Optimisation of Biofouling Control in Industrial Water Systems

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Report to the Water Research Commission on the Project
"Optimisation of Biofouling Control in Industrial Water Systems"

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EXECUTIVE SUMMARY

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Supplement to WRC Report No. 318 which is the full
report to the Water Research Commission on the Project
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EXECUTIVE SUMMARY

Both water consumption and discharge in industrial water systems are currently minimised. The circulation of such water results in concentration of dissolved and suspended substances, promoting the growth of waterborne microbes, biofouling and subsequent macrofouling of the system and concomitant microbially induced corrosion.

A number of reviews have been published on the mechanisms of microbially induced corrosion and the organisms involved. The subject of biofilm formation has also been well covered in the literature. A lack of information on the community structure and physiology is, however, apparent. Many advances have, nonetheless, been made from planktonic bacterial monitoring to sessile bacterial monitoring. This led to the introduction of a variety of different sessile monitoring techniques. Much experience has since been obtained on the use and limitations of these techniques and, to date, one of the main problem areas remaining is the monitoring of biofouling.

Research has also indicated the problem of microbial resistance to nonoxidising biocides. This has suggested that some of these compounds may be mutagens. From an environmental point of view, it has become very important to verify this. This has also indicated the need to develop biocides which do not induce resistance in micro-organisms, and to investigate whether oxidising biocides are also capable of inducing resistance in micro-organisms. Recent studies have indicated that biofilm ecosystems respond to stress (i.e. biocides) in ways similar to macro-ecosystems. Generally, there is a decline in species diversity and a selection of more tolerant isolates.

These developments have placed the spotlight on alternative technologies, like biodispersants, which have shown potential as biofouling control agents, and which should be investigated further. Physical control measures are currently still limited to pigging, although a number of other technologies show promise. Although fluid dynamics and their effect on biofouling control programmes have been well reported in the literature, it remains an aspect which is neglected by industry in terms of practical applications.

The objectives of this study were therefore:

* To develop an *in situ* biocide concentration monitoring technique which could be used by field personnel. This technique would be aimed at determining biocidal activity, rather than pure chemical analysis. This technique would also be used for determining the influence of other water treatment chemicals on the biocidal activity of biocides.

* To determine the sessile microbiological tolerance level before biofouling sets in. This technique would in practice be used to monitor the efficacy of biocide programmes.

* To determine whether bacteria develop a resistance to biocides and how this would influence biocide programmes.

* To develop a rapid, easy to use technique for identifying an enumerating sulphate reducing bacteria (SRB) *in situ* and to determine their role in microbial induced corrosion (MIC).

* To determine the effect of nutrients on biofilm activity in industrial water systems and

* To investigate the development of cross resistance amongst different bactericides.

DETERMINATION OF *IN SITU* BIOCIDE CONCENTRATIONS

Biofouling in industrial water systems is normally prevented by the use of bactericides. However, bactericide programmes often fail owing to the lack of suitable techniques for determining the *in situ* bactericide concentration and this usually results in either inadequate or excessive bactericide concentrations. In this study, the Sterikon(R) bioindicator was evaluated for determining the minimum inhibitory concentrations of 5 industrial bactericides (dichlorophen, sulphone, thiocarbamate, isothiazolone and a quaternary ammonium compound) for the monitoring of the concentrations of these compounds in industrial water

systems. The results indicated that the Sterikon(R) bioindicator can be used for the determination of bactericide concentrations.

RESISTANCE OF BACTERIA FROM COOLING WATERS TO BACTERICIDES

Bacteria from cooling water systems developed resistance to three different bactericides, i.e. quarternary ammonium compounds (QAC), isothiazolone and thiocarbamate. Resistance was induced by exposing isolates to increasing sublethal concentrations for a period of 10 weeks. *Bacillus subtilis* became resistant to 1000 mg.ℓ^{-1} QAC. Cross resistance was also detected, e.g. isothiazolone induced resistance to QAC and thiocarbamate.

The relationship between culture age and resistance was investigated, as well as the resistance of attached cells. Resistance of *Pseudomonas stutzeri* and of *Bacillus cereus* doubled to all water treatment bactericides evaluated. Mucoid mutants did not exhibit increased tolerance to bactericides, indicating that extracellular polysaccharide does not confer increased resistance to bacteria in biofilms. However, attached cells were more resistant than free-living cells within 15 min following attachment. Cell age also had a marked influence on resistance, where actively growing cells were most resistant and late stationary phase cells were least resistant.

A culture of *Pseudomonas aeruginosa* isolated from a cooling water system was grown in the presence of sub-inhibitory concentrations of the water treatment bactericide 2,2'-Methylenebis(4-chlorophenol) (MBC). It grew in the presence of increasing concentrations by a mechanism of adaptation. The initial minimum inhibitory concentration was 36 μg/mℓ^{-1} and the highest attained was 80 μg.mℓ^{-1}. Resistant cultures exhibited a higher survival rate when exposed to 320 μg.mℓ^{1-} than did the original strain.

Lipopolysaccharide and outer membrane protein profiles were determined by SDS PAGE. No changes were detected in the LPS profile. The quantity of OprP, a phosphate uptake protein in the outer membrane decreased to a low level, correlating with decreased phosphate (P_i) uptake during growth. It is proposed that OprP is the place of entry for MBC through the outer membrane of *P. aeruginosa* and that the cell can adapt to growth in its presence

by decreasing the level of OprP in the outer membrane.

Resistance and the development thereof in *Pseudomonas aeruginosa* to the bactericide sodium dimethyl dithiocarbamate (SMT) was investigated. *Ps. aeruginosa* was cultured in nutrient-poor broth in the presence of sub-inhibitory concentrations of SMT. It adapted over 21 days of exposure from 250 $\mu g.m\ell^{-1}$ to 490 $\mu g.m\ell^{-1}$. The initial high MIC was ascribed to exclusion of SMT by the lipopolysaccharide layer since removal thereof by EDTA rendered cells highly susceptible. The alginate-producing mutant PAO 579 was much more susceptible to SMT than was its parent PAO 381, indicating that extracellular polysaccharide does not act as an exclusion barrier to SMT. Following 24 h exposure to SMT, *Ps. aeruginosa* had an altered profile of outer membrane proteins as determined by SDS PAGE. Resistant cells had a further altered profile. Resistance of *Ps. aeruginosa* is ascribed to a change in the outer membrane protein profile, leading to improved exclusion of SMT.

P. aeruginosa was cultured in nutrient limited broth in the presence of sub-inhibitory concentrations of isothiazolone (a mixture of 1.15% 5-chloro-N-methylisothiazolone (CMIT) and 0,35% N-methylisothiazolone (MIT)). Three cultures tested in parallel adapted gradually over 15 days of exposure from an initial minimum inhibitory concentration (MIC) of 300 $\mu\ell.\ell^{-1}$ to 607 $\mu\ell.\ell^{-1}$. The three parallel cultures adapted at similar rates, therefore the adaptation was not ascribed to mutation but to a specific mechanism. Resistant cells did not produce any extracellular isothiazolone-quenching compounds nor undergo detectable alterations in their lipopolysaccharide layer. In wild cells a 35 kD outer membrane protein (protein S) was detectable, whereas resistant cells lacked this protein. Production of protein S was suppressed within 24 h of exposure to isothiazolone. It was still suppressed after 72 h of growth in isothiazolone-free medium. We propose that *P. aeruginosa* acquires resistance to isothiazolone by a process of adaptation where the outer membrane protein S is suppressed.

Hypochlorous acid and hydrogen peroxide are employed as biofouling control agents and as surface disinfectants. Whereas the bacterial development of resistance to non-oxidising bactericides is well established, the question whether bacteria develop resistance to oxidising agents has not been answered. The minimum inhibitory concentration (MIC) of HClO for

P. aeruginosa fluctuated during repeated culturing in presence of sub-inhibitory concentrations of HClO. The MIC did, however, not increase during the 49 d period. No detectable stress response to either HClO or H_2O_2 was found in *P. aeruginosa* following exposure to relevant oxidant stress. Rather, cultures became more susceptible to oxidant following such stress. *P. aeruginosa* exhibited a degree of recovery to H_2O_2 60 min after exposure to stress, but this protective mechanism was not maintained. Inhibition of *de novo* protein synthesis by chloramphenicol prior to exposure to H_2O_2 stress lead to decreased susceptibility of *P. aeruginosa*. The converse was true in the case of HClO, with an intact protein-synthesising system leading to a lesser increase in susceptibility. *Escherichia coli* demonstrated a stress response to H_2O_2, and to a lesser degree to HClO. This response to HClO lead to increased protection to inhibition, but not to cell death.

TECHNIQUES FOR ENUMERATING AND IDENTIFYING SRBs IN INDUSTRIAL WATER SYSTEMS

The importance of sulphate-reducing bacteria (SRB) in microbial induced corrosion has been widely recognized for many years. However, little is known about the ecology of SRB in industrial cooling water systems. The problem has been in detecting and quantifying these organisms. There are many shortcomings in the use of culture media for this purpose. As an alternative, immunological techniques were evaluated as a method for detection and identification of SRB in industrial cooling water systems. Antisera were prepared against whole cells of different species of SRB and evaluated for detection and identification of these organisms in industrial cooling water systems. Antisera prepared against the surface antigens of SRB were species specific and the different species shared no antigenic determinants. In addition, culture conditions influenced the expression of surface antigens causing the antisera to be extremely specific and unsuitable for the identification of SRB enriched from industrial cooling water systems. These results were confirmed by the SDS PAGE profiles of membrane proteins.

The agar plate method is still the method of choice for quantifying viable bacteria in water, especially in routine laboratories. Eleven nutrient agar media were compared for colony producing ability by incubating at 25°C and at 30°C. Appearance of colonies was recorded

against time and colony forming curves were constructed. The highest counts after five days (*ca* 75% of maximum count) were obtained on R3A agar incubated at 30°C. R3A agar at 30°C also allowed the most rapid colony development, with most of the colonies appearing between two and five days. Both Plate Count Agar (PCA) and Standard I Agar (Std I) performed poorly. PCA yielded 30% of the highest count after five days at 30°C and at 25°C. Std I yielded 27% at 30°C and 23% at 25°C. The standard procedure of 48 hours at 30°C on PCA yielded 14% of the final highest count, *i.e.* one log less.

In contrast to PCA and Std I, R3A agar contained magnesium and pyruvate, both of which on their own were shown to increase the plate count. R3A also had a lower nitrogen content and nitrogen to carbon ratio. The highest count after 14 days were achieved on double strength R3A (R4A) at 20°C. R4A also showed the highest colony diversity. It was therefore seen to be the most suitable for population studies. However, colony development was slower. This makes it unsuitable for plate counts, whereas R3A lets most cells develop into colonies within the first five days of incubation.

The reactions of a cooling water sample and a tap water sample to storage at various temperatures were determined. Samples were stored at 4, 10, 20 and 30°C for 24, 48, 72 and 216 h and the total culturable count and population structure of each were determined. The culturable count in both samples varied over time, even at 4°C after 24 h. In the cooling water sample, the dominant isolates throughout were *Pseudomonas stutzeri* and an unidentified pink isolate although the other isolates were present randomly. No direct tendencies of rate of decrease or increase could be detected in any of the samples, either in the culturable count or in population structure. Therefore results of analysis after storage cannot be adapted by a known factor. They must be interpreted with extreme caution, as they do not of necessity reflect the bacterial composition of the sample as drawn, both in terms of total numbers and in terms of population structure. Only counts performed on fresh samples yield reliable results on the total culturable count, and only population structures performed immediately, reflect the state of the population in the system from which the sample was drawn.

The importance of sulphate-reducing bacteria (SRB) in microbiological induced corrosion

(MIC) has been widely recognized for many years [1]. There are many formulations of culture media used for enumerating SRB [2, 3]. Previous studies indicated that viable count procedures underestimated the *in situ* population by a factor of approximately 1000 compared to *in situ* sulphate reduction activity [1, 4]. The aim of this study was to evaluate different isolation media and carbon sources for the isolation and detection of SRB in South African industrial water systems. Modified Iron sulphite (IS) medium yielded the highest numbers when used to enumerate SRB from pure cultures and industrial water samples. When comparing API, SABS, IS, Oxoid and modified synthetic medium using pure cultures of *Desulfovibrio desulfuricans* IS-medium gave a 12.1%, 40%, 53.3% and 60.3% higher recovery than SABS-, Postgate-, API- and synthetic medium, respectively (P @ 0.05). IS-medium gave a 20.1%, 61.8% and 100% higher recovery than SABS-, API- and Oxoid medium, respectively, when using pure cultures of *Desulfotomaculum orientis* (P @ 0.05). The dominant sulphide-producing bacteria isolated from the industry using IS-medium were facultative aerobic Gram-negative rods that were able to produce sulphide from sulphite under strictly anaerobic conditions. IS-medium was therefore not selective for SRB only. H_2S-producing bacteria that utilize lactate, acetate, formate or palmitic acid as different carbon sources were also isolated from industrial water samples when using synthetic medium.

Several Gram-negative facultative aerobic bacteria cultures that were capable of corroding mild steel were isolated from oil field water (Obuekwe *et al.*, 1981). These bacteria and other iron reducing bacteria, isolated from oil field water were classified as *Shewanella putrefaciens* (Semple and Westlake, 1987). Gram-negative facultative aerobic rods capable of anaerobic growth and H_2S-production on lactate using sulphite as electron acceptors, were the dominant bacteria isolated by De Bruyn and Cloete (1993) from industrial cooling water systems using Iron Sulphite medium (Mara and Williams, 1970). In this study these bacteria were identified as *S. putrefaciens*. This is the first time that *S. putrefaciens*, a potentially corrosive bacterium, has been isolated from industrial cooling water systems.

According to various authors the most widely distributed and economical important organisms associated with microbial induced corrosion (MIC) are the dissimilatory sulphate-reducing bacteria (SRB). However, there has been increasing evidence that organisms, other than

SRB have been involved in the corrosion process. Using iron sulphide medium *Shewanella putrefaciens* was the dominant sulphide producing bacteria isolated from industrial cooling water systems. It was therefore considered necessary to determine the role of these organisms in MIC. Silicon plates coated with a thin layer of copper or nickel were used for rapid screening of the corrosivity of *S. putrefaciens* isolates, *Desulfovibrio desulfuricans*, *Pseudomonas fluorescens* and *Escherichia coli* in liquid medium under anaerobic conditions or on solid medium under aerobic conditions. The results obtained demonstrated the importance of iron sulphide production in the corrosion process. The degree of corrosion varied with the bacterial species and media used. The most severe corrosion obtained on solid media under aerobic conditions was with *S. putrefaciens* cultured on nutrient agar. When using benzyl viologen as evidence for cathodic depolarization it was demonstrated that *S. putrefaciens* has the ability to utilize cathodic hydrogen. Microscope studies were also performed to determine the role in MIC of *S. putrefaciens* and *D. desulfuricans* respectively, cultured in iron sulphide medium with simultaneous production of iron sulphide, an 3CR12 metal coupons. After exposure of the coupon to a *D. desulphuricans* culture, a thin layer of iron sulphide covered the metal surface, whereas exposure of the metal to a *S. putrefaciens* culture resulted in bulk iron sulphide-like deposits. This study indicated that *S. putrefaciens* could play an important role in MIC.

Microbiological induced corrosion (MIC) makes an important contribution to corrosion in various industries. Considerable success has been achieved by the use of biocides. Little information for controlling MIC is however available on the effectivity of biocides against SRB due to the difficulties of culturing these organisms using conventional techniques. Conductance changes monitored using the Malthus system was evaluated as an alternative method of estimating numbers of *Desulfovibrio desulfuricans* for laboratory biocide evaluation. The correlation of $\log_{10}$ counts of *Desulfovibrio* cells in IS-medium using conventional techniques with detection times using the Malthus systems was highly significant ($r = 0.974$), indicating that the Malthus system can be used as a alternative method to conventional media for the enumeration of SRB. Growth studies of *Desulfovibrio* using the Malthus system were useful in the evaluation of biocides.

PHYSIOLOGICAL EFFECTS OF BACTERIAL ATTACHMENT TO SURFACES AND THE EFFECT OF NUTRIENTS ON BIOFILM ACTIVITY

The study of biofilms has indicated that bacteria behave fundamentally differently in the biofilm habitat than in suspension. The aim of the study reported here was to determine whether available carbon (COD) had an effect on the rate of attachment of bacteria to surfaces, and whether *P. aeruginosa* PAO underwent any changes during attachment. There was a relationship between available glucose and rate of attachment during the first 20 min of exposure. Glucose concentrations between 0.05 and 0.001 g.ℓ^{-1} yielded *ca.* 10 attached cells per field. Concentrations of between 0.5 and 10 g.ℓ^{-1} yielded *ca.* 100 attached cells per field. Within 1 h of exposure, however, there was little difference in the numbers of attached cells between various levels of available carbon. The level of available carbon has little effect on the rate of bacterial attachment to surfaces. A method for the study of *de novo* protein synthesis in attached bacteria has been developed. This was used to demonstrate that the synthesis of several proteins in *P. aeruginosa* PAO was influenced by attachment of cells. Attachment influenced the synthesis of at least 11 proteins during various stages after attachment. Attachment *per se* was demonstrated to exert some effect on the regulation of certain genes. It is not clear whether this is at the transcriptional of translational level, and what the nature of the attachment-mediated signal is.

The results of the research carried out during the project on optimisation of biofouling control in industrial water systems have far-reaching implications for the control and treatment of industrial water systems. These include new monitoring methods, information regarding mechanisms of action and information leading to better treatment programmes.

Monitoring methods

Aerobic bacteria in industrial water systems are best enumerated using R2A agar and incubating for 5 d at 30°C. Sulphate-reducing bacteria are best enumerated using IS medium and it is recommended that this medium be used for SRB enumeration in water cooling systems. Because water samples are dynamic, both the culturable count and the community structure change during storage, irrespective of temperature. Samples must be analysed as

soon as possible after taking in order to ensure a representative result. Biocide concentrations in water systems can be determined using the Sterikon^R bioindicator system. An investigation into the commercialization of this method is recommended. The Malthus system, using conductance measurements, can be used for enumerating sulphate-reducing bacteria in pure culture, *i.e.* also for biocide evaluations. Antisera prepared against the surface antigens of sulphate-reducing bacteria (SRB) cultured in IS medium, were species specific and could not be used to identify SRB enriched from natural systems, since surface antigens are subject to environmental conditions.

Resistance to biocides

A variety of bacteria dominant in industrial water systems do develop resistance to various water treatment biocides. In certain cases, resistance is concurrent with cross-resistance. Resistance to the biocides 2,2-methylenebis(4-chlorophenol), Na dimethyl dithiocarbamate and isothiazolone increases gradually during exposure to sub-inhibitory concentrations. This is due to alterations in the outer membrane protein profiles. Resistance to tetradecyl-benzyl-dimethyl ammonium chloride develops after approximately 1 month's exposure to sub-inhibitory concentrations. Extracellular polysaccharides are not the cause of increased resistance of bacteria in biofilms. Attachment *per se* confers increased resistance to bacteria due to an as yet uncharacterised phenomenon. Bacteria do not develop resistance to the oxidising biocides hypochlorous acid and hydrogen peroxide. This suggests that biofouling controlling programmes should where possible always incorporate oxidising biocides as well as non-oxidising biocides.

A new group of bacteria involved in corrosion

Most of the sulphide-producing bacteria isolated using IS medium were found to be Gram-negative facultative anaerobes belonging to the genus *Shewanella*. Most were *Shewanella putrefaciens*. *Shewanella putrefaciens* plays an important role in microbially induced corrosion, mainly by way of cathodic depolarisation (utilisation of cathodic hydrogen). More research re the characteristics and role of these organisms in MIC should be conducted.

Bacterial attachment

The available carbon in water does not have an influence on the rate of bacterial attachment

to surfaces. Attachment of bacteria has a marked influence on the structure and function of the cell.

In view of the important findings and implications of this research it is recommended that a technology transfer document be drawn up and distributed to industry.

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Mr P. Weideman	Water Research Commission (Secretary)
Mr J. Pressly	Anglo American Researcg Laboratories
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BOD	Biochemical oxygen demand
Cfu	Colony forming units
COD	Chemical oxygen demand
D	Dalton
EPS	Extracellular polysaccharide
IS	Iron sulphide
LPS	Lipopolysaccharide
MGLA	Minimal glucose leucine agar
MIC	Minimum inhibitory concentration
MLC	Minimum lethal concentration
SMT	Sodium dimethyl dithiocarbamate
SPB	Sulphide-producing bacteria
SRB	Sulphate-reducing bacteria
TNTC	To numerous to count

Chapter 1: Literature Survey

1 BACTERIA IN WATER

Micro-organisms always live in an aqueous environment, whether it be in the film of water around a soil particle, in the blood of a mammalian host or in lakewater (Hobbie and Fletcher, 1988). Bacteria all require water in their immediate surroundings, and even the extreme halophiles require an external water activity of at least 0.75 (VanDemark and Batzing, 1987). The microbial habitat is an aqueous one. Each bacterial species has a different set of abilities and can grow in a different set of niches (Atlas and Bartha, 1987a), *i.e.* in water having a different composition. Actually all bacteria are water-organisms. However, water in the strict sense of habitat is water visible to the naked eye, a habitat termed the hydro-ecosphere (Atlas and Bartha, 1987d). The hydro-ecosphere includes lakes, dams, rivers, temporary ponds, estuaries, and the open oceans which contain 97 percent of the earth's water (Grant and Long, 1981).

