of 20 samples with a 75% correlation.

Group B consisted of 8 samples with a 82% correlation. This group was divided into subgroups 1 and 2. Subgroup 1 consisted of 3 samples with 83% correlation. Subgroup 2 correlated with 87% and consisted of 5 samples, representing the three zones during week 30, and the anoxic and aerobic zones during week 33. Group C consisted of 7 samples showed a similarity of 79%. Two subgroups were distinguished. Subgroup 1 consisted of all three zones during week 16 with an 86% correlation. Subgroup 2 with 4 samples correlated at 87%. Group D consisted of 2 samples (Week 11 anaerobic and Week 10 Anoxic) with a 84% correlation.

A joint similarity of 59% was observed between Section I and II. The 9 samples in Section II showed a 72% correlation. Two groups were identified. Group A with two samples and a 76% correlation and Group B with 7 samples and a 83% similarity. The latter group was divided into subgroup 1, which showed a 95% correlation and Subgroup 2 with a 92% correlation.

Section III showed a correlation of 41% with regards to Sections I and II. Section IV, V, and VI joined at similarity values < 38%. The samples in section V, showed a 74% similarity.

No specific pattern was observed, this indicated that protein profiles did not change due to seasonal changes. Also the protein patterns between the different zones showed no major differences, which indicated that the same bacterial community was present throughout the activated sludge process.

In Figure 5 the protein profiles of the aerobic zones and the corresponding phosphorus concentrations were compared. The general shape of the dendrogram stayed the same as that of Figure 4. Two sections with a 42% similarity were identified with section I consisting of 5 samples with an average phosphorus concentration of 5.3 $mg.l^{-1}$ and a correlation of 45%. Section II consisted of 28 samples with a 60% relatedness. Section II was sub-divided into 4 groups. Group A with 79% correlation included 7 samples with an average phosphorus concentration of 2.23 $mg.l^{-1}$. Group B with 7 samples correlated with 79% and showed an average P concentration of 2.6 $mg.l^{-1}$. Group C correlated at 76% and included 10 samples with an average P concentration of 3.26 $mg.l^{-1}$. Group D with only three samples with a 83% similarity had an average P concentration of 5.47 $mg.l^{-1}$. When comparing the average P concentration of the samples there seems to be a tendency for the protein profiles of samples with better P removal to cluster together. But when looking at the individual P removal concentrations of each sample there are several samples with P concentration values that were higher than the average value.

Conclusions

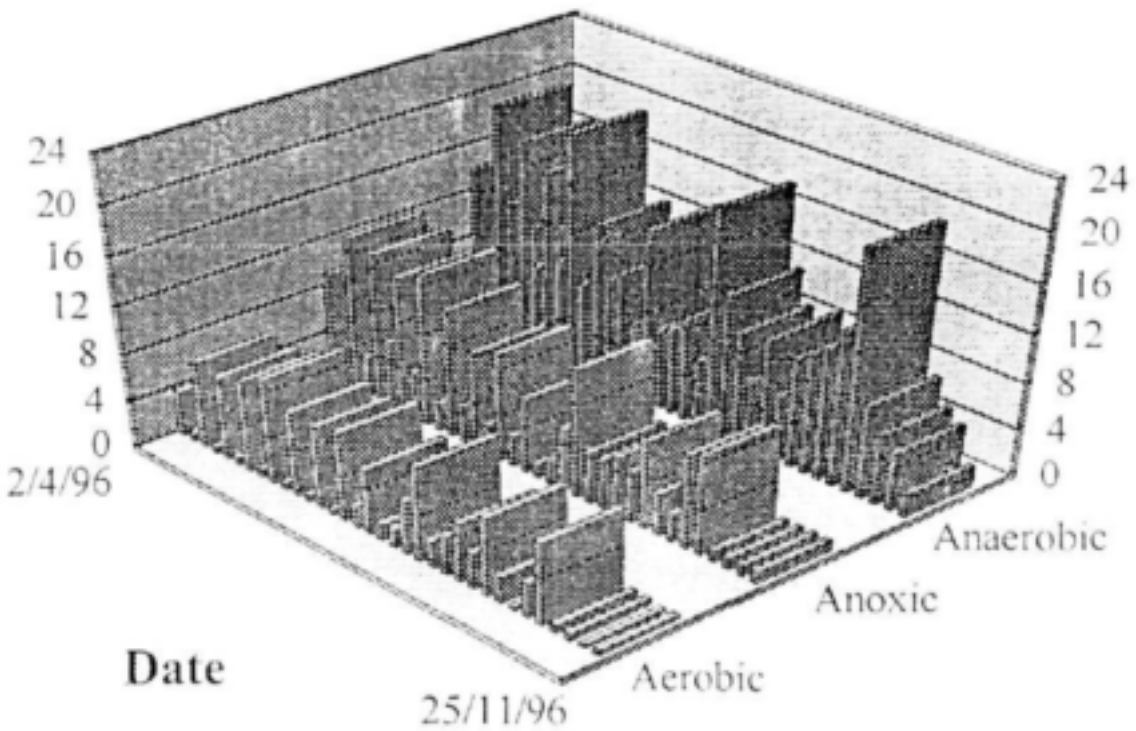
* The majority of the protein profiles indicated a correlation of > 63%, with no specific protein pattern due to seasonal changes or between the different zones, indicating a stable microbial community structure throughout the study period. Bond *et al.*, (1995) also suggested that the same bacterial community is present throughout the activated sludge process.

TABLE 3: **Phosphorus concentrations ($mg.l^{-1}$) in the anaerobic, anoxic and aerobic zones of the Daspoort Activated sludge plant analyzed on a weekly basis for a period of 34 weeks (April - November 1996).**

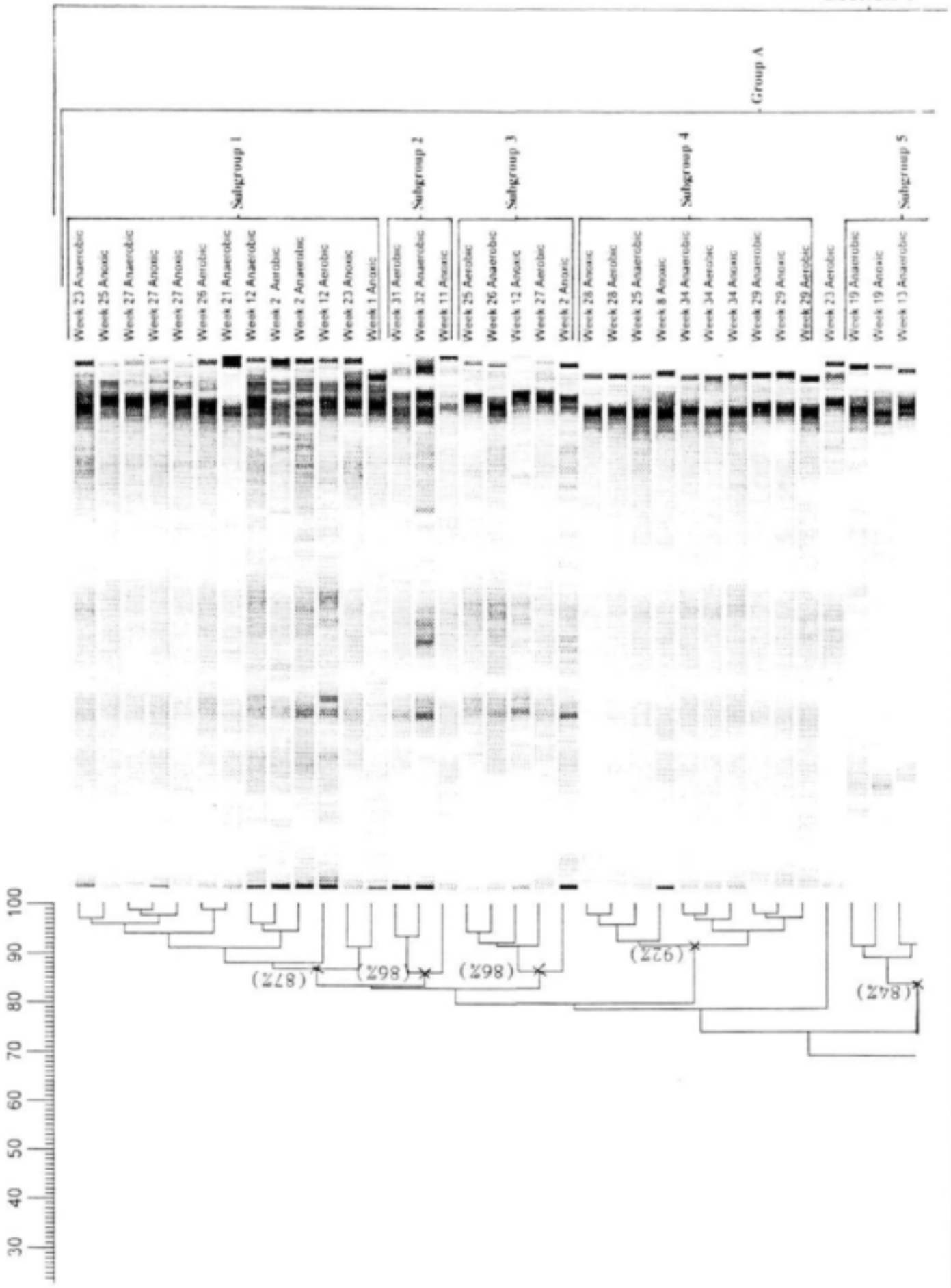
Week	Date	△P	Anaerobic	Anoxic	Aerobic
1	2/4	10.14	14	9.2	3.86
2	16/4	5.92	6.65	1	0.73
3	22/4	8.91	12.35	7.4	3.44
4	29/4	13.5	20.3	13.2	6.8
5	7/5	10.76	17.9	12.7	7.14
6	14/5	15.58	16.2	11.1	0.62
7	20/5	3.92	11.7	9.5	7.78
8	27/5	8.39	9.06	4.13	0.67
9	3/6	11.15	20.14	12.76	8.99
10	10/6	7.44	8	2.28	0.56
11	18/6	6.2	13.43	10.84	7.23
12	24/6	8.79	9.35	3.1	0.59
13	1/7	3.21	10.27	8.25	7.06
14	11/7	5.89	6.33	2.59	0.44
15	15/7	7.12	14.76	9.2	7.64
16	22/7	4.95	6.85	2.65	1.9
17	29/7	1.76	7.4	6.38	5.64
18	5/8	8.2	8.65	1.3	0.45
19	12/8	4.02	5.68	2.05	1.66
20	19/8	14.18	18.75	5.38	4.57
21	26/8	13.95	15.15	3.3	1.2
22	2/9	7.15	9.6	4.48	2.45
23	9/9	2.73	6.07	4.39	3.34
24	16/9	4.94	9.95	5.12	5.01
25	23/9	2.61	5.71	4.40	3.1

TABLE 3: **Phosphorus concentrations ($mg.l^{-1}$) in the anaerobic, anoxic and aerobic zones of the Daspoort Activated sludge plant analyzed on a weekly basis for a period of 34 weeks (April - November 1996) (continued).**

Week	Date	△P	Anaerobic	Anoxic	Aerobic
26	30/9	2.69	9.38	7.5	6.69
27	7/10	7.99	10.27	3.59	2.28
28	14/10	10.93	11.25	2.45	0.32
29	21/10	10	13	8	3
30	28/10	13	20	8.5	7
31	4/11	7.66	8	1	0.34
32	11/11	5.42	6	1.73	0.58
33	18/11	4.13	5	1	0.87
34	25/11	1.54	2	1.03	0.46



Graph 1: **Daspoort activated sludge system phosphorus removal over 34 weeks (April - November 1996).**


