COMPARING THE PROTEIN PROFILES OF 21 DIFFERENT ACTIVATED SLUDGE SYSTEMS AFTER SDS-PAGE

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Abbreviations—Aer, aerobic, Anaer, anaerobic, Anox, anoxic, DVT, Daveyton, EBPR, enhanced biological phosphate removal, ERWAT, East Rand Waterboard, ESTR, Ester park Water Treatment Plant, HBFT, Hartebeesfontein Water Treatment Plant, HDLB, Heidelberg Water Treatment Plant, JPMS, J. P. Marais Water Treatment Plant, MDFN, Modderfontein Water Treatment Plant, NYLS, Nylstroom Water Treatment Plant, OLFT, Olifantsfontein Water Treatment Plant, P, phosphorus, P-N/P-S, Rynfield Water Treatment Plant, POTG, Potgietersrus Water Treatment Plant, RDLT, Rondebult Water Treatment Plant, SDS-PAGE, sodium dodecyl sulphate polyacrylamide, TSKN, Tsakane Water Treatment Plant, UPGMA, unweighted pair group method of arithmetic averages, VLKP, Vlakplaats Water Treatment Plant, WTV, Waterval Water Treatment Plant, ZKG, Zeekoeigat Water Treatment Plant

INTRODUCTION

Physical forces as well as chemical and biological processes drive the treatment of wastewater. The removal of nutrients such as nitrogen (N) and phosphorus (P) from wastewater can be obtained in activated sludge under specific conditions by chemical and/or biological means. Activated sludge systems modified for enhanced phosphorus removal are well described and are a method of biological P removal. 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.

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. Bond *et al.* (1995) reported that while *Acinetobacter* appeared to be important in P-removing sludges in laboratory reactors, it was unimportant in full scale systems. The present design for P removal, termed enhanced biological phosphate removal (EBPR), requires the activated sludge wastewater to pass through an initial anaerobic treatment and then an aerobic stage, during which P removal takes place, provided the sludge is now surphased, P is effectively removed from the wastewater (Bond *et al.,* 1995).

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 and 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 removed from the biomass in an anaerobic stage, phosphate is reincor-porated in the biomass during aeration, together with part or all of the influent phosphorus (Toerien *et al,* 1990; Van Veen *et al,* 1993). The primary purpose of the stored poly-P in most bacteria is that it serves as a phosphorus source for periods of phosphorus starvation (Wanner, 1994).

Knowledge obtained explaining the chemical transformations that occur throughout the different stages of wastewater treatment has provided insight into the biological mechanism of EBPR. The phos-phate community needs to be established and may not occur merely as a result of conditioning of the existing population (Okada *et al,* 1987).