1.1 Biofilms

The most important environment in flowing waters is the surface micro-environment (Hobbie and Fletcher, 1988). It is protective and offers resident bacteria a nutritional advantage so that surfaces are the major site of microbial activity (Van Loosdrecht, Lyklema, Norde and Zehnder, 1990). Harvey and Young (1980) showed that almost all bacteria involved in mineralisation of estuarine carbon were associated with particles. Kirchman and Mitchell (1982) found the contribution of particle-bound bacteria to total heterotrophic activity to be four times as high as expected from the fraction of attached cells. Many aquatic bacteria depend on attachment to surfaces for species survival, with attached cells growing and dividing at nutrient concentrations too low to permit growth in the aqueous phase (Kjelleberg, Humphrey, and Marshall, 1982). As a consequence most surfaces exposed to water are covered in bacterial conglomerates termed biofilms (Lewin, 1984).

Bacteria in natural systems seldom if ever occur in pure culture (Karl, 1986). They form part of food chains and many are only found as part of consortia and never on their own (Costerton, Cheng, Geesey, Ladd, Nickel, Dasgupta and Marrie, 1987). Bacterial consortia are often highly structured (Costerton *et.al.*, 1987), catalyzing breakdown of complex substrates to CO_2. Bacteria as members of biofilms benefit in various ways: they have enhanced access to nutrients, are close to cells with which they are in mutualistic or synergistic association (*eg.* as part of a consortium breaking down cellulose), and are protected to a high degree from various antimicrobial mechanisms *i.e.* bactericides, antibiotics, antibodies and predators (Costerton and Lappin-Scott, 1989).

According to members of the Dahlem conference (Marshall, 1984) "a biofilm is, in general, a collection of microorganisms and extracellular products associated with a solid surface, termed a substratum".

1.1.1 Attachment to surfaces

In order to remain in the proximity of a surface, bacteria must adhere or attach. The Dahlem conference (Marshall, 1984) proposed the term adhesion, defining it as follows. "Adhesion can be defined unambiguously only in terms of the energy involved in the formation of the adhesive junction. Thus, two surfaces may be said to have adhered when work is required to separate them to their original condition". Physically speaking, adhesion is an example of particle adsorption on a substrate (Rutter and Vincent, 1988). Bacteria usually carry a net negative charge, and so do the surfaces to which they adsorb (Van Loosdrecht *et.al.*, 1990). They can further be considered colloidal particles, though they are not inert and can change characteristics with altering environment (Van Loosdrecht, Lyklema, Norde and Zehnder, 1989).

Various authors have expanded on the physicochemical thermodynamic approach, including Absolom, Lamberti, Policova, Zingg, Van Oss and Neumann (1983), Rutter and Vincent (1984), and Van Loosdrecht *et.al.* (1989). Absolom *et.al.* (1983) employed a concept of short-range interaction. Bacteria are seen to be in direct contact with the substratum and the Gibbs free energy is estimated from the interfacial tension. Alternatively the contact angle can be used. Rutter and Vincent (1984, 1988) use the long-range interaction concept based on DLVO theory for colloidal stability. Here the interaction Gibbs free energy between particle and surface is a function of the distance between the two. As both bacteria and surface usually cary a net-negative charge, there is a net repulsive interaction between cells and surface (Van Loosdrecht *et.al.*, 1989).

Other authors have taken an empirical approach, observing attachment microscopically, and modelling the adsorption process thereon. Among them are Bryers (1988), Characklis (1983), Costerton *et.al.* (1987) and Hamilton (1987). Attachment is usually studied by image analysis of video signals of thin flowcells. The most important technique described to date is image analysis of images obtained by confocal laser microscopy, developed by Caldwell and co-workers (Caldwell and Lawrence, 1989).

A synthesis of the two approaches was pleaded for at the Dahlem conference (Marshall, 1984), resulting in a good picture of bacterial attachment to surfaces. Figure 1 shows an accepted model of the sequence of steps leading to attachment.

Hamilton (1987) argues that new surfaces in aqueous surrounding adsorb organic molecules, resulting in a so-called conditioning film. Bacteria can then attach to this layer, and not to the metal surface *per se*. Lawrence, Delaquis, Korber and Caldwell (1987) described the mechanics of

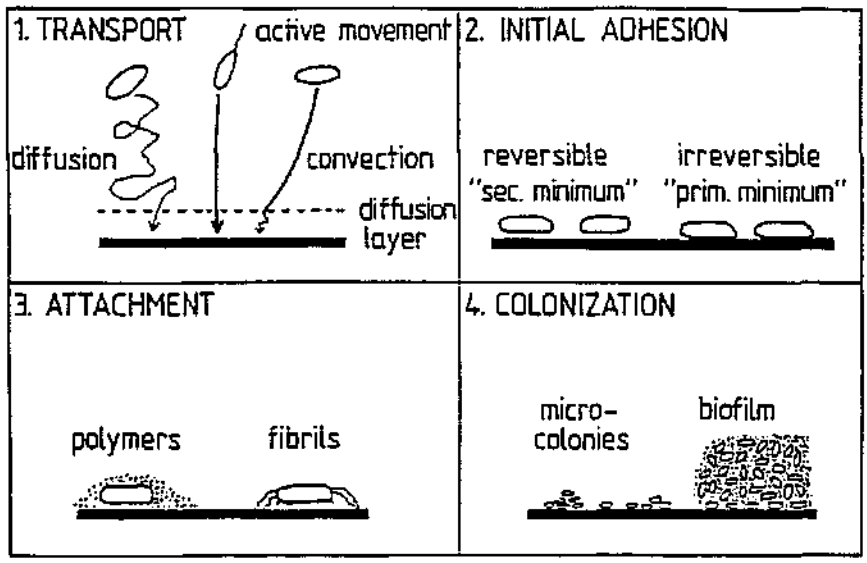

Figure 1: Schematic representation of the steps leading to irreversible attachment of bacteria to surfaces (after Van Loosdrecht *et.al.*, 1990).

attachment of *Pseudomonas fluorescens* to surfaces in the hydrodynamic environment: Motile cells move down and upstream, even against a laminar flow velocity of 200 $\mu m.s^{-1}$ within the surface microenvironment. They approach the surface and achieve contact by the polar flagellum and at an angle of between 40° and 45°. Attached cells rotate either clockwise or counterclockwise about the point of attachment for an average duration of 2 minutes. At this stage some detach and reattach elsewhere. After 2 minutes rotation slows and ceases, and the cell becomes attached along its longitudinal axis. The cell is now in the secondary minimum Gibbs energy dip. The distance between cell and surface depends on the concentration, valency and charge density of cations (Van Loosdrecht *et.al.*, 1990), but is in the order of 70 Angstroms (Lewin, 1984). Extracellular polysaccharides are produced which fill the area between cell and surface, and attach the cell irreversibly (Costerton and Lappin-Scott, 1989; Lewin, 1984).

1.1.2 Development of biofilms

Attached cells grow and divide, sliding in next to each other in an obliquely adjacent position (Lawrence *et.al.*, 1987). This occurs three times, yielding a microcolony of eight cells. Then some members of the microcolony start a vibrational movement, which leads to some cells leaving the surface (Lawrence *et.al.*, 1987). Korber, Lawrence, Sutton and Caldwell (1989) showed that motile strains of *Pseudomonas fluorescens* (Mot^+ strains) reattached more often than Mot^- strains, even in flow as low as 8 $\mu m.s^{-1}$. Mot^+ cells also dispersed more evenly over the surface, resulting in more complete coverage of the surface in microcolonies (Korber *et.al.*, 1989).

Cells in the microcolonies produce extracellular polysaccharide (EPS) (Costerton *et.al.*, 1987). Costerton, Irvin and Cheng (1981) termed all EPS, whether capsule or a loose network, a glycocalyx. The bacterial cell is at a set distance from the surface, depending solely on the radius of curvature, and protrusions such as flagella, pili and loose strands of EPS can penetrate the energy barrier (Lewin, 1984). EPS are complex polymers, highly hydrated mucupolysaccharides, that vary not only between isolates, but also under different culture conditions and have recently been reviewed by Sutherland (1985) and by Whitfield (1988). They fill the space between cell and surface, thereby mediating attachment. During subculture on agar the ability to produce EPS is often lost (Beveridge, 1989).

As the surface is covered, lateral interactions between particles must be taken into account. If adsorbing particles are all of the same type, lateral interactions will be repulsive at low electrolyte concentrations, but attractive at high ones (Rutter and Vincent, 1984). Electrolytes actually decrease the repulsive effect of the electrical double layer sufficiently by changing the surface charge of particles. Ionic strength is strongly influenced by the charge of ions, as can be seen from the definition: $I = \frac{1}{2} \sum_1 c_i z_i^2$ where z represents charge (Laidler and Meiser, 1982). Therefore divalent cations such as Ca^{2+} and Mg^{2+} have a much larger effect. Ca^{2+} is rather insoluble in the basic surrounding of carbohydrates, and also with SO_4^{2-}. South African cooling waters contain between 254 and 514 ppm (6.35 - 12.85 mM) Ca^{2+} (Howarth and McEwan, 1989) and between 70 and 4120 ppm (0.7 - 43 mM) SO_4^{2-} (Kincer, Scheers and McEwan, 1989). Biofilms can therefore be expected to develop at a much faster rate in cooling waters than in natural waters.

The primary colonizers are mainly *Pseudomonas* species as they attach well and possess polar flagella (Jacques, Marrie and Costerton, 1987). During film development considerable amounts of EPS material is synthesized such that the mature biofilm contains only ten percent or less of its dry mass in the form of cells (Hamilton, 1985). Oxygen in the film is depleted by respiration and filamentous forms, mainly members of the genera *Hyphomicrobium, Sphaerotilus, Leptothrix* and *Beggiatoa* gain ecological advantage as they can obtain oxygen by protruding into the flow (Characklis, 1983). Young biofilms contain few species, reflecting the low diversity of pioneer populations (Atlas, 1984), but diversity increases to form a stable climax community. Diversity is always underestimated due to the selectivity and inadequacy of pure-culture isolation techniques (*cf.* 4.1.2).

Attached bacteria have different properties to those of free swimming or agar-grown bacteria. They exhibit profoundly different metabolism, and are often highly dependant on other members of the consortium (Costerton, Nickel and Ladd, 1986). Most morphological and physiological information has been determined on planktonic bacteria. Yet these make out less than 0.1 percent of most eco-systems (Costerton *et.al.*, 1986). Valeur, Tunlid and Odham (1988) showed that free-living

Gram-negative bacteria had different lipid compositions and a much higher poly-ß-hydroxybutyrate (PHB) content than attached bacteria. Attached bacteria had a higher ratio of unsaturated to saturated fatty acids and a higher ratio of C_{16} to C_{18} fatty acids.

As the biofilm develops, various gradients develop across it, as exchange only occurs on one side (Characklis, 1983). A nutrient gradient develops, with respiration at the upper layer and fermentation in the middle layer with the resulting release of fermentaion products such as ethanol, lactate and succinate (Pfennig, 1984). An oxygen gradient also develops, due to respiration of bacteria in the upper layer (Hamilton, 1987). When the biofilm has reached a thickness of 10 - 25 μm, conditions at its base are anaerobic (Hamilton, 1987). The biofilm is now approaching a state of maturity, with a high species diversity and consequent stability (Hamilton, 1987).

1.1.3 Physiology of biofilms

Mature biofilms are composed mainly of EPS (Hamilton, 1987) and glycoproteins (Characklis, 1983). The polymeric matrix has highly adsorptive capability, adsorbing cations into its structure which help to stabilize it (Characklis, 1983). Nutrients from the flow are also adsorbed onto microfibrils of the polymeric matrix, and this adsorptive ability is advantageous to surface-associated bacteria which have a higher metabolic rate than suspended bacteria (Wardell, Brown and Flannigan, 1983). Attached cells are further exposed to a larger flux of nutrients, as water flows over the surface continuously (Hobbie and Fletcher, 1988).

The high diversity results in more effective utilzation of substrates, especially of polymers. Extracellular enzymes are concentrated in the biofilm, supplying monomers at a much higher concentration than in the flow (Costerton *et.al.*, 1987). Due to the large gene pool, many synergistic interactions can be expected. A biofilm having a complex structure or high species richness needs a lower amount of energy to maintain itself (Atlas and Bartha, 1987a). There is a strong relationship between diversity and stability (Atlas, 1984), so the high degree of diversity of mature biofilms leads to a stable structure.

Mature biofilms finally mineralize organic compounds (Pfennig, 1984), and only inorganic compounds are produced, either as end products (eg. CO_2) or as reduced electron acceptors (eg. H_2S). They are vital in natural systems, as they mineralize organic substrates, thereby making elements available again (Hobbie and Fletcher, 1988). Examples are sediments in lakes and biofilms in rivers. Trickling filters in sewage works employ the same principle.

1.1.4 Detachment of cells from the biofilm

Allison, Evans, Brown and Gilbert (1990) showed that daughter cells of sessile *Escherichia coli* released into the planktonic phase had a much lower hydrophobicity than those remaining attached. They showed that late- exponential cells were less hydrophobic than lag phase or stationary phase cells. Delaquis, Caldwell, Lawrence and McCurdy (1989) and Allison, Brown, Evans and Gilbert, (1990) reported an increase in hydrophobicity of *Pseudomonas fluorescens* actively detaching from biofilm. Allison *et.al.* (1990a) proposed the following mechanism for release from biofilms: Surface appendages associated with adhesion increase or at least alter surface hydrophobicity, and such structures are minimized during and immediately after cell division, leading to separation and dispersal of one daughter cell. Wirth and Wanner (1989) showed that *Enterococcus faecalis* cell aggregation is controled by a pheromone which induces production of a dense hairlike layer called the aggregation substance.

Kjelleberg *et.al.* (1982) showed that a marine *Vibrio* which had attached due to low nutrient concentration regrew and left the surface upon increase in nutrient concentration. The cells became motile within seven minutes. Some of them divided while being attached to the surface. However Delaquis *et.al.* (1989) showed that *P. fluorescens* actively detached from biofilm upon nutrient limitation. Bacterial surface behaviour seems to be nutrient-induced. At high nutrient concentration, release from the biofilm aids in spreading of the species, and at to low concentration cells are released to search for nutrients. This hypothesis for low nutrient release is supported by the fact that *Bacillus* species become motile by producing flagella upon nutrient depletion (Doi, 1989). Release from mature biofilms seems to be periodic, *i.e.* different strains releasing progeny at different times (J. W. Costerton, personal communication[1]).

1.2 Planktonic Bacteria

Is the primary habitat of aquatic bacteria water or is it biofilm ? Costerton *et.al.* (1986) stated that planktonic bacteria make up *ca.* 0.1 percent of bacteria in nature. Van Loosdrecht *et.al.*, (1990) stated that the ability of micro-organisms to attach to a substratum often determines its ability to survive in a particular habitat. A common feature of pioneer organisms is their effective dispersal mechanism (Atlas and Bartha, 1987a). Primary colonizers of surfaces disperse by water flow. Therefore they must occur in the planktonic phase.

Planktonic bacteria are usually transient, depending on attachment to surfaces for survival (1.2). Even the srictly oligotrophic *Caulobacter* and *Asticacaulis* reproduce by attachment to suspended particles

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(Poitdexter, 1989). However certain bacteria, *eg. Prosthecobacter* are strictly planktonic (Poindexter and Staley, 1989), as are many of the appendaged bacteria. Although most bacteria are actually surface-growing, many can adapt well to planktonic growth, as proven by all the experimental work on growth-rate of bacteria in chemostats. They also proliferate in nutrient-rich liquids such as foodstuffs and effluent.

1.2.1 Natural interactions among planktonic bacteria

In nature pure cultures of bacteria are seldom encountered (Costerton *et.al*, 1987). Exceptions are infected animals where sterile conditions are contaminated by a pathogen circumventing host defence systems (Costerton *et.al*, 1981). Even extreme environments cary more than a single strain of bacteria (Jannasch and Mottl, 1985). Microbes involved in the anoxic degradation of organic matter exist in consortia, each group performing part of the degradation, and passing the end-product on to the next (Pfennig, 1984). This is known as synergism, but where the relationship is obligatory, it is mutualism (Atlas.and Bartha, 1987b). The same holds for biofilms (Costerton *et.al*, 1987).

Where a community is composed of many different species (high species diversity) the energy required for maintenance is lower (Atlas and Bartha, 1987a). The more diverse the population, the more readily it responds to change. Where species diversity is high, the system is biologically controlled, and abiotic stress is more easily overcome (Atlas and Bartha, 1987a). The more diverse the community, the more stable it is. According to Atlas (1984) stability occurs when several interacting populations co-operate to best exploit the available resources.

1.2.2 Dynamics in the aqueous systems

Bacteria have evolved strategies for survival and maintenance within communities (Atlas and Bartha, 1987a), attempting to maximize their fitness for survival either in crowded (K-selection) or in uncrowded (r-selection) environments (Andrews and Harris, 1986). Table 1 gives a summary of characteristics of r- and K-strategists compiled from Andrews and Harris (1986).

From table 1 it is clear that biofilm inhabitants are K-strategists, whereas planktonic bacteria are r-strategists. Although the r- K- division is one of extremes and most bacteria will occur on a scale between the two (Andrews and Harris, 1986), it is clear that r-strategists will occur chiefly in the planktonic phase, as they cannot compete well for nutrients and live under uncrowded conditions. K-strategists could not survive in the planktonic phase due to their low growth rate and decreased ability to adapt to fluctuating conditions. This could explain why planktonic bacterial population structures differ from biofilm population structures and why certain bacteria occur only in biofilms (Costerton *et.al.*, 1986).

Table 1: Properties of r- and K-strategists compiled from Andrews and Harris (1986).

	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 requirement:	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 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.

Planktonic bacteria include biofilm bacteria released by one of the following mechanisms: (i) as swarmer cells (Kjelleberg *et.al.*, 1982), (ii) bacteria released due to shear stress - where biofilm thickness exceeds the laminar sublayer, shear-stress increases dramatically (Bryers, 1988), and (iii) sloughing or periodic release of entire sections of biofilm due to production of volatile acids in the deep, anaerobic section (Bryers, 1988).

Planktonic bacteria are also subject to grazing by flagelates and ciliates (Gonzalez, Sherr and Sherr, 1990), whereas attached bacteria are afforded a degree of protection (Costerton *et.al.*, 1987). Bacteriophages also play a role in planktonic populations, but their presence does not bring about sterilization (Ogunseitan, Sayler and Miller, 1990). Biofilm bacteria seem to be protected from bacteriophage action (Costerton *et.al.*, 1987).

1.3 Reactions of Bacterial Populations under Stress

Microbial cells are in a dynamic state, adapting readily to shifts in environmental parameters (Roszak and Colwell, 1987). Stress is any factor which influences a bacterial cell in a limiting fashion. The cell is shifted away from its optimal growth conditions. Therefore stress is dependant on the growth requirements of the specific bacteria involved. In aqueous systems stress is usually due to nutrient limitations, or to substances toxic to the cell. *Caulobacter*, being a strict oligotroph, experiences high nutrient concentrations as stress (Pfennig, 1984).

Bacteria react differently to various stress conditions. *Arthrobacter* responds rapidly to low nutrient conditions by reducing endogenous respiration to extremely low levels (Pfennig, 1984). Many aquatic bacteria respond to low nutrient conditions by decreasing in size (Roszak and Colwell, 1987). Novitsky and Morita (1976) reported a marine *Vibrio* Ant-300 to change from rod to coccus and pass through a filter with pore size of 0.4 μm within 3 weeks of starvation. Such socalled minicells do not readily grow in laboratory media. Although Tuckett and Moore (1959) state that they cannot regrow, subsequent authors did report regrowth after long incubation at minimal nutrient concentrations (Tabor, Ohwada and Colwell, 1981). Other bacteria attach to surfaces upon low nutrient stress as discussed under 1.1 and in 1.1.4.

Many bacteria under environmental stress produce copious quantities of EPS (Beveridge, 1989). This serves as protectant against antagonistic molecules, host defense systems and abrasive forces. It also facilitates attachment to surfaces. When the stress is relieved, EPS production often ceases (Beveridge, 1989).

1.4 When is a Bacterial Cell Alive ?

The definition of procaryote viability is not simple. Postgate (1969) defined bacterial viability pragmatically as "the property possessed by that portion of a bacterial population capable of multiplying when provided with optimal conditions for growth." This definition is problematic in aquatic bacteriology as it is not always known what constitutes an optimal environment. An inherent char-

acteristic of many aquatic bacteria is their extremely low growth rate. Some bacteria have a mean generation time of 53 hours (Jannasch, 1969), and 48 or 72 hours' incubation would not detect such bacteria.

Many bacteria are sublethaly injured but not dead, and will grow actively after resuscitation (Roszak and Colwell, 1987). Heinmets, Taylor and Lehman (1953) showed that synthetic and metabolic growth processes continue in the absence of cell division due to heat or chemical stress. Depending on the nature of injury, the resuscitation required would be different. In quantification resuscitation should only aid in restoring cells, and not allow multiplication (Roszak and Colwell, 1987). Calcott and Postgate (1972) coined the term "pseudosenescent" for a state where bacteria have lost the ability to multiply as a result of certain stresses, but otherwise remain completely functional. There is sufficient evidence that metabolically active populations exist that do not divide, the so-called viable but non-culturable state (Roszak and Colwell, 1987). The traditional growth curves usually show a death phase after the stationary phase. A large proportion of these so-called dead cells is in the viable but nonculturable state (Roszak and Colwell, 1987). Such cells make a contribution to respiration and subsequent production in systems, although they are usually not detected.