Group A
Subgroup 1
Subgroup 2
Subgroup 3
Subgroup 4
Subgroup 5
Week 23 Anaerobic
Week 25 Anoxic
Week 27 Anaerobic
Week 27 Anoxic
Week 27 Anoxic
Week 26 Aerobic
Week 21 Anaerobic
Week 12 Anaerobic
Week 2 Aerobic
Week 2 Anaerobic
Week 12 Aerobic
Week 23 Anoxic
Week 1 Anoxic
Week 31 Aerobic
Week 32 Anaerobic
Week 11 Anoxic
Week 25 Aerobic
Week 26 Anaerobic
Week 12 Anoxic
Week 27 Aerobic
Week 2 Anoxic
Week 28 Anoxic
Week 28 Aerobic
Week 25 Anaerobic
Week 8 Anoxic
Week 34 Anaerobic
Week 34 Aerobic
Week 34 Anoxic
Week 29 Anaerobic
Week 29 Anoxic
Week 29 Aerobic
Week 23 Aerobic
Week 19 Anaerobic
Week 19 Anoxic
Week 13 Anaerobic
(87%)
(86%)
(86%)
(92%)
(84%)
100
90
80
70
60
50
40
30

(59%)
(63%)
(69%)
(75%)
(82%)
(83%)
(86%)
Week 21 Aerobic
Week 21 Anoxic
Week 24 Aerobic
Week 7 Aerobic
Week 9 Anoxic
Week 6 Anoxic
Week 4 Anaerobic
Week 4 Anoxic
Week 18 Anaerobic
Week 18 Aerobic
Week 18 Anoxic
Week 24 Anaerobic
Week 24 Anoxic
Week 22 Anaerobic
Week 22 Anoxic
Week 22 Aerobic
Week 31 Anaerobic
Week 7 Anaerobic
Week 10 Aerobic
Week 14 Anaerobic
Week 14 Aerobic
Week 15 Anaerobic
Subgroup 6
Week 11 Aerobic
Week 17 Aerobic
Week 26 Anoxic
Week 32 Aerobic
Subgroup 1
Week 30 Anaerobic
Week 33 Aerobic
Week 30 Anoxic
Week 30 Aerobic
Week 33 Anoxic
Subgroup 2
Group B
Week 10 Anaerobic
Week 16 Anoxic
Week 16 Aerobic
Week 16 Anaerobic
Subgroup 1
Group C

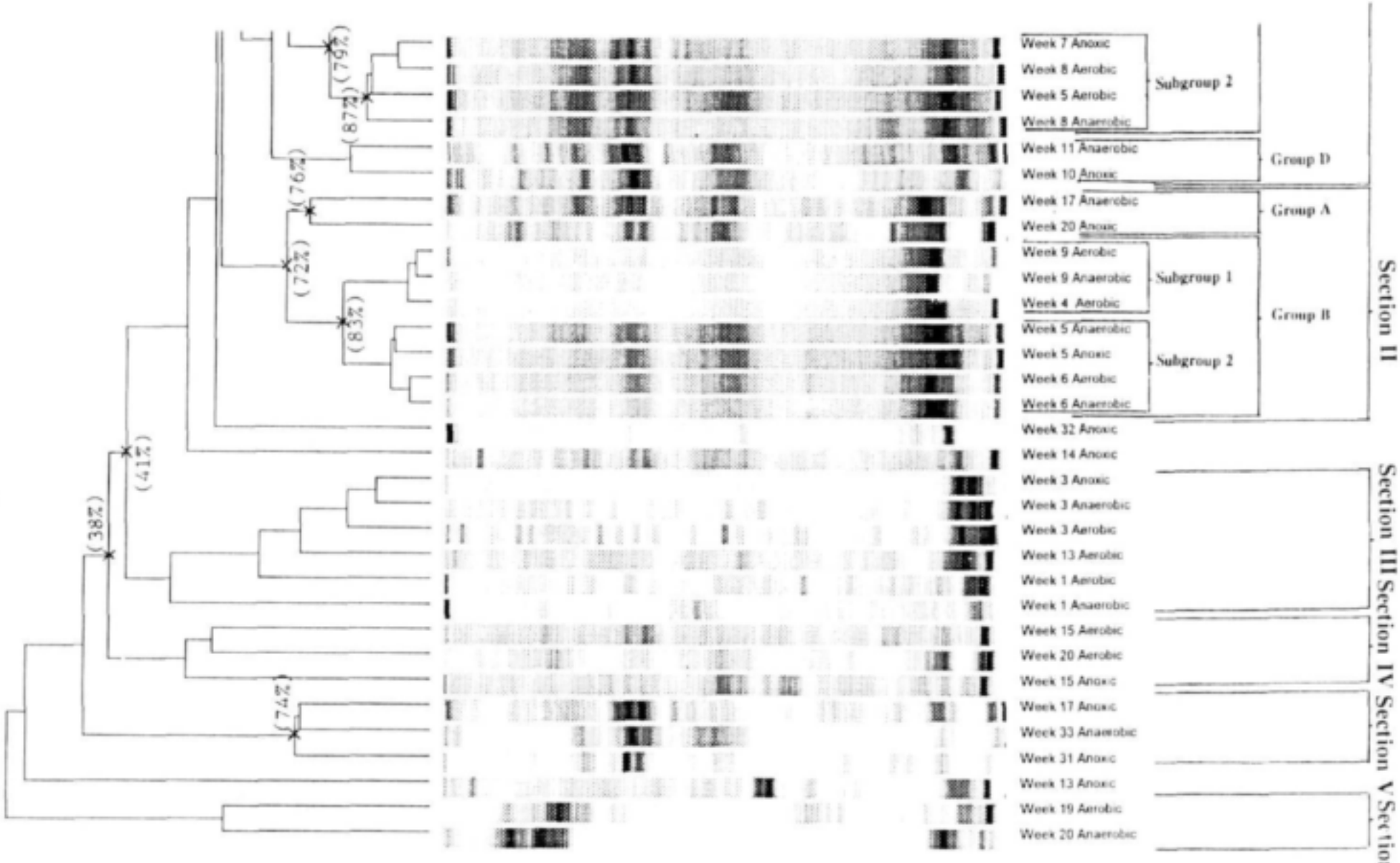


Figure 4: Dendrogram of the electrophoretic patterns of the three zones of the Daspoort Activated Plant collected on a weekly basis for 34 weeks, based on UPGMA analysis of the correlation coefficients (r) of the protein patterns.

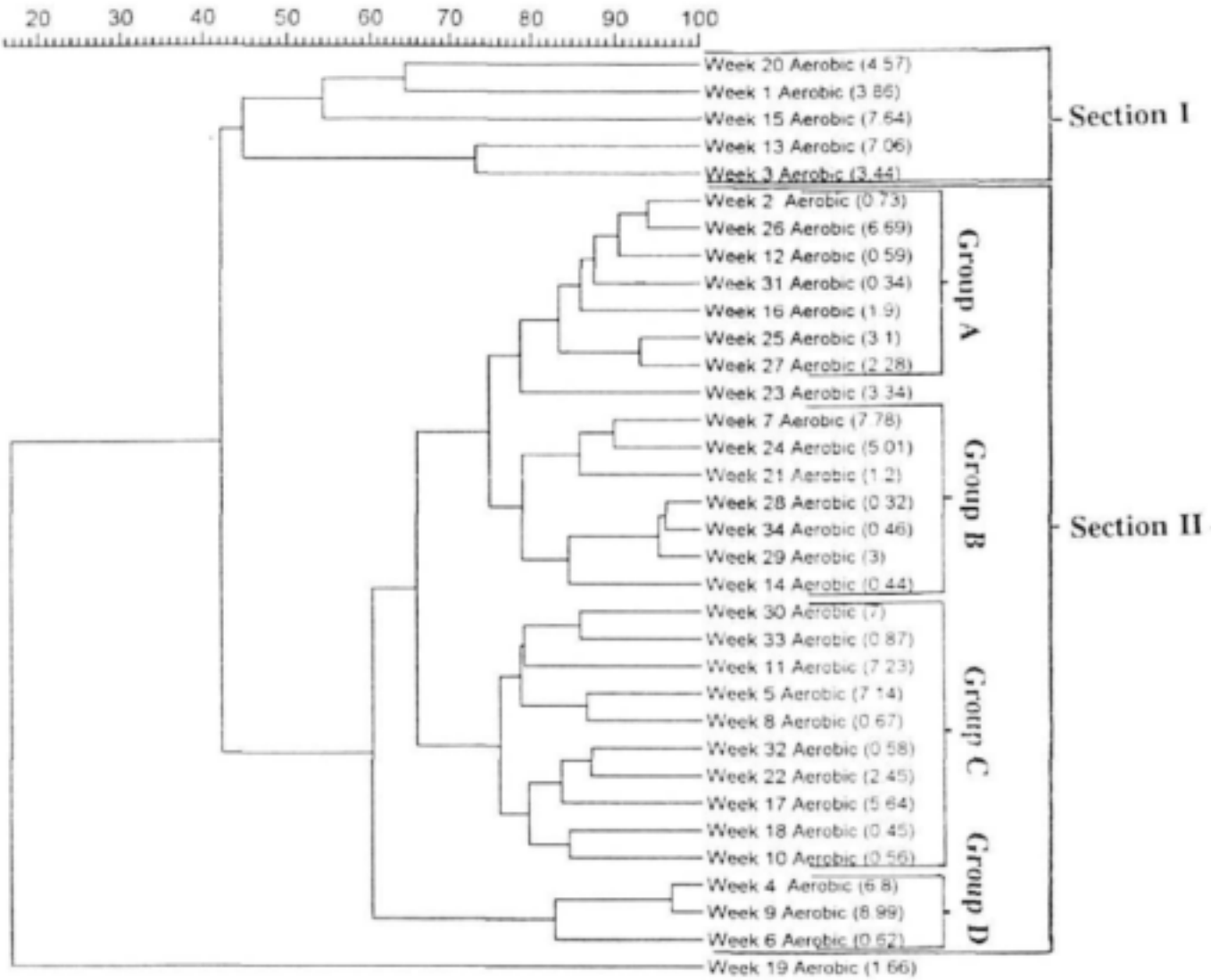


Figure 5: **Dendrogram of the electrophoretic patterns of the aerobic zones of the Daspoort Activated Plant samples and their P-concentration as collected on a weekly basis for 34 weeks, based on UPGMA analysis of the correlation coefficients (r) of the protein patterns.**

4.4 Determination of microbial community structure of P-, and N-removing laboratory scale activated sludge systems using SDS-PAGE.

The protein profiles as represented in the dendrogram were divided into four clusters (Fig. 6). Section I consisted of 56 samples which were 60% related. This section was further divided into 3 groups. Group A, with a 72% correlation, representing 18 samples, consisted of protein profiles of both N- and P-removing and N removing systems. This group was further sub-divided into four groups. Subgroup 1, with a 84% correlation, consisted of 4 samples which represented samples from the N-and P-removing systems. Subgroup 2, also with a 84% correlation, representing 8 samples, represented a combination of N-and P-removing and N-removing systems. Subgroup 3, with only three samples, showed a 88% correlation. Subgroup 4, with 94% correlation consisted of three samples. Group B, with a 75% correlation, was sub-divided into three groups. Subgroup 1, included 10 samples with a 88% correlation. Subgroup 2, representing 11 samples with a 81% correlation, represented both N- and P-removing and N-removing systems. In Subgroup 3, representing 3 samples a 87% correlation was observed. Group C, showed a 95% similarity and represented 13 samples from both the N- and P-removing and N-removing systems. However, virtually no protein bands were observed. This was ascribed to problems with sampling or sample preparation, since the data in Table 4 indicated that the systems were operating successfully.

Section II, clustered at 60% similarity, in respect to the other sections. A 73% correlation existed between the 5 samples from the MS, MT and MU systems, which was further sub-divided into two groups. Group A, with two samples and a correlation of 78% and Group B, with 3 samples which showed a 82% similarity. P-removing and N-removing samples were represented in this section.

The third section clustered at 55% similarity. In Section III, 5 samples were present with a 92% correlation. Section III could be divided into two groups. Group A, with 2 samples which were 94% related and Group B, with 3 samples 96% related. Only two of the three P-removing systems were present in this section.

The similarity of Section IV in relation to the other Sections was only 41%. The two

samples of the aerobic zones of a P-removing and N-removing systems indicated a 87% correlation. A similarity value of > 60% showed a high correlation between the protein profiles of the samples.

