Direct extractions of proteins to monitor an activated sludge system on a weekly basis for 34 weeks using SDS-page

Marthie M Ehlers* and TE Cloete
Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria 0001, South Africa

Abstract

The diversity and dynamics of microbial communities of phosphorus-removing and non-phosphorus-removing activated sludge systems have mostly been analysed by culture-dependent methods. A more direct method is the isolation of the total protein content of samples of activated sludge systems and separating the proteins with SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Total proteins were analysed and used as fingerprints to type and compare the diversity of the bacterial community. The objectives of this study were to determine if there were any differences between the anaerobic, anoxic and aerobic zones of an activated sludge plant as well as the effect of seasonal changes on the bacterial community structure of an activated sludge plant over a 34-week period. The protein profiles, over this study period, indicated a relatively high (> 63%) similarity between the samples. The results indicated no specific protein pattern in the different zones or due to seasonal changes. This implicated that a stable bacterial community was present throughout the study period.

Abbreviations

BDM	2-β-mercaptoethanol
COD	Chemical oxygen demand
EBPR	Enhanced biological phosphate removal
LMG	Laboratorium voor Microbiologie Ghent Culture Collection, State University Ghent, Belgium
N	Nitrogen
P	Phosphorus
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
STB	Sample treatment buffer
UPGMA	Unweighted pair group method of arithmetic averages

Introduction

In terms of wastewater treatment, the activated sludge process is probably today's most important biotechnological process (Wagner et al., 1993). Nutrients such N and P can be removed from wastewater under specific conditions. The present design for P removal, namely EBPR, requires wastewater to pass through an initial anaerobic treatment process and thereafter an aerobic stage, during which P removal takes place (Bond et al., 1995).

The need for nutrient removal from effluents 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 et al., 1990). This leads to the growth of aquatic photosynthetic plants, notably algae. To prevent eutrophication, phosphate removal from effluents is necessary, whether it is by chemical and/or biological means (Toerien et al., 1990). Substantial savings are also achieved through biological rather than chemical P removal (Toerien et al., 1990).

* To whom all correspondence should be addressed.
☎ (012) 420-2995; fax (012) 420 -3266; e-mail mehlers@nsnper1.up.ac.za
Received 9 April1998; accepted in revised form 3 July 1998.

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 is still very limited. Diversity and dynamics of the microbial consortia in activated sludge have mostly been analysed by culture-dependent methods (Wagner et al., 1993). The literature indicates that 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 and Steyn, 1987, Wagner et al., 1993). EBPR from activated sludge has been well documented, but attempts to elucidate the exact mechanism have not been successful as a result of these inadequate microbiological techniques (Srinath et al., 1959; Shapiro, 1967; Shapiro et al., 1967).

The need excists to better understand the EBPR process, since it is not optimised and routinely fails (Bond et al., 1995). To achieve this, a more complete knowledge of microbial phosphate metabolism in activated sludge is required. Since conventional techniques offer limited possibilities, an alternative method was investigated in this study. Protein electrophoresis is a sensitive technique, yielding valuable information on the similarity or dissimilarity amongst bacterial cultures during taxonomical studies. Until now no direct method has been developed to analyse the protein products in gene expressions of environmental samples (Ogunseitan, 1993). This method could therefore, possibly also be used to determine the similarity or dissimilarity between different environmental samples containing micro-organisms. SDS-PAGE of whole-cell soluble proteins, prepared under standard conditions, produced a complex banding pattern (termed 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 P- and non-P-removing microbial communities present in activated sludge systems, since these profiles would indicate similarity or dissimilarity in samples obtained from this system.

The total bacterial protein content of activated sludge samples was therefore, used as a fingerprint to give insight into the

TABLE 1
PHOSPHORUS CONCENTRATIONS (mg·ℓ$^{-1}$) IN THE ANAEROBIC, ANOXIC AND AEROBIC ZONES OF THE DASPOORT ACTIVATED SLUDGE PLANT ANALYSED 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	9.20	3.86
2	16/4	5.92	6.65	1	0.73
3	22/4	8.91	12.35	7.40	3.44
4	29/4	13.50	20.30	13.20	6.80
5	7/5	10.76	17.90	12.70	7.14
6	14/5	15.58	16.20	11.10	0.62
7	20/5	3.92	11.70	9.50	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	6.20	13.43	10.84	7.23
12	24/6	8.76	9.35	3.10	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	9.20	7.64
16	22/7	4.95	6.85	2.65	1.90
17	29/7	1.76	7.40	6.38	5.64
18	5/8	8.2	8.65	1.30	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	3.30	1.20
22	2/9	7.15	9.60	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	3.10
26	30/9	2.69	9.38	7.50	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	8.50	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

metabolic variation over a period of 34 weeks. Such fingerprints can possibly be used to monitor the deterioration or enrichment of species diversity, or even more specific changes during P- and non-P-removing periods. This may be indicated by differences in the protein patterns of P- and non-P-removing systems.

