A bacterial population structure study of water cooling systems in South Africa

T E Cloete,[1] * V S Brözel[1] and J Pressly[2]

[1] *Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, South Africa.*

[2] *Anglo American Research Laboratories, P O Box 106, Crown Mines, Johannesburg, South Africa.*

Abstract

Bacteria forming biofilms occur in all open water cooling systems where they accelerate metallic corrosion, reduce flow rate and decrease heat energy transfer rate. A population structure study of seven systems was conducted. The isolate most frequently encountered was *Pseudomonas fluorescens* (35,5%), the species commonly used in research regarding biofilm formation. This was followed by *Chromobacter violaceum, P. pickettii, P. stutzeri* and *P. putida,* each amounting to 6,6%. The dominant organisms occurred in two groups of over 85% relatedness between their biochemical reaction patterns. Overall four distinguishable groups occurred on the 90% similarity level.

Introduction

The main research effort in the field of biofouling has been to develop empirically or by design, methods for the prevention of biodeterioration of materials, thereby preserving their value and service life for as long as possible. Planktonic microorganisms which grow in industrial systems often attach firmly to surfaces (Savage and Fletcher, 1985). These immobilised cells produce extracellular polymers, consisting of a varying protein to polysaccharide mass ratio of between 0 to 10. These form a tangled mass of fibres termed a biofilm (Characklis and Cooksey, 1983). Although biofilms are beneficial in certain natural and modulated environments by removing undesirable substances from waters, e.g. in rivers or waste-water treatment systems, they are responsible for a variety of effects commonly termed biofouling. The biofilms produced in water cooling systems cause acceleration of metallic corrosion (Iversen, 1987); increased resistance to heat energy transfer (Characklis and Cooksey, 1983); and increased fluid frictional resistance when film thickness surpasses the monolayer (McCoy *et al.*, 1981).

The use of biocides to control biofouling is an accepted practice. Although biocides are employed to reduce bacterial numbers, mere use of the correct biocide does not necessarily reduce the fouling rate. It is essential to apply the correct dosage at the correct frequency. Wrong use of biocides gives poor results and is expensive. The application of biocides has developed into a field of expertise in its own right. Therefore the building blocks of a successful biocide programme are ideally considered to be:

- knowledge of the organisms to be killed (Allsop and Seal, 1986);
- selection of the correct biocide or combinations and their respective concentrations (Allsop and Seal, 1986; Freedman, 1979);
- scientific determination of dosage frequency (Freedman, 1979);
- monitoring the control of microorganisms through analysis and data processing (Freedman, 1979; Young-Bandala and Boho, 1987; Cloete *et al.*, 1988); and
- monitoring microbiological attachment to surfaces (Savage and Fletcher, 1985).

Selection of the correct biocide programme depends mainly on the variety of bacteria encountered and on their respective numbers. Very few studies have been conducted to determine the microbial population structure in water cooling systems in South Africa. Consequently biocides, dosage concentrations and frequency of dosage have often been selected on an arbitrary basis, resulting in ineffective programmes for the control of biofouling. Knowledge of the different kinds of microorganisms in these water systems will greatly assist in selecting the correct biocides. The minimum inhibitory concentration (MIC) of different biocides and the contact time that a particular biocide requires for a specific kill percentage against a particular organism (biocide fingerprint) can be determined only once the microbial population structure in a system is known. These data would predetermine the minimum contact time required for a biocide to kill bacteria and therefore directly influence the dosage concentration.

The aim of this study was, therefore, to determine the bacterial population structure in seven water cooling systems in South Africa.

Materials and methods

Systems studied

- Vaal Reefs (VR) No. 2 Shaft compressor (Tvl);
- VR surface fridge plant No. 2 Shaft;
- Elandsrand Surface Refrigeration Plant;
- Western Deep Levels North No. 3 Shaft compressor pond (West Rand);
- Freddies's No. 5 Shaft compressors (OFS);
- President Brand No. 1 Shaft spray pond (OFS); and
- President Brand No. 1 Shaft BAC tower (OFS).