The protein profiles of the aerobic zones and their P concentrations ($mg.l^{-1}$) were compared (Fig.7). The P concentrations were used to determine if there was any possible correlation between the protein profiles and P concentrations. Three sections could be identified. Section I, was divided into two distinct groups. Group A, with 11 samples and a 63% correlation and Group B, with 14 samples and 74% correlation. Sections II showed a 41% similarity and Section III correlated < 41%. Both these sections had two samples.

The protein patterns of the activated sludge samples showed a diverse bacterial community structure, which consists of many different bacterial species. This agrees with recent literature, suggesting that the same bacterial community is present throughout the activated sludge process (Bond, Hugenholtz, Keller & Blackall, 1995).

Conclusions

* The protein profiles indicated a > 70% correlation for all the systems (N- and P-removing and N-removing included), in each group or subgroup. No specific protein pattern with regard to the different zones was observed. We can therefore, conclude that the same bacterial communities were present in both the N- and P-and N removing systems, and the different zones of these systems.

TABLE 4: **Phosphorus concentration and chemical analysis ($mg.l^{-1}$) of the different zones of the UCT laboratory scale activated sludge systems (June - November 1996)**

System	Date	COD	△P	△P/ COD_{in}	Anaer-obic	Anoxic	Aerobic
HM	27/5	720	4.36	0.0061	14.48	10.37	10.12
(30°C)	17/7	679	4.92	0.0072	15.28	10.93	10.36
	11/8	665	4.47	0.0067	13.86	9.71	9.39
	12/9	686	4.69	0.0068	14.28	10.07	9.59
	16/10	705	3.00	0.0043	14.32	11.82	11.32
	7/11	672	4.64	0.0069	15.14	11.14	10.5
ML	12/8	767	16.17	0.0218	37.89	18.27	6.52
(20°C)	17/10	760	18.48	0.0243	39.30	19.83	5.57
+ leachate	28/11	789	19.64	0.0249	49.60	25.40	4.82
MS	28/5	663	9.61	0.0145	20.80	11.29	6.07
(20°C)	12/8	629	9.09	0.0145	25.17	17.30	13.71
- leachate	17/10	607	10.48	0.0173	25.31	17.75	13.46
	8/11	666	16.10	0.0242	38.05	21.62	10.35
MT	27/5	720	8.46	0.0118	16.46	8.77	6.02
(30°C)	17/7	679	7.43	0.0109	16.73	9.42	7.85
	11/8	665	8.94	0.0134	16.66	7.89	4.92
	17/9	686	10.29	0.0150	23.10	9.92	4.49
	16/10	705	9.58	0.0136	24.49	11.06	4.74
	7/11	672	13.45	0.0200	23.96	7.84	1.69
MU	28/5	458	2.75	0.0060	ND	51	6.7
(20°C)	12/8	638	2.5-3		ND	57	6.5-7.0
	13/9	557	2.5-3		ND	37	6.5-7.0
	17/10	542	2.5-3		ND	3	6.5-7.0
	8/11	487	2.65	0.0054	ND	1.5	7.02

ND = does not include anaerobic
System PW = Results outstanding
P-Removing systems: ML, MS, MT.
N-Removing systems: HM, PW, MU.

40
50
60
70
80
90
100
(84%)
(84%)
(72%)
(88%)
(94%)
(88%)
(60%)
MS3 Anaerobic
ML2 Anoxic
ML2 Anaerobic
MT5 Anoxic
Subgroup 1
PW4 Aerobic
MS3 Aerobic
MS4 Anaerobic
HM5 Aerobic
PW4 Anoxic
MS3 Anoxic
HM5 Anoxic
HM4 Aerobic
Subgroup 2
MT4 Anoxic
MT4 Aerobic
MT4 Anaerobic
Subgroup 3
MU4 Aerobic
MU4 Anoxic
ML2 Aerobic
Subgroup 4
Group A
MT6 Anoxic
MT2 Anaerobic
HM6 Anoxic
MS4 Anoxic
HM5 Aerobic
PW5 Anoxic
PW5 Aerobic
MT6 Anaerobic
MT6 Aerobic
HM4 Anoxic
Subgroup 1
MT5 Anaerobic
HM1 Aerobic
HM1 Anoxic
PW1 Anoxic
MU1 Anoxic
MU1 Aerobic
PW1 Aerobic
Subgroup 2
Group B
Section 1

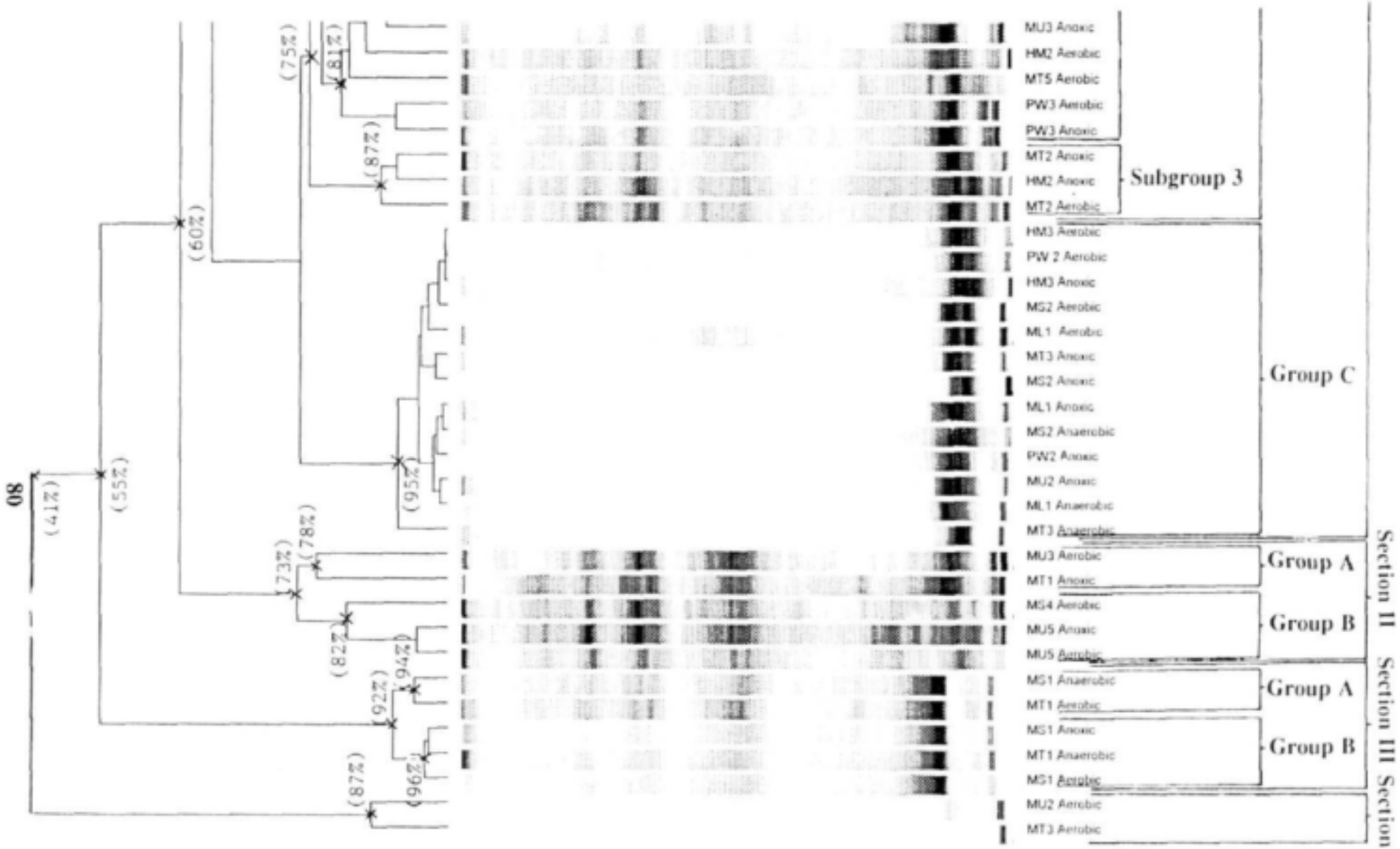


Figure 6: **Dendrogram of the electrophoretic patterns of P-removing and N-removing activated sludge samples of the anaerobic, anoxic and aerobic zones (UCT), based on UPGMA analysis of the correlation coefficients (r) of the protein patterns.**

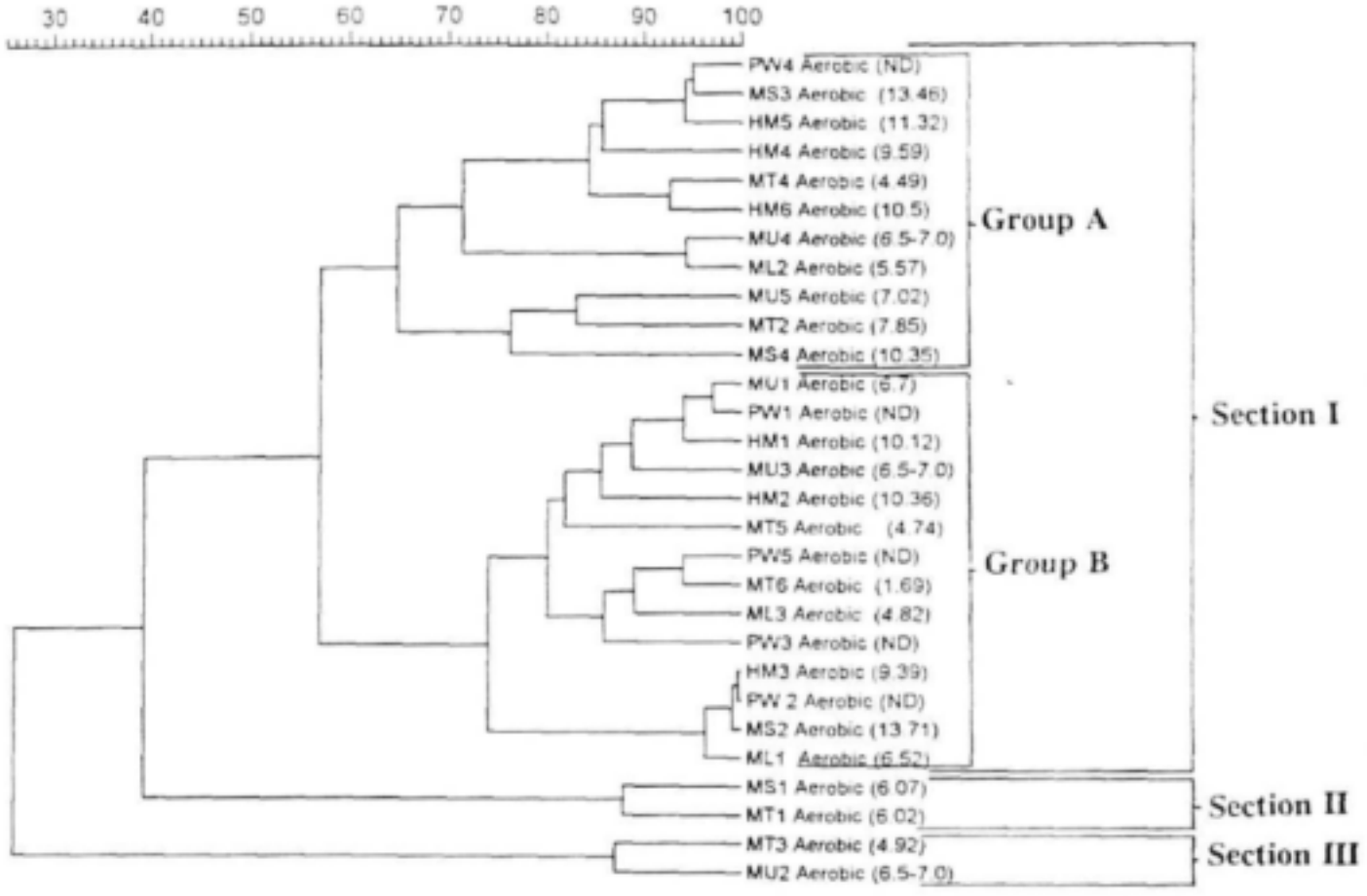


Figure 7: **Dendrogram of the electrophoretic patterns of the aerobic zones of P-removing and N-removing activated sludge samples (UCT) (with the P-concentrations included), based on UPGMA analysis of the correlation coefficients (r) of the protein patterns.**

4.5 Determination of microbial community structure of P-, and non-P removing full scale activated sludge systems using SDS-PAGE.