2 MICROBIALLY INDUCED CORROSION

Microbially induced corrosion (MIC) is the corrosion of metals induced by the activity of one or more micro-organisms (Ford and Mitchell, 1990; Iverson, 1987). It occurs by any of a number of mechanisms, either directly or indirectly, but all these mechanisms relate to micro-organisms. Ford and Mitchell (1990) listed five factors responsible for MIC: These are (i) formation of oxygen concentration cells, (ii) formation of ion concentration cells, (iii) influence of iron- and manganese-oxidizing bacteria, (iv) acid production, and (v) creation of anaerobic niches. Because this form of metal corrosion is caused by actively metabolizing cells, *i.e.* by a biological process, Videla (1987) termed it biologically induced corrosion (BIC). Yet the most commonly coined term for the phenomenon is MIC. Pope, Zintel, Cookingham, Morris, Howard, Day, Frank and Pogemiller (1989) described MIC as the influence microbes exert by helping to establish conditions under which normal corrosion mechanisms operate, or by producing materials which affect the rate of corrosion, *eg.* organic acids, H_2S, *etc.*

MIC is caused by various micro-organisms including bacteria, fungi, and algae (Iverson, 1987). Sulphur oxidizers, mainly of the genus *Thiobacillus* form sulphuric acid which is a strongly corrosive agent, especially in concrete structures where steel is corroded and the carbonates are solubilized (Golovacheva, Rozanova and Karavaiko, 1986). The aerobic iron bacteria in the genera *Gallionella*, *Pedomicrobium* and *Siderococcus* oxidize ferrous iron to ferric iron ($Fe^{2+} \rightarrow Fe^{3+} + e$), catalyzing the deposition of tubercles, especially on stainless steel weld-seams (Iverson, 1987; Staley, Bryant, Pfennig and Holt, 1989). Members of the filamentous genus *Leptothrix* deposits ferric oxides in its

sheath (Mulder and Deinema, 1981; Staley *et. al.*, 1989). *Metallogenium*, *Pedomicrobium* and *Leptothrix* species oxidize manganese (Mn^{2+}) and deposit Mn^{4+} salts outside the cell wall (Sly, Hodgkinson and Arunpairojana, 1988; Staley *et.al.*,1989). Various *Pseudomonas* isolates have been implicated in reduction of ferric to ferrous iron, exposing steel to further oxidation as ferrous iron compounds are much more soluble and the protective ferric iron layer is solubilized (Obuekwe, Westlake, Cook and Costerton, 1981).

The fungi *Hormoconis resinae* and *Aspergillus fumigatus* cause corrosion in fuel tanks by profuse production of organic acids (Iverson, 1987). These fungi utilize C_9 - C_{16} hydrocarbons in fuel oils as carbon source, but require water as environment; as a consequence they grow at the oil-water interface (Smith and Maw, 1987). Various algae growing on metal surfaces promote corrosion by forming concentration cells, producing organic acids, and by supplying nutrients to other corrosion causing organisms (Iverson, 1987).

Sulphate-reducing bacteria (SRB) have been cited as the chief culprits of MIC (Hamilton, 1985; Iverson, 1987). In their presence steel and other alloys in anoxygenic aqueous surrounding corrode up to four times as fast as by normal oxygen-promoted corrosion (Brözel and Cloete, 1989; Hamilton, 1985). SRB associated corrosion is predominant where metal structures are covered by biofilm, and on metal structures in the seabed (Cord-Ruwisch and Widdel, 1986; Iverson, 1987). It is accompanied by sulphide deposits, chiefly ferrous sulphides (King, Miller and Wakerley, 1972). Hydrogen sulphide and some of the iron sulphides produced as a result of sulphate reduction are also corrosive (King, Miller and Smith, 1973).

2.1 Mechanisms of Corrosion

With the exception of some of the noble metals which may occur in the elemental state, metals are usually found in nature in their oxidized state, principally as oxides or sulphides (Chilton, 1973). On the contrary, metals employed in the omnipresent man-made structures used in our day and age are in the elementary state, a state of high energy which easily reverts in the presence of a suitable electron acceptor (Chilton, 1973). Corrosion is the electrochemical phenomenon occurring on metal surfaces, where atoms are exposed to an electron acceptor with a higher affinity than that of the potential donor. The result is a metal oxide or other salt having little structural ability, and the metal is rendered useless (Chilton, 1973).

Oxidation occurs as follows:

$$M \rightarrow M^{x+} + xe \quad \text{..... (1)}$$

The position of oxidation is termed the anodic site and the position of reduction or electron acceptance the cathodic site. As both metals and water are competent electron carriers, the anode and cathode are not of necessity in close proximity. At neutral pH and under oxygenic surrounding, the cathodic reaction is as follows:

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$$M \rightarrow M^{x+} + x\grave{e} \quad \text{.....} \quad (1)$$

The position of oxidation is termed the anodic site and the position of reduction or electron acceptance the cathodic site. As both metals and water are competent electron carriers, the anode and cathode are not of necessity in close proximity. At neutral pH and under oxygenic surrounding, the cathodic reaction is as follows:

$$H_2O + \tfrac{1}{2}O_2 + 2\grave{e} \rightarrow 2OH^- \quad \text{.....} \quad (2)$$

At lower pH the oxidant is the proton pool as follows:

$$2H^+ + 2\grave{e} \rightarrow 2H \rightarrow H_2 \quad \text{.....} \quad (3)$$

Corrosion under anoxygenic conditions is halted very quickly due to the build-up of hydrogen which is formed by reduction of protons derived from disproportionated water:

$$M \rightarrow M^{x+} + x\grave{e} \quad \text{.....} \quad (4)$$
$$xH_2O \rightarrow xOH^- + xH^+ \quad \text{.....} \quad (5)$$
$$xH^+ + x\grave{e} \rightarrow xH \quad \text{.....} \quad (6)$$

$$M + xH_2O \rightarrow M(OH)_x + xH \quad \text{.....} \quad (7)$$

From Nernst's theory (Laidler and Meiser, 1982) it follows that with increasing hydrogen concentration ([H]), the potential of the cathode will increase to equal that of the anode, and corrosion will stop. The hydrogen atoms formed dimerise to form hydrogen gas. The corrosion products Fe_xS_y, often poison dimerisation of hydrogen radicals (King *et al.*, 1973), and these radicals enter the metal structure, causing embrittlement and hydrogen cracking of the structure (Iverson, 1987).

2.2 Corrosion by Microorganisms

2.2.1 Sulphate-reducing bacteria and anaerobic corrosion

As shown in equation (7), oxidation of iron in the absence of oxygen is halted by the build-up of hydrogen. Where corrosion does take place, an alternative mechanism/s of electron acceptance must

be functioning. This entails two categories, either removal of hydrogen to maintain a favourable potential for corrosion, or production of an alternative oxidant. Research has revealed three mechanisms, all of which contribute to anoxygenic corrosion of iron.

2.2.1.1 Removal of cathodic hydrogen

Von Wolzogen-Kühr and Van de Vlugt (Hamilton, 1985) proposed the first accepted theory to explain corrosion of iron by SRB. Here sulphate is the actual oxidant in the corrosion of iron, and hydrogen acts only as an intermediate electron carrier. The end product is ferrous sulphide as shown in the following reaction sequence:

$$4Fe \rightarrow 4Fe^{2+} + 8\grave{e} \quad (8)$$

$$8H_2O \rightarrow 8OH^- + 8H^+ \quad (9)$$

$$8H^+ + 8\grave{e} \rightarrow 8H \quad (10)$$

$$SO_4^{2-} + 8H \rightarrow 4H_2O + S^{2-} \quad (11)$$

$$Fe^{2+} + S^{2-} \rightarrow FeS \quad (12)$$

$$3Fe^{2+} + 6OH^- \rightarrow 3Fe(OH)_2 \quad (13)$$

$$4Fe + 4H_2O + SO_4^{2-} \rightarrow FeS + 3Fe(OH)_2 + 2OH^- \quad (14)$$

SRB proliferate in the anaerobic section of biofilms (Hamilton, 1987), but why ? Firstly biofilms are anaerobic at their base (*cf.* 1.1.2). Secondly iron in an aqueous anoxygenic surrounding has a positive hydrogen potential at its surface (Iverson, 1987). Many SRB can utilise hydrogen as an energy source (Badziong and Thauer, 1980), or at least catalyze oxidation of hydrogen (Cord-Ruwisch and Widdel, 1986). Bryant and Laishley (1990) reported that cathodic hydrogen was removed by bacterial hydrogenase, an enzyme produced by most SRB and certain *Clostridium* species. Certain SRB can grow on hydrogen and sulphate as sole energy source (Brandis and Thauer, 1981). Although there was much controversy about utilisation of cathodic hydrogen in the older literature, it now appears to be an accepted fact (Brözel and Cloete, 1989).

2.2.1.2 Corrosion by iron sulphides

At low free ferrous iron concentrations a ferrous sulphide layer is formed on the metal surface by Fe^{2+} reacting with H_2S produced by SRB (King *et.al.*, 1972). Although this film initially protects the surface, decreasing the corrosion rate, it is dependent on the free ferrous iron concentration. At low concentration (25 uM) it transforms to the more corrosive greigite (Fe_3S_4) and crumbles at higher concentrations (125 uM) (King, Dittmer and Miller, 1976), exposing the surface for further corrosion.

King and Miller (1971) found conclusively that iron sulphide, and not cathodic iron was the cathode, hydrogen evolution occuring there. King *et.al.* (1976) later showed that iron sulphides caused mass-loss of mild steel in sterile surroundings. The crystaline nature of iron sulphide had a profound influence, pyrite (FeS_2) corroding iron in a 1:1.1 ratio. The iron sulphide cathode is the catalyst for dimerisation of atomic hydrogen, but is poisoned by adsorbed molecular hydrogen. Therefore the corrosive potential of the crystalline state is inversely proportional to its hydrogen adsorption rate. This finding does not exonerate SRB from their purported corrosion-promoting action as they are the only quantitative producers of sulphide reported from biofilms (Brözel and Cloete, 1989).

2.2.1.3 Corrosion by reduced phosphorus compounds

Phosphate is similar in structure to sulphate, the phosphorus atom having a redox state of +V. Iverson (1968) reported that *Desulfovibrio desulfuricans* produced colloidal iron phosphate in yeast extract broth in presence of steel and a hydrogen atmosphere. Weimer, Van Kavelaar, Michel and Ng (1988) reported that phosphate increased the rate of corrosion of carbon steel in presence of *D. desulfuricans*, especially when the organic energy source was the limiting substrate.

Although the main corrosion product was mackinawite (Fe_9S_8), significant amounts of phosphorus compounds were also present. Some phosphate occurred in a more reduced form. Furthermore the bacteria contained electron dense granules containing iron and phosphorus but not in polyphosphate form. Because reduced phosphorus is highly reactive, it could contribute to corrosion of iron. Its production by SRB has not been conclusively shown to date.

2.2.1.4 Sulphate-reducing bacteria and the myths

Much of the data on SRB in the scientific literature is unreliable due to use of wrong culture media (Widdel, 1988). Counts of less than one $cfu.ml^{-1}$ are not proof of the absence of SRB. Production of H_2S is also not proof of the presence of SRB as this could be produced by various other bacteria, eg. *Proteus, Salmonella, Pseudomonas* etc. Many anaerobic heterotrophs produce H_2S from cysteine or methionine (Stanier, Ingraham, Wheelis, and Painter, 1986). SRB also produce EPS and this is stabilized by Fe^{2+} and not by Ca^{2+} or Mg^{2+} (Beech and Gaylarde, 1989). Therefore it cannot be dislodged by EDTA. Corrosion therefore stabilises biofilms containing SRB.

SRB can grow in otherwise aerobic surrounding, and have been isolated out of microniches 50 - 200 μm in diameter (Cypionka, Widdel and Pfennig, 1985). Against popular belief SRB are not strict anaerobes. *Desulfovibrio desulfuricans* strain CSN reduced up to 5mM O_2 in sulfide- and sulphate-free medium (Dilling and Cypionka, 1990). Various SRB survived six minutes' exposure to oxygen, after

which oxygen stress was irreversible (Cypionka *et.al.*, 1985). However low redox potential as poised by reducing agents does not protect SRB from oxygen toxicity and enumeration of stressed cells must occur under oxygen-free conditions (Cypionka *et.al.*, 1985).

2.2.2 Acid-producing bacteria

The most directly corrosive metabolites produced by microorganisms seem to be acids (Ford and Mitchell, 1990). Acetic and butyric acids are examples of such corrosive microbial products (Ford and Mitchell, 1990). Clostridia produce organic acids as end-products of fermentation, and Pope, Zintel, Kuruvilla and Siebert (1988) reported corrosion of carbon steel by *Clostridium*-produced acids. However SRB utilize a wide range of organic acids as carbon source (Widdel, 1988). As SRB are present in mature biofilms where SO_4^{2-} is present, the contribution of acid-producing clostridia to MIC is questionable. The sulphur-oxidizing bacteria produce sulfuric acid from sulphur or sulphide (Iverson, 1987). There appears to be evidence for corrosion of concrete and steel pipes carrying municipal water (Ford and Mitchell, 1990).

2.2.3 Iron-reducing bacteria

Various pseudomonads have been implicated in reduction of ferric (Fe^{3+}) to ferrous iron (Fe^{2+}) (Obuekwe *et.al.*, 1981). *Shewanella putrefaciens* has been shown to reduce Fe^{3+} under anaerobic conditions *via* both a constitutive and an inducible Fe(III)-reducing system (Arnold, Hoffmann, DiChristina and Picardal, 1990). As ferric iron is insoluble except at very low pH (Huheey, 1978), ferric salts protect the metal surface from further corrosion due to chemical activity. Ferrous salts are mostly soluble and therefore reduction of ferric salts results in removal of the protective layer. Therefore iron-reducing pseudomonads promote corrosion indirectly. Certain bacteria are capable of reducing Fe^{3+} anoxically (Ghiorse, 1988). These include members of the following genera: *Bacillus, Pseudomonas, Paracoccus, Micrococcus, Corynebacterium, Escherichia, Enterobacter, Citrobacter, Serratia, Proteus, Alcaligenes, Vibrio, Clostridium, Bacteroides* and *Desulfovibrio*. Most of these stop reducing Fe^{3+} in the presence of either O_2 or NO_3^-.

2.2.4 Iron-oxidizing bacteria

The aerobic iron bacteria in the genera *Gallionella, Pedomicrobium* and *Siderococcus* oxidize ferrous (Fe^{2+}) to ferric iron (Fe^{3+}) (Staley *et.al.*, 1989). Tubercles are initially formed by the deposition of iron and manganese oxides (Ford and Mitchell, 1990). These appear mainly at weld seams (Iverson, 1987). The bacteria produce exopolymers which bind the mineral deposit together, forming an oxygen-proof structure. *Pedomicrobium manganicum* oxidizes manganese in drinking water systems,

and binds colloidal MnO_2 in the extracellular polysaccharide (Sly, Arunpairojana and Dixon, 1990). They can even oxidize ferrous iron on stainless steel (Iverson, 1987). The tubercles have a steep pH gradient, the pH inside being very low (Ford and Mitchell, 1990).

Various filamentous iron-accumulating bacteria have been isolated from water systems (Ford and Mitchell, 1990). Examples are *Leptothrix*, *Crenothrix* and *Sphaerotilus*. Mulder (1989) maintains that although *Leptothrix* accumulates Fe^{3+}, this is no proof of oxidation of Fe^{2+}, and that the organism merely accumulates ferric oxide in its sheath.

2.2.5 Algae

Algae play an important role in MIC by virtue of their capability to produce molecular oxygen, corrosive organic acids, slime, and nutrients for other microorganisms involved in MIC (Ford and Mitchell, 1990). Two species of blue-green algae (*Nostoc paramelioides* and *Anabaena sphaerica*) have been implicated in corrosion of mild- and stainles-steel weld-seems (Iverson, 1987). Various hydrogenase-positive *Chlorophyta* and *Cyanophyta* species utilize cathodic hydrogen (Iverson, 1987).

2.3 Concentration Cells

Various microstructural factors influence corrosion of metals, as do the solubilities of their hydroxides (Iverson, 1987). Materials deposited on the surface and impermeable to oxygen (eg. insoluble hydroxides) cause anodic sites. The areas directly adjacent act as cathode. Such structures are called oxygen concentration cells, and cause pitting corrosion (Iverson, 1987). The EPS material excreted by attached bacteria causes sites of differing cathodic activity, resulting in an electrical potential across the surface (Beech and Gaylarde, 1989). Bradley and Gaylarde (1988) showed that *Desulfovibrio vulgaris* lipopolysaccharide has a specific iron-binding site within its polysaccharide side chain. Whether or not this plays a role in cathodic polarisation is not clear as yet.

2.4 Fouling

Mature biofilms vary in thickness, but in water systems they are between 10 and 100 μm thick (Iverson, 1987). Such a biofilm can, if it covers the hull of a ship, retard its spead by 20 percent, or reduce flow in a thin pipe (12.5 mm diameter) by 50 percent (Lewin, 1984). Fluid frictional resistance is increased in all water systems when film thickness surpasses the monolayer (McCoy, Bryers, Robbins and Costerton, 1981). The conductivity of biofilms is similar to that of stationary water, but much lower than that of metals (Characklis, 1983). It therefore acts as an insulator, increasing heat transfer resistance. Table 2 compares the thermal conductivities of certain materials.

Biofilms are stabilised by deposition of cations in the acidic polysaccharide (Costerton *et.al.*, 1987). *Pedomicrobium manganicum* oxidizes manganese, and binds colloidal MnO_2 in the extracellular polysaccharide (Sly *et.al.*, 1990).

Table 2: Thermal conductivity of biofilms and other selected materials relevant to biofouling of heat exchangers. (After Characklis, 1983).

Material	Thermal conductivity ($W.m^{-1}.K^{-1}$)	Temperature (^oC)
Biofilm (various)	0.68 - 0.27	28.3 ± 0.3
	0.71 - 0.39	26.7 ± 0.3
	0.57 - 0.10	28.3 ± 0.3
Water	0.61	26.7
Carbon steel	51.92	0 - 100
Steel	46.86	18
Stainless steel (316)	16.30	0 - 100
Copper	384	18
Glass	0.6 - 0.9	-

3 MICROBIOLOGICAL MONITORING TECHNIQUES

3.1 Quantification of Bacteria in Water Systems

The determination of bacterial numbers is a basic prerequisite in microbiology and yet the study of microorganisms in nature remains one of the most poorly quantitated areas of microbiological research (Karl, 1986). To date our attempts at understanding microbial ecology are limited by the lack of suitable methods (Karl, 1986). Quantification of live bacterial cells in medical or food samples can be performed using reliable counting techniques such as the agar plate count (VanDemark and Batzing, 1987). However live bacteria from aquatic and soil environments cannot be accurately enumerated (Roszak, Grimes and Colwell, 1984; Atlas and Bartha, 1987c). Non-critical use of so-called standard methods may yield data of questionable accuracy as microorganisms live in microenvironments having physicochemical properties which may be distinctly different from those of the surrounding environment (Karl, 1986).

3.1.1 Available techniques of enumeration

A range of direct counting techniques have been developed to count dead and living cells separately. These have been listed by Roszak and Colwell (1987) and all are based on the indication of metabolic activity of the living cells. Some techniques require culturing of the sample on a thin nutrient agar

film and subsequent microscopic counting of micro-colonies formed (Torella and Morita, 1981). Others are based on inhibition of cell division by inhibitors such as nalidixic and oxolinic acid (Kogure, Simidu and Taga, 1978). Cells elongate or enlarge, but do not divide. Nalidixic and oxolinic acids both inhibit DNA synthesis in Gram negative bacteria by inhibiting the ATP-dependant action of DNA gyrase (Franklin and Snow, 1981). They are ineffective against Gram positive bacteria, and Gram negative ones can become resistant by mutations in the *NalA* gene (Franklin and Snow, 1981). Therefore this approach is only advisable where samples contain mostly Gram negative bacteria. A promising microscopic approach employs the formation of intracellular granules after reduction of tetrazolium salts by cellular hydrogenases (Zimmerman, Iturriaga and Becker-Birck, 1978). Examples are INT (2-[4-Iodophenyl]-3-[4-nitrophenyl]-5-phenyl- tetrazolium chloride, TTC (2,3,5-Triphenyltetrazolium Chloride) and MTT (3-[4,5-Dimethyl- thiazol-2-yl]-2,5-diphenyltetrazolium bromide) of which INT seems to give the best results due to minimal reaction with bactericides and other substances in cooling water (this laboratory). An accurate technique is differential staining by DNA-RNA stains (Francisco, Mah and Rabin, 1973), but probes specific to the bacteria in question must be available. Uptake or release of radioactive compounds is often employed in natural systems (Hoppe, 1976). Examples are incorporation of tritiated thymidine ([methyl-^{3}H]thymidine) into bacterial DNA (Wicks and Robarts, 1987), measurement of $Zn^{35}S$ produced by SRB from $^{35}SO_4^{2-}$ (Maxwell and Hamilton, 1986), and determination of $^{14}CO_2$ produced by respiration of glucose (Roszak and Colwell, 1987). Roszak and Colwell (1987) list many more. All of the latter techniques encompass pre-treatment of cell suspensions and require either complex equipment or highly skilled operators. Determination of the numbers of viable bacteria in water on a routine basis is still performed by the agar plate count method in most South African laboratories.