Sampling procedure

Circulating cooling water in the abovementioned systems was sampled by collecting 250 mℓ aliquots into sterile "whirlpak" bags. All samples were kept below 4°C in transit to the laboratory where dilutions were prepared and spread out on nutrient agar petri dishes.

The Harrison's disk method (Harrigan and McCane, 1976) was used to isolate colonies at random from the highest dilutions of plates yielding growth from each system. This procedure yielded forty-five pure cultures.

*To whom all correspondence should be addressed.

Received 17 May 1988.

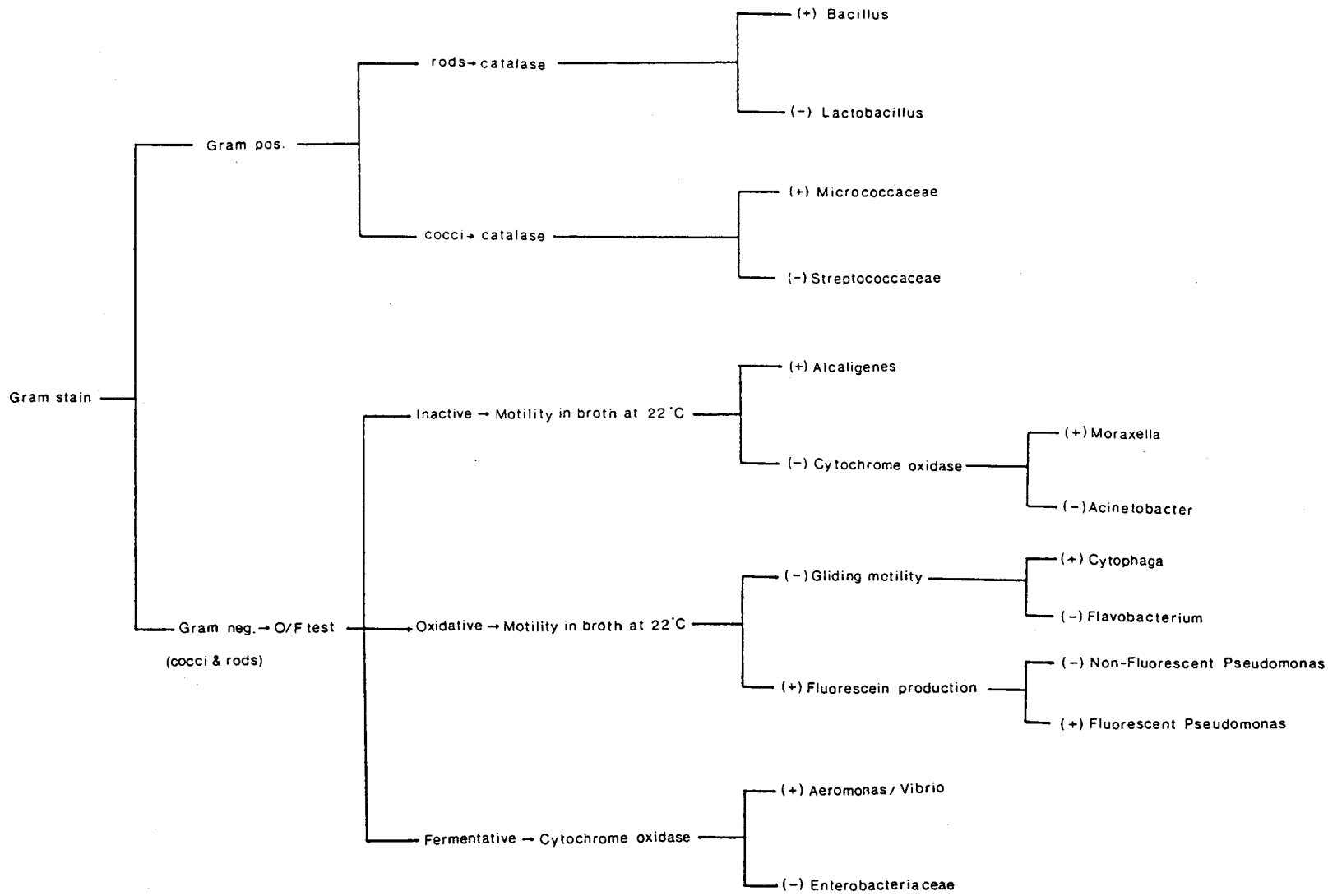


FIGURE 1
Simplified flowchart used for the preliminary identification of bacterial isolates. (Fischer et al., *1986)*