This dendrogram represents the different systems with different designs. Three sections were identified (Fig. 8). Section I, with a 50% correlation, included 66 samples. Nine groups were distinguished in this section. Group A, with 7 samples at a 93% correlation, included the Nylstroom system (a carousel type of system) treating domestic waste and the Modderfontein system (a 3-stage system) treating both industrial and domestic waste and samples of Rynfield South (a 5-stage phoredox systems) which treated domestic waste. Group B, with 95% correlation, included 7 samples of Rynfield North and South systems. Group C, with a 85% correlation, consisted of 7 samples of the 3-stage Olifantsfontein Module 1 and 2 system, which treated industrial and domestic waste. Group D, with 90% correlation, included all three zones of the following 3 stage systems: Heidelberg, which treats industrial and domestic waste, JP. Marais and Potgietersrus both treating domestic waste. Group E, with 3 samples represented two Esterpark samples (anoxic and aerobic) and treating domestic waste and one Modderfontein anaerobic sample treating industrial and domestic waste with a 86% correlation. Both systems were 3-stage plants. Group F, included 8 samples and showed a 77% similarity. All three zones of Tsakane and Zeekoeigat were included in this group. These systems were 3-stage systems treating domestic waste. In Group G, 7 samples of the primary and secondary stages of Hartbeesfontein which treats industrial and domestic waste correlated at 70%. Group H including 12 samples correlated at 58% consisted of al the samples of the 2-stage Waterval-South and Waterval-North modules 1 and 2, which treated industrial and domestic waste. In Group I, two Daveyton samples correlated at 88%.

Section II clustered at 39% similarity with regard to the other two sections. This Section consisted of only one Vlakplaats Anoxic sample. Section III consisted of 3 Waterval-North module 3 samples correlating at 90%. No explanation can be given as to why this system did not cluster with the other two modules of the Waterval system, because it treats the same type of waste as the other two modules of this system.

The resulting dendrogram indicated two Sections when only the aerobic zones of the different

systems were compared (Fig. 9). Section I was divided into Group A, which included most of the aerobic zone samples of the different systems and with an average P concentration of 7 $mg.l^{-1}$. The P concentration for individual samples again showed a lot of variation. Group B with an average P concentration of 0.76 $mg.l^{-1}$, consisted only of samples of the Waterval system. Section II consisted of only two Waterval Module 3 samples which showed a 90% similarity.

Conclusions

* Most of the activated sludge samples of the different systems grouped together in Section I, indicating an overall similarity between them of 50%.
* Protein profiles do not differ significantly in any of the systems, suggesting that the bacterial community remains constant.
* System design and the type of waste treated by the plant did not result in altered protein patterns. This indicates that the microbial community structure of activated sludge plants are closely related and that each sludge community contains the potential or capacity (genetic information or species diversity) to treat almost any kind of waste.

Table 5: **Phosphorus concentrations ($mg.l^{-1}$) of 21 different activated sludge systems (1996).**

System	Design	Waste Type	Anaerobic	Anoxic 1	Aerobic 1	Anoxic 2	Aerobic 2
Daveyton **(DVT)**	3-stage	Domestic	14.43	25.6	1.12	ND	ND
Ester Park **(ESTR)**	3-stage	Domestic	7.44	7.22	7.62	ND	ND
Hartebees-fontein **(HBFT)**	5-stage	Industrial and Domestic	9.25	10.6	8.34	ND	ND
Heidelberg **(HDLB)**	3-stage	Industrial and Domestic	18.6	5.04	5.08	ND	ND
JP. Marais **(JPMS)**	3-stage	Domestic	11.46	6.3	5.98	ND	ND
Modder-fontein **(MDFN)**	3-stage	Industrial and Domestic	0.11	0.11	0.12	ND	ND
Nylstroom **(NYLS)**	carousel	Domestic	26	22	14	ND	ND
Olifants-fontein 1 **(OLFT1)**	3-stage	Industrial and Domestic	0.19	0.17	0.09	ND	ND
Olifants-fontein 2 **(OLFT2)**	3-stage	Industrial and Domestic	0.18	0.15	0.08	ND	ND
Potgieters-rus **(POTG)**	3-stage	Domestic	31.5	29.5	28.5	ND	ND
Rondebult **(RDLT)**	1-stage	Industrial and Domestic	ND	ND	0.29	ND	ND

Table 5: Phosphorus concentrations ($mg.l^{-1}$) of 21 different activated sludge systems (1996)(continued).

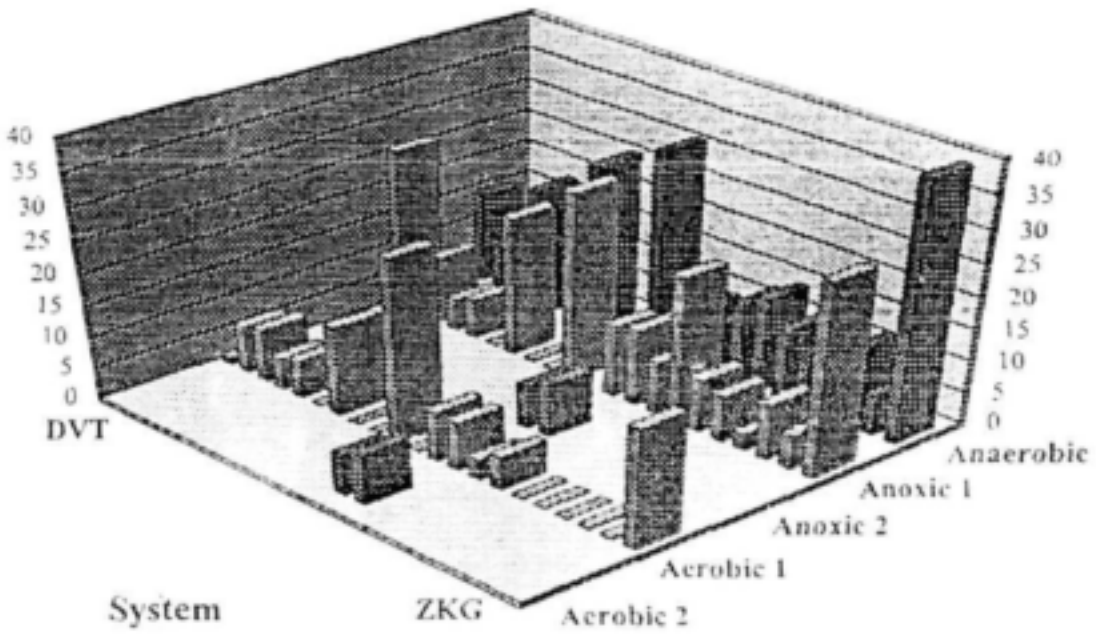
System	Design	Waste Type	Anaerobic	Anoxic 1	Aerobic 1	Anoxic 2	Aerobic 2
Rynfield-PRDX-N **(P-N)**	5-stage phoredox	Domestic	9.56	11.27	7.86	6.77	6.63
Rynfield-PRDX-S **(P-S)**	5-stage phoredox	Domestic	7.92	11.97	7.63	8.28	8.18
Tsakane **(TSKN)**	3-stage	Domestic	12.85	8.28	3.29	ND	ND
Vlakplaats **(VLKP)**	3-stage	Industrial and Domestic	14.8	22.92	5.39	ND	ND
Waterval-N Mod 1 **(WTV-N-Mod1)**	2-stage	Industrial and Domestic	11.1	ND	0.3	ND	ND
Waterval-S Mod 1 **(WTV-S-Mod1)**	2-stage	Industrial and Domestic	9.13	ND	0.29	ND	ND
Waterval-N Mod 2 **(WTV-N-Mod2)**	2-stage	Industrial and Domestic	6.75	ND	0.22	ND	ND
Waterval-S Mod 2 **(WTV-S-Mod2)**	2-stage	Industrial and Domestic	12.23	ND	0.44	ND	ND
Waterval-N Mod 3 **(WTV-N-Mod3)**	2-stage	Industrial and Domestic	6.2	ND	0.38	ND	ND
Zeekoegat **(ZKG)**	3-stage	Domestic	40	30	19	ND	ND

ND: Not determined

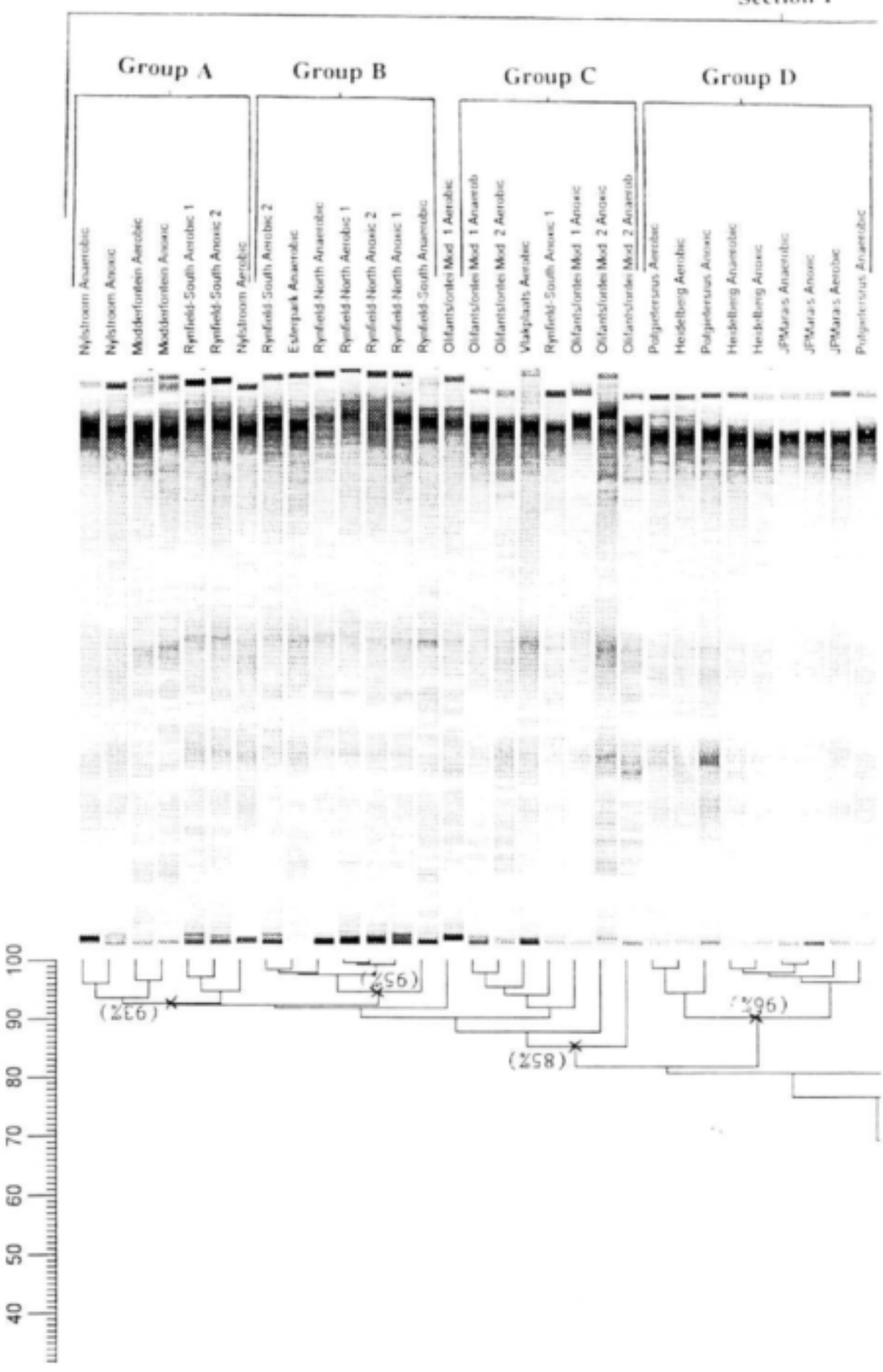
Table 6: Chemical analysis ($mg.l^{-1}$) of the different activated sludge systems (1996).