Materials and methods

Sampling

Grab wastewater samples from the anaerobic, anoxic and aerobic zones of the Daspoort activated sludge system (3-stage Bardenpho) in Pretoria, Gauteng Province, South Africa, were collected on a weekly basis for a period of 34 weeks (April - November 1996). The Daspoort system treats both domestic and industrial wastewater. Samples were analysed within 2 h of collection. P concentrations (mg·ℓ$^{-1}$) were determined with the SQ 118 spectroquant (Merck) (Table 1). Average values for the following chemical analyses as determined by the Daspoort plant laboratories were: $COD_{(raw)}$ (408 mg·ℓ$^{-1}$); ammonia (14.61 mg·ℓ$^{-1}$) and nitrate (0.39 mg·ℓ$^{-1}$).

Sample preparation

Protein extraction method developed during this study

Protein extractions were carried out with the use of different centrifuging and buffer washing steps. 100 mℓ activated sludge samples and 70 g glass beads where homogenised for 10 min. The supernatant was centrifuged for 15 min at 1 000 r·min^{-1} in a Hermle 360 K centrifuge. Thereafter the resulting supernatant was pelleted by centrifuging for 15 min at 7 000 r·min^{-1}. Pellets were resuspended in 2 mℓ 40 mM Tris pH 7.4. Percoll (1 mℓ) (Merck) was added to each sample mixed and centrifuged for 10 min at 12 000 r·min^{-1} 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 after each washing for 10 min at 12 000 r·min^{-1} to remove the percoll.

Polyacrylamide gel electrophoresis of proteins

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 r·min^{-1}. 75 µℓ of STB [0.5 M Tris-HCl pH 6.8, 5% (v/v) 2-β-mercaptoethanol (BDH), 10% (v/v) glycerol (Merck), and 2% (m/v) SDS (Univar)], was added to each pellet whereafter the mixture was boiled for 5 min at 94°C. Cell pellets were kept on ice and cells were ruptured by sonication using a Cole-Parmer Ultrasonic Homogeniser (Series 4710) at 50% maximum output (40 watt) for a maximum of 45 s using 15 s pulses. A second volume of sample buffer (75 µ) was added and mixed with the ruptured cell suspension. Cell debris were removed by centrifuging at 12 000 r·min^{-1} for 8 min. The clear supernatant was stored at -20°C until required.

Standard conditions for SDS-PAGE

SDS-PAGE was performed by using the method described by Laemmli (1970), modified according to Kiredjian et al., 1986. Proteins were separated on gels (1.5 mm thick and 125 mm long) by using 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 per 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).

Analysis of protein patterns

Gels containing the protein profiles were analysed after normalisation by using a Hoefer GS300 densitometer. Data obtained were directly stored on a computer and analysed using the GelCompar 4.0 computer program (Applied Maths, Kortrijk, Belgium), which calculated the % similarities and differences between each protein profile, with the Pearson product moment correlation coefficient (r) between samples, to construct a matrix. The samples were then clustered using the unweighted pair group method of arithmetic averages (UPGMA). *Psychrobacter immobilis* LMG 1125 was used as reference patter on each gel. Reproducibility of electrophoresis was determined by comparing the reference with a *Psychrobacter immobilis* protein profile selected in the GelCompar 4.0 programme as standard. A relationship of >90% between gels was presumed as acceptable.

Results and discussion

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. 1 and Table 2). Section I and II were 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 thus indicated that the community composition remained similar irrespective of the season.