Identification and characterisation

Preliminary identification of the isolates was carried out by following the steps illustrated in Fig. 1. Oxidative metabolism of the Gram negative isolates was determined by inoculating into the Hugh and Leifson medium. All Gram negative oxidation isolates were then identified using the biochemical identification kit API 20 NE (API system S.A. — La Balme Les Grottes — 38390 Montalieu Vercieu, France). Cells were in logarithmic phase of growth and suspended in Ringers solution (1/4 strength) to a turbidity of 0,5 on the McFarland scale (Cloete, 1984).

All identification work was carried out at 25°C. Isolates were also subjected to temperatures of 7, 15, 30 and 37°C to determine temperature ranges. All isolates were also tested for nitrate reduction by inoculating into nitrate broth.

Statistical analysis

The relatedness of biochemical reaction patterns of the various Gram negative isolates was determined by the simple matching coefficient (S_{sm}) method (Sneath and Sokal, 1973). The results from the API 20 NE identification strips were used as biochemical reaction patterns. From the resulting data a dendogram was compiled using average linkage clustering by the unweighted pair group method (Austin and Priest, 1986).

Results

The results obtained for each system are shown in Table 1.

TABLE 1
IDENTIFICATION OF ALL GRAM NEGATIVE ISOLATES OBTAINED FROM THE SEVEN SYSTEMS

System and isolate identification	Percentage representation
Vaal Reefs No. 2 Shaft compressor	
Pseudomonas pickettii	11,1
P. fluorescens	11,1
Moraxella lacunata	11,1
Alcaligenes faecalis	11,1
Acinetobacter calcoaceticus	11,1
P. putida	11,1
P. alcalis	11,1
Gram positive bacteria (2 isolates)	22,2
Vaal Reefs surface fridge plant No. 2 Shaft	
P. fluorescens (3 isolates)	75,0
Chromobacter violaceum	25,0
Elandsrand surface refrigeration plant	
P. fluorescens (4 isolates)	100,0
Western Deep Levels North No. 3 compressor pond	
P. putida (2 isolates)	50,0
P. mendocina	25,0
P. fluorescens	25,0
Freddies No. 5 Shaft compressors	
P. fluorescens (2 isolates)	28,6
C. violaceum (2 isolates)	28,6
Gram positive bacteria (3 isolates)	42,9
President Brand No. 1 Shaft spray pond	
P. stutzeri (3 isolates)	37,5
P. fluorescens	12,5
Achromobacter xylosoxidans	12,5
P. alcalis	12,5
CDC fr V E-1	12,5
Gram positive bacteria	12,5
President Brand No. 1 Shaft BAC tower	
P. fluorescens (4 isolates)	44,4
M. urethralis	11,1
P. vesicularis	11,1
A. calcoaceticus var. *lwoffii*	11,1
P. pickettii (2 isolates)	22,2

Population structure

Seven of the isolates from the Vaal Reefs No. 2 Shaft compressor were Gram negative bacteria and two isolates Gram positive. Seven different species were identified, all occurring in equal quantities (Table 1). All four isolates obtained from the Vaal Reefs No. 2 Shaft surface fridge plant were Gram negative. *Pseudomonas fluorescens* was the dominant bacterium with a 75% representation. The results in Table 1 indicate that *P. fluorescens* was the predominant bacterium in the Elandsrand surface refrigeration plant. *P. putida* was the predominant organism in the Western Deep Levels North No. 3 Shaft compressor pond followed by *P. mendocina* and *P. fluorescens*. *P. stutzeri* was the predominant organism in the President Brand No. 1 Shaft spray pond. In President Brand No. 1 Shaft BAC Tower, *P. fluorescens* was the predominant bacterium (Table 1).

Altogether thirty-nine isolates from seven systems studied were identified. Fig. 2 shows the overall combined population structure of these systems.