System	⌂P	COD (raw)	COD (final)	NH_3/N (raw)	NH_3/N (final)	NO_3 (final)
Daveyton	13.31	1170	36	27.1	5.6	1.00
Ester Park	-0.18	847	10	73.3	0.1	16.87
Hartebees-fontein	0.91	557	70	24.6	9.2	1.08
Heidelberg	13.52	615	39	34.7	6.8	1.43
JP. Marais	5.48	570	73	46.2	0.00	6.38
Modder-fontein	-0.01	253	36	32.2	22.6	27.80
Nylstroom	12	ND	ND	ND	ND	ND
Olifants-fontein	0.1	791	53	31.8	1.2	2.64
Potgietersrus	3	ND	ND	ND	ND	ND
Rondebult	0.29	985	105	18.9	6.9	0.23
Rynfield	2.93	277	95	24.3	0.8	1.2
Tsakane	9.56	370	24	25.4	0.00	3.24
Vlakplaats	9.41	253	20	22.6	9.7	1.17
Waterval	10.3	741	102	17.2	9.2	0.18
Zeekoegat	21	ND	ND	ND	ND	ND

ND: Not determined



Graph 2: **Phosphorus concentrations ($mg.l^{-1}$) from 21 activated sludge systems.**


Group A
Group B
Group C
Group D
Nylstroom Anaerobic
Nylstroom Anoxic
Middelfontein Aerobic
Middelfontein Anoxic
Rynfield-South Aerobic 1
Rynfield-South Anoxic 2
Nylstroom Aerobic
Rynfield-South Aerobic 2
Esterpark Anaerobic
Rynfield-North Anaerobic
Rynfield-North Aerobic 1
Rynfield-North Anoxic 2
Rynfield-North Anoxic 1
Rynfield-South Anaerobic
Olifantsfontein Mod 1 Aerobic
Olifantsfontein Mod 1 Anaerob
Olifantsfontein Mod 2 Aerobic
Vlakplaats Aerobic
Rynfield-South Anoxic 1
Olifantsfontein Mod 1 Anoxic
Olifantsfontein Mod 2 Anoxic
Olifantsfontein Mod 2 Anaerob
Polgietersrus Aerobic
Heidelberg Aerobic
Polgietersrus Anoxic
Heidelberg Anaerobic
Heidelberg Anoxic
JPMaras Anaerobic
JPMaras Anoxic
JPMaras Aerobic
Polgietersrus Anaerobic
(93%)
(95%)
(85%)
(96%)
100
90
80
70
60
50
40

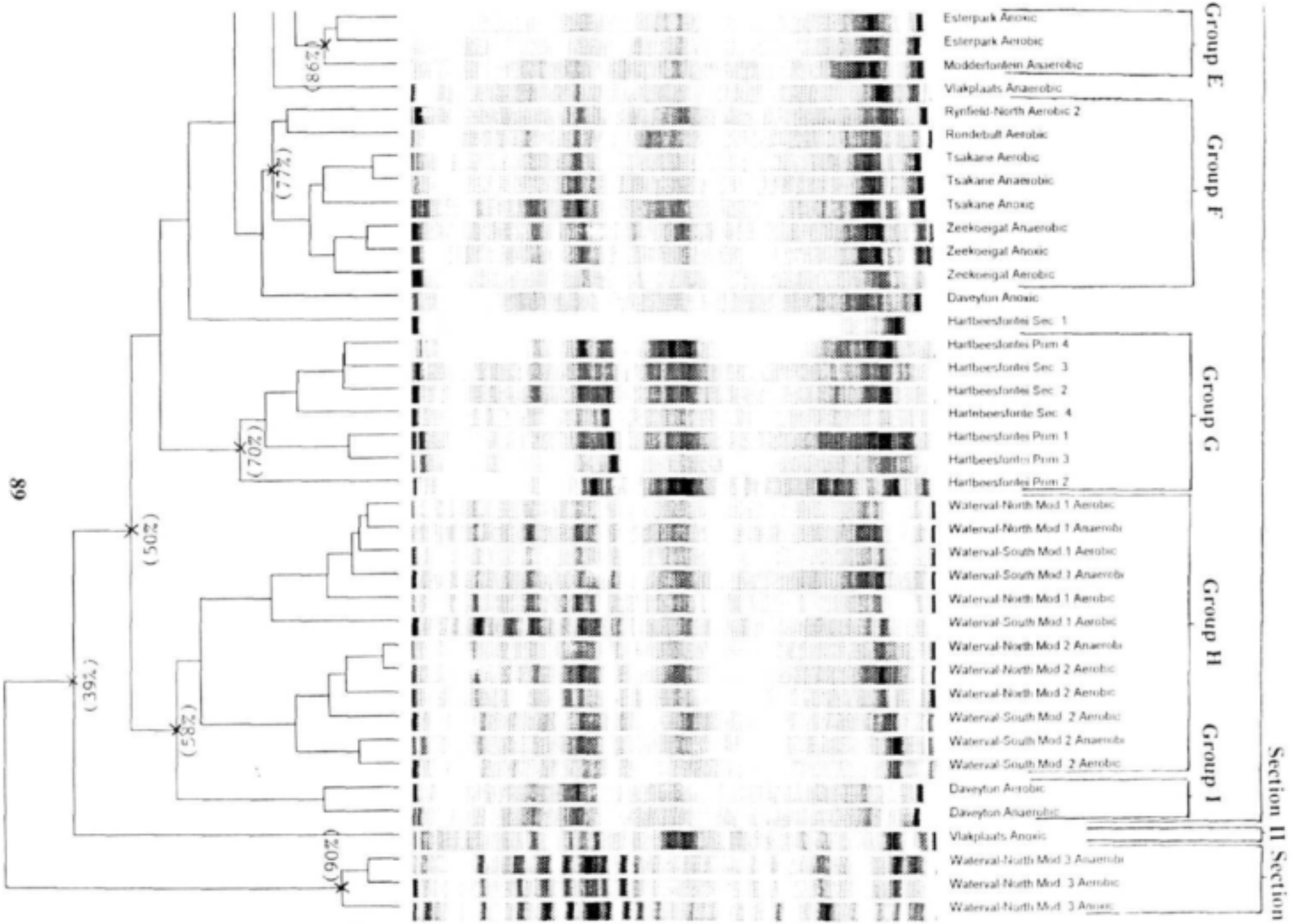


Figure 8: Dendrogram of the electrophoretic patterns of the anaerobic, anoxic and aerobic zones of 21 different activated sludge systems, based on UPGMA analysis of the correlation coefficients (r) of the protein patterns.

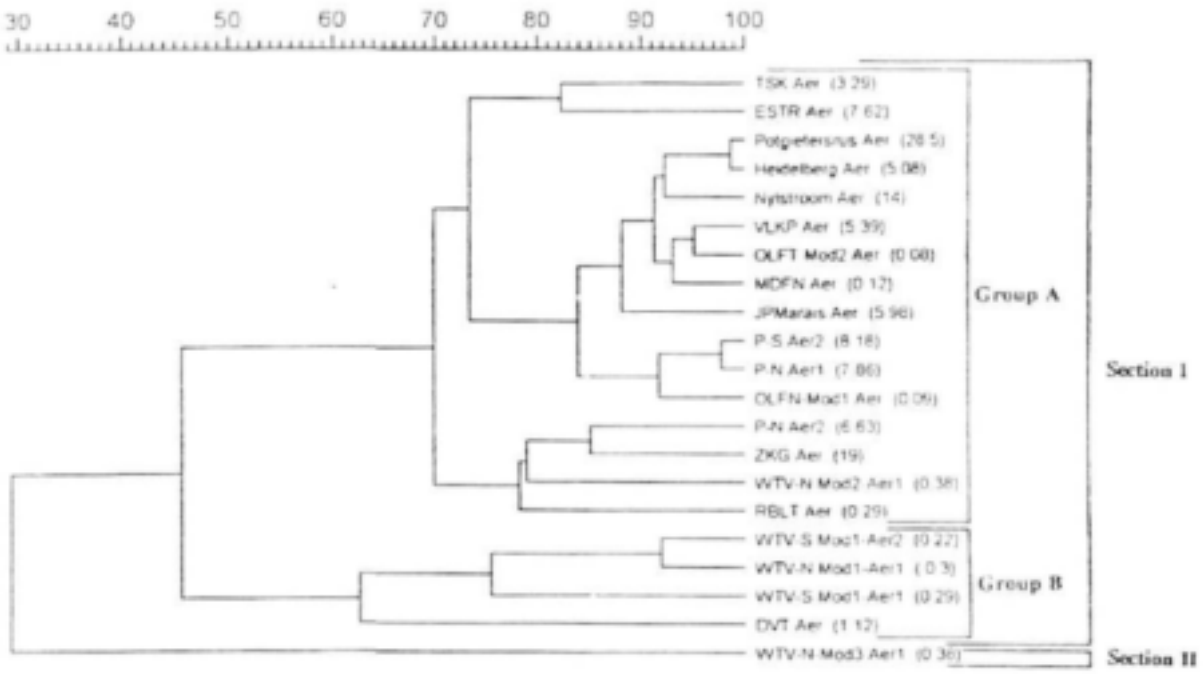


Figure 9: **Dendrogram of the electrophoretic patterns of the aerobic zones and P concentrations of 21 different activated sludge systems, based on UPGMA analysis of the correlation coefficients (r) of the protein patterns.**

CHAPTER 5

CONCLUSIONS

The results indicated that SDS-PAGE was a sensitive tool for the determination of the bacterial population structure of activated sludge. Due to the lack of methods to determine bacterial community structure of environmental samples, there is a constant search for new methods to investigate and better understand the functioning of microorganisms in their natural environment. Conventional microbial techniques have provided a misleading picture of bacterial community structure of environmental samples. SDS-PAGE was used because the method alleviated the need for culturing and samples were analyzed in a more direct manner which prevents the selection of specific organisms. Other advantages of this method was that it was relatively easy and many samples can be analyzed at the same time. It is also not as expensive as DNA:DNA hybridization. However, the results obtained by SDS-PAGE of whole-cell proteins discriminates at much the same level as DNA:DNA hybridization (Priest & Austen, 1993).

Resulting protein profiles, after SDS-PAGE were normalized and analyzed with the Gelcompar 4.0 programme. This programme calculated the % similarities and differences between each protein profile, with the Pearsons product moment correlation coefficient (r) between samples to construct a matrix. The samples were then clustered using the unweighted pair group method of arithmetic average (UPGMA) which resulted in a dendrogram.

Researchers tend to construct dendrograms consisting of only a few samples and then base the identification of a new genus or species on their findings. When samples are added to smaller dendrograms the dendrogram is more likely to vary. However, the larger the dendrogram, the more value can be attached to the results. When new samples are added, the groups will stay the same and only a small variation in the % correlation might appear. Each dendrogram must be evaluated on its own and not be compared with other dendrograms. These are the main reasons why no definite value of > 80 % for the same species and > 60 % for the same genus can be attached to a dendrogram. Percentage

correlation between the samples must only be an indication of similarity.

One disadvantage of the SDS-PAGE method is that it needs to be standardized. Results between different laboratories may differ if standard methods are not followed. An exact value can not be attached to the % similarity or correlation of the resulting dendrogram after SDS-PAGE. The % similarity can rather be used as an guideline. SDS-PAGE can therefore, not discriminate between the bacterial populations of the different activated sludge samples, it can only indicate samples with a high % similarity or not.