3.1.2 The aerobic plate count

The agar plate count is based on the asumption that a viable bacterial cell is capable of multiplying to form two progeny and so on under conditions that are "optimal" for the cell concerned (Postgate, 1969). Continued growth under such conditions on agar would result in a vissible colony (Hattori, 1988). The count therefore reflects the number of cells capable of dividing under the given conditions, and not necessarily the total viable number. Due to the physiological diversity of microbial groups and the physicochemical diversity of their microenvironments, no single medium has ever been able to provide for the growth requirements of more than a small percentage of the total number of bacteria in a natural habitat (Karl, 1986). Direct microscopic counts of aquatic samples are always much higher than plate counts (Jannasch and Jones, 1959; Kogure *et.al.*, 1978; Cloete *et.al.*, 1989). Kogure *et al.* (1978) reported the agar plate count of open sea samples to be 0.1 percent of the direct count. As living and dead particles could not be distinguished directly, this ratio could however have been too low (Kogure *et al*, 1978). Costerton *et.al.* (1986) state that *ca.* 0.1 percent of biofilm bacteria grow on nutrient agars.

The bacterial culture media commonly used in laboratories are mostly designed for the cultivation of human pathogens or other fastidious bacteria and contain mainly proteinaceous substrates such as peptones and meat- and yeast extract (Stolp and Starr, 1981). Agars commonly used to determine viable bacterial numbers in waters, *eg* Plate Count Agar and Standard I nutrient agar, fall in this category. Yet planktonic bacteria in water systems have different nutrient requirements to the fastidious bacteria mentioned above (Pointdexter, 1981). Most nutrient media contain more than 2 grammes of carbon per liter, whereas oligotrophic habitats contain in the order of 1 - 15 milligrammes (Roszak and Colwell, 1987). Heinmets *et.al.*, 1953 and Reasoner and Geldreich (1985) reported that many non-culturable aquatic bacteria grow on media containing certain fermentation products, *eg.* pyruvate or acetate. Consequently many viable bacteria do not form visible colonies on certain agars, resulting in a false low count (Roszak *et.al*, 1984; Sørheim, Torsvik and Goksøyr, 1989). Inability of bacteria to form colonies on agar may also be due to substrate-induced death or to other cell conditions discussed in 4.1.1.1 and 4.1.1.2 below.

3.1.2.1 Colony forming ability

The number of colonies formed in the standard plate count are normally counted after 48 or 72 hours (Gibbs and Hayes, 1988 ;Greenberg, Trusell and Clesceri, 1985). Yet water contains many slow-growing bacteria which take longer to form a visible colony (Hattori, 1988; Reasoner and Geldreich, 1985). There is also a time lag before initiation of cell division takes place on agar surfaces, similar but longer than in liquid culture (Hattori, 1988). This time lag is not identical for every cell, but is distributed around a mean value (Hattori, 1988). Hattori (1988) devised the technique of plotting the number of visible colonies formed against time on semi-log paper. The resulting plots are termed colony forming curves. From this the kinetics of colony formation can be calculated and the optimal incubation time determined.

Cells in the viable but nonculturable state do not form colonies on agar (Roszak and Colwell, 1987). This is supported by the large discrepancy between agar counts and viable counts determined microscopically (Kogure *et.al.*, 1978). Bacteria exposed to high osmotic stress by NaCl could regain colony-forming ability by pretreatment with betaine (Roth, Leckie and Dietzler, 1988). *Salmonella enteritidis* induced to lose colony-forming ability by low nutrient stress, regained it after 25 hours' exposure to heart infusion broth (Roszak *et.al.*, 1984).

Stress can also be exerted by the enumeration method. Klein and Wu (1974) showed that heterotrophic bacteria in water samples are susceptible to heat stress by warm agar during the pour plate procedure. Starvation seemed to make cells more susceptible to heat stress. Certain nutrients are toxic to certain bacteria, whereas others are necessary. Although this may sound trivial, many microbiologists ignore the environment of bacteria when attempting to isolate or enumerate on agar (Roszak and Colwell,

1987). If a specific nutrient is withheld from a cell where all others are abundantly available, unbalanced growth occurs; the cell grows but does not divide and subsequently dies (Dawes, 1989). The composition of a nutrient agar for enumeration of viable bacterial cells in a specific environment should therefore be tailored to the specific nutritional requirements of the relevant bacteria or of the majority thereof.

3.1.2.2 Substrate-induced death

Substrate-induced death was first reported by Postgate and Hunter (1964). They termed it substrate accelerated death. They found that starved cells which remained viable died upon exposure to certain carbon sources. The limiting nutrient determined which carbon source would bring about death. Although the phenomenon of substrate-induced death has not been explained to date, it plays a role in decreasing bacterial counts on nutrient-rich agars.

3.2 Bactericide Activity

The efficacy of a bactericide programme relies on the active concentration of bactericide in the system. EPS adsorb bactericides, rendering them inactive (LeChevalier *et.al.*, 1988). Secondly bactericide activity is not linearly related to concentration (Hill, Hill and Robbins, 1989).

3.2.1 Bactericide concentration

The concentration of available bactericide in water can be determined chemically, although this is not easy for most compounds. Determination of most organic bactericides at low concentrations is an expensive and laborious exercise. However substances such as chlorine and peroxides can be measured easily at low concentrations by one of a number of kits available (*e.g.* Merck (Pty) Ltd.).

3.2.2 Bactericide activity

Bactericide activity is a measure of whether or not the bactericide kills bacteria at the present concentration (Cloete, Brözel and Da Silva, 1990). A number of kits to determine bactericide activity are available (Hill *et al.*, 1989). These involve bacterial spores immobilized onto a test strip with an indicator such as a tertrazolium salt. If the bactericide concentration is to low, the spores germinate and grow, reducing the tetrazolium to a coloured form. A colour change indicates to low a concentration. Other bio-indicators are currently being evaluated in this laboratory. An example is Sterikon(R) from Merck (Pty) Ltd. This contains spores of *Bacillus stearothermophilus* in nutrient broth with bromocresol purple as indicator, and was originally designed for testing heat-sterilization processes.

4 COMPONENTS INVOLVED IN BACTERIAL RESISTANCE

Bactericides are antimicrobial agents employed in various spheres of human activity to prevent, inhibit or eliminate bacterial growth. By virtue of the dynamic of life, organisms have been equipped with various mechanisms to survive. According to Whitehead (1929), organisms have two poles on their environment: They have a physical pole, in which they collect their reaction from the given world from their perspective on it; and they have a mental pole, in which they react, "feel and organise themselves to have an influence on the future." Bacteria can react to stimuli challenging their survival, and some do adapt to a "resistant" form. But let us take a closer look , at three components involved in bacterial survival in cooling water systems, *viz.* the aqueous environment, the bacterial cell itself, and the bactericides.

4.1 Bacteria in the Aqueous Environment of Cooling Systems

All bacteria require an aqueous environment for growth because water is the basis for the colloidal nature of the cytoplasm (Stryer, 1981). However, some bacteria have evolved to grow in water bodies as their natural habitat (Hobbie & Fletcher, 1988). Cooling water systems are water bodies, the properties of which are conducive to the growth of a range of bacteria. They are well aerated so that aerobes can grow well in the water phase as well as at surfaces. Convection currents which serve to cool the water carry in dust from the surroundings, so that the water contains a variety of organic nutrients such as cellulose and starch. The incoming water contains salts which are concentrated by evaporation. Many systems are maintained at 16 cycles of concentration (Poulton, 1992 - Personal communication[2]) so that they contain up to 514 ppm Ca^{2+} and 4120 ppm SO_4^{2-} (Brözel, 1990). The water is passed over large surface areas in pipelines and heat exchangers, providing favourable conditions for bacterial attachment. Surfaces offer bacteria a nutritional advantage by attracting amphipathic molecules such as proteins, phospholipids and glycoproteins (Fletcher, 1991; Van Loosdrecht *et al.*, 1990). Most planktonic bacteria attach readily to most surfaces upon approaching these (Lawrence *et al.*, 1987). Bacterial attachment and subsequent growth leads to the formation of biofilms which lead to fouling of the systems and to increased corrosion (Cloete *et al.*, 1992; Ford & Mitchell, 1990).

During biofilm development considerable amounts of EPS material is synthesized such that the mature biofilm contains only ten percent or less of its dry weight in the form of cells (Christensen & Characklis, 1990; Hamilton, 1985). Mature biofilms are composed mainly of EPS (Hamilton, 1987) and glycoproteins (Characklis, 1983). The polymeric matrix has highly adsorptive capability, adsorbing cations into its structure which help to stabilize it (Characklis, 1983). Nutrients from the flow are also adsorbed onto microfibrils of the polymeric matrix, and this adsorptive ability is advantageous to surface-as-

2 ESKOM Engineering Investigations, Rosherville, Johannesburg, South Africa.

sociated bacteria which have a higher metabolic rate than suspended bacteria (Wardell *et al.*, 1983). Attached cells are further exposed to a larger flux of nutrients, as water flows over the surface continuously (Hobbie & Fletcher, 1988).

4.1.1 Microbially induced corrosion

Bacterial activity in biofilms influences corrosion of underlying metal surfaces by a number of mechanisms, a phenomenon termed microbially induced corrosion (Cloete *et al.*, 1992). Ford and Mitchell (1990) listed five factors responsible for microbially induced corrosion: These are (i) formation of oxygen concentration cells, (ii) formation of ion concentration cells, (iii) influence of iron- and manganese-oxidizing bacteria, (iv) acid production, and (v) creation of anaerobic niches harbouring sulphate-reducing bacteria. Pope *et al.* (1989) described MIC as the influence microbes exert by helping to establish conditions under which normal corrosion mechanisms operate, or by producing materials which affect the rate of corrosion, *eg.* organic acids, H_2S, *etc.*

Sulphate-reducing bacteria (SRB) have been cited as the chief culprits of MIC (Ford & Mitchell, 1990; Hamilton, 1985). In their presence steel and other alloys in anoxygenic aqueous surrounding corrode up to four times as fast as by normal oxygen-promoted corrosion (Brözel & Cloete, 1989; Hamilton, 1985). SRB-associated corrosion is predominant where metal structures are covered by biofilm, and on metal structures in the seabed (Cord-Ruwisch & Widdel, 1986; Iverson, 1987). Removal or death of respiring aerobic bacteria renders the lower biofilm layers aerobic, leading to inactivation and death of SRB. Biofouling control is the science of the removal of bacteria from surfaces in order to halt microbially induced corrosion and fouling (Cloete *et al.*, 1992).

4.1.2 Bacteria under stress

Microbial cells are in a dynamic state, and many can adapt readily to changes in environmental parameters (Roszak & Colwell, 1987). The aqueous environment of cooling water systems has fluctuating parameters so that bacteria are exposed to fluctuating conditions (Cloete *et al.*, 1992). Conditions which are different to the optimal growth requirements of any bacterial culture exert stress on the cells where stress is any factor that influences a bacterial cell in a negative fashion. Bacteria react differently to various stress conditions (Nyström *et al.*, 1992). In aqueous systems, stress is usually due to nutrient limitations or to substances toxic to the cell. *Escherichia coli* can produce a variety of stress proteins upon exposure to toxic substances or to high temperature (Blom *et al.*, 1992). The functions of some stress proteins have been elucidated, *eg.* heat shock response proteins and SOS response proteins in *E. coli* (Warner-Bartnicki & Miller, 1992) but the functions of many stress proteins are, as yet, unknown. Nyström *et al.* (1992) found that a marine *Vibrio* produced various stress proteins 20 min after carbon, nitrogen or phosphorous starvation. *Arthrobacter* responds rapidly to low nutrient conditions by reducing endogenous respiration to extremely low levels (Pfennig,

1984). Many aquatic bacteria respond to low nutrient conditions by decreasing in size (Roszak & Colwell, 1987). Novitsky and Morita (1976) reported a marine *Vibrio* Ant-300 to change from rod to coccus morphology and pass through a filter with pore size of 0.4 μm within 3 weeks of starvation.

A general low nutrient stress response of aquatic bacteria is attachment to surfaces (Kjelleberg *et al.*, 1982). Many bacteria under environmental stress produce copious quantities of EPS (Beveridge, 1989). This serves as protectant against antagonistic molecules, host defense systems and abrasive forces. It also facilitates attachment to surfaces and the concentration of nutrients from the surroundings (Costerton *et al.*, 1981). When the nutrient stress is relieved, EPS production often ceases. This holds especially where nitrogen stress is relieved (Beveridge, 1989).

4.2 The Structure of the Bacterial Cell Envelope

The bacterial cell could be defined in many ways, but it has three decisive components, *viz.* a genome controlling all functions, a cytoplasm facilitating protein synthesis and general metabolic activity, and a cell envelope delineating and upholding that which is the cell from the immediate environment. For purposes of this review, only the eubacterial cell structure will be discussed.

Bacteria, being unicellular and potentially free-living entities, must be able to perform all functions necessary for survival and procreation as single cell entities (Moat & Foster, 1988). In this regard membranes play the central role in the structure and function, defining the compartment and determining the nature of all communication between the cell and its immediate environment (Gennis, 1989). All those structures involved in delineating the cell contents to the immediate environment, are collectively termed the cell envelope. The cell envelope regulates the passage of salts and organic molecules, upholding the osmotic potential, supplying the respiratory process with compatible sources of energy, excluding deleterious substances and determining cell shape (Gennis, 1989). The eubacteria can be divided into two distinct morphological groupings on the grounds of their cell envelope structure; gram negative and gram positive. Although the division rests on a basic staining procedure devised by the Danish microbiologist, Dr Hans Christian Gram, various ultrastructural studies have revealed that the stain reflects two cell envelope structures of considerable difference to a remarkably accurate degree (Moat & Foster, 1988). The common denominator of the two morphotypes is a cytoplasmic membrane which is composed of a phospholipid bilayer containing various proteins. The membrane houses the enzyme system of the electron transport chain and for oxidative phosphorylation, protein systems for the transport of solutes, attachment point for DNA replication, and the synthetic apparatus for the production of polymers for structures outside of the cytoplasmic membrane

(Hammond *et al.*, 1984; Moat & Foster, 1988). Further, certain gram positive and negative bacteria have an externally located S-protein layer (Meissner & Sleytr, 1992), and can produce extracellular polysaccharide material (Costerton *et al.*, 1987).

The gram positive cell envelope is considerably more simple than that of the gram negative cell. It is made of an external cell wall and a cytoplasmic membrane (Hammond *et al.*, 1984). Gram positive cells are therefore less selective; they are less protected from the entry of deleterious substances. The cell wall contains approximately 50 % of peptidoglycan. The rest of the material is an array of accessory polymers; teichoic and teichuronic acids and a few proteins (Hammond *et al.*, 1984). The cytoplasmic membrane is similar to that in gram negative bacteria. Because gram positive cells are far less resistant to bactericides than are gram negative ones, the following will concentrate on components of the latter.

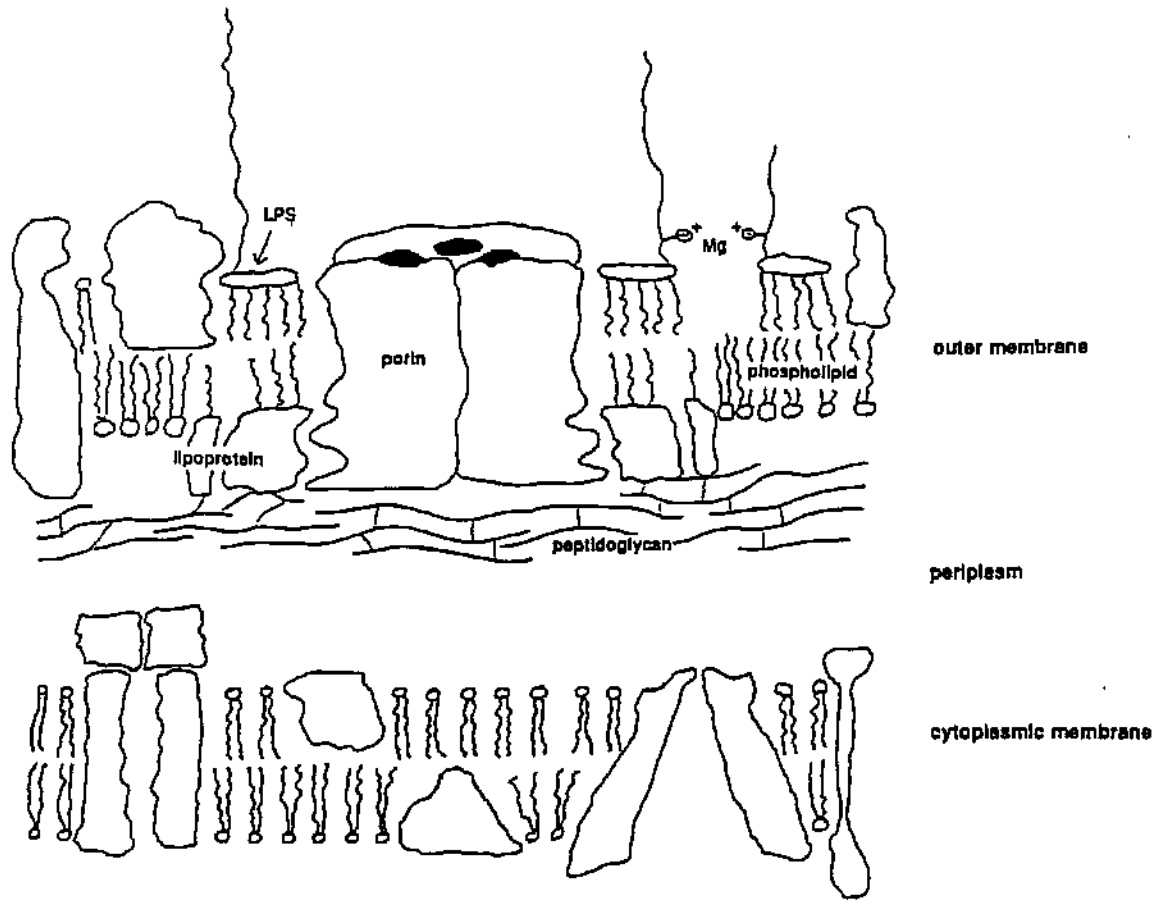


Figure 2: Diagrammatic representation of the cell envelope of a gram negative cell (from Hancock, 1991).

The gram negative cell envelope is composed of three distinct layers, *viz.* the outer membrane, peptidoglycan layer and the cytoplasmic membrane on the inside (Hammond *et al.*, 1984). The exact topology of these three layers is not clear to date, as can be seen from the various diagrams published in recent years. Examples can be found in Hammond *et al.* (1984, p 58), Hancock (1991, p 175), Moat and Foster (1989, p72), Russell and Chopra (1990, p 20), and Tommassen (1988, p 352). The point of discrepancy is in regard to the positioning of the peptidoglycan layer between the outer and cytoplasmic membranes.

Whereas most authors place it adjacent to the cytoplasmic membrane and depict it as supported by Braun's lipoproteins (anchored in the outer membrane), Hancock (1991) shows it to be adjacent to the outer membrane (Fig. 2). Peptidoglycan is a gel-like structure formed by cross-linking of short pieces of the polymer (Hancock, 1991). It is permeable to the constituents of the periplasmic space. Most of the work has been performed using *Escherichia coli* as a model, and extrapolating the information to all other gram negative families. Whereas in *E. coli*, Braun's lipoprotein is linked to peptidoglycan, it is not covalently linked in *Pseudomonas aeruginosa* (Hancock *et al.*, 1990). This could indicate that there are more differences between the cell envelopes of various gram negative species, a point which is supported by the differences between the outer membrane proteins of *E. coli* and *P. aeruginosa* (Nikaido, 1992). The rationale is that, what text books say is usually true for *E. coli*, but it is not necessarily true for other gram negative bacteria.

4.2.1 The outer membrane

The outer membrane is the interface between the immediate environment and the interior of the cell (Hancock, 1991). It is much more selective than the old textbooks taught, and its main function is to exclude a variety of potentially deleterious molecules while permitting selective uptake of others (Nikaido & Vaara, 1987). It is an asymmetric bilayer with lipopolysaccharides forming the outer leaflet, and phospholipids forming the inner leaflet (Gennis, 1989; Hammond *et al.*, 1984). Proteins having various functions are inserted in the bilayer. An average *E. coli* cell contains 2 X 10^5 outer membrane proteins which cover 1.8 μm^2 or 27 % of the surface area. The largest part of the surface area, 4.9 μm^2 or 73 % is covered by an average of 34.6 X 10^5 molecules of lipopolysaccharides (LPS) (Nikaido & Vaara, 1987). Hydrophilic molecules above a certain size, as well as hydrophobic molecules, are excluded from the cell by the LPS-protein combination.