Relatedness between Gram negative isolates

Extracellular polymers were formed profusely by all the isolates studied under laboratory conditions, when kept for longer than 72 h. This implicates their association with biofilm formation in the systems from which they were isolated. The question therefore arises: to what degree are these organisms related in terms of their biochemical capabilities? The degree of relatedness between the various biochemical reaction patterns of Gram negative isolates yielded the dendogram shown in Fig. 3.

Table 2 lists all the isolates similar to a degree of 85% or greater in two groups.

Three of the dominant organisms, i.e. *P. putida*, *P. pickettii* and *P. stutzeri* cluster well together. *P. fluorescens* clusters into a second group, occurring together with *Chromobacter violaceum*. *P. fluorescens* was distinct from the other *Pseudomonas* species, and clustered into two subgroups on the 90% similarity level.

The isolates were all capable of growth over a wide temperature range, adapting after 72 h to growth temperatures of between 7°C and 37°C. Also 56% of all Gram negative isolates reduced nitrate to nitrite or to nitrogen.

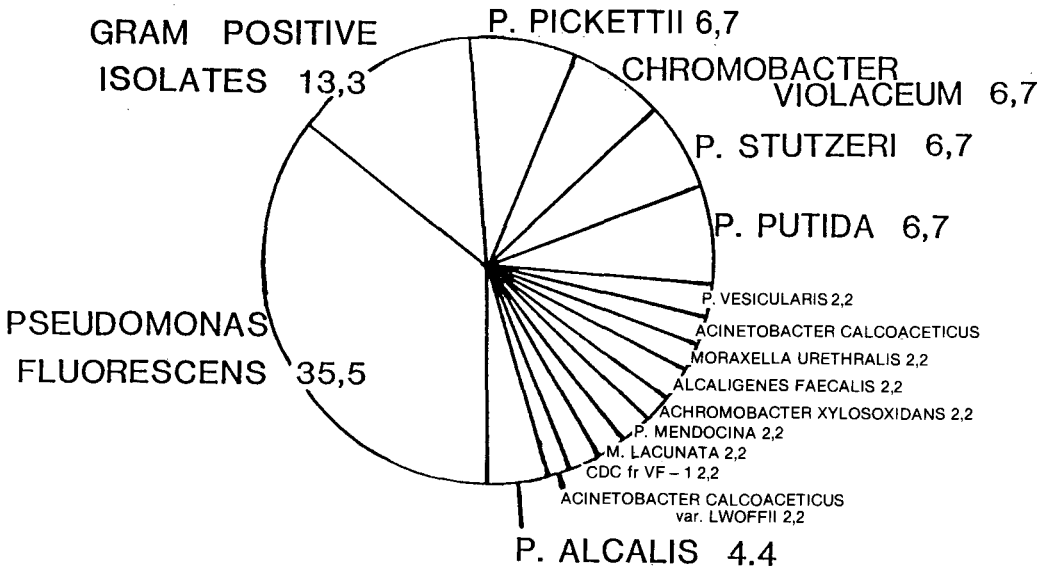


FIGURE 2
Combined population structure study of the systems studied.

TABLE 2
GRAM NEGATIVE ISOLATES FROM SEVEN SOUTH AFRICAN WATER COOLING SYSTEMS. ISOLATES ARE GROUPED WHERE THE DEGREE OF SIMILARITY EXCEEDS 85%

Group 1	
D6	*Acinetobacter calcoaceticus*
I4	*Pseudomonas putida*
N3	*P. stutzeri*
L4	*P. stutzeri*
L5	*P. stutzeri*
M1	*P. pickettii*
D9	*P. putida*
I3	*P. putida*
I5	*P. mendocina*
D4	*P. pickettii*
M5	*P. pickettii*
O3	*A. calcoaceticus* var. *lwoffii*
N2	*P. alcalis*
M2	*Moraxella urethralis*
D7	*Alcaligenes faecalis*
F7	*P. alcalis*
Group 2	
I4	*P. fluorescens*
J8	*Chromobacter violaceum*
J6	*C. violaceum*
L1	*P. fluorescens*
J7	*P. fluorescens*
H3	*P. fluorescens*
G6	*P. violaceum*
O2	*P. fluorescens*
K3	*P. fluorescens*
G2	*P. fluorescens*
G5	*P. fluorescens*
D8	*P. fluorescens*
O6	*P. fluorescens*
M4	*P. fluorescens*
M7	*P. fluorescens*
G4	*P. fluorescens*
H2	*P. fluorescens*
H7	*P. fluorescens*
H6	*P. fluorescens*
Isolates less related	
O1	*P. vesicularis*
D5	*M. lacunata*
N5	*CDC gr V E-1*
L3	*Achromobacter xylosoxidans*