Valuable information concerning the bacterial population structure of activated sludge was obtained when SDS-PAGE was used. The results confirmed previous studies performed by Cloete & Steyn (1987) which indicated that the bacterial population of activated sludge stayed the same throughout the system. The main drawback of this technique was that it was not sensitive enough to determine the difference in protein profiles of P-removing and non-P-removing bacterial populations. SDS-PAGE studies, however, could be useful when monitoring a specific environment over time. Should a stress situation develop altered protein patterns or low % similarity will indicate this.

Future studies on the bacterial structure of activated sludge or any environmental sample should include the use of a combination of methods such as standard culturing and identification techniques, SDS-PAGE and 16S rRNA. A method such as 16S rRNA may result in the same problems as phenotypic methods because the work is restricted to the system on which the initial work was performed as the probes only detect those isolates for which they are made. The unculturable species of the community will remain undetected. However, Bond *et al.*, (1995) used 16S rRNA methods to determine the difference between P-removing and non-P-removing activated sludge systems. They obtained interesting results but further research as well as the combination of different techniques, as well as the role of biomass in the P-removal process need to be investigated.

5.1 SDS-PAGE was a sensitive method to determine the bacterial community structure of activated sludge sample.

5.2 This technique has the advantage that samples can be analyzed directly without prior cultivation.

5.3 The results suggested that there was no difference between the bacterial community structures of the different activated sludge zones.

5.4 The bacterial communities of P-, and N-removing laboratory scale systems indicated no difference amongst their protein profiles after SDS-PAGE.

5.5 Each system seemed to have its own specific microbial community structure which does not differ much from those of other systems.

5.6 System design and the type of waste treated by the plant did not result in altered protein patterns. This indicates that the microbial community structure of activated sludge plants is closely related and that each sludge community contains the potential or capacity (genetic information) to treat a variety of waste types.

5.7 Seasonal changes did not have an affect on the protein profiles of the bacterial community of the Daspoort activated sludge plant when monitored over a 34 week period.

5.8 The protein profiles of a P-removing and non-P-removing system indicated a high percentage of correlation indicating little variation in their bacterial communities.

5.9 Activated sludge systems are not dominated by one or a few specific bacterial species but consist of a combination of different bacterial species which co-exist and function together in a complex community.

5.10 Antigen preparation from the anoxic and aerobic zones contained intact and lysed bacterial cells. Besides conventional immunization, subtractive immunization using cyclophosphoamide was also used to focus the immune response on unique epitopes in the zones. Neither strategy provided antibodies capable of distinguishing phenotypic diversity between the two zones, emphasizing the homogeneity of the microbial populations in the different zones of phosphate removing activated sludge systems. Nine stable hybridoma lines were established, all secreting IgM cross reactive to both antigen preparations but differing in the antigen specificity. Monoclonal antibody 7B9, putatively protein-directed, could clearly distinguish between the aerobic zones of the two activated sludge systems differing only in phosphate removal ability: immunoblot showed five discrete bands in the system successful at phosphate removal. The molecular weights of the bands appeared to be

multiples of 18 kDa, indicating possible involvement of an 18 kDa proteinaceous monomer in phosphate uptake.

The ultimate method to determine the bacterial community structure has not been developed. New methods should therefore, be investigated until one or a combination of methods are found to better understand microbial ecology.

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

Previous studies also indicated that biomass was related to phosphorus removal. The higher the "biomass" the better the P-removal. This suggested that the main difference between P-removing and non P-removing systems is biomass related and not due to the microbial community structure. The aims for future studies are therefore: i) To determine the relationship between biomass and P removing and non-P removing systems ii) To determine the P removal capacity of a system based on biomass iii) To determine the effect of bioaugmentation on phosphorus removal in a conventional activated sludge system by adding biosupplements and/or anaerobic sludge in order to increase the biomass.

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APPENDIX

IMMUNUCHEMICAL INVESTIGATION OF ENHANCES PHOSPHATE REMOVAL BY ACTIVATED SLUDGE

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SUMMARY

Phosphate in wastewater effluent is implicated in eutrophication of water reserves. Enhanced biological phosphate removal by activated sludge is attributed to polyphosphate accumulating bacteria, which release phosphate during anaerobiosis and reincorporate it during aerobiosis. The aim of the study was to investigate whether the process of phosphate removal by activated sludge could be probed immunochemically. Antigen preparations from the aerobic and preceding anoxic zones of a phosphate removing system contained intact and lysed bacterial cells. Neither conventional nor subtractive immunization strategies, the latter employing cyclophosphamide to immunofocus on unique epitopes in the zones, provided antibodies capable of distinguishing between these zones. However, a putatively protein-directed monoclonal antibody could distinguish between the aerobic zones of two activated sludge systems, differing only in phosphate removal ability: immunoblot showed five discrete bands, with molecular weights appearing to be multiples of 18 kDa, unique to the system successful at phosphate removal.

Key words: phosphate; activated sludge; monoclonal; antibodies; cyclophosphamide.

INTRODUCTION

Phosphate has been implicated in eutrophication, an undesirable phenomenon of overgrowth of water plants and algae in water systems, necessitating legal restrictions on the levels of soluble phosphate in effluents from wastewater treatment systems (Cooper *et al.*, 1994). The

success of enhanced biological phosphate removal (EBPR) by polyphosphate accumulating (polyP) bacteria in activated sludge has spawned much research interest, but its mechanisms remain far from fully elucidated.

Many variations of the EBPR process exist, all utilizing the same mechanism, but applied in different ways to select for polyP organisms and achieve phosphate removal. A primary requirement for phosphate removal is the recirculation of the sludge through alternating anaerobic and aerobic stages, anaerobic indicating an environment free of dissolved oxygen, nitrate or nitrite, i.e., absence of an electron acceptor. These conditions suit acidogenic microflora, which convert organic substrate to short-chain volatile fatty acids such as acetate. The polyP bacteria take up the acetate and store it as polyhydroxy-butyrate (PHB). The energy for transport and storage is provided by the hydrolysis of an intracellular polyphosphate reserve to phosphate, resulting in phosphate release from the cell. In the aerobic zone the bacteria utilize the accumulated PHB reserves to generate energy for the synthesis of new biomass and for polyphosphate synthesis, accompanied by the uptake of phosphate from the sludge liquor (Kortstee *et al.*, 1994). In plants where nitrate removal is also required, an additional anoxic stage for denitrification is needed. The term "anoxic" indicates an environment free of dissolved oxygen, but oxygen is available as part of the nitrate ion (Schön *et al.*, 1993).

This study was aimed at the development of immunochemical probes to determine and monitor phenotypic traits of the activated sludge phosphate removal process. The antibodies were used to evaluate the phenotypic status pertaining to different zones in a phosphate removing system.

Monoclonal antibody production is the method of choice to obtain immunoglobulins against proteins that cannot be purified or that are available in low amounts (Drenckhahn *et al.*, 1993). Antibodies have been used in investigations of samples from activated sludge systems, although such studies are not numerous. Cloete & Steyn used a fluorescent antibody technique for the identification and enumeration of *Acinetobacter* in activated sludge (Cloete & Steyn, 1988). Monoclonal antibodies specific for *Nitrobacter* and *Nitrosomonas* respectively were produced and used for estimation of cell numbers in activated sludge

(Sandén *et al.*, 1994).

Conventional as well as focused immunization strategies were used in this study to maximize the possibility of producing antibodies with the required ability to discern between the antigen preparations. The cytotoxic, immunosuppressant drug cyclophosphamide was used as tolerizing agent to manipulate the bias of the normal immune response. All proliferating lymphocytes stimulated by a preceding immunization are eliminated by cyclophosphamide administration, thus suppressing the immune response of mice immunized against either of the antigens. Subsequent immunization of such mice with the alternative antigen preparation should direct the immune response at those determinants that had been absent from the former antigen preparation used. Matthew & Patterson (1983) were the first to effectively employ this approach to develop highly specific antibodies to impure material.

The possibility was also investigated that monoclonal antibodies could distinguish between activated sludge systems with differing phosphate removal abilities.

MATERIALS AND METHODS

Samples of activated sludge

For immunization and screening. Samples were taken from the second anoxic and the aerobic zones of a stable laboratory scale activated sludge system of the modified UCT configuration (Toerien *et al.*, 1990). The system showed good phosphate removal (11,43 mg PO_4^--P ℓ^{-1} at the time of sampling).

For comparative studies of systems with different phosphate removal abilities. Samples were simultaneously taken from the aerobic zones of two laboratory scale systems receiving identical daily inflow. The systems differed only in configuration: the first, being a UCT (University of Cape Town) system, removing phosphate successfully; the other, lacking the anaerobic zone at the head of the system, comparatively unsuccessful at phosphate removal. Table 7 shows the operational parameters of the two systems. After collection, samples were immediately fixed by addition of formalin to 0,25 %(v/v), incubated at 10°C for 24 h, aliquoted, and stored at 4°C.

Antigen preparation

Flocs were disrupted by shaking anoxic or aerobic zone samples with glass beads in a mechanical shake apparatus (Edmund Bühler Type Vi2, Tübinger). Loose cells were separated from remaining flocs and other organic material by low speed centrifugation (45 g, Hermle Z320 centrifuge, Germany) for 10 min. Washing of cell fractions was performed three times by centrifugation at 10 000 g for 20 min (Beckman L7-55 Ultracentrifuge, Germany) to 10^6-fold dilution of contaminants using double distilled deionized water. As a relative measure of cell density, A_{540} of 0,1 was arbitrarily taken as 10^8 cells/mℓ. Cell suspension volumes of 0,5 mℓ each were lysed by sonication on ice for 4 min, 50 % pulsed, at maximal energy input (Sonifier Cell Disruptor B-30, Branson, U.S.A.). Whole cell fractions were combined with lysed fractions in a 1:1 ratio to obtain final antigen preparations.

Immunization

Two groups of C57Bl/6J mice (H.A. Grové Research Centre, Pretoria) were used, four to six weeks old at the start of the immunization programme. These were intraperitoneally injected with 0,5 mℓ of either the aerobic or the anoxic antigen preparation, freshly prepared and diluted with O,9 %(w/v) NaCl to 2 x 10^7 cells/mℓ. Mice were immunized at 0, 2, 6, 10 and 14 weeks.

Immunofocusing. A subset of the mice in the two groups was subjected to immunofocusing (based on the protocol followed by Crause, 1993). Four days after the week 14 immunization, 40 mg of cyclophosphamide (Sigma, U.S.A.) per kg body weight in 0,5 mℓ 0,9 %(w/v) NaCl was administered intraperitoneally. Subsequent immunizations using the alternative antigen preparation were performed ten days after immunosuppression, and again at 18, 22, 28 and 32 weeks. Antisera were obtained by bleeding mice one week after immunization.

Hybridoma production

The general method of Galfré and Milstein (1981) was used to produce hybridomas, both from the unfocused mice as well as the immunofocused mice.

ELISA procedures

Screening. Aerobic or anoxic zone antigen preparations, freshly prepared and diluted with PBS to 2 x 10^8 cells/mℓ were used for coating of microtitre plates (Sterilin, England) at 100 $\mu\ell$ per well. The plates were dried by means of a heating lamp and fan. After fixation with 200 $\mu\ell$ of 70 % methanol per well for 10 min, the methanol was shaken out and the plates kept at 4°C until use (Smith, 1988). Casein (Merck, Germany) at 0,5 %(w/v) in PBS was used for blocking against non-specific binding, washing, and dilution of sera and immunoglobulin peroxidase conjugates. In some experiments (see legends of figures), 5 % horse serum was included in the blocking buffer as this diminished non-specific binding presenting as high background signals. Antigen coated wells were blocked for one to two hours before incubation with undiluted hybridoma culture supernatants at 50 μl per well for 45 min, all at room temperature. The plates were washed three times, aspirated and incubated with a 1/4000 dilution of goat anti-mouse IgG (heavy and light chain) peroxidase conjugate (Cappel, USA) at 50 $\mu\ell$ per well for 30 min at room temperature. The plates were again washed three times and aspirated. Colour development followed at room temperature by adding to each well 50 $\mu\ell$ of a substrate solution consisting of 0,1 %(w/v) *o*-phenylenediamine (Sigma, U.S.A.) and 0,08 %(w/v) urea hydrogen peroxide (BDH Chemicals, England) in 0,1 mol l^{-1} citrate buffer, pH 4,5. Absorbance was read at 450 nm on an ELISA spectrophotometer (SLT Labinstruments, Austria).