4.2.1.1 Outer membrane proteins

Bacterial outer membrane proteins are vital structures of gram negative cell function. Three functions have been identified. The majority are permeation channels and form the link between the periplasmic space and the immediate environment by forming channels or pores through which solutes of up to 600 D can pass (Nikaido & Vaara, 1987; Tommassen, 1988). In *E. coli* most permeation channels are non-specific and have only two selective properties, *ie.* maximum size of the solute and charge of

the solute. Solutes are not transported actively as is the case with transport across the cytoplasmic membrane (Hancock, 1991; Nikaido & Vaara, 1987). Outer membrane protein nomenclature has been standardised. In *E. coli* proteins are termed Omp*X* (Nikaido & Vaara, 1987), and in *P. aeruginosa* they are termed Opr*X* (Hancock *et al.*, 1990). *P. aeruginosa* has only one general porin, OprF, and this permits only a slow rate of solute diffusion, probably due to a large proportion of porins in a collapsed state (Nikaido, 1992). It does have a larger inside diameter than the porins of *E. coli*, affording passage for molecules over 2000 D, including tetrasaccharides (Bellido *et al.*, 1992). In contrast to *E. coli*, most *P. aeruginosa* permeation channels are what Nikaido (1992) termed specific channels. They are selective for the passage of either specific solutes, *eg.* OprP which selects for PO_4^{-2} (Hancock *et al.*, 1987), or for groups of solutes, *eg.* OprD which selects for basic amino acids (Hancock *et al.*, 1990). Structural analogues of specific solutes can also pass through. An example is the antibiotic imipenem which passes through OprD (Quinn *et al.*, 1986).

Whereas *E. coli* has only two dominant outer membrane proteins and a few inducible ones, *P. aeruginosa* produces a wide array and often in equal quantities (Nikaido, 1992). Of the proteins reported to date, only OprF, OprL, OprI and a 55 kD esterase are constitutive (Hancock *et al.*, 1990).

Outer membrane proteins are abundant; together OmpF and OmpC, the general porins of *E. coli*, make up 2 % of the total cell protein (Nikaido & Vaara, 1987). Many outer membrane proteins are under environmental control (Nikaido, 1992). Production of OmpF is suppressed at 37 °C or by high osmolarity, conditions mimicking the interior of the digestive tract of most animals. Whereas cholic acid, a powerful detergent, can pass through OmpF, it cannot pass through the smaller channel of OmpC. *E. coli* can therefore grow well under otherwise unfavourable conditions (Nikaido & Vaara, 1987). Pho E, the phosphate uptake porin is produced under conditions of low phosphate, whereas Lam B, the maltose uptake porin is produced where maltose is present (Nikaido & Vaara, 1987). In *P. aeruginosa*, many outer membrane proteins are under environmental control. OprP is only produced under conditions of low phosphate, and OprB is produced where glucose is available (Hancock *et al.*, 1990).

OprH is a structural protein in *P. aeruginosa* which replaces the function of divalent cations such as Ca^{2+} or Mg^{2+} in habitats where these are deficient (Hancock & Carey, 1979). Cells with a high OprH content are not affected by treatment with ethylenediaminetetraacetate (EDTA) (Nicas & Hancock, 1980). OprH also inhibits self-promoted antibiotic uptake by replacing divalent cations at negatively charged sites on LPS (Nicas & Hancock, 1980). Isolates of *P. aeruginosa* resistant to gentamicin, an antibiotic entering the cell by self-promoted uptake, produce large quantities of OprH, replacing divalent cations. Thereby they prevent the self-promoted uptake of gentamicin (Bell *et al.*, 1991).

Lipoproteins are the third type of proteins in the outer membrane. In *E. coli* they are called Braun's lipoprotein and in *P. aeruginosa* they are OprI. Braun's lipoprotein is associated with peptidoglycan, partially covalently bound and partially non-covalently associated (Nikaido & Vaara, 1987). In *P. aeruginosa* the binding to peptidoglycan is not clear (Hancock, 1991; Mizuno & Kageyama, 1979). OprL is strongly bound to peptidoglycan via covalently bound fatty acyl chains (Hancock *et al.*, 1990).

Table 3: Outer membrane proteins of *Pseudomonas aeruginosa* described to date (compiled from Grabert *et al.*, 1990; Hancock *et al.*, 1990; Hancock *et al.*, 1992; Huang *et al.*, 1992).

Protein	Genetic name	Mr*	Conditions favouring production	Function
IROMP	-	78 - 87	Low iron	Fe^{3+} - siderophore- binding
C	OprC	70	ND	Porin ?
Esterase	-	55	Constitutive	Growth on acyl esters
Alginate	-	54	ND	Biosynthesis / excretion of alginate
O	OprO	50	ND	Diphosphate uptake
P	OprP	48	Low phosphate	Monophosphate transport
D1	OprB	46	Glucose	Glucose uptake
D2	OprD	45.5	Certain C sources	Basic amino acids
E	OprE	44	ND	Porin
F	OprF	38	Constitutive	Porin
G	OprG	25	High iron, certain carbon sources	Unknown
H1	OprH	21	Low divalent cation	Stabilising Mg^{2+} deprived cells
H2	OprL	20.5	Constitutive	Structural lipoprotein
FBP	Fbp	9 - 14	Low iron	Ferripyochelin binding and transport
I	OprI	9	Constitutive	Structural lipoprotein

ND = not determined

* = mass in kD as estimated by SDS PAGE

4.2.1.2 Lipopolysaccharides

Lipopolysaccharides (LPS) are amphiphilic molecules composed of sugars and lipids (Hammond *et al.*, 1984). In bacteria, lipopolysaccharides are located in the outer leaflet of the outer membrane (Nikaido & Vaara, 1987). Their structure determines colony morphology; strains producing full LPS (smooth strains) develop into colonies with a smooth glossy appearance whereas those with incomplete LPS (rough strains) form colonies having an irregular appearance (Hammond *et al.*, 1984). Bacterial LPS has four structural zones having varying degrees of conservation between species (Fig. 3). The zone inserted into the outer leaflet of the outer membrane is lipid A, and is highly conserved. Four fatty acid chains are linked to a diglucosamine backbone (Hammond *et al.*, 1984). The intermediate zone is a complex oligosaccharide chain. Two 3-deoxy-D-manno-2-octulosonate (KDO) residues form the link between lipid A and the outer core (Nikaido & Vaara, 1987). LPS mutants having only lipid A and KDO are termed deep rough mutants (Hancock, 1991). Rough mutants have a complete core section which is composed of a linear covalently linked series of saccharides with side chain saccharides (Hammond *et al.*, 1984). These are rich in charged groups, especially negative ones (Nikaido & Vaara, 1987). Smooth strains have long chains of repeating tri- or tetrasaccharides, the O-specific chain or O-antigen, which give gram negative bacteria their serological characteristics (Kastowsky *et al.*, 1992; Nikaido & Vaara, 1987). The many serovars of *Salmonella* differ only in the composition of their O-specific chain (Hammond *et al.*, 1984).

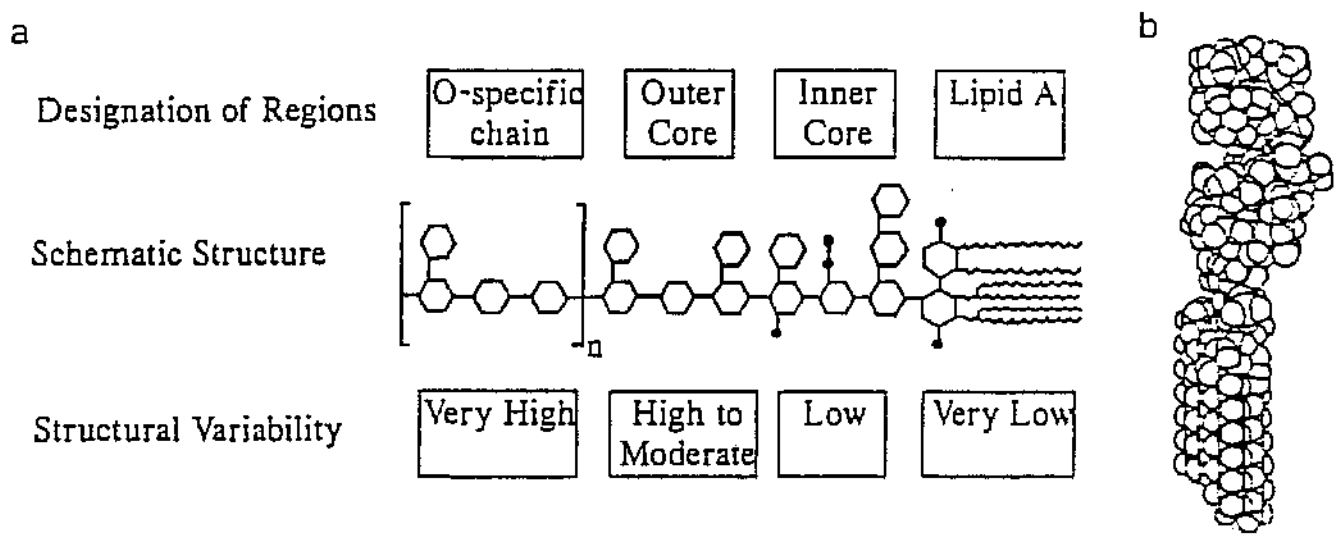


Figure 3: Schematic representation of the general structure of bacterial lipopolysaccharide (LPS) (a) and of a space-filling model of LPS (b) (after Kastowsky *et al.*, 1992)

LPS plays the primary role in exclusion of hydrophobic compounds. The basis for this exclusion ability is twofold. Firstly, there is a strong association between LPS due to cation bridging, constituting a physical barrier (Hancock, 1991). Secondly, both the long O-specific chain and the outer core prevent passage of hydrophobic molecules due to their charged nature. Most gram negative strains isolated from natural environments are smooth (Hancock, 1991). Lipid A is an exclusion barrier to hydrophilic compounds due to its hydrophobic nature and interaction with lipid chains of phospholipids of the inner leaflet (Gennis, 1989).

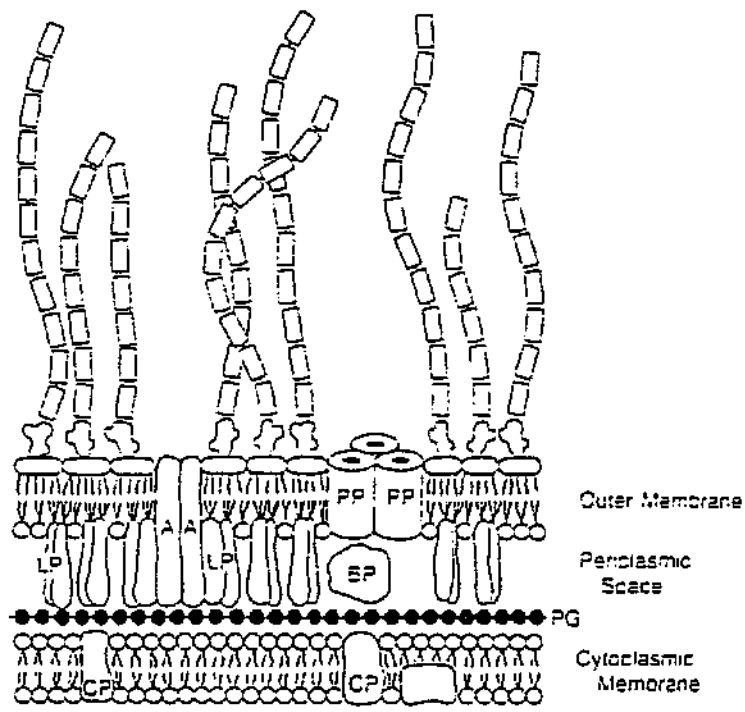


Figure 4: Schematic representation of the outer membrane of a gram negative cell with special reference to lipopolysaccharide (after Gennis, 1989). L = lipopolysaccharide, PP = porin protein, LP = lipopolysaccharide, CP = cytoplasmic protein, PG = peptidoglycan, A = OmpA, BP = solute transport binding protein.

4.2.1.3 Lipids

Lipids of prokaryotic membranes are all amphipathic phospholipids (Goldfine, 1988). In the gram negative outer membrane only the inner leaflet is phospholipid, whereas the outer leaflet is LPS (Hancock, 1991; Nikaido & Vaara, 1987). The predominant group (phosphatidylglycerol, diphosphatidylglycerol, phosphatidylcholine, *etc.*) have a negative polar head group and are bilayer-forming phospholipids due to their cylindrical shape (Gennis, 1989). To allow for curvature of the membrane, some cone-shaped phospholipids having a dipolar ionic head are also present (*eg.* phosphatidylethanolamine and diphosphatidylglycerol - Ca^{2+}) (Hammond *et al.*, 1984). The resultant membrane is a stable bilayer where phospholipids diffuse laterally within leaflets, but cannot transfer between leaflets (Gennis, 1989).

The three-dimensional structures of the various proteins embedded in lipid bilayers are dependant on interactions with the adjacent lipids, and on membrane fluidity (Cronan *et al.*, 1987). Depending on temperature, phospholipids associate in one of three phases; gel (rigid), ripple (transition) or liquid crystalline phase (Gennis, 1989). After transition to the liquid crystalline state (melting), phospholipids remain arranged in a bilayer state, but the bilayer thins due to greater vibrational energy and consequently covers a larger surface area (Cronan *et al.*, 1987). The single most important contributing factor to phase transition temperatures is the fatty acyl chain (Gennis, 1989). Phase transition temperature is decreased by unsaturated C-C bonds (a *cis* double bond has a larger effect than a *trans* double bond), by cyclopropane groups, and by shorter chain length (Hammond *et al.*, 1984). Changes in conditions of growth (temperature, presence of organic solvents) within the range permitting growth affect the structure of fatty acyl chains synthesized. Bacteria are able to regulate the degree of saturation and the chain length of fatty acyl chains as a response to external stimuli in order to maintain a fluid but stable membrane (Goldfine, 1988).

4.2.2 Cytoplasmic membrane

This is a true phospholipid bilayer carrying the enzyme systems for the electron transport chain (the cytochromes) and for oxidative phosphorylation, as well as various systems for active transport of solutes (Hammond *et al.*, 1984). The phospholipid bilayer provides the hydrophobic layer required to allow differential concentration of protons and molecules, which is necessary for cell function (Cronan *et al.*, 1987). The cytoplasmic membrane contains *ca.* 100 different proteins with a variety of functions and making up 60 % of the cell envelope protein (Hammond *et al.*, 1984).

4.2.3 Peptidoglycan

This is the material which lends all eubacteria their characteristic shapes (Koch, 1988). It is a mixed polymer of carbohydrates and amino acids found only in bacteria (Harold, 1990). It consists of linear

polymers of alternating ß(1 -> 4) linked N-acetylglucosamine and N-acetylmuramic acid residues cross-linked with tetrapeptides (Hammond *et al.*, 1984; Park, 1987). Covalent bonding is often so complete that the cell is surrounded by a single peptidoglycan molecule to form a sacculus (Harold, 1990; Park, 1987). The internal pressure is *ca.* 5 atm in gram negative cells, and *ca.* 20 atm in gram positive cells, and is borne by the peptidoglycan sacculus (Harold, 1990).

4.3 Extracellular Polysaccharides

Many gram negative bacteria can produce extracellular polysaccharides (EPS) (Hammond *et al.*, 1984). In some cases EPS is tightly associated with the cell and has a clearly delineated border, termed a capsule. In other cases the EPS is a loose network appearing as slime (Costerton *et al.*, 1981). The colloidal nature of the EPS is determined by its monosaccharide composition, and by the salt content and pH of the environment (Christensen & Characklis, 1990; Neu & Marshall, 1990). Some bacteria produce defined homopolysaccharides under all conditions where the mechanism of synthesis can be defined; *eg.* EPS of *E. coli* K1 which is a sialic acid homopolymer (Hammond *et al.*, 1984). Most bacteria produce heteropolysaccharides as EPS. The group of monomers remains the same, but the molar ratio does vary considerably according to strains and cultural differences (Whitfield, 1988). *P. aeruginosa* produces the acidic EPS alginate which is composed of segments of ß-1,4-linked poly-D-mannuronic and poly-L-guluronic acids (Maharaj *et al.*, 1992; Sutherland, 1985). Bacterial EPS production is controlled by environmental parameters in certain bacteria. During subculture of biofilm isolates on agar the ability to produce EPS is often lost (Beveridge, 1989). *P. aeruginosa* PAO produces alginate under conditions of high osmolarity (Deretic *et al.*, 1989). High concentrations of Na^+, K^+, Ca^{2+} and Cl^-, as well as 1 % ethanol activate transcription of *algD* by an osmoregulatory DNA-binding protein, AlgR1 (Berry *et al.*, 1989; Maharaj *et al.*, 1992). EPS production by *Xanthomonas* sp. is regulated by the C/N ratio of the environment (Sutherland, 1985).

In natural biofilms where various bacterial species are present, the EPS (glycocalyx) is composed mainly of mannans, glucans and uronic acids (Hamilton, 1987). Most bacterial EPS is anionic (Whitfield, 1988), so that the glycocalyx of biofilms is polyanionic. Some authors have equated it with an anion exchange matrix serving to concentrate organic nutrients while limiting the penetration of charged molecules (Hamilton, 1987; Costerton *et al.*, 1981). However, not much is known about the physical properties of EPS and many authors ascibe properties or functions which have not been demonstrated. Christensen and Characklis (1990) refer to this as "the magic substances that mediate otherwise unexplained phenomena". Yet biofilm phenomena are determined by the gel matrix of EPS surrounding cells and stabilising them on surfaces (Christensen & Characklis, 1990). Diffusion coefficients in biofilms are *ca.* 80 % of those for water for most substances (Christensen & Characklis, 1990). However, diffusion coefficients of larger molecules out of the same biofilms are much slower

(Christensen & Characklis, 1990). This implies that the glycocalyx does act as a trap or sink for organic molecules. Further research will have to be performed on the effect of EPS on the passage of substances to and from bacterial cells.

4.4 Bactericides

Bactericides are antimicrobial agents employed in various spheres of human activity to prevent, inhibit or eliminate microbial growth. They can be divided into two groups; those occurring naturally and mostly produced by prokaryotic organisms (termed antibiotics), and those not occurring readily in nature (termed antiseptics, disinfectants, biocides, bactericides, sanitisers and preservatives). Members of the second group are classified, depending either on their chemical nature, but more often on their specific field of application (Table 4).

Table 4: Classification of antimicrobial agents according to their application

Field	Specific field	Term
Medical	Human disease Skin Hospital surfaces	antibiotic antiseptic disinfectant
Food	Product Factory surfaces	preservative disinfectant / sanitiser
Domestic	Drains and toilets Baby nappies	sanitiser disinfectant
Swimming pool	Clear clean water	pool cleaner
Potable water	Disinfection	sanitizer
Industry	Water cooling Paper industry Leather tanning Metal working Petrochemical	water treatment biocide slimicide preservative biocide biocide

The use of bactericides to control biofouling in water cooling systems is an accepted practice (Cloete *et al.*, 1992). A recent market survey indicated that the direct prevention costs in terms of bactericide usage in South Africa was R 19.6 million (Brözel, 1990). Although bactericides are employed to reduce bacterial numbers, mere use of the correct bactericides does not necessarily reduce the fouling rate. It is essential to apply the correct dosage at the correct frequency to maintain antibacterial activity in the water.

The modes of action of a plethora of antibiotics have been investigated in detail. Various journals are devoted to this topic, examples being *Antimicrobial Agents and Chemotherapy* and *Journal of Antimicrobial Chemotherapy*. Little has been published on the mechanisms of action of most bactericides and antiseptics. Exceptions are quaternary ammonium compounds and biguanides. Generally, bactericides are not as site-specific as are the antibiotics (Heinzel, 1988).

Bactericides attack functional cell components, placing the bacterium under stress (Wainwright, 1988). At low concentrations bactericides often act bacteriostatically, and are only bacteriocidal at higher concentrations (Woodcock, 1988). Targets of bactericide action are components of the cytoplasmic membrane or of the cytoplasm (Gilbert & Wright, 1987; Russel & Chopra, 1990). For bactericides to be effective, they must attain a sufficiently high concentration at the target site in order to exert their antibacterial action. In order to reach their target site(s), they must traverse the outer membrane. Therefore different bacteria react differently to bactericides due to differing cell wall properties (Nikaido, 1992; Paulus, 1987). Bacteria with effective penetration barriers to bactericides display a higher inherent resistance than those bacteria which are readily penetrated (Heinzel, 1988). The rate of penetration is linked to concentration, so that a sufficiently high bactericide concentration will kill bacteria with enhanced penetration barriers (Russell & Chopra, 1990). An example is the antimicrobial activity of thiocarbamate against 18 isolates from cooling water systems (Brözel and Cloete, 1991a). At 50 ppm only certain isolates were killed, whereas 174 ppm lead to the death of isolates with improved barrier properties such as *P. fluorescens* and *P. pickettii* (Fig. 5).