Discussion

The dendogram shows that 92% of the Gram negative isolates had biochemical reaction patterns similar to a degree of 80%. This would be expected in nutrient-restricted systems such as those studied. Although the group comprised only 39 isolates, its specialised nature does warrant demonstration of relatedness within so small a group (Austin and Priest, 1986).

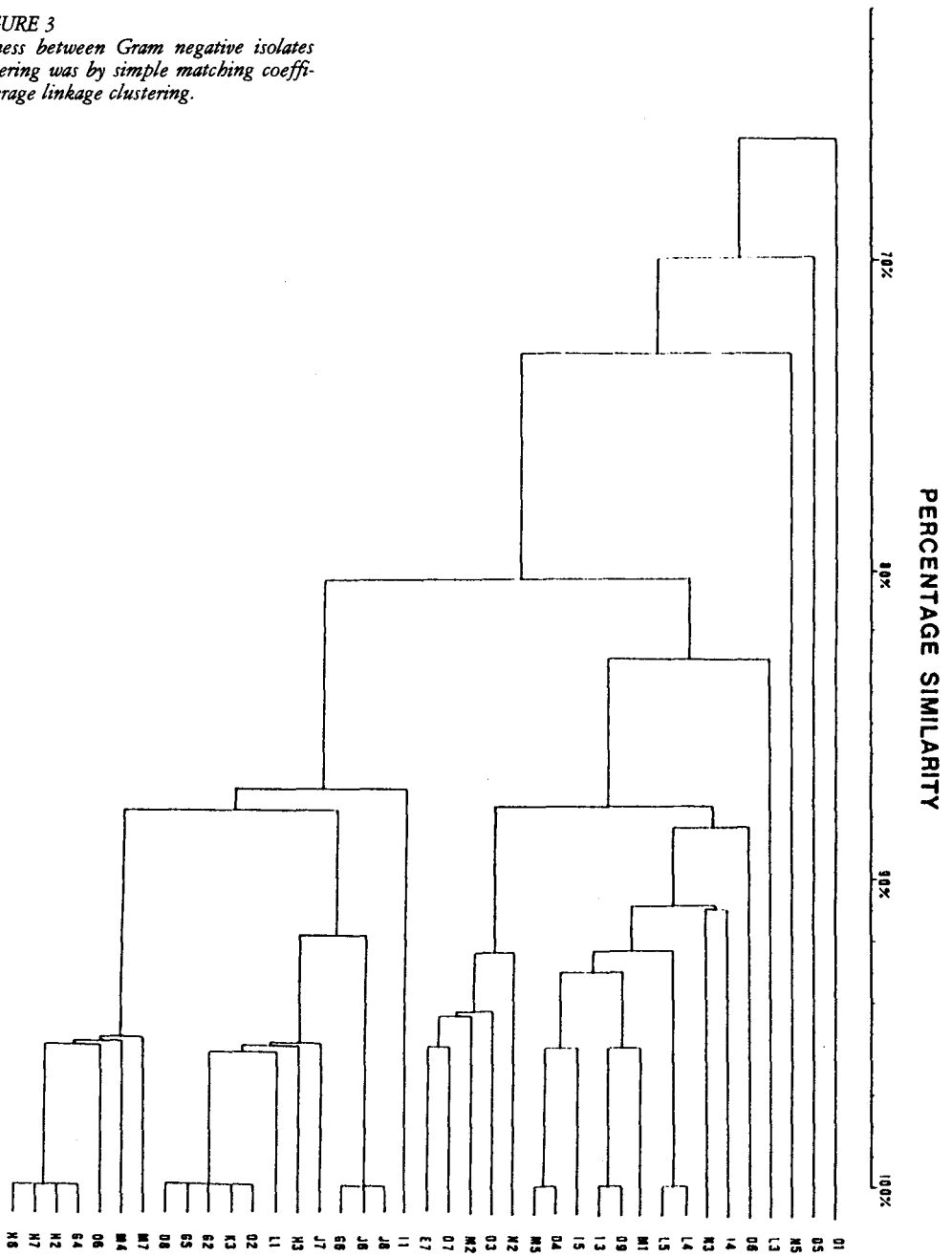
Nitrates are often used as passivating agents in open water cooling systems. The presence of denitrifying bacteria would catalyse breakdown of this passivating agent, rendering it inactive. Conversely, the population would adapt to the presence of nitrates, and select for denitrifying bacteria. The wide temperature tolerance of the dominant bacteria indicates that they, having been selected by the differing temperature zones in water cooling systems, are able to grow and form biofilms in various sections of the plant.

