Protein profiles of phosphorus- and nitrate-removing activated sludge systems

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Abstract

Samples from laboratory-scale activated sludge systems operating under specific conditions which differ in phosphate-removing capabilities were obtained from the University of Cape Town. The total protein content of samples of these systems was used and the proteins separated with SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Analysis of the total proteins was employed as a fingerprint to type and compare the diversity of the bacterial communities of P-and N-removing systems. Samples of six activated sludge systems (three N- and P-removing and three N-removing) were used in this study. Protein profiles indicated a high (>70%) correlation for all the systems. No difference was observed in the protein profiles of the bacterial communities of N- and P-removing or N-removing systems. We can therefore conclude that the same bacterial communities were present in P- and N-removing systems.

Abbreviations

BPR	biological phosphorus removal
EBPR	enhanced biological phosphate removal
LMG	Laboratorium voor Microbiologie Gent Culture Collection, State UniversityGent, Belgium
N	nitrogen
P	phosphorus
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
UCT	University of Cape Town
UPGMA	unweighted pair group method of arithmetic averages

Introduction

Biological phosphorus removal in activated sludge is of interest due to low sludge production and the fertiliser value of the sludge (Henze, 1996). Substantial savings are also achieved through biological, rather than chemical P-removal (Toerien et al., 1990).

Biological phosphorus removal can be performed by a rather broad group of micro-organisms referred to as the phosphate-accumulating organisms (PAOs or polyP-organisms) (Henze, 1996). The micro-organisms 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 necessarily showing any biological P-removal activity, until being activated (Henze, 1996). Optimisation of enhanced P-removal processes therefore depends on a complete understanding of the ecophysiology of poly-P organisms.

The mechanism of enhanced P-removal in activated sludge systems must therefore depend on a group of organisms (the poly-P organisms) which in nature are favoured by fluctuating conditions of aerobiosis-anaerobiosis. Their selective advantages require: their presence; alternating aerobiosis-anaerobiosis; degradable organic matter which can be fermented by acidogenic bacteria; and the presence of a sufficient quantity of P to allow uptake of fatty acids during anaerobiosis and P-uptake during aerobiosis (Toerien et al., 1990). However, the precise role of the consortia of micro-organisms of enhanced P-removal in activated sludge systems has not yet been clarified.

The bacterial genome contains information involved in the production of some 2 000 proteins, which function either enzymatically or structurally (Kersters, 1990; Priest and Austin, 1993). Electrophoresis of the total cellular proteins in polyacrylamide gels (PAGE) provides a partial separation in which individual bands mostly represent several proteins (Kersters, 1990). This complex pattern represents a "fingerprint" of a specific strain (Priest and Austin, 1993; Van Damme et al., 1996). Previously this technique has been used for comparative taxonomical studies. More recently sodium dodecyl sulphate (SDS) PAGE has found application for use in comparative studies of environmental samples such as activated sludge (Ogunseitan, 1993).

Expression of genes in micro-organisms correlates 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 available to analyse the protein products of gene expressions of environmental samples (Ogunseitan, 1993).

Protein electrophoresis is a sensitive 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 micro-organisms. SDS-PAGE of whole-cell soluble proteins, prepared under standardised conditions, produces 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 P-removing and non-P-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 P-removing, non-P-removing and N-removing systems.

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Due to the problems derived when using culture-dependent methods, the direct extraction of proteins and their analysis was used to elucidate the possible difference between the microbial community structure of P-removing and non-P-removing activated sludge systems.

Materials and methods

Sampling

Samples were taken from six activated sludge systems of the University of Cape Town (UCT). These systems were operated under specific controlled conditions. Three UCT type systems for N- and P-removing (ML: operated at 20°C with supplement of unstabilised landfill leachate; MS: operated at 20°C without leachate; MT: operated at 30°C) and three modified Ludzack Ettinger (MLE) N-removing (HM: operated at 30°C; PW: operated at 20°C; MU: operated at 20°C) were analysed. The three P-removal UCT configurations also removed N. A total of at least four samples of each system were received at monthly intervals (June to November 1996). The systems treated raw municipal wastewater from Mitchells Plain Treatment Plant (Cape Town, South Africa), diluted with tap water to a selected COD concentration (Table 1). These laboratory-scale systems were operated under constant flow and load, sludge ages, recycles, reactor volumes, etc.