Section I was furthermore divided into group A representing 57 samples with a 69% correlation. This group was further subdivided into Subgroup 1 which consisted of 13 samples with an 87% correlation. This subgroup also represented different sampling weeks and zones. Subgroup 2, consisting of only 3 samples, representing all three zones, showed an 86% correlation. Subgroup 3 consisted of 5 samples with an 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

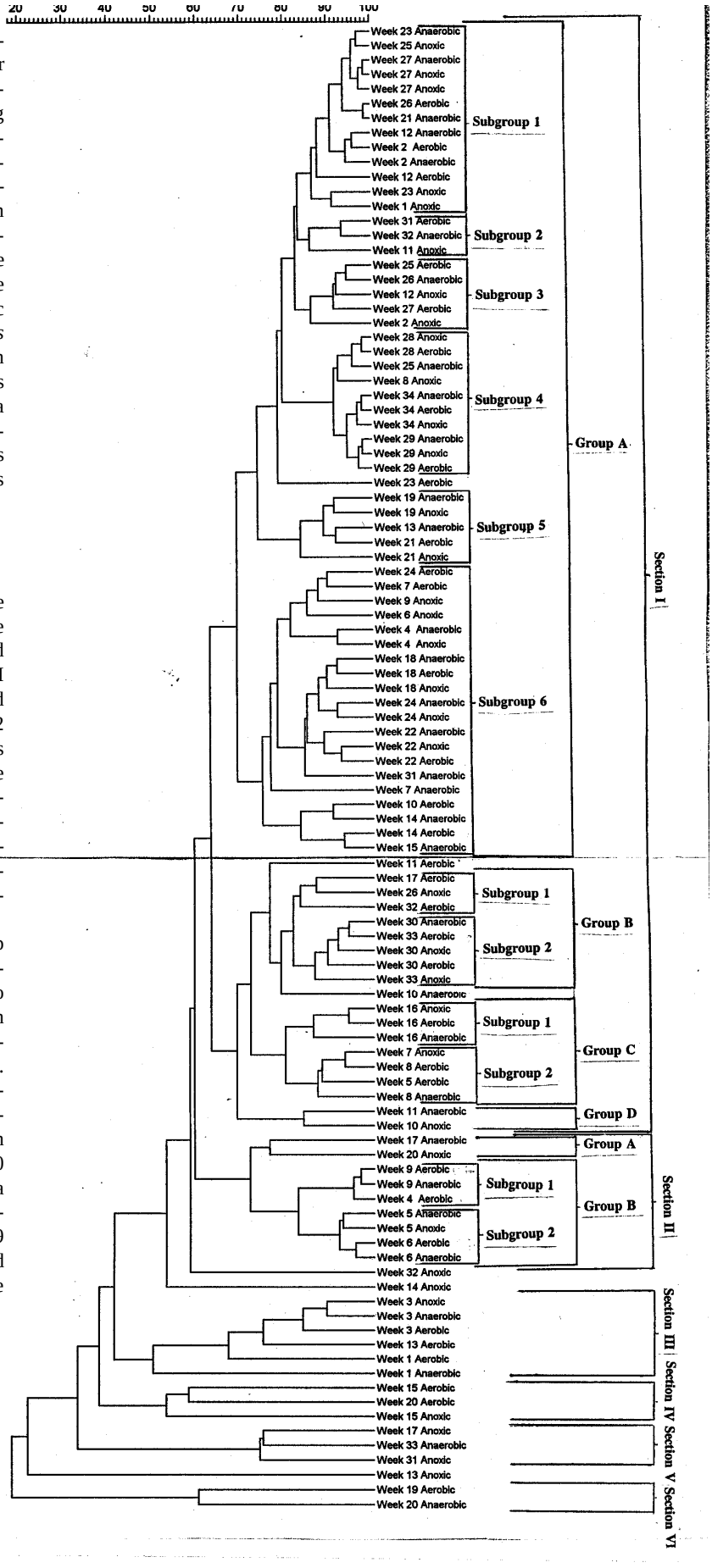

Figure 1 (right)
Dendrogram of the electrophoretic patterns comprising the three zones of the Daspoort activated plant collected on a weekly basis for a 34-week period, based on UPGMA analysis of the correlation coefficients (r) of the protein profiles.