Isotyping of antibodies. A similar ELISA procedure as described above was used, but utilizing either a rabbit anti-mouse γ-chain peroxidase conjugate, or a rabbit anti-mouse μ-chain peroxidase conjugate (Cappel, USA).

Antigen characterization

Trypsin digestion of antigen. Freshly prepared aerobic zone antigen preparations were diluted with PBS to 2 x 10^8 cells/mℓ. Trypsin (Boehringer Mannheim, Germany), reconstituted in 0,1 mM HCl, was immediately added to a concentration of 0,2 %(w/v) and incubated for 16 h at room temperature (Boshoff, 1991). Trypsin was withheld from control specimens. Presence or absence of proteolytic activity was confirmed by adding a drop of the reaction solution to a few drops of a substrate solution (40 mM N-benzoyl-DL-arginine 4-nitroanilide-HCl (Merck, Germany) in DMSO, diluted ten-fold before use). Development

of a yellow colour indicated activity (Geiger & Fritz, 1984). Trypsinated antigen preparations were used for coating of microtitre plates, and ELISA was carried out as described for screening.

Periodate oxidation of antigen. Freshly prepared aerobic zone antigen preparations were diluted to concentrations of 2 x 10^8 cells/mℓ in 50 mM sodium glutamate buffer, pH 4,5, containing 20 mM periodic acid (Merck, Germany). Periodic acid was withheld from control specimens. After one hour incubation at 4°C, glycerol was added to 20 mM and the pH adjusted to 7,2 with 1 mol l^{-1} sodium hydroxide to stop the oxidation (Vogel, 1989). Presence or absence of periodate oxidative activity was confirmed before and after this step by adding a drop of the reaction solution to a few drops of 0,1 mol l^{-1} potassium iodide solution: development of a yellow colour indicating a positive result (Boshoff, 1991). A 0,125 % starch solution was effectively oxidised by this treatment, indicated by the yellow colour that developed after adding an equal volume of iodine-potassium iodide solution (0,3 g I_2 and 3 g KI in 50 ml water, diluted 2%(v/v) with 0,3 mol l^{-1} HCl before use). A control starch solution (no HIO_4 present) coloured blue with this test, showing no oxidation of starch (Moreno *et al.*, 1994). Periodate oxidised antigen preparations were used for coating of microtitre plates, and ELISA was carried out as described for screening.

SDS-PAGE and immunoblotting

SDS-PAGE was performed as described by Laemmli (1970). The separating gel was composed of 0,38 mol l^{-1} Tris-HCl, pH 8,8, 0,1 %(w/v) SDS, 12 %(w/v) acrylamide and 0,3 %(w/v) N'N'-Bis-methylene-acrylamide. The stacking gel was composed of 0,13 mol l^{-1} Tris-HCl, pH 6,8, 0,1 %(w/v) SDS, 4 %(w/v) acrylamide and 0,1 %(w/v) N'N'-Bis-methylene-acrylamide. The gel solutions were degassed under vacuum for 15 min. Polymerization was induced by addition of ammonium persulfate and N,N,N',N'-tetramethyl-ethylenediamine (TEMED) to final concentrations of 0,05 % for the separating gel, and 0,1 % for the stacking gel. Electrophoresis buffer, pH 8,3, consisted of 0,025 mol l^{-1} Tris, 0,2 mol l^{-1} glycine and 0,1 %(w/v) SDS.

The whole cell antigen preparations were pelleted at 12 000 g in a microfuge (Eppendorf, Germany) for 15 min. Pellets were resuspended in reducing sample buffer composed of 0,06

mol l^{-1} Tris-HCl, pH 6,8, 10 %(v/v) glycerol, 2 %(w/v) SDS, 5 %(v/v) mercaptoethanol and 0,001 %(w/v) bromophenol blue to 1 x 10^6 cells per mℓ. After lysis by sonication for 4 min, 50 % pulsed, at maximal energy input, samples were heated at 95°C for 5 min. Insoluble debris was pelleted by centrifugation at 12 000 g for 3 min and the supernatant (100 $\mu\ell$/well) was loaded onto the gel. Low M_r markers (Pharmacia, U.S.A.) and BSA were applied in separate lanes. Pre-electrophoresis was carried out at a constant voltage of 60 V for one hour, followed by electrophoresis at 100 V for 3 h (Pharmacia, U.S.A.).

The gel was equilibrated in a 10 mM CAPS (Sigma, U.S.A.) buffer, pH 9,0, for 15 min before electroblotting the proteins onto a PVDF membrane (Millipore, U.S.A.) pre-wetted with methanol, followed by CAPS buffer. A Transblot semidry blot apparatus (Bio-Rad, U.S.A.) was used at 10 V for 45 min.

The strip of PVDF membrane containing the lanes with the M_r markers and the BSA was cut off and stained in destaining solution (methanol, acetic acid and distilled water in a volume ratio of 1:2:1) containing 0,25 % Coomassie-Blue (Bio-Rad, U.S.A.), then destained overnight in destaining solution. The sample-containing part of the membrane was cut as required and blocked for one hour in TBS (Tris buffered saline) (20 mM Tris, 9 %(w/v) NaCl, pH 7,4) containing 1 % skimmed milk powder (Elite, Roodepoort, South Africa) and 0,05 % Tween-20. The membrane strips were incubated separately overnight at room temperature in the individual mature hybridoma culture supernatants. Each strip was washed three times in washing buffer (0,1 % skimmed milk powder in TBS) for 5 min before incubation at room temperature in goat anti-mouse IgG (heavy and light chain) peroxidase conjugate (Cappel, USA) diluted 1:1000 with the blocking buffer. After washing as before, the strips were developed in substrate buffer (0,05 %(w/v) 4-chloronaphthol, cold methanol to 17 %(v/v) and 0,05 %(v/v) hydrogen peroxide in TBS) until bands showed clearly. Development was stopped by rinsing with distilled water and the strips were photographed.

RESULTS

The preparation of immunogen from activated sludge proved more complex than is usually the case with bacterial pure cultures. Bacterial cells in activated sludge are largely enmeshed

in flocs, requiring dispersion of these aggregates before immunization. Washing of activated sludge cells before immunizing was required to remove material exuded into the surrounding sludge liquor such as enzymes and exoglycans, to minimize non-specific responses. The optimized procedure for antigen preparation from activated sludge is detailed in Materials & Methods. After floc disruption, washing of cells with water resulted in minimal re-aggregation. The sonication regimen yielded a maximal decrease in particulates as well as adequate protein release (results not shown).

The antigen preparations were used for immunization in both the conventional and the immunofocused antiserum production protocols described in Materials & Methods. Antisera obtained from either strategy were unable to distinguish antigenically between the the anoxic and aerobic zones of a phosphate removing activated sludge system (results not shown), prompting a monoclonal approach. This yielded similar results: no monoclonal antibodies able to distinguish such antigenic differences could be produced, not even from the immunofocused mice (Table 8). The chance of finding a stable hybridoma clone secreting antibody with such unique specificity appeared to be less than 0,1 % (i.e., no such clones found out of 1000 wells screened), suggesting low abundance of antigens exclusive to either zone.

Nine stable hybridoma clones, secreting cross-reactive monoclonal antibodies, were used in attempts to distinguish between antigen preparations from the aerobic zones of two activated sludge systems with different phosphate removal abilities. One monoclonal antibody, 7B9, derived from an aerobic zone immunofocused mouse and being of the IgM class, showed selectivity for the antigen preparation from the system successful at phosphate removal (Figure 10). This was confirmed by western blot (Figure 11). The antibody showed five compact bands occurring uniquely in the system successful at phosphate removal. Although limited by the resolution achieved in the separation, the M_r of the bands appeared to be multiples of 18 kDa, probably representing an oligomeric protein in different stages of covalent oligomerization.

To establish the molecular nature of the epitope recognised by antibody 7B9, epitope degradation strategies were employed (Figure 12). ELISA signals were practically

unchanged after periodate oxidation, but much diminished after trypsination, indicating the character of the epitope to be proteinaceous.

DISCUSSION

Sound environmental management hinges on effective wastewater treatment. In a country like South Africa with limited water resources, it is of cardinal importance that pollutants be prohibited from entering sensitive reservoirs and rivers. Phosphate has been implicated in eutrophication, an undesirable phenomenon of overgrowth of water plants and algae in water systems, necessitating legal restrictions on the levels of soluble phosphate in effluents from wastewater treatment systems. The success of enhanced biological phosphate removal (EBPR) by polyphosphate accumulating bacteria in activated sludge has spawned much research interest, but its mechanisms remain far from elucidated.

This study was aimed at the development of immunochemical probes to determine and monitor phenotypic traits of the activated sludge phosphate removal process. The antibodies were used to evaluate the phenotypic status pertaining to different zones in a phosphate removing system (configured to contain anaerobic, anoxic and aerobic stages), as well as to equivalent zones (aerobic) in activated sludge systems differing in configuration and thus phosphate removal ability.

Antigenic differences between the anoxic and aerobic zones of a phosphate removing activated sludge system were not distinguished either by polyclonal or monoclonal antibodies. This was confirmatory of expectations that such differences would be small, if in fact they existed at all, as flow-through and mixing of zonal contents was continual in an activated sludge system. The finding also corroborated those of Muyima (1995) and Louw (1996). The former showed reversible phosphate uptake and release in alginate-immobilized *Acinetobacter calcoaceticus*, depending on the environmental conditions to which the cells were exposed. It was concluded that under aerobic and anaerobic conditions the same activated sludge microbial community was responsible for the overall EBPR process since similar patterns were displayed regarding phosphate uptake and release behaviour (Muyima, 1995). Examining SDS-PAGE protein profiles of total protein extractions from activated

sludge, Louw (1996) found little metabolic diversity between phosphate removing (aerobic) and non-phosphate removing (anaerobic) periods, concluding that no microbial population shift occurred between the different zones of a phosphate removing activated sludge system, and that the enzyme systems required for phosphate uptake occurred in all the zones of the system. It may be deduced that if phenotypic differences were present during that study, these were too slight to be resolved by SDS-PAGE protein profiles. The absence of immunochemical resolution as demonstrated in the current study tends to confirm the findings of these workers.

The search for antigenic differences between the anoxic and aerobic zones as conducted in this study was aimed at detection of an antigen specifically involved in phosphate uptake in the aerobic zone, possibly induced by the introduction of oxygen to the environment. In the anaerobic zone the lack of a terminal electron acceptor results in an increase in the NADH level, inhibiting oxidative metabolism (such as the tricarboxylic acid cycle). The resultant increase in acetyl CoA levels stimulates polyhydroxybutyrate (PHB) synthesis, while polyphosphate degradation is stimulated by the decrease in the ATP/ADP ratio in the absence of oxidative phosphorylation (Smolders *et al.*, 1994a). In the aerobic zone, oxygen is utilized as final electron acceptor for the electron transport system by which the high NADH level is reduced. Inhibition of the tricarboxylic acid cycle is relieved permitting oxidation of stored substrate (polyhydroxybytyrate). Oxidative phosphorylation proceeds and ATP is produced. Phosphate is actively transported into the cell and polyphosphate synthesis proceeds (Smolders *et al.*, 1994b). Only recently has the phenomenon of denitrification by polyphosphate organisms, and therefore the uptake of phosphate in the anoxic zone, been incorporated in modelling of the activated sludge process (Mino *et al.*, 1995). This was due to the observation that phosphate is sometimes taken up in the sludge under anoxic conditions. The existence of two groups of phosphate accumulating bacteria was postulated: one group capable of utilizing only oxygen as electron acceptor and the other group capable of utilizing both oxygen and nitrate as electron acceptor, i.e., denitrifying polyP organisms (Mino *et al.*, 1995; Sorm *et al.*, 1996). The anoxic zone sample utilized in the current study was obtained from the second anoxic zone of a modified UCT system. This zone receives nitrified mixed liquor from the aerobic zone directly (Knight *et al.*, 1995a), so that adequate levels of nitrate to sustain the uptake of phosphate are likely to be present. The difficulty

experienced in finding antibodies against antigens unique to the aerobic zone might be explained by the possible presence of such denitrifying polyP organisms in this system: antigens expressed by these for the purpose of phosphate uptake would already be present in the anoxic zone. The extent of phenotypic similarity between the anoxic and aerobic zones would be increased, thereby further obscuring those antigens uniquely expressed in the aerobic zone by that group of polyP organisms restricted to oxygen as final electron acceptor.