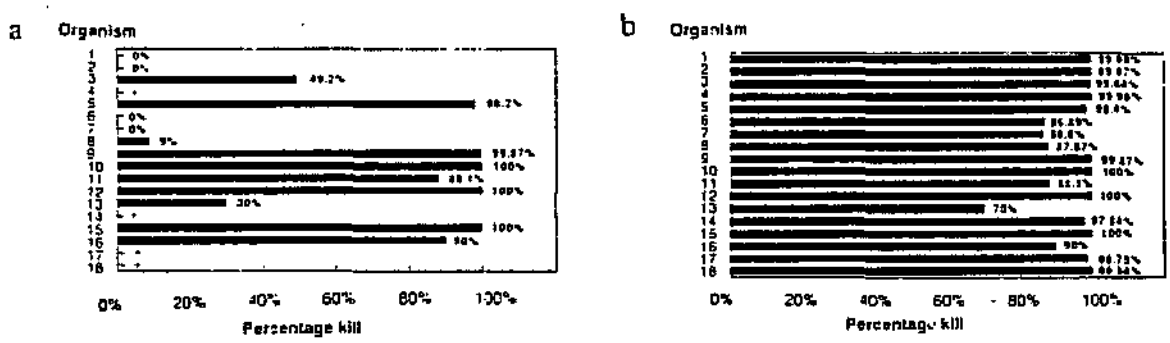


Figure 5: Fingerprints of thiocarmabate (50 ppm)(a) and thiocarbamate (174 ppm)(b) determined for 18 bacterial isolates dominant in South African Cooling water. Culture 2 is *P. fluorescens*, and culture 6 is *P. pickettii* (after Brözel & Cloete, 1991a).

Water treatment bactericides fall into two categories, oxidising (*eg.* chlorine and hydrogen peroxide) and non-oxidising. Non-oxidising bactericides can be divided into five groups based on their chemical nature or mode of action, and these will be discussed below.

4.4.1 Detergent type bactericides

Three groups of surface-active antimicrobial agents have been documented to date; anionic, cationic, and amphoteric (Wallhäußer, 1988). Anionic antimicrobials are only effective at pH < 3.0 and include the aliphatic acids such as Na dodecyl sulphate (Wallhäußer, 1988). The cationic antimicrobial agents are the quarternary ammonium compounds which are well documented and widely used. The best known one is benzalkonium chloride which is actually a group of compounds with varying chain lengths of the aliphatic chain (C_8 - C_{18}) (Wallhäußer, 1988) and has the following structure (Fig 6):

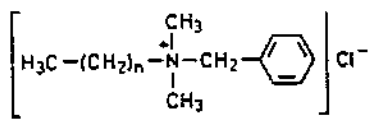


Figure 6: Chemical structure of the quarternary ammonium compound benzalkonium chloride (Wallhäußer, 1988).

Benzalkonium chloride adsorbs to the cell surface of negatively charged cells (pH > 7.0) in an irreversible way (Russel & Chopra, 1990). The pH minimum for any antimicrobial activity is 3.0. It is membrane active and induces leakage of cytoplasmic constituents. Upon exposure to benzalkonium chloride, membranes of *P. cepacia* appeared irregular, indicating membrane damage (Richards & Cavill, 1980). At 37 °C it is twice as active as at 20 °C (Wallhäußer, 1988). It is active against gram positive but also against gram negative cells, but not against spores (Russel & Chopra, 1990). Cations such as Ca^{2+} and Fe^{3+} decrease its activity, as does NaCl (Wallhäußer, 1988).

4.4.2 Biguanides

Biguanides are polymer derivatives of a general guanidine structure (Fig. 7):

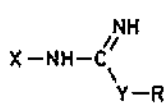
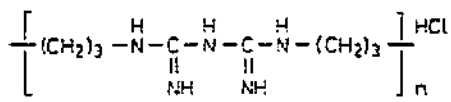


Figure 7: General chemical structure of guanidines (Wallhäußer, 1988).

Two biguanides are currently used as industrial bactericides. These are polyhexamethylene biguanide (PHMB) and 1,6-di-(4-chlorophenyldiguanido)-hexane, better known as chlorhexidine (Wallhäußer, 1988). Both are non corrosive and all are well suited for application in cooling water (Woodcock, 1988).

a



b $C_{22}H_{30}Cl_2N_{10}$ MG: 505.48 F: 134 °C

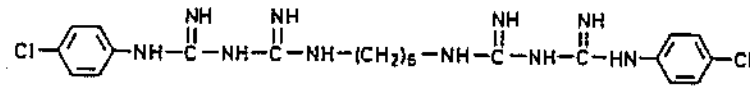


Figure 8: Chemical structure of PHMB (a) and of chlorhexidine digluconate (b) (Wallhäußer, 1988).

Biguanides are bacteriostatic at low concentrations and bactericidal at higher concentrations, and have a wide spectrum of activity, especially against gram negative bacteria (Wallhäußer, 1988). They are membrane active agents and attach rapidly to negatively charged cell surfaces (pH neutral or alkaline). Fitzgerald *et al.* (1992), using ^{14}C-radiolabelled PHMB, showed that it was absorbed into cells of *E. coli* within 20 s after exposure. Bactericidal action did, however, require a few min. Biguanides compete with divalent cations for negative sites at LPS, displacing these. PHMB then interacts by elecrostatic interactions with the charged headgroups of phosphatidylglycerol and diphosphatidylglycerol (negative), but not with the neutral phosphatidylethanolamine (Broxton *et al.*, 1984). By binding to phospholipids of the inner leaflet of the outer membrane and of the outer leaflet of the inner membrane, the two membranes attain net positive charges and are repelled from each other, causing membrane damage by distortion (Broxton *et al.*, 1984). This is supported by TEM studies on *P. cepacia* where both membranes acquired a distinct irregular appearance after treatment with chlorhexidine (Richards & Cavill, 1980). Cytoplasmic constituents start leaking out of the cell due to rupturing of the membranes, and the cell loses its viability.

4.4.3 Aldehyde type bactericides

Two aldehydes are commonly used as antimicrobial agents, *ie.* formaldehyde and gluteraldehyde. Further there is a range of bactericides such as the hydroxyethyl- and ethyltriazine- bactericides available which all release formaldehyde (Sondossi *et al.*, 1986). Formaldehyde has a high polarity and high nucleophilic reactivity, so that it reacts primarily with free primary amino groups, but also with amines, amides, sulfides, purines and pyrimidines (Rossmoore & Sondossi, 1988). In water it hydrates to methylene glycol. Reaction with primary amino groups leads to the formation of methyloamines which react further with cellular components (Rossmoore & Sondossi, 1988). Formaldehyde damages the transport properties of membrane porins, decreasing the rate of proline uptake and of enzyme synthesis (Barnes & Eagon, 1986). It is active over a wide pH spectrum (3.0 - 10.0), and is sporicidal (Wallhäußer, 1988).

Gluteraldehyde also reacts with amino and sulfhydryl groups (Russell & Chopra, 1990). It is stable in acid solution but is only active at pH 7.5 - 8.5, so it must be alkalinified before application (Wallhäußer, 1988). A 2 % solution at the correct pH is ten times more bactericidal than a 4 %

solution of formaldehyde (Wallhäußer, 1988). Its reactivity is related to temperature; a 2 % solution kills spores of *Bacillus anthracis* in 15 min at 20 °C, whereas it requires only 2 min at 40 °C. In gram positive bacteria it reacts with, and binds to, peptidoglycan and teichoic acid, and is also sporicidal (Russell & Chopra, 1990). In gram negative bacteria it reacts primarily with lipoproteins of the outer membrane, preventing the release of membrane-bound enzymes (Russell & Chopra, 1990).

4.4.4 Phenol derivatives

Phenol was the antimicrobial agent which revolutionised invasive surgery, and was pioneered by Lister in 1870 (Franklin & Snow, 1981). It enters the cell by dissolving in the membrane, and upon entry into the cytoplasm, precipitates proteins (Wallhäußer, 1988). It is, however, harmfull to humans, and its antibacterial activity is not very high. A range of halogenated phenols, cresols, diphenyls and bisphenols have been developed from phenol, and have excellent antimicrobial activity, many being applied in the preservation of pharmaceutical products (Wallhäußer, 1988). Halogenation increases the antimicrobial activity of phenol, as does the addition of aliphatic and aromatic groups. Bisphenols have the highest antimicrobial activity of the phenol derivatives, especially halogen substituted ones. Hexachlorophen and 2,2'-methylenebis(4-chlorophenol) (dichlorophen) fall into this group (Wallhäußer, 1988). Growth of *P. aeruginosa* is inhibited by 36 $\mu g.ml^{-1}$ of the bisphenol dichlorophen whereas it is only inhibited by 1000 $\mu g.ml^{-1}$ of the diphenyl o-phenylphenol (Brözel & Cloete, 1993; Wallhäußer, 1988).

Phenol derivatives are membrane active agents. They penetrate into the lipid phase of the cytoplasmic membrane, inducing leakage of cytoplasmic constituents (Russell & Chopra, 1990). 3- and 4-chlorophenol uncouple oxidative phosphorylation from respiration by increasing the permeability of the cytoplasmic membrane to protons (Gilbert & Brown, 1978).

4.4.5 Thiol-oxidising bactericides

Thiols on amino acids such as cysteine are important groups which influence the tertiary structure of proteins by the forming disulphide bridges (Stryer, 1981). Three groups of antimicrobial agents, isothiazolones, Bronopol (2-bromo-2-nitropropane-1,3-diol) and mercury and other heavy-metal compounds, react with accessible thiols, altering the three dimensional structure of enzymes and structural proteins (Collier *et al.*, 1991; Russell & Chopra, 1990). Mercury interacts with sulfhydryl groups by complexing with sulphur (Russel & Chopra, 1990; Wallhäußer, 1988). Bronopol oxidises thiols to disulphides, reacting especially with the active center of hydrogenase enzymes (Wallhäußer, 1988).

Three isothiazolones possess antibacterial activity; 5-chloro-N-methylisothiazolone (CMIT), N-methylisothiazolone (MIT) and benzisothiazolone (BIT) (Collier *et al.*, 1990a; Wallhäußer, 1988). Isothiazolones react oxidatively with accessible thiols such as cysteine and glutathione (Fig. 10) (Collier *et al.*, 1990b). These thiols are reduced to their disulphide adjuncts which, in the case of cysteine, leads to an alteration of protein conformation and functionality. Isothiazolone is hereby oxidised to mercaptoacrylamide, which in the case of CMIT tautomerises to thioacyl chloride, the latter reacting with amines such as histidine and valine (Collier *et al.*, 1991). Isothiazolones are primarily bacteriostatic, and are only bactericidal at high concentrations (Collier *et al.*, 1991).

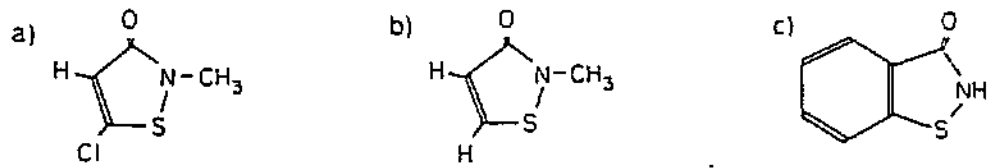


Figure 9: Structures of 5-chloro-N-methylisothiazolone (a), N-methylisothiazolone (b) and benzisothiazolone (c) (Collier *et al.*, 1990b).

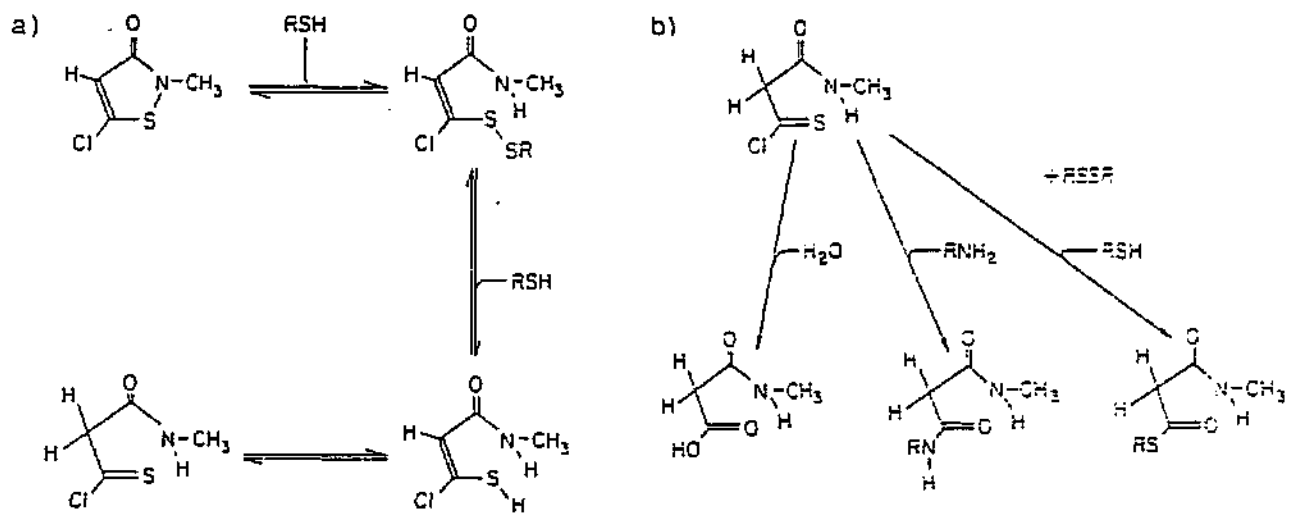


Figure 10: Reaction mechanism of 5-chloro-N-methylisothiazolone with accesible thiols (RSH) (a), and the proposed pathway of interaction of thioacylchlorides with amines, thiols and water (b) (after Collier *et* al., 1990b).

4.4.6 Miscellaneous bactericides

The mechanisms of action of various antimicrobial agents, employed to control bacterial growth in cooling water systems, have not been formally published to date. These include phosphonium chloride

(tetra-alkyl-phosphonium chloride), thiocarbamates (Na diethyldithiocarbamate) and MBT (methylene bis-thiocyanate). Phosphonium chloride probably has surfactant properties, damaging the bacterial cell envelope. The mechanism of antimicrobial action of MBT is not known to date.

Thiocarbamates have the following structure (Fig. 11)

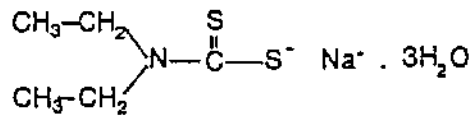


Figure 11: Chemical structure of Na diethyldithiocarbamate.

Thiocarbamates are used as agents for the extraction of trace metals such as Fe, Cd, Co, Cu, Mn, Ni, Pb and Zn (Lo *et al.*, 1982). This would imply that it chelates iron, a vital trace element of most bacteria (Visca *et al.*, 1992). The nucleophilic sulphur atom indicates potential reactivity with accesible thiols, but this would have to be investigated further.

4.4.7 Factors affecting bactericide effectivity

The antibacterial activity of bactericides is determined by their chemical reactivity with certain organic groups. Bactericides do not select between free and cell-bound groups. Therefore oxidising bactericides react with any readily oxidisable organic compound, and not only with live cells. Bactericide activity is influenced by the chemistry of the surroundings where it is employed (Wallhäußer, 1988). Factors effecting bactericide effectivity are the following:

* pH
* water hardness
* organic compounds such as proteins or saccharides
* additives such as antiscaling agents or corrosion inhibitors

These factors affect different bactericides to different degrees. Table 5 summarises the operating parameters for various bactericides.

Some bactericides are not very stable in concentrated form and undergo changes. Formaldehyde polymerises when exposed to polar compounds (acids or alkalis) or high temperature and oxidises to formic acid when exposed to air (Wallhäußer, 1988). Isothiazolones are unstable at temperatures above 40 °C and chlorhexidine is unstable above 70 °C (Wallhäußer, 1988). A decrease in the efficacy

of a bactericide treatment programme can be due to a decrease in bactericide activity, or due to inactivation by adverse conditions, and does not always indicate bacterial resistance (Cloete *et al.*, 1992).

Table 5: Operating parameters for selected bactericides (compiled from Collier *et al.*, 1990a; Wallhäußer, 1988).

Bactericide	Reaction time	pH range	Bactericidal	Sporicidal
Chlorhexidine	Rapid	5 - 9; increasing from 5	+	-
Formaldehyde	Slow	3 - 10	+	+
Formaldehyde releasers	Very slow	4 - 8	+	+
Gluteraldehyde	Rapid	7.5 - 8.5	+	+
Isothiazolone	Slow	4 - 7	+	-
Phenol derivatives	Rapid	2 - 9; decreasing from 4	+	-
PHMB	Rapid	5 - 9; increasing from 5	+	-
Quaternary ammonium	Slow	5 - 9	+	-

Table 6: Toxicity and mutagenicity data for selected bactericides (after Collier *et al.*, 1990a; Wallhäußer, 1988).

Bactericide	LD_{50} value	Mutagenicity
Chlorhexidine	Mouse: 2000 $mg.kg^{-1}$	Ames +
Formaldehyde	Rat: 800 $mg.kg^{-1}$	Ames - Mouse lymphoma test +
Gluteraldehyde	Rat: 60 $mg.kg^{-1}$	Ames -
Isothiazolone(Kathon CG)	Rat: 3350 $mg.kg^{-1}$	Ames + Mouse lymphoma test +
Dichlorophen	Rat: 2690 $mg.kg^{-1}$	No data
PHMB	Rat: 5000 $mg.kg^{-1}$	No data
Quaternary ammonium	Mouse: 300 $mg.kg^{-1}$	Ames -
Thiocarbamate	No data	No data

Some bactericides, being toxic substances, have a broader spectrum of toxicity than merely bacteria. Some are mutagens or even carcinogenic (Wallhäußer, 1988). Table 6 summarises the LD_{50} values for the bactericides discussed in section 1.4, as well as mutagenicity data where available.

4.5 Test Procedures for the Determination of Bactericide Efficacy

There are many fields of applications for antimicrobial agents (Table 4) and the conditions in all these fields vary (Eigener, 1988). Therefore tests designed to determine the antimicrobial efficacy of antimicrobial agents must be tailored to reflect the efficiency of the agent for the relevant application (Russell *et al.*, 1982). There can be no general method of evaluation of efficacy. Tests must, however, be described and performed in a clear manner so that results can be compared (Eigener, 1988). A variety of tests are used in various countries so that comparison of results is often inappropriate (Eigener, 1988; Russell *et al.*, 1982; Wallhäußer, 1988).

The tests described can be divided into three groups, *ie.* suspension tests, capacity tests and carrier tests (Russell *et al.*, 1982). Suspension tests entail the suspension of a bacterial culture in a diluent containing a known concentration of the antimicrobial agent. After a set time, the number of viable cells are determined (Eigener, 1988). Tests such as the phenol coefficient, the Rideal-Walker and Chick-Martin tests are all suspension tests (Eigener, 1988). Tests to determine bacteriostatic activity are also suspension tests. Bacterial cultures are suspended in a series of tubes of increasing concentrations of the antimicrobial agent (Russell *et al.*, 1982). Growth is determined after a set incubation period, and the lowest concentration of antimicrobial agent inhibiting growth is the minimum inhibitory concentration. The best known capacity test is the Kelsey-Sykes test. The aim of this test is to determine the capacity of the antimicrobial agent for an increasing dirt load (Russell, *et al.*, 1982). Carrier tests entail the antimicrobial effectivity of the agent against attached bacteria, and are designed to evaluate surface disinfectants (Russell, *et al.*, 1982). The evaluation of bactericides for application in cooling water systems is performed in various ways, but the most suitable method would be an inhibition test as the aim of bactericide treatment is the inhibition of bacterial growth (Cloete *et al.*, 1992). Alternatively a suspension kill test could be performed, but this should entail a long exposure time (6 h) as bactericides remain in systems until they are inactivated or fully diluted (Cloete *et al.*, 1992).

Temperature has an effect on bactericide activity and the relationship can be expressed by the temperature coefficient θ. The temperature coefficient can be calculated as follows:

$$\theta^{(T_2-T_1)} = \frac{k_2}{k_1}$$

k2 and k1 are the death rate constants at T2 and T1 respectively (Bloomfield, 1991). The temperature of evaluation must therefore be kept constant so that results are comparable.

Where time plays a role in the test procedure, the activity of the antimicrobial agent must be arrested at the moment of sampling (Bloomfield, 1991). A range of neutralising agents have been described in the literature, and these may be added to the diluent or sampling container, and must be non-toxic to the relevant bacteria (Bloomfield, 1991). Table 7 lists neutralising agents for a series of non-oxidising bactericides. Some bactericides adsorb onto bacterial cell surfaces, or enter cells long before they kill these cells. A example is PHMB (Woodcock, 1988). The addition of a neutralising agent only inactivates unbound PHMB, whereas bound PHMB continues its antimicrobial activity (Broxton *et al.*, 1984).

Table 7: Neutralising agents for selected antibacterial agents (compiled from Bloomfield, 1991 and Wallhäußer, 1988).

Antibacterial agent	**Neutraliser**
Bronopol	Cystein (0.1 %), thioglycolate
Chlorhexidine	Tween 80 (3 %) + Lecithin (0.3 %)
Formaldehyde	Histidine (0.5 %), dimedone (0.05 %), morpholine
Gluteraldehyde	Sodium bisulphite, glycine
Iodine and iodophores	Sodium thiosulphate
Isothiazolone	Sodium glycolate, chlorine, amines (*eg.* histidine) and sulphides.
Phenol and derivatives	Tween 80 (1 %), dilution
PHMB	Tween 80 (3 %) + Lecithin (0.3 %), anionic detergents, certain rubber compounds, precipitated by complex phosphates.
Quaternary ammonium compounds	Tween 80 (3 %) + Lecithin (0.3 %), Lubrol, organic substances such as milk, NaCl, Fe^{2+}, anionic tensides.