TABLE 2
THE % SIMILARITY OF THE 102 PROTEIN PROFILES OF FIG. 1 FOR THE THREE ZONES OF THE DASPOORT ACTIVATED SLUDGE PLANT AS COMPILED OVER 34 WEEKS

Sections, groups and subgroups	Sample quantity	% Similarity
Section I:	**76**	**63**
Group A	57	69
Subgroup 1	13	87
Subgroup 2	3	86
Subgroup 3	5	86
Subgroup 4	10	92
Subgroup 5	5	84
Subgroup 6	20	75
Group B	8	82
Subgroup 1	3	83
Subgroup 2	5	87
Group C	7	79
Subgroup 1	3	86
Subgroup 2	4	87
Group D	2	84
Section II:	**9**	**72**
Group A	2	76
Group B	7	83
Subgroup 1	3	95
Subgroup 2	4	92
Section III:	**6**	**41**
Section IV:	**3**	**38**
Section V:	**3**	**38**
Section VI:	**2**	**38**

TABLE 3
THE % SIMILARITY OF THE PROTEIN PROFILES AND P CONCENTRATIONS OF FIG. 2 FOR THE AEROBIC ZONE OF THE DASPOORT ACTIVATED SLUDGE PLANT AS COMPILED OVER 34 WEEKS

Sections, groups and subgroups	Sample quantity	Average P concentations ($mg \cdot \ell^{-1}$)	% Similarity
Section I:	**5**	**5.30**	**45**
Section II:	**28**	**3.39**	**60**
Group A	7	2.23	79
Group B	7	2.60	79
Group C	10	3.26	76
Group D	3	5.47	83

anoxic sample taken during the 8th week. The three different zones all present in this subgroup were represented in Weeks 29 and 34, indicating almost identical protein profiles for the different zones, thus implicating a stable bacterial community structure in the activated sludge plant. Subgroup 5 (5 samples) showed an 84% correlation and consisted of 5 samples. Subgroup 6 consisted of 20 samples with a 75% correlation. Group B consisted of 8 samples with an 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% relatedness (5 samples), representing all three zones during week 30, and the anoxic and aerobic zones during week 33. Group C, consisting 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 consisting of only 2 samples (Week 11 anaerobic and Week 10 anoxic) with an 84% correlation.

By analysing Sections I and II, a 59% relatedness of the protein profiles was observed. In Section II the 9 samples showed a 72% correlation. Two groups were identified. Group A consisting of 2 samples with a 76% correlation and Group B (7 samples) and an 83% similarity. The latter group was divided into Subgroups 1 and 2, showing a 95% and 92% correlation, respectively.

Section III showed a correlation of 41% compared with Sections I and II. Section IV, V, and VI joined at similarity values < 38%. However, Section V, showed a 74% similarity.

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

In Fig. 2 (Table 3) the protein profiles of the aerobic zones and the corresponding P concentrations were compared to establish if samples with the same P concentrations will cluster together (Table 1). The general profile of the dendrogram was found to be similar to Fig. 1. Two sections with a 42% similarity were identified with Section I consisting of 5 samples with an average P concentration of 5.3 $mg \cdot \ell^{-1}$ and a correlation of 45% (Table 3). Section II comprised 28 samples with a 60% relatedness. Section II was subdivided into 4 groups. Group A with 79% correlation included 7 samples with an average P concentration of 2.23 $mg \cdot \ell^{-1}$. Group B with 7 samples correlated with 79% and showed an average P concentration of 2.6 $mg \cdot \ell^{-1}$. Group C correlated at 76% and included 10 samples with an average P concentration of 3.26 $mg \cdot \ell^{-1}$. Group D included only three samples with an 83% similarity and had an average P concentration of 5.47 $mg \cdot \ell^{-1}$. When comparing the average P concentrations of the samples there seems to be a tendency for the protein profiles of samples with a higher percentage P concentration to cluster together. However, studying the individual P concentrations, several samples have a P concentration higher than the average value.