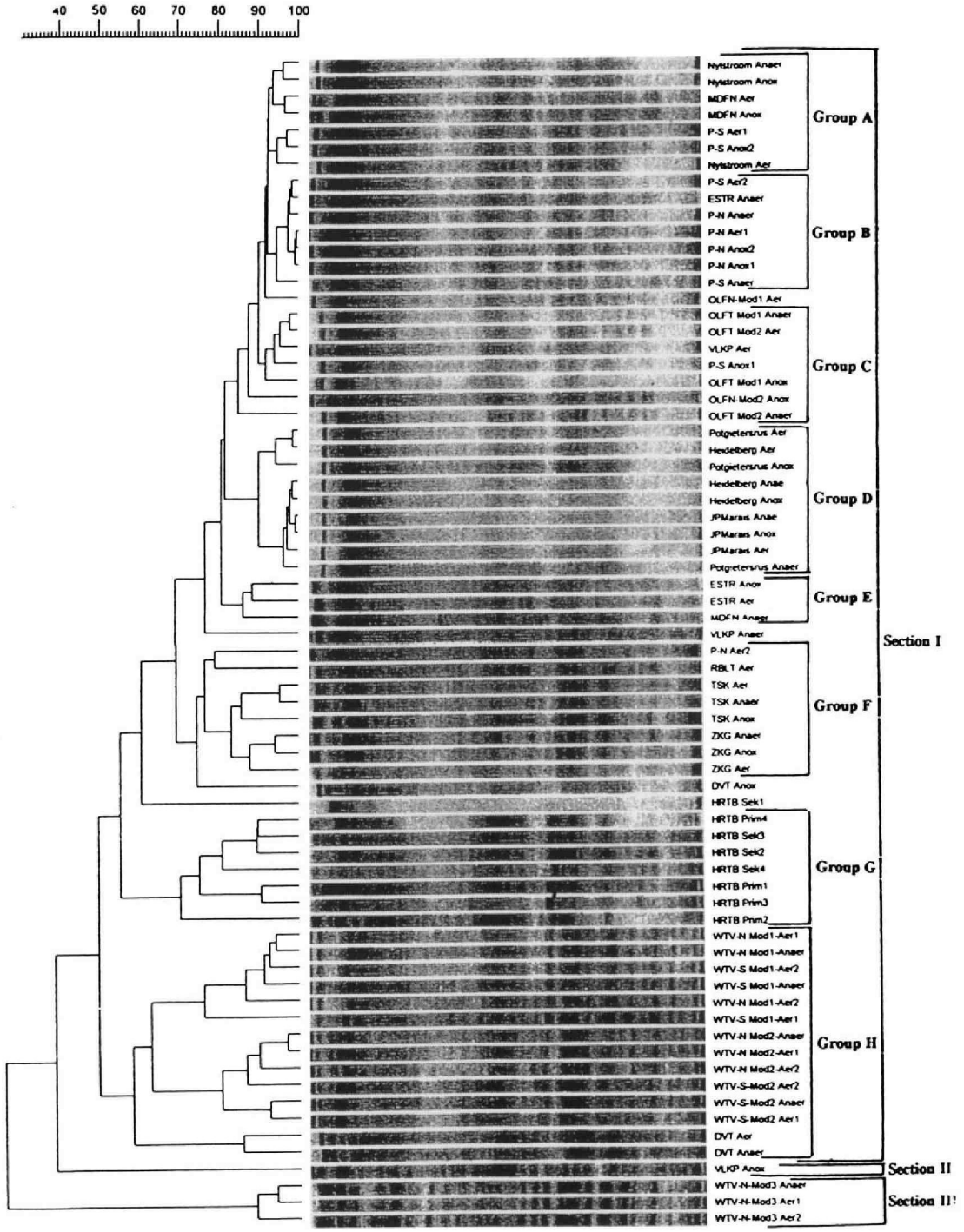

Fig. 1. Dendrogram of the electrophoretic patterns of the anaerobic, anoxic and aerobic zones of 21 different activated sludge systems, based on UPGMA analysis of the correlation coefficients (r) of the protein patterns.

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 available to analyze 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 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 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 and (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 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.

MATERIALS AND METHODS

Sampling

Wastewater samples from the anaerobic, anoxic and aerobic zones of 21 activated sludge systems in the Gauteng and Northern province in South Africa, were collected. Samples from the Gauteng province were received from the East Rand Waterboard (ERWAT). Samples were analyzed within 2 h of collection [chemical and phosphorus analysis (mg. T^{1}) is shown in Tables 1 and 2].

Sample preparation

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 homogenized for 10 min. The supernatant was centrifuged for 15min 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

Table 1. Phosphorus concentrations (mg. 1^{-1}) of 21 different activated sludge systems

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
Hartebeesfontein (HBFT)	2-stage	industrial & domestic	9.25	10.6	8.34	ND	ND
Heidelberg (HDLB)	3-stage	industrial & domestic	18.6	5.04	5.08	ND	ND
JP Marais (JPMS)	3-stage	domestic	11.46	6.3	5.98	ND	ND
Modderfontein (MDFN	3-stage	industrial & domestic	0.11	0.11	0.12	ND	ND
Nylstroom (NYLS)	carousel	domestic	26	22	14	ND	ND
Olifantsfontein 1 (OLFT1)	3-stage	industrial & domestic	0.19	0.17	0.09	ND	ND
Olifantsfontein 2 (OLFT2)	3-stage	industrial & domestic	0.18	0.15	0.08	ND	ND
Potgietersrus (POTG)	3-stage	domestic	31.5	29.5	28.5	ND	ND
Rondebult (RDLT)	1-stage	industrial & domestic	ND	ND	0.29	ND	ND
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 & domestic	14.8	22.92	5.39	ND	ND
Waterval-N Mod 1 (WTV-N-Mod1)	2-stage	industrial & domestic	11.1		0.3	ND	ND
Waterval-S Mod 1 (WTV-S-Mod1)	2-stage	industrial & domestic	9.13		0.29	ND	ND
Waterval-N Mod 2 (WTV-N-Mod2	2-stage	industrial & domestic	6.75		0.22	ND	ND
Waterval-S Mod 2 (WTV-S-Mod2)	2-stage	industrial & domestic	12.23		0.44	ND	ND
Waterval-N Mod 3 (WTV-N-Mod3)	2-stage	industrial & domestic	6.2		0.38	ND	ND
Zeekoeigat (ZKG)	3-stage	domestic	40	30	19	ND	ND

Table 2. Chemical analysis (mg l^{-1}) of the different activated sludge systems (1996)

System	[unavailable] P	COD (raw)	COD (final)	NH_3//N (raw)	NH_3/N (final)	$N0_3$ (final)
Daveyton	13.31	1170	36	27.1	5.6	1.00
Ester Park	-0.18	847	10	73.3	0.1	16.87
Harteheesfontein	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
Modderfontein	-0.01	253	36	32.2	22.6	27.80
Nylstroom	12	ND	ND	ND	ND	ND
Olifantsfontein	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
Rynfleld	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
Zeekoeigat	21	ND	ND	ND	ND	ND

ND: not determined.

mixed and centrifuged for 10 min at 12000 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 12000 rpm 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 12000 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 sonification using a Cole-Parmer Ultrasonic Homogenizer (Series 4710) at 50% maximum output (40 W) 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 15000 rpm for 8 min. The clear supernatant was stored at -20°C until required.