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 with the use of different centrifuging and buffer-washing steps. 100 mℓ activated sludge samples and 70 g glass beads were homogenised for 10 min. The supernatant was centrifuged for 15 min at 1 000 r·min^{-1} (24 g) in a Hermle 360 K centrifuge. The supernatants were pelleted by centrifuging for 15 min at 7 000 r·min^{-1}. Pellets were resuspended in 2 mℓ 40 mM Tris pH 7.4. One mℓ of percoll (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 each time 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 sample treatment buffer (STB) [0.5 M Tris-HCl pH 6.8, 5% (v/v) 2-ß-mercaptoethanol (BDH), 10% (v/v) glycerol (Merck), and 2% (m/v) SDS (Univar)], were added to each pellet and heated 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 W) for up to 45 s using 15 s pulses. The second volume of 75μℓ sample buffer was added and mixed. 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 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) 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).

Analysis of protein patterns

The protein profiles were analysed with a Hoefer GS300 densitometer. Data obtained were directly stored on a computer and analysed with the GelCompar 4.0 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 *P. immobilis* protein profile selected in the GelCompar 4.0 programme as standard. A relationship of >90% between gels was considered acceptable for reproducible gels.

Results and discussions

The protein profiles as represented in the dendrogram were divided into four major clusters namely Sections I to IV (Fig. 1). Section I consisted of 56 samples which were 60% related. This section was further divided into 3 groups (Groups A to C). 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 subdivided into four groups (Subgroups 1 to 4). Subgroup 1, with an 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, all from the N- and P-removing system, showed a 88% correlation. Subgroup 4, with 94% correlation consisted of three samples, from N- and P-removing and N-removing systems. Group B, with a 75% correlation, was subdivided into three groups. Subgroup 1, included 10 samples from N- and P-removing and N-removing systems, 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 from N- and P-removing and N-removing systems, 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 can be ascribed to a problem with sample taking or preparation of extracts since the data in Table 1 indicated that the systems were operating successfully.