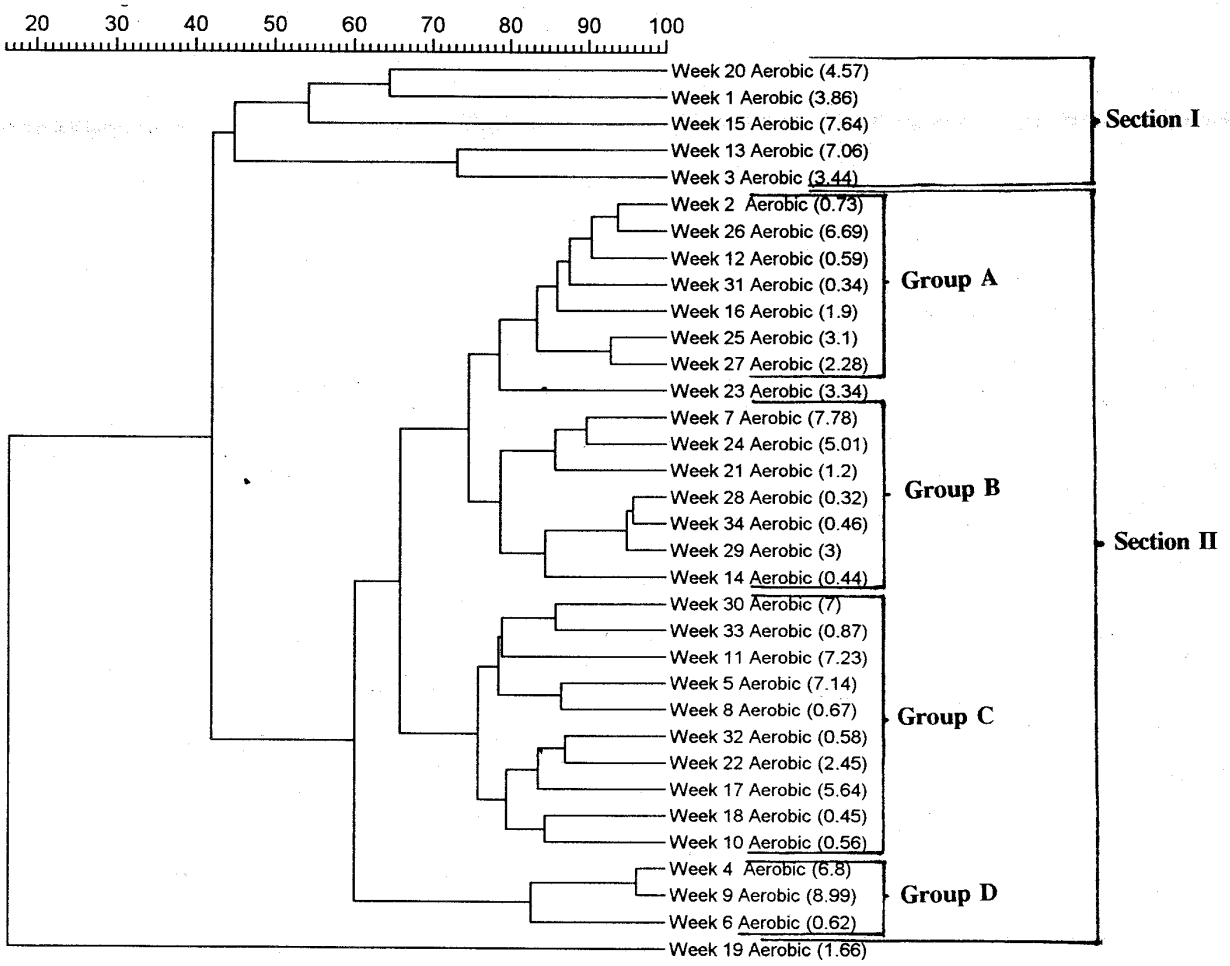

Figure 2

Dendrogram of the electrophoretic patterns of the aerobic zones of the Daspoort activated sludge plant and the corresponding P-concentration, based on UPGMA analysis of the correlation coefficients (r) of the protein profiles

Conclusions

- SDS-PAGE was found to be a sensitive tool for the determination of the bacterial population structure of activated sludge. Due to the lack of methods currently available to determine bacterial community structure of environmental samples, there is a constant search for new methods to investigate and better understand the functioning of micro-organisms in their natural environment. Conventional microbial techniques have provided a misleading picture of bacterial community structure of environmental samples (Cloete & Steyn, 1987; Lee et al., 1996). SDS-PAGE was used as the method to alleviate the need for culturing resulting in a more direct manner of analysing samples and thereby preventing the selection of specific organisms. The main advantage of this method is the relative ease as well as the quantity of samples that can be analysed at the same time. It is also more cost-effective than DNA:DNA hybridisation. The results obtained by SDS-PAGE of whole-cell proteins discriminates at much the same level as DNA:DNA hybridisation (Priest & Austen, 1993).

- The majority of the protein profiles indicated a high percentage relatedness (> 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. Similarly Bond et al. (1995) also suggested that the same bacterial community is present throughout the activated sludge process.

- The current tendency is to construct dendrograms consisting of only a few samples and then base the identification of a new genus or species on the findings. By adding more samples to these 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 probably stay the same with only a small variation in the % correlation. Each dendrogram should be evaluated individually 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 samples should only be used as an indication of similarity.