Conclusion

Pseudomonas fluorescens was the predominant bacterium encountered in the majority of systems studied, followed by *P. pickettii, P. putida, P. stutzeri* and *Chromobacter violaceum.* The other organisms encountered occurred at much lower frequencies and in very few systems. The dominant organisms occurred in two groups as can be seen from Table 3. *P. stutzeri, P. putida* and *P. pickettii* were biochemically well related in a group and *C. violaceum* and *P. fluorescens* in a second. Altogether four clusters occurred. Denitrifying isolates were dominant.

FIGURE 3

Dendogram showing the relatedness between Gram negative isolates from water cooling systems. Clustering was by simple matching coefficient (S_{sm}) using average linkage clustering.



Acknowledgement

We thank D Tönsing for writing the program for the cluster analysis and Anglo American Laboratories for making this study possible.

References

ALLSOP, D and SEAL, KJ (1986) *Introduction to Biodeterioration.* Edward Arnold, London.

AUSTIN, B and PRIEST, FG (1986) *Modern Bacterial Taxonomy.* Van Nostrand Reinhold (UK) Co. Ltd Berkshire, England.

CHARACKLIS, WG and COOKSEY, KE (1983) Biofilms and microbial fouling. *Advances in Applied Microbiology* **29** 93-138.

CLOETE, TE (1984) Possible role of *Acinetobacter calcoaceticus* as a biological phosphorus removing agent in activated sludge. Ph.D. Thesis, University of Pretoria.

CLOETE, TE, STEYN, PL and SMITH, L (1988) Planktonic bacteria and the evaluation of biocides. (Accepted for publication in *International Biodeterioration*).

FISCHER, PL, JOOSTE, PJ and NOVELLO, JC (1986) Evaluation of rapid plate counting technique for the enumeration of psychrotrophic bacteria in raw milk. *SA J. Dairy Sci.* **18** 137-141.

FREEDMAN, L (1979) Using chemicals for biological control in cooling water systems; some practical considerations. *Industrial Water Engineering* **16** (5) 14-17.

HARRIGAN, WF and McCANCE, ME (1976) *Laboratory Methods in Food and Dairy Microbiology.* Academic Press, NY.

IVERSEN, WP (1987) Microbial corrosion of metals. *Advances in Applied Microbiology* **32** 1-36.
McCOY, WF, BRYERS, JD, ROBBINS, J and COSTERON, JW (1981) Observations of fouling biofilm formation. *Canadian Journal of Microbiology* **27** 910-917.
SAVAGE, DC and FLETCHER, M (1985) *Bacterial Adhesion.* Plennum Press, New York.
SNEATH, PHA and SOKAL, RR (1973) *Numerical Taxonomy: The Principles and Practice of Numerical Classification.* WH Freeman, San Francisco.
YOUNG-BANDALA, L and BOHO, MJ (1987) An innovative method for monitoring microbiological deposits in pulp and paper mills. *Tappi Journal* **70** (1) 68-71.