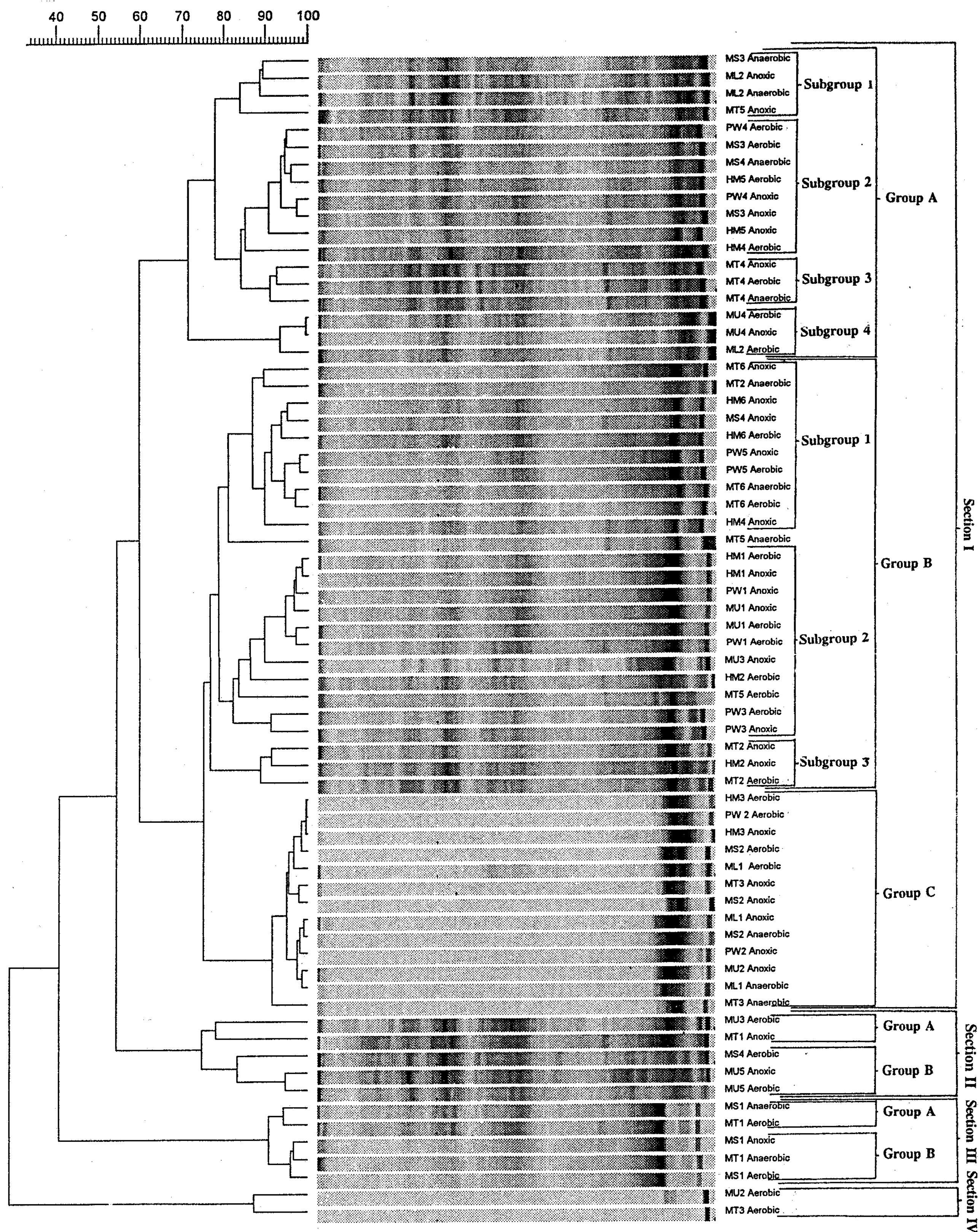

Figure 1

Dendrogram of the electrophoretic patterns of N- and 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

TABLE 1 PHOSPHORUS CONCENTRATIONS AND CHEMICAL ANALYSIS (mg·ℓ^{-1}) OF THE DIFFERENT ZONES OF THE UCT LABORATORY-SCALE ACTIVATED SLUDGE SYSTEMS (JUNE TO NOVEMBER 1996)							
System	**Date**	**COD**	**Δ-P**	**Δ-P/ COD_{in}**	**Anaer-obic**	**Anoxic**	**Aerobic**
HM (30EC)	27/5	720	4.36	0.0061	14.48	10.37	10.12
	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 (20EC) + leachate	12/8	767	16.17	0.0218	37.89	18.27	6.52
	17/10	760	18.48	0.0243	39.30	19.83	5.57
	28/11	789	19.64	0.0249	49.60	25.40	4.82
MS (20EC) - leachate	28/5	663	9.61	0.0145	20.80	11.29	6.07
	12/8	629	9.09	0.0145	25.17	17.30	13.71
	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 (30EC)	27/5	720	8.46	0.0118	16.46	8.77	6.02
	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 (20EC)	28/5	458	2.75	0.0060	ND	51	6.7
	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.							

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 2 samples and a correlation of 78% and Group B, with 3 samples which showed a 82% similarity. Both N- and 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 N- and P-removing and N-removing systems indicated an 87% correlation. A >60% similarity value was indicated between the protein profiles of the samples.

The protein profiles and the aerobic P concentrations (indicated in brackets as mg·ℓ^{-1}) were compared (Fig. 2). The P concentration was used to determine if there was any possible correlation between the protein profiles and P concentration. 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. Section 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 consisted of many different bacterial species. This agrees with recent literature, suggesting that the same bacterial community is present throughout the activated sludge process (Bond et al., 1995). When comparing the average P concentrations of the samples there seems to be a tendency for the protein profiles of samples with higher P concentrations to cluster together (Fig. 2). But when looking at the individual P concentration of each sample there are several samples with P concentration values that were higher than the average value.