In order to increase the probability of finding antibodies to rare antigens in the anoxic and especially the aerobic zones, both conventional and immunofocused strategies were employed for immunization. The use of more than one immunization schedule was, in fact, highly recommended by Galfré & Milstein (1981) in their classic protocol for hybridoma production. Neither improvement in resolution nor enhancement of the immune response by the immunofocusing was clearly seen. Although it had not been an aim of the study to evaluate the subtractive immunization method of immunofocusing in terms of its success in producing the desired antibodies, certain comments may be made in this regard. Williams and co-workers (1992) compared various subtractive immunization techniques and concluded that chemical immunosuppression with cyclophosphamide (as used in this study) was the most effective. They found, however, that the dosage regimen of cyclophosphamide administration was crucial to the success of reducing immunological responsiveness to a specific set of antigens: multiple high doses of cyclophosphamide (100 mg/kg 15 min, 24 h and 48 h after antigen exposure) were very effective while lower single doses (40 mg/kg), as had been used in this study, were entirely ineffective. Matthew and Sandrock (1987) used such a protocol, while more recently similar multiple high dose regimens employed by Brooks and co-workers (1993) and Varecková and co-workers (1995) yielded good results. Although other workers had success with the lower single dosage regimen (Matthew & Patterson, 1983; Balkema & Dräger, 1985 and Crause, 1993), using the multiple high dose regimen in this study might have produced better results. It may be noted, however, that the monoclonal antibody capable of resolving phenotypic differences between two activated sludge systems as discussed below is a product of the immunofocused immunization strategy. Whether this was manifested by chance or design cannot be determined.

A significant finding emerging from this study was based on the observation that antigenic

differences were clearly detected between the aerobic zones of two activated sludge systems with differing phosphate removal ability. A monoclonal antibody was found that was capable of distinguishing between the two systems both by ELISA and immunoblot. Characterization of the antigen recognised suggested a protein nature. The antibody showed five compact bands on the blot, with M_r appearing to be multiples of 18 kDa. The epitope recognised probably occurred on a 18 kDa proteinaceous monomer, represented on the blot in incremental steps of subunit association. Of note is the fact that the two activated sludge systems compared received identical influents, and that they differed only in their configuration: the non-phosphate removing system lacked an anaerobic zone, this zone being the key to establishing the EBPR process in activated sludge (Kortstee *et al.*, 1994). The *raison d'être* of the unique antigen in the phosphate removing system may be speculated upon: did the specific conditions prevailing in the phosphate removing system as opposed to the non-phosphate removing system induce its expression, or did the anaerobic zone select for a unique bacterial population in the phosphate removing system which is recognised by the antibody, but is not necessarily correlated with phosphate removal? Could the state of oligomerization of the 18 kDa protein correlate to the phosphate removing ability of the sludge?

It would therefore be of value to examine the significance of the unique protein with respect to phosphate removal. Various channels of investigation are being pursued:

* Does the 18 kDa protein differ in its state of oligomerization between the anoxic and aerobic zones of a phosphate removing system?
* Does this phenotype occur consistently in systems configured for phosphate removal, and is it consistently absent in non-phosphate removing systems?
* By which activated sludge organism is this expressed, and is this organism a polyphosphate accumulating organism? If so, is it a polyP organism of significance or merely a minor league player, according to the information available in the literature on these organisms?
* Does this phenotype disappear when failure of the EBPR process sets in?
  Nakamura *et al.* (1995) is quoted: "*Since biological phosphorus removal is achieved by the dominant growth of polyphosphate-accumulating bacteria in activated sludge, control of the composition of the microbial population in the activated sludge is very*

important for maintaining a sufficient level of phosphorus removal activity. However, our level of understanding of the characteristics of polyphosphate-accumulating bacteria is still too low to enable appropriate control of the microbial population."

For this purpose a monoclonal antibody directed against a single antigen produced by a single polyP organism is probably too selective, even if it were a polyP organism of major significance in the EBPR process. A panel of monoclonal antibodies against antigens with proven participation in the process, and representing at least the major polyP organisms, would be ideal for use in a phosphate removal indicator system. Meanwhile, the potential applications of this single monoclonal antibody may be investigated, should it become evident that it recognises a noteworthy antigen. Some possibilities are discussed:

* Cells or antigenic molecules may be quantitatively estimated by various immunoassay techniques or by flow cytometry.
* Immunofluorescence microscopy may be used to observe and quantitatively determine relevant cells in activated sludge systems. This approach has been used on activated sludge samples to identify a species of filamentous bacteria (Howgrave-Graham & Steyn, 1988), to identify and enumerate *Acinetobacter* (Cloete & Steyn, 1988), and to quantify a chlorophenol-degrading bacterial strain (Jacobsen, 1995). The technique would be useful, as conventional techniques for detection and quantification are difficult to use on activated sludge samples.
* Antibody-coupled bacteria could be harvested from activated sludge samples by techniques such as flow cytometry in combination with cell sorting, or immunomagnetism. After release from the antibodies, the purified cultures could be used for research purposes or for enriching activated sludge populations with the selected organisms.
* Biosensor technology harnesses a variety of scientific disciplines and is represented by a spectrum of devices, as reviewed by Romito, 1993. These incorporate immobilized biological material in contact with a device able to convert a biochemical signal into an electrical one. Sandén and co-workers (1994) prepared monoclonal antibodies against nitrifying bacteria from activated sludge systems, their goal being to ultimately develop a biosensor that can measure bacteria numbers in parallel with

measurements of their activity. They postulated that this could be used to detect disturbances in the biological process and give information to apply relevant measures. The monoclonal antibody established in the current study might also be utilized this way, to set in place a means of early warning for optimal system performance.

Finally, a study similar to that reported here may be conducted to determine antigenic diversity between the anaerobic and the aerobic zones of a phosphate removing activated sludge system. Antigens pertaining to the phosphate release aspect of the EBPR process might be identified and studied, and an indication of the degree of phenotypic difference between the phosphate release and uptake processes be gained. The information gleaned from investigations sprouting from such studies and from this report may provide a window by which a clearer view of the complexities of biological phosphate removal may be had.

ACKNOWLEDGEMENTS

The authors are indebted to Prof M C Wentzel, Department of Civil Engineering, University of Cape Town, South Africa, for providing samples; and the Water Research Commission of South Africa for financial support.

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TABLE 7: **Operational parameters of two activated sludge systems with different phosphate removal ability.**

Parameter	System removing phosphate successfully	System removing phosphate unsuccessfully
Sludge age (d)	10	10
Temperature (°C)	30	30
Chemical oxygen demand (mg COD/ℓ):		
Influent	760	760
Effluent	55	50
Total phosphate (mg PO_4^{-}-P ℓ^{-1}):		
Influent	16,5	16,5
Effluent	1,7	8,6
Phosphate removal (mg PO_4^{-}-P ℓ^{-1}):	14,8	7,9

TABLE 8: **Specificities of secreting hybridoma clones produced from spleen cells of mice with immune responses focused against either the anoxic zone or the aerobic zone antigen preparations and of mice with unfocused immune responses. Antisera from all groups of mice were cross-reactive with the antigens. Results shown are for positive hybridoma cell lines stable after at least two clonings.**

Fusion of origin	Number of cultures screened; percentage of total number of wells plated out indicated in brackets	Number of cultures cross-reactive	Number of cultures reactive to anoxic zone antigen only	Number of cultures reactive to aerobic zone antigen only
Unfocused	906 (94%)	5	-	-
Anti-anoxic zone focused	480 (100%)	1	-	-
Anti-aerobic zone focused	480 (100%)	3	-	-

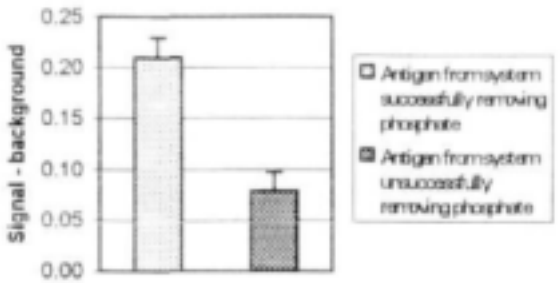

Figure 10: Comparison of activated sludge systems with differing phosphate removing abilities by ELISA using monoclonal antibody 7B9. The procedure is detailed in Materials and Methods, using casein buffer supplemented with horse serum. Supernatant from Sp2/0 cultures was used as negative serum control. Results are the average ± S.D. of eight values.

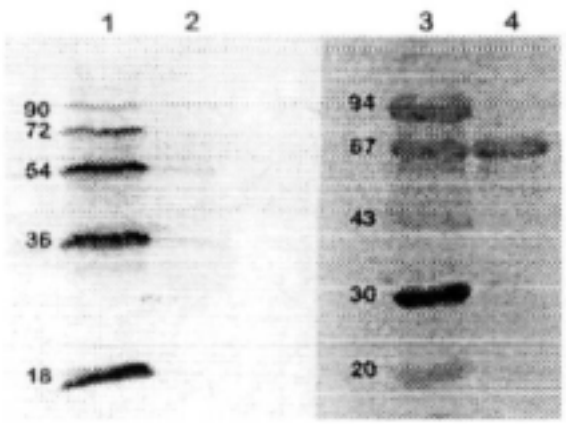

Figure 11: Immunoblot comparison of reduced-SDS-PAGE antigen preparations from activated sludge systems with differing phosphate removing abilities. Lane 1: the system successful at phosphate removal; lane 2: the system less successful at phosphate removal. Antigens were probed with monoclonal antibody 7B9. Coomassie Blue stained low M_r markers (kDa) and BSA are shown in lanes 3 and 4 respectively.

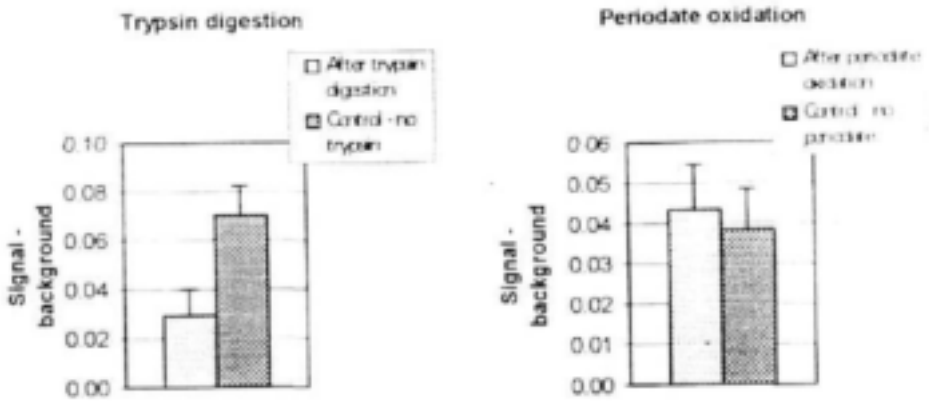

Figure 12: **Effect of epitope degradation by proteolysis or polysaccharide oxidation on the affinity of monoclonal antibody 7B9 for antigen. The procedure is detailed in Materials and Methods, using casein buffer supplemented with horse serum. Supernatant from Sp2/0 cultures was used as negative serum control. Results are the average ± S.D. of five values.**