- As an exact value cannot be attached to the % similarity or correlation of the resulting dendrogram after SDS-PAGE, the % similarity should rather be used as a guideline. SDS-PAGE can therefore not discriminate between the bacterial

populations of the different activated sludge samples; it can only indicate samples with a low or a high % similarity.

- Another disadvantage of the SDS-PAGE method is that it needs to be standardised. Results between different laboratories may differ if standard methods are not followed. 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 and 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.

- It is thus recommended that 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. However, a method such as 16S rRNA may result in similar problems as phenotypic methods as the work is restricted to the system on which the initial work was performed. The probes only detect those isolates for which they are made, thus implicating that the unculturable species of the community will remain undetected. However, Bond et al., (1995) used 16S rRNA methods successfully to determine the difference between P- and non-P-removing laboratory scale activated sludge systems. However, further research as well as the combination of different techniques, and the role of biomass in the P-removal process, need to be investigated.

- In conclusion the ultimate method to determine the bacterial community structure of environmental samples still has to be developed. Therefore, each possible method should be investigated, until one or a combination of methods is found, that can assist in the better understanding of microbial ecology.

Acknowledgements

The authors would like to thank the following:
The Water Research Commission of South Africa for funding this project; and Daspoort Water Treatment Plant for activated sludge samples and analyses provided.

References

BOND PL, HUGENHOLTZ P, KELLER J and BLACKALL LL (1995) Bacterial community structure of non-phosphate-removing activated sludges from sequencing batch reactors. *Appl. Environ. Microbiol.* **61** 1910-1916.

CLOETE TE and STEYN PL (1987) A combined fluorescent antibody-membrane filter technique for enumerating *Acinetobacter* in activated sludge. In: R Ramadori (ed.) *Advances in Water Pollution Control, Biological Phosphate Removal from Wastewaters* , Pergamon Press, Oxford. 335-338.

DAGUTAT H (1990) Taksonomiese Ondersoek van *Pseudomonas angulata* Verwante *Pseudomonas* Spesies met Behulp van Poli-akrielamied Jel Elektroforese. M.Sc. Thesis. University of Pretoria, Pretoria, Republic of South Africa. (Taxonomical investigation of *Pseudomonas angulata* related *Pseudomonas* species with the use of polyacrylamide gel electrophoresis).

KERSTERS K (1990) Polyacrylamide gel electrophoresis of bacterial protein. In: Clement Z, Rudolph K and Sands DC (eds.) *Methods in Phytobacteriology*. Akadëmiai, Kiado, Budapest.

KIREDJIAN M, HOLMES B, KERSTERS K, GUILVOUT I and DE LEY J (1986) *Alcaligenes piechaudii*, a new species of human clinical specimens and the environment. *Int. J. Syst. Bacteriol.* **36** 282-287.

LAEMMLI UK (1970) Cleaving of the structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** 680-685.

LEE D-H, ZO Y-G and KIM S-J (1996) Nonradioactive method to study genetic profiles of natural bacterial communities by PCR-single-stranded-conformation polymorphism. *Appl. Environ. Microbiol.* **62** 3112-3120.

OGUNSEITAN OA (1993) Direct extraction of proteins from environmental samples. *J. Microbiol. Methods* **17** 273-281.

PRIEST FG and AUSTEN B (1993) *Modern Bacterial Taxonomy* (2nd edn.) Chapman & Hall. London.

SHAPIRO J (1967) Induced rapid release and uptake of phosphate by microorganisms. *Sci*. **155** 1269-1271.

SHAPIRO J, LEVIN GV and ZEA HG (1967) Anoxically induced release of phosphate in wastewater treatment. *J. Water Pollut. Control Fed.* **39** 1810-1818.

SRINATH EG, SASTRY CA and PILLAI SC (1959) Rapid removal of phosphorus from sewage by activated sludge. *Exper.* **15** 339-340.

SLIM JA (1987) Some developments in the water industry in South Africa. *Water Pollut. Control* **86** 262-271.

TOERIEN DF, GERBER A, LöTTER LH and CLOETE TE (1990) Enhanced biological phosphorus removal in activated sludge systems. *Adv. Microb. Ecol.* **11** 173-230.

WAGNER M, AMANN R, LEMMER H and SCHLEIFER K (1993) Probing activated sludge with oligonucleotides specific for proteobacteria: Inadequacy of culture-dependent methods for describing microbial community structure. *Appl. Environ. Microbiol.* **59** 1520-1525.