The results indicated that SDS-PAGE is a sensitive tool for the

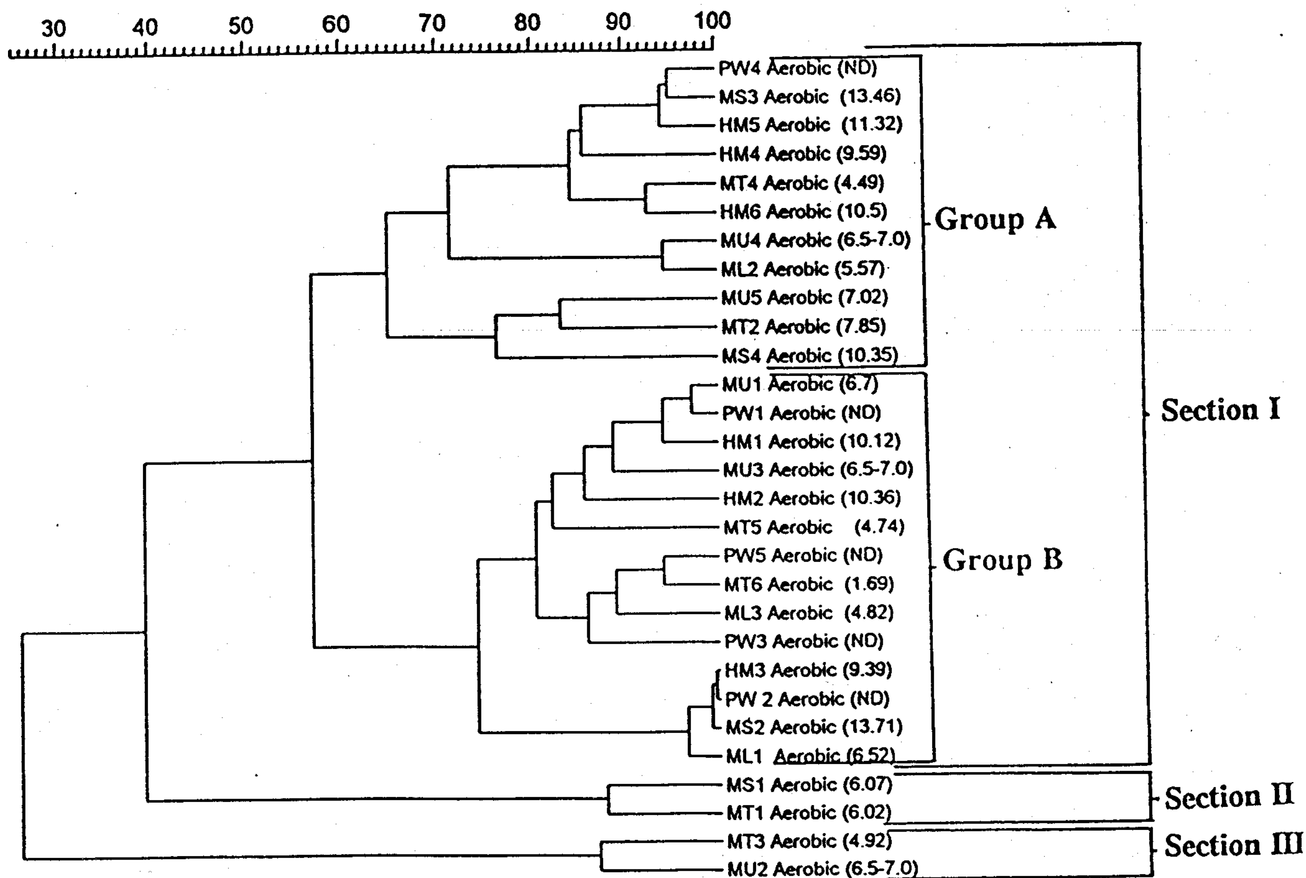

Figure 2

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

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 micro-organisms 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 alleviates the need for culturing and samples are analysed in a more direct manner which prevents the selection of specific organisms. Other advantages of this method are that it is relatively easy and many samples can be analysed at the same time. It is also not as expensive as DNA:DNA hybridisation. However, the results obtained by SDS-PAGE of whole-cell proteins discriminates at much the same level as DNA:DNA hybridisation (Priest and Austin, 1993).

Resulting protein profiles, after SDS-PAGE, were normalised and analysed 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.

There is a tendency 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 should only be an indication of similarity.

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

Conclusions

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-remov-

ing 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.

The protein profiles indicated a high (> 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.

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 is required, and the combination of different techniques. The role of biomass in the P-removal process also needs to be investigated.

The main conclusion that can be drawn is that we still have not developed the ultimate method to determine the bacterial community structure of environmental samples. We should therefore, endeavour to investigate each possible method, until we find one or a combination of methods, that can help us to better understand microbial ecology of activated sludge systems.

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