5 RESISTANCE TO BACTERICIDES

Bactericide treatment regimes for cooling water systems often fail, posing the question of bacterial resistance to the bactericide (Battersby *et al.*, 1985; Brözel & Cloete, 1992a; Costerton & Lashen, 1983). Certain authors have argued that failure of treatment programmes was due to selection for resistant strains (Characklis, 1990). We have however shown that susceptible bacterial isolates did acquire increased tolerance to bactericides following serial transfer in sub-inhibitory concentrations (Brözel & Cloete, 1991b). Resistance has been defined as the temporary or permanent ability of an organism and its progeny to remain viable and / or multiply under conditions that would destroy or inhibit other members of the strain (Gilbert & Wright, 1987). Russell and Chopra (1990) defined bacteria as resistant when they were not susceptible to a concentration of antibacterial agent used in practice. Traditionally, resistance refers to instances where the basis of increased tollerance is a genetic change, and where the biochemical basis is known (Russel & Chopra, 1990). Whereas the basis of bacterial resistance to antibiotics is well known, that of resistance to antiseptics, disinfectants and food preservatives is less well understood (Russell, 1991). The basis of resistance to water treatment bactericides is little known (Brözel & Cloete, 1991b). The phenomenological (how) and informational (why) aspects will be discussed.

5.1 Mechanisms of Resistance

Two reasons exist why the efficacy of bactericide treatment programmes can decrease at times. The one is a decrease in the activity of the bactericide (*cf.* 1.4.7), and the other is a decrease in the bacterial susceptibility towards the bactericide. Three mechanisms of resistance have been reported in the field of antibiotic study:

* Inaccessibility of the antimicrobial agent to its site of action,
* Absence of the susceptible site, or alteration to a non-susceptible form, and
* Inactivation of the antibacterial agent (Franklin & Snow, 1981; Lancini & Parenti, 1982).

Bactericides are less specific in their action than some antibiotics, so that the alteration of a reactive site or the substitution of an amino acid in a protein will not render bacterial cells resistant. Therefore, inaccessibility and inactivation will be reviewed as possible mechanisms of resistance.

5.1.1 Inaccessibility of the cell to the antimicrobial agent

The initial stage of bactericide action is binding to the bacterial cell surface (Russell & Chopra, 1990). Then it must traverse the cell wall (gram positive) or outer membrane (gram negative) to reach its site of action at the cytoplasmic membrane or cytoplasm (Gilbert & Wright, 1987). In gram positive bacteria there are no specific receptor molecules or permeases to assist or block bactericide penetration. The cell wall of *Bacillus megaterium* is permeable to molecules up to 30 kD (Russell, 1991). Intrinsic resistance of gram positive bacteria to bactericides is therefore low. The gram negative cell

envelope has, however, evolved to regulate the passage of substances into and out of the cell to a remarkable degree of specificity (Gennis, 1989; Hancock, 1991; Nikaido & Vaara, 1987). All the components of the cell envelope except peptidoglycan play a role in the barrier mechanisms because peptidoglycan is spongy and therefore permeable (Hancock, 1991). *P. aeruginosa* is the most resistant non-sporeforming bacteria to most bactericides, due to the superior barrier properties of its outer membrane (Russell & Chopra, 1990). In a recent study Igarashi and Watanabe (1992) compared the antimicrobial activity of a series of new 2-arylthio-*N*-alkylmaleimides and found many to be active against *Staphylococcus aureus*, *Bacillus subtilis* and *E. coli*. Only one of the 51 derivatives tested was marginally active against *P. aeruginosa*.

The physiological state of cells and the nature of the habitat can lead to considerable variation in the susceptibility of bacteria to bactericides (Gilbert & Wright, 1987). The composition of the bacterial cell envelope does change as a response to available or limiting nutrients, so that the barrier properties of the envelope are affected (Gilbert & Brown, 1978). Exposure to sub-inhibitory concentrations of bactericides can lead to phenotypic adaptation, resulting in a resistant cell population (Brözel & Cloete, 1993; Jenkins *et al.*, 1988). In *E. coli* certain proteins induced by heat or starvation stress also confer resistance to H_2O_2 and to UV light (Jenkins *et al.*, 1988). According to Russell (1991), most bactericide-resistance is due to adaptation, and the resistant phenotype is mostly lost upon removal of the bactericide.

5.1.1.1 Barrier properties afforded by lipopolysaccharides

The largest part of the surface of gram negative bacteria is covered by LPS (Nikaido & Vaara, 1987). There is a high degree of association between LPS molecules due to cation linking at the inner core (KDO) section (Hancock, 1991). This prevents the penetration of all but very small molecules, and only hydrophobic ones can pass through the lipid A section (Russell, 1991). Deep rough mutants are therefore susceptible to hydrophobic bactericides. The outer core sugars of rough mutants are charged, so that the section has a net negative charge (Nikaido & Vaara, 1987). This prevents the entry of hydrophobic as well as negatively charged antimicrobials (Hancock, 1991). This is why gram negative bacteria are resistant to the antimicrobial action of common detergents such as Na dodecyl sulphate except at low pH (< 3.0) where the outer core is neutral (Nickerson & Aspedon, 1992). Smooth strains have a thick layer of O-side chain sugars which further decreases the permeability for hydrophobic compounds (Russell, 1991). Quarternary ammonium compounds are excluded from smooth cells due to incompatibility of their long aliphatic side chain with the O-section sugars (Russell, 1991). The entry of biguanides is not affected by LPS as they are amphiphilic and promote their own uptake, making use especially of the core polysaccharides (Gilbert *et al.*, 1990).

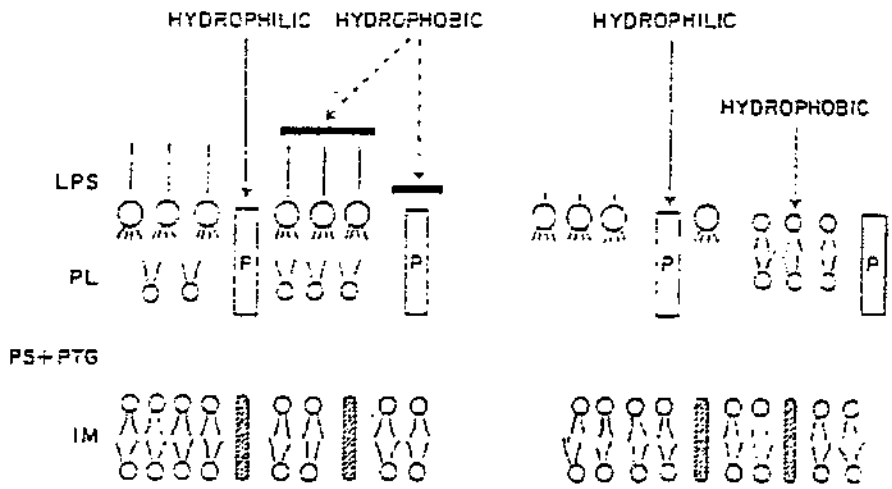


Figure 12: Diagrammatic representation of the penetration of hydrophobic bactericides into smooth (a) and deep rough (b) gram negative cells. LPS = lipopolysaccharide, P = protein, PL = phospholipid, PTG = peptidoglycan, IM = inner membrane (after Russell, 1991).

Temperature of growth has an effect on the structure of the LPS of *P. aeruginosa* PAO (Kropinski *et al.*, 1987). 15 °C stimulates the production of long O-chains, whereas at 45 °C these chains are short. The side-chain substitution of core sections was low at low temperature and stimulated by high temperature. Growth temperature would therefore influence the permeability of hydrophobic antimicrobials through the outer membrane.

5.1.1.2 Barrier properties afforded by altered outer membrane protein profiles

Outer membrane proteins play an important role in the resistance of gram negative bacteria, especially of *P. aeruginosa* to hydrophilic bactericides. This is because most porins are specific, and only OprF is a general uptake porin, although with a low contribution to the total uptake (Nikaido, 1992). Most reports regarding resistance mechanisms due to outer membrane proteins have dealt with antibiotic resistance. The best studied phenomenon is resistance to imipenem (Trias *et al.*, 1989; Tzouvelekis *et al.*, 1992). Both *P. aeruginosa* and *Enterobacter aerogenes* resistant to imipenem lack an outer membrane protein, OprD in the first case and a 43 kD protein in the second. Gentamycin resistance has been related to the production of large quantities of OprH, a cationic protein replacing divalent cations (Bell *et al.*, 1991). These mutants were also resistant to the action of EDTA. It has been found that cultures of *P. aeruginosa* resistant to 2,2'-methylenebis(4-chlorophenol) lack OprP, the phosphate uptake porin (Brözel & Cloete, 1993). Suppression of OprP was inducible. Although resistance is usually due to the absence of a specific outer membrane protein, this does not hold where the bactericide attacks all proteins, *eg.* in the case of formaldehyde. Resistance of *Serratia marcescens*

and of *E. coli* to formaldehyde was due to a decreased protein content of the outer membrane, and not to alterations in the profile (Kaulfers *et al.*, 1987). Therefore the absence of outer membrane proteins plays an important role in resistance to bactericides.

Growth conditions influence the outer membrane proteins synthesized. The best documented example is OmpF which is the larger of the two *E. coli* porins, allowing passage of solutes up to 600 D. Production of OmpF is regulated by osmolarity and temperature; it is not produced at 37 °C or at 1 % NaCl (Nikaido & Vaara, 1987). These are conditions in the intestinal tract of mammals, one of the habitats of *E. coli*, a habitat rich in the detergent cholic acid. Cholic acid has a mass of 430,6 D, and cannot pass through the other porin OmpC. In this way *E. coli* can absorb most nutrients while surviving in the presence of cholic acid. A less understood mechanism is that of resistance to the zwitterionic carbapenem antibiotic, imipenem, where the cause of resistance is the absence of OprD (Huang *et al.*, 1992). OprD is produced in the presence of basic amino acids so that the question arises whether imipenem resistance is due to mutants (as advocated by Hancock and co-workers), or whether it is due to a regulatory mechanism where the antibiotic induces repression of production. This would be supported by the high rate at which "resistant isolates" arise following clinical therapy (Quinn *et al.*, 1986). Where *P. aeruginosa* grows in the absence of Mg^{2+}, it overproduces OprH which confers resistance to EDTA (Nicas & Hancock, 1980).

5.1.1.3 Barrier properties afforded by fatty acids and phospholipids

Phospholipids do not form part of the outer leaflet of the outer membrane, so they should not play the primary role in the prevention of bactericide passage across the outer membrane. Yet much research has been performed specifically on the phospholipid profiles of bacteria resistant to antiseptics, *eg.* chlorhexidine (Sakagami *et al.*, 1989b), benzalkonium chloride (Sakagami *et al.*, 1989a) and phenol (Keweloh *et al.*, 1991). These authors (and others) only analysed for phospholipids and for KDO, and ascibed the resistance mechanisms solely to changes in the phospholipid profiles. The general trend in all cases was that resistant cells contained more free fatty acids. Jones *et al.* (1989) indicated that both biguanide- as well as QAC-resistant cells had increased levels of mono-unsaturated fatty acids and decreased levels of saturated C_{17} and C_{19} fatty acids. This would cause an increase in membrane fluidity, but how this contributes to resistance is not clear. Phenol resistant cells of *E. coli* had a higher percentage of saturated lipids to compensate for increased membrane fluidity due to phenol (Keweloh *et al.*, 1991). *P. aeruginosa* resistant to chlorhexidine had increased lipid and phospholipid content, but less LPS, implying that sections of the outer leaflet of the outer membrane were phospholipid (Sakagami *et al.*, 1989b). Changes in the degree of saturation and in the relative quantities of outer membrane phospho- and neutral lipids do contribute to resistance to membrane-active bactericides.

Phosphate limitation of growing cells of *E. coli* causes a decrease of the phospholipids phosphatidyl glycerol and phosphatidylethanolamine, and an increase in free fatty acid content of the cell membranes (Broxton *et al.*, 1984; Gilbert & Brown, 1978). Such cells are more resistant to biguanides because biguanides adsorb preferably to phosphatidyl glycerol and phosphatidylethanolamine (Broxton *et al.*, 1984). They are also more resistant to the antimicrobial action of 3- and 4-chlorophenol (Gilbert & Brown, 1978). Growth temperature influences the fatty acid profile of bacteria to facilitate a constant degree of membrane fluidity (Gennis, 1989). At lower temperatures the membrane contains a higher percentage of unsaturated fatty acids. This would afford the cells increased resistance to biguanides and to QAC's as resistance to these is linked to a higher unsaturated fatty acid content (Jones *et al.*, 1989).

5.1.1.4 Barrier properties afforded by extracellular polysaccharides

EPS does decrease the bactericide susceptibility of bacteria embedded in it (Costerton *et al.*, 1987). This phenomenon has been ascribed to the anionic nature of EPS which changes the orientation and order of water molecules and this has an effect on transport of substances such as bactericides (Christensen & Characklis, 1990; Costerton *et al.*, 1987). The protection afforded by EPS depends on the chemical nature of the bactericide. Possitively charged molecules such as quarternary ammonium compounds should enter easily into the anionic matrix, as should molecules of a less polar nature due to the lower polarity of the water matrix (Christensen & Characklis, 1990). *Legionella pneumophila* growing in a biofilm was indicated less susceptible to isothiazolone as well as to Bronopol (Wright *et al.*, 1991). Mucoid *Staphylococcus aureus* was more resistant to a range of phenol derivatives and to chlorhexidine than were non-mucoid cultures (Kolawole, 1984). Chlorine reacts with the alginate of *P. aeruginosa* and is thereby depleted so that the alginate is sacrificed but the cells survive (Learn *et al.*, 1987). Certain authors have indicated that attached cells are more resistant than are suspended cells covered in slime (Cargill *et al.*, 1992; Costerton *et al.*, 1987). This has been ascribed to changed physiology of attached cells, and to the protection afforded by the large cell density in the biofilm. In all these cases it is not clear whether the bactericide is prevented from passing through the EPS matrix, or whether it is bound or inactivated by the EPS, preventing access to the cell envelope. Further research in this regard is necessary.

Bacterial EPS production is influenced by various growth conditions (Sutherland, 1985). These include temperature (Alves *et al.*, 1991), osmolarity (Berry *et al.*, 1989), and the carbon to nitrogen ratio of the medium (Sutherland, 1985). Resistance will therefore depend on the interaction between EPS and the bactericide. In some cases the presence of EPS would cause an increase in resistance to the bactericide, whereas certain bactericides could act better against EPS-covered cells. Growth conditions influence the resistance of *Legionella pneumophila* to chlorination and iodination, as

attached cells were most resistant, followed by water-grown cells. Agar-grown cells were the least resistant ones (Cargill *et al.*, 1992). The same holds for chlorination of biofilm bacteria in general (LeChevallier *et al.*, 1988).

5.1.2 Enzymatic inactivation of bactericides

Resistance to antimicrobial agents can be due to enzymes transforming the bactericide to non-toxic form (Heinzel, 1988). The phenomenon is usually investigated from the biodegradation point of view, *ie.* the biodegradation of toxic pollutants. A host of aromatic, phenolic and other compounds toxic to many bacteria (some of which are employed as bactericides) can be degraded by certain bacteria. The topic has been reviewed by various authors and the current literature reports extensively on biodegradative pathways (Commandeur & Parsons, 1990; Engesser and Fisher, 1991; Smith, 1990).

Two types of enzyme-mediated resistance mechanims have been documented, *ie.* heavy metal resistance and formaldehyde resistance. Resistance to heavy metals includes resistance to the following: mercury, antimony, nickel, cadmium, arsenate, cobalt, zinc, lead, tellurite, copper, chromate and silver (Russell & Chopra, 1990; Summers, 1986). Detoxification is usually by enzymatic reduction of the cation to the metal (Heinzel, 1988). Where some heavy metal resistance genes are carried on plasmids (Rouch *et al.*, 1985; Summers, 1986), others are chromosomal (Kaur *et al.*, 1990). The resistant phenotype is usually inducible by the presence of the heavy metal (Kaur *et al.*, 1990; Rouch *et al.*, 1985). Some heavy metals induce resistance to a broader spectrum of heavy metals. Arsenate, arsenite and antimony, for example, induce resistance to each other in *E. coli* (Russell & Chopra, 1990).

The detoxification of formaldehyde by *P. aeruginosa* and *P. putida* has been studied extensively by the groups of Eagon and of Rossmoore. Formaldehyde is reduced by an NAD^+- gluthathione-dependant dehydrogenase, formaldehyde NAD^+ oxidoreductase (Eagon & Barnes, 1986). This enzyme is probably plasmid-encoded (Hall & Eagon, 1985), and appears to be constitutively expressed (Eagon & Barnes, 1986). Resistance to most formaldehyde-releasing formaldehyde condensates is also due to formaldehyde dehydrogenase activity as the antibacterial mechanism of these condensates appears to be via formaldehyde (Sondossi *et al.*, 1986).

5.1.3 Active removal of the bactericide

Bacteria can actively pump compounds out of the cell via membrane efflux systems. Only one type of bactericide-efflux system has been described to date, the QAC efflux system of *Staphylococcus aureus* (Sasatsu *et al.*, 1992). This efflux system is coded by two gene systems. *qacA* and *qacB* encode

for a high level of resistance, and *qacC* and *qacD* encode for a low level of resistance. *qacC* and *qacD* are further identical to the *ebr* gene encoding for resistance to ethidium bromide in *S. aureus*, explaining why resistance to QAC is often concurrent with resistance to ethidium bromide (Sasatsu *et al.*, 1992). The *qacA* gene codes for a 50 kD protein which mediates energy-dependant efflux of both benzalkonium chloride and ethidium bromide (Tennent *et al.*, 1989). The *qacC* gene also mediates energy-dependant efflux of benzalkonium chloride and ethidium bromide (Sasatsu *et al.*, 1992). Two different but isofunctional gene systems appear to have evolved in *S. aureus* (Sasatsu *et al.*, 1992).

5.2 Informational Aspects of Bacterial Resistance

According to Jacoby (1985) bacteria appear to have adapted to co-exist with antibiotics for a long time. Tetracycline-resistant bacteria have been isolated off human bones at least 1400 years old (Jacoby, 1985). Antibiotics, after all, have co-evolved with bacteria. Bactericides, disinfectants and antiseptics are synthetic compounds of human innovation, and most of these were developed over the past 100 years. Yet bacteria acquire resistance to most of these substances over time. Some authors state that bacteria develop resistance to any antimicrobial agent in the laboratory by successive transfer into subinhibitory concentrations (Jacoby, 1985; Russell & Chopra, 1990). How do bacteria acquire the information to react to a new stimulus in a directed fashion in order to become resistant and survive ?

Bacterial development of resistance has often been viewed as "survival of the fittest". Cells would be subject to a set rate of random mutations as was determined by Luria and Delbrück (1943). Those few cells containing sensible mutations conferring resistance to the bactericide would survive and multiply to form a resistant population. In 1988 the group of John Cairns reported the increased rate of mutations in response to environmental challenges (Cairns *et al.*, 1988). They have shown that mutations take place more often when favourable than when not. Hereby mutations are "directed" to assist the population in adapting to new environmental challenges. This has since been supported by the work of Barry Hall (1990). To return to Whitehead (1929), "organisms have a mental pole in which they feel and organise themselves to have an influence on the future". The old adage by Beijerinck "Everything is everywhere, the environment selects" (Atlas & Bartha, 1987) could therefore be reformulated to; "Everything develops everywhere, the environment selects." Certain bacteria can apparently determine the nature of certain new stimuli such as bactericide (Russell, 1991) or a new carbon source (Hall, 1990), and react in a "sensible" manner by changing their cell wall properties or by producing new enzymes.

Three basic types of resistance have been described, natural or intrinsic resistance, adaptation (a transient state lost in absence of the bactericide) and acquired or stable resistance (Heinzel, 1988).

The various phenomena of resistance will be divided into two categories, one where the information is contained (has the information), and another where it is acquired (acquires the information).

5.2.1 Resistance due to the genotype

Where resistance is due to the genotype all the information is already present on the genome. The phenotype could be constitutive, and the cells would be inherently resistant. This is the case with *P. aeruginosa* which are inherently resistant to a variety of antimicrobial agents due to the nature of their cell envelope (Nikaido, 1992; Wallhäußer, 1988). Alternatively the phenotype could be inducible, a description which holds for the phenomenon of adaptation. Because most cases of bacterial resistance to bactericides appear to be due to adaptation (Brözel & Cloete, 1993; Jones *et al.*, 1989; Russell, 1991), bacteria must contain the genetic information to adapt to a wide range of possible stress factors. The ability to adapt to stress factors which are new in the environment (*eg.* bactericides) is intriguing, but no plausible explanations have been published to date.

5.2.2 Resistance due to alteration of the genotype

Where resistance is due to alteration of the genotype, new information has been aquired. This can be due to transfer of genetic material, to rearrangement of genetic material (transposon) or to a mutation. The resistance of *S. aureus* to benzalkonium chloride can be due to the transfer of plasmid-borne *qacA & qacB* or *qacC & qacD* operons (Tennent *et al.*, 1989). Jacoby (1985) supported the view that exposure of bacteria to sub-inhibitory concentrations of antimicrobial agents leads to increased resistance due to multiple mutations. This would only hold if mutation was "directed", and would take place at an accelerated rate, supported by the work of Hall (1990) and of Cairns (1988). However, no cases of bactericide resistance have been shown to be due to this mechanism to date.