Standard conditions for SDS-PAGE. SDS-PAGE were 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 (Uni-Var) 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 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 a 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.

RESULTS AND DISCUSSION

This dendrogram represents the different systems with different designs. Three sections were identified (Fig. 1). Section I, with a 50% correlation, included 66 samples. Eight 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 92% 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 sludge, JP. Marais and Potgietersrus both treating domestic sludge. Group E, with 3 samples represented two ESTR samples (anoxic and aerobic) and treating domestic waste and one MDFT 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 HRTB which treats industrial and domestic waste correlated at 70%. Group H including 12 samples correlated at 58% consisted of all the samples of the 2-stage WTV-S and WTV-N modules 1 and 2, which treated industrial and domestic waste.

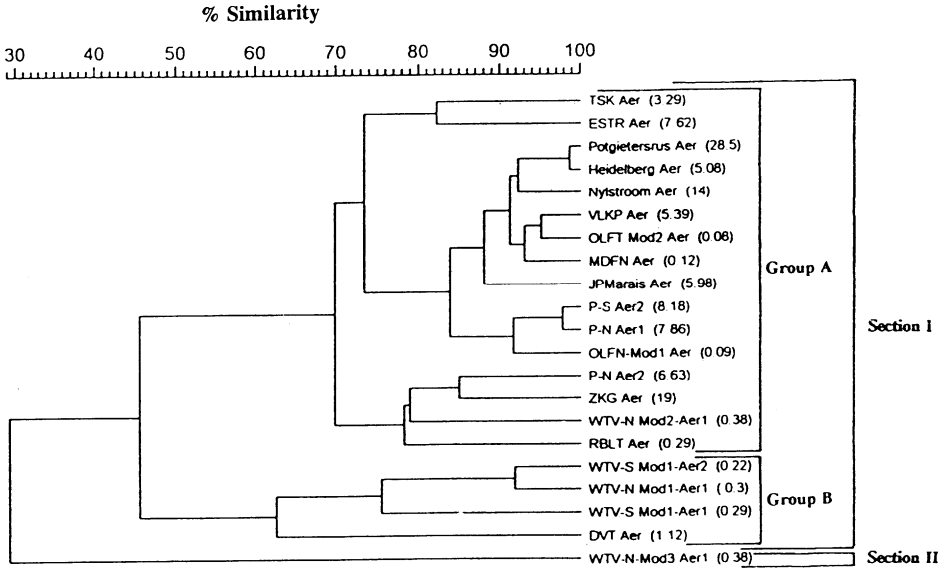

Fig. 2. Dendrogram of the electrophoretic patterns of the aerobic zones and P concentrations (mg. P^{1}) of 21 different activated sludge systems, based on UPGMA analysis of the correlation coefficients (r) of the protein patterns.

Section II clustered at 39% similarity with regards to the other two sections. This section consisted of only one VLKP Anox sample. Section III consisted of 3 WTV-N module 3 samples. No explanation can be given 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. In samples from the WTV system, the different modules monitored showed a high correlation (65%).

The resulting dendrogram indicated two sections when only the aerobic zones of the different systems were compared (Fig. 2). 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 variation. Group B with an average P concentration of 0.76 mg. l^{-1}, consisted only of samples of the WTV system. Section II consisted of only two WTV module 3 samples which showed a 90% similarity. No correlation between [unavailable]P and the clustering of specific activated sludge samples were observed (Table 2). However, interesting information concerning the population dynamics of activated sludge was obtained. 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 bacterial community structure of activated sludge sample. 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 cannot be attached to the % similarity or correlation of the resulting dendrogram after SDS-PAGE. The % similarity can rather be used as a guideline to 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 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. 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.

CONCLUSIONS

- Most of the activated sludge samples of the different systems grouped together in section I, indicating a high overall similarity between them.
- The protein profiles of the same systems showed a high correlation (>80%) and could clearly be distinguished from each other.
- The results again agree with the literature, indicating that there is no difference between the bacterial community structures of the different zones. Although each system seemed to have its own specific microbial community structure, this did not differ significantly from those of other systems when comparing protein profiles.
- System design and the type of waste treated by the plant also did not result in altered protein patterns. This indicates that the microbial community structure of activated sludge plants are closely related apparently regardless of the type of waste.

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