6 RELATIONSHIP BETWEEN CARBON CONTENT AND BACTERIAL ATTACHMENT

6.1 Chemical Oxygen Demand

6.1.1 Application of COD

The chemical oxygen demand (COD) test is widely used to determine the organic content of wastewater (Ballinger, *et al.*, 1982). The COD of a sample is defined as the amount of oxygen consumed when the sample or a dilution is heated with acidified dichromate solution for two hours. The amount of oxygen consumed is determined by measurement of the remaining dichromate, either by titration with acidified iron (II) solution or by a spectrophotometric determination (Edwards and Allen, 1984).

Along with the measurement of biochemical oxygen demand (BOD), total oxygen demand (TOD), total organic carbon (TOC) and dissolved organic carbon (DOC), the measurement of COD is particularly important for the characterization of industrial and municipal effluents and their treatment (Dasgupta and Petersen, 1990).

In 1841, (Phelps, 1944) the broad intent of the BOD test was to determine the extent to which a waste sample would consume oxygen and thereby affect the ecosystem recieving such a discharge. Unfortunately, even the presently shortened form of the BOD test requires five days. The need for a faster comparable procedure resulted in the COD test which measures the number of equivalents of an oxidant consumed.

6.1.2 Determination of COD

For nearly forty years , the dicromate open reflux method for the determination of the COD has been, and still is, used in water analysis, although it has several shortcomings:
a) It requires large amounts of reagents and samples,
b) It is space demanding,
c) Time consuming and
d) Its sensitivity (about 5 $mg.l^{-1}$ when using 0,0417 M $K_2Cr_2O_7$) is too low for application to waters with low organic matter contents such as drinking, ground and non-polluted surface waters (Hejzlar and Kopácek, 1990).

Alternative methods of COD determination on a semi-micro scale have been introduced. The digestion conditions are largely identical with those used in the original method, however, the boiling under a reflux condencer is usually replaced by heating of samples in thermostated blocks. The quantification is often based on the spectrophotometric determination of Cr^{3+} ions or on the amperometric titration of the unconsumed dichromate (Hejzlar and Kopácek, 1990).

As with the open reflux method, all inorganic substances capable of oxidation, such as Fe^{2+}, S^{2-} or nitrate, interfere with the semi-micro method. Furthemore, turbidity of the reaction mixture caused by inorganic particulate substances present in some surface water samples, increases the absorbance, causing a negative error in the determination (Hejzlar and Kopácek, 1990).

Although a variety of oxidizing agents such as permanganate, cerium, iodate, Fenton's reagent, persulfate, etc. have been investigated, stongly acidic Cr(VI) at reflux temperatures is the preferred reagent. Cr(VI) absorbs more strongly than Cr(III) while optically monitoring Cr(VI) provides the more sensitive procedure and is therefore the present preference in automated methods (Dasgupta and Petersen, 1990)

6.1.3 Problems Associated with COD

The main problem with the standard COD test is that the oxidizing agent, potassium dichromate, used in the strong sulphuric acid matrix, oxidizes cloride to chlorine:

$$Cr_2O_7^{2-} + 6Cl^- + 14H^+ \longrightarrow 3Cl_2 + 2Cr^{3+} + 7H_2O$$

It has been observed by numerous workers that the effect of chloride, significantly decreases as the COD increases. This can be attributed to the formation of chromium(III) which complexes any free chloride ions in the digest solution (Thompson *et al.*, 1986).

Samples containing high chloride concentrations (especially those with low COD values) can show falsely high results due to the interference of chloride. In the presence of large amounts of chloride, the presence of ammonia and amines can cause high results. The chlorine formed reacts with the nitrogen compounds to form chloramines which decompose, reforming chloride which is then reoxidized to chlorine.

All oxidizable compounds are not oxidized to the same extent. For example, reducing sugars such as glucose are oxidized even without heating while, heterocycles such as pyridine are hardly oxidized ever after refluxing at 140 °C for two hours.

The oxidation potential of the dichromate ion varies proportionially with the logarithm of its concentration and is also dependant on the acidity; while the oxidizability of organic compounds varies widely. Inorganic reducing agents usually react with dichromate faster than organic matter and so contribute to the oxygen demand. If present in large amounts, they can rapidly reduce the dichromate concentration and so affect the oxidizability of organic matter in the test.

Similarly, while difficultly oxidizable organic compounds may not react when alone and dilute, their oxidation may be partial when mixed with readily oxidized organic substances which react more rapidly with dichromates.

6.1.4 Suppression of Chloride Interference

The use of mercury(II)sulphate as a complexing agent to decrease the concentration of free chloride ions was introduced and thus reduce the availibility of chloride for reaction with dichromate. As mercury salts are highly toxic, it would be desirable to find an alternative means of suppressing chloride interference (Ballinger *et al.*, 1982)

Other than Hg(II), the addition of Cr(III) has been suggested as an alternative for preventing the oxidation of chloride to chlorine. Although complexation of chloride by Cr(III) has been suggested to be the operative basis of this procedure, other workers feel that the operative factor is likely a decrease in the oxidizing ability of the dichromate due to lowering of the oxidation potential. This may also reduce the rate of oxidation of some compounds (Dasgupta and Petersen, 1990).

The use of silver sulphate as a suppressant for chloride has been investigated and it is currently used by many workers. This alternative is attractive because of the comparatively low toxicity of silver compounds in biological systems and the ease of recovery of silver from waste solutions (Ballinger *et al.*, 1982)

6.2 Biological Oxygen Demand

6.2.1 Application of BOD

The biological oxygen demand (BOD) analysis was traditionally used for the evaluation of treatment processes and assessment of the pollution load to a recieving body of water (Hill and Spiegel, 1980).

In water pollution control, BOD is commonly used for detecting the effect of wastewaters, treated or untreated, on the biological condition of the recieving waters.

The BOD test is not a measure of the quantity of organic material in wastewater, but instead is a bioassay procedure for assessment of the amount of oxygen required for the respiratory functions of the microorganisms (seed material) in the BOD bottle(Stover and McCartney, 1984).

6.2.2 Bacterial Oxidation

In an aqueous environment bacterial oxidation is brought about by microorganisms which utilise the available organic matter as sources of carbon and nitrogen while consuming the available oxygen. The action of these microorganisms is the basis of the self purifying process which occurs when polluting organic matter is discharged to water courses, lakes and the sea. The rate of purification depends on many factors including the ambient temperature, sunlight levels, flow patterns, the nature of the polluting matter and the types of flora and fauna present. The BOD test clearly indicates the important role of biological action on the substrate to be tested. Without the microbes, there is no BOD to speak of. With proper pH, temperature, nutrients, dissolved oxygen and with the other physical and chemical components of the environment under control, the biological oxidation process

of the substrate may be explained in the following manner:

$$\text{Substrate} + O_2 \xrightarrow{\text{microbes}} \text{More cells} + \text{Oxidized products} + \text{Less } O_2 + \text{Unoxidized residue}$$

6.2.3 The 5-Day BOD Test

The basis of the method is that an air-saturated sample is diluted and if appropriate, "seeded" with a suitable source of microorganisms, incubated in a full container for 5 days at 20 °C (in the dark). The amount of oxygen absorbed is determined by means of appropriate dissolved oxygen measurements (volumetric, *eg.* titration or insrumental, *eg.* spectrophotometry).

The uptake of dissolved oxygen (mg/l) by a sample of natural or waste waters during 5 days at 20 °C are determined. This test has a range of application up to 6,0 mg/l (without dilution). All larger values are determined by appropriate dilution. For an undiluted sample a corrected dissolved oxygen absorbtion of 0,1 mg/l represents a BOD of 0,1 mg/l(HMSO- BOD).

During biological growth in a batch process, the oxygen uptake (BOD exertion) curve roughly parallels the shape of the growth curve. The specific rate of growth has been shown to be dependant on the concentration of the substrate or carbon source. Therefor the specific rate of oxygen uptake should also be dependant on the concentration of the carbon suorce. In other words, the specific rate of BOD exertion depends on the dilution factor of the sample used in the BOD bottle. The lower the dilution factor (higher sample volume corresponding to higher carbon concentration), the faster the rate of BOD exertion up to the maximum rate, provided there is not inhibition of biological activity (Stover and McCartney, 1984).

6.2.4 Problems Associated with BOD

Substances could influence the test either by inhibiting the activity of the microorganisms or by causing an enhanced utilization of oxygen. In the former category are:

1) Free chlorine
2) High or low pH
3) Phenols
4) Pesticides
5) Metals toxic to bacteria such as copper, zinc, lead, chromium, nickel and cadnium. These metals inactivate the bio-degradation activity of bacteria which results in lower BOD values.
6) Any other substance toxic to microorganisms.

In the latter category are:

1) Algae
2) Ammonia and nitrogen compounds, utilized by nitrifying organisms, may enhance the oxygen uptake by nitrification and will give high results
3) An immediate oxygen demand may be exerted by ferrous iron, sulphite, sulphide or aldehydes.

A low BOD value of an effluent is regarded as good, while a high BOD is frequently sufficient reason for some form of 'retribution'. Many industries pay sewage charges to municipalities on the basis of gallonage and BOD loading. But, a low BOD value may not always be good. Chloride added to water during sewage chlorination has become an indicator of pollution. Chloride is added to the sewage to destroy pathogenic bacteria, but in reality this is keeping the 5-day BOD value at a false low.

It is well known that sufficient oxygen must be supplied in order to obtain the oxidation of the substrate. Little has been emphasized as to :

1) The proper number and types of active microbes needed to assimilate and
2) The unit concentration of the substrate present in the water sample (Howe *et al.*, 1981).

It has also been accepted in theory and practice that the microbes generate or elaborate enzymes for speeding up the breakdown of the substrate through the oxidation process. Assuming that proper microbes are cultivated that produce the specific enzymes needed for the oxidation of the substrate, the rate of oxidation depends on the proper amounts of enzymes or the number of microbes present in the system (Howe *et al.*, 1981).

For a system with more microbes, the survival probability, acclimatization and active oxidation will be better than for a system having fewer microbes present. Results from a system having high concentrations of microbes, will therefore differ from a system with the same substrate concentration but with fewer microbes (Howe *et al.*, 1981).

The rate of the BOD process is dependant upon the substrate concentration or the dilution factor. Some variability in the BOD determinations could be expected due to the dilutions employed, since the BOD exertion rate is affected by the dilution factor and lag periods in substrate utilization may occur, especially with non-acclimated seeds (Stover and McCartney, 1984).

Factors other than the above mentioned that could influence the BOD test are:

1) Use of acclimated or non-acclimated biological seed material and the corresponding lag periods prior to oxygen consumption.

2) Dissolve oxygen (DO) due to the dilution water itself. The greater the depletion of the dilution water, the greater the variability of the BOD values determined by dilution.

3) Inhibition of biological activity can occur in the bottle due to the dilution effects on the substance : microorganism ratio. Inhibition in the BOD bottle is however, not representative of the conditions in the treatment plant or recieving stream (Stover and McCartney, 1984).

4) The amount of oxygen consumed for oxidation of organic carbon in the wastewater and for endogenous respiration and/or maintenance activities of the seed material is variable depending on the substrate-to-microorganism ratio and the amount and type of seed material employed from one test to another (Stover and McCartney, 1984).

7 ECOLOGY AND PHYSIOLOGY OF SULPHATE-REDUCING BACTERIA

7.1 Dissimilatory Sulphate Reduction

Most bacteria, fungi and plants reduce sulphate to sulphide before incorporation of sulphur into amino acids. This process is termed assimilatory sulphate-reduction and is purely a biosynthetic process. During dissimilatory sulphate reduction, sulphate is utilized as an oxidant for the degradation of organic material. Dissimilatory sulphate-reduction is carried out by a specialized group of anaerobes: the sulphate-reducing bacteria (SRB) (Gibson, 1990).

SRB are a phylogenetic and morphological diverse group of strictly anaerobic eubacteria that utilize, as electron acceptors, sulphate, other oxidized sulphur compounds or elemental sulphur, and reduce it to H_2S. This reaction is coupled to the oxidation of a variety of organic compounds. Genera and species differ with respect to their utilization of organic compounds (electron donors). Many species carry out an incomplete oxidation of substrates such as lactate to CO_2 and acetate. Other species are capable of oxidizing acetate and other organic compounds completely to CO_2 (Widdel and Pfennig, 1984). A wide range of different carbon sources can be utilized by SRB (Widdel, 1988). The enzyme hydrogenase is present in many SRB-species and hydrogen is an important substrate for SRB. Certain strains of SRB are able to grow on H_2 and sulphate as sole energy source (Badziong *et al.*, 1978, Brandis and Thauer, 1981). Some SRB-species grow with hydrogen as the energy source and acetate and CO_2 as carbon substrate (Hansen, 1988).

The initial step in the biochemical sulphate-reduction pathway is the transport of exogenous sulphate across the bacterial membrane into the cell. Once inside the cell, sulphate dissimilation proceeds by the action of adenosine tri-phosphate (ATP) sulphurylase which combines sulphate with ATP to produce the highly activated molecule adenosine phosphosulphate (APS), as well as pyrophosphate. APS is then rapidly converted to sulphite by the cytoplasmic enzyme APS reductase. Sulphite may then be reduced by sulphite reductase via a variety of intermediates to form the sulphide ion (Gibson, 1990).

7.2 Ecological Distribution of Sulphate-Reducers

SRB have been isolated from the anaerobic regions of marine and estuarine sediments as well as saline ponds. A number of SRB are able to grow in non saline environments such as anaerobic mud and sediment of freshwater and brackish water. SRB are able to multiply in the gastro-intestinal track of man or animals (Widdel and Pfennig, 1984). Other habitats in which SRB have been detected include environments such as sour whey digesters, spoiled foods, anaerobic water purification plants and sewage plants. Although these bacteria are strictly anaerobic, their presence has been detected in many ostensibly aerobic regions (Gibson, 1990).

SRB enriched from sea water using either lactate or propionate based media were tentatively identified as strains of *Desulfovibrio vulgaris* (Hardy, 1981). Colony counts of acetate, propionate and lactate oxidizing SRB in freshwater and marine sediments were performed by Laanbroek and Pfennig(1981). In marine environments, 5 different SRB-strains were isolated using acetate as carbon source, 5 different SRB-strains were isolated using propionate as carbon source and 9 different SRB-strains were isolated using lactate as carbon source. In freshwater environments 2 different SRB-strains were isolated using propionate as carbon source and 3 different SRB-strains isolated using lactate as carbon source. Acetate-oxidizing SRB could only be isolated from marine sediments. They belonged to the genus *Desulfobacter*. Lactate-oxidizing SRB belonged to the species *Desulfovibrio desulfuricans* and propionate-oxidizing SRB to the genus *Desulfobulbus* (Laanbroek and Pfennig, 1981). Eight different SRB-strains were isolated from oil field water. These organisms were identified as two strains of *D. desulfuricans*, *Desulfovibrio africanus*, two strains of *Desulfotomaculum nigrificans* and three strains of *Desulfotomaculum* sp. This work illustrated the wide variety of microorganisms loosely classified together as SRB (Antloga and Griffin, 1985). Freshwater genera were; *Desulfotomaculum* (6 species), *Desulfovibrio* (10 species), *Desulfomonas pigra, Desulfobulbus propionicus, Desulfovibrio thermophilus* and *Thermodesulfobacterium commune. Desulfotomaculum acetoxidans* was present in freshwater contaminated with manure. SRB from freshwater environments metabolized a limited number of substrates (Pfennig, 1989). Different populations of SRB were identified within marine sediment systems, using fatty acid biomarkers (Taylor and Parkes, 1985). The viable populations of SRB in the littoral sediments of a lake was investigated using enrichment

and enumeration techniques. The community of SRB had a characteristic population structure consisting of: 87.7% H_2-utilizing SRB (resembling *Desulfovibrio* species), 12% propionate utilizers (*Desulfobulbus* species), 0.3% long chain fatty acid-oxidizing *Desulfovibrio sapovorans* species and less than 0.05% acetate-utilizing *Desulfotomaculum acetoxidans* (Back and Pfennig, 1991).

According to Sorensen *et al.* (1981), acetate, H_2, propionate and butyrate accounted for 4050, 5-10 and 10-20%, respectively, of the substrates available for sulphate reduction and together accounted for about 80% of the total sulphate reduction within slurries of marine sediment (Sorensen *et al.*, 1981). In marine and estuarine sediments from three different sites, acetate was the major substrate for sulphate reduction. In addition to acetate, 17 individual substrates were involved in sulphate reduction, these included lactate, H_2, propionate, iso- and *n*-butyrate, *iso*- and *n*-valerate, 2-methylbutyrate and amino acids (Parkes *et al.*, 1989).

8 METHODS FOR THE DETECTION AND ENUMERATION OF SULPHATE-REDUCING BACTERIA

8.1 Isolation and Growth of Sulphate-Reducing Bacteria

8.1.1 Incubation conditions

SRB are strict anaerobes (Widdel and Pfennig, 1984). Handling and cultivation of SRB require techniques to effectively remove oxygen (air) from both the medium and the gas phase in contact with the medium as well as lowering the redox potential. Gases used in anaerobic work generally are CO_2, H_2, N_2 or mixtures of these gases. Cylinder gases contain small amounts of O_2, which must be removed. This can be done by (a) passing the gas through a column containing copper wire electrically heated to about 350 °C, (b) by using a gas mixture containing 3% H_2 and passing the gas mixture through a titanium (III) citrate solution or (c) by passing a gas mixture containing 3% H_2 through a paladium column (Costilow, 1981).u

Since SRB growth continues in sulphate-containing media, the production of sulphides ensures the absence of O_2 and maintains a reduced environment. With growth in sulphate-free media this is not the case and a negative redox potential has to be maintained in other ways. The addition of redox-poising agents is one method commonly used for the exclusion of oxygen and the establishment of reducing conditions. A negative redox potential of - 100 mV (Eh) is recommended for successful growth of SRB (Herbert and Gilbert, 1984). Various reducing agents are available for lowering the redox potential of media, for example ascorbate (Eh = 80 mV), cysteine hydrochloride (Eh = -210), dithiothreitol (Eh = -330) and titanium(III) citrate (Eh = -480). Since reducing agents react with oxygen to form toxic substances, the preparation of reducing agents must take place under anaerobic conditions. The inclusion of redox dyes such as resazurin, which has an Eh of -51 mV, gives a visible

indication of the redox state of the medium. Resazurin changes from blue to pink to completely colorless when the redox potential is lowered to about -110 mV or lower. When reoxidized, resazurin becomes pink and does not turn to blue. Once a negative redox potential has been obtained, entry of O_2 may be prevented by using alkaline pyrogallol plugs to absorb O_2, purging with N_2, or using entirely sealed vessels. Agar plates can be incubated in the conventional manner using an anaerobic cabinet with an anaerobic atmosphere (Costilow, 1981).

8.1.2 Isolation media

Various media and modifications of these media are available for the detection and isolation of SRB (Fedorak *et al.*, 1987, Gibson *et al.*, 1987, Hardy, 1981, Herbert and Gilbert, 1984, Pankhurst, 1971, Postgate, 1984). These media contain sodium lactate as a carbon source, ferrous salt as an indicator of sulphide production, redoxpoising agents and yeast extract. The pH range most commonly used in media for the growth of SRB is 7.2 - 7.6. The temperature required for incubation, depends upon the SRB-species. Thermophilic *Desulfotomaculum* species are generally incubated at 55 ^{o}C and mesophilic *Desulfovibrio* species are incubated at 30 ^{o}C. SRB found in freshwater and marine environments have different salinity requirements (Herbert and Gilbert, 1984).

The use of lactate was in conformity with the classical view that the range of carbon and energy sources for SRB were narrow. This has changed with the isolation of SRB that grew on a wider range of carbon compounds. (Herbert and Gilbert, 1984). Acetate, propionate, butyrate and hydrogen are important *in situ* substrates for sulphate reduction within marine sediments (Gibson *et al.*, 1987). Media for the isolation of SRB that grew on a wider range of carbon sources or on lactate alone were presented by Pfennig *et al.* (1981). SRB are enumerated by the most probable number (MPN) technique (Battersby *et al.*, 1985, Fedorak *et al.*, 1987, Hardy, 1981). Other methods that are being used are broth containers, agar depths and melt agar tubes. There are various advantages coupled to the use of these methods (Tatnall, *et al.*, 1988). However, strictly anaerobic conditions are generally not maintained during inoculation of media or during culturing of the organisms. The use of the MPN-technique hamper the selective enumeration and isolation of SRB. SRB in sewage digestors were enumerated by the anaerobic roll tube method (Ueki *et al.*, 1980). The roll tube method facilitates the isolation of single colonies and has the advantage of maintaining good anaerobiosis for growth of strict anaerobes.

The API (American Petroleum Institute Recommendation RP-38) broth medium is the most widely used culture medium (Tatnall *et al.*, 1988). The recovery of known viable populations of SRB belonging to the genera *Desulfovibrio, Desulfobacter* and *Desulfobulbus* from inoculated, sterile anoxic estuarine

sediments has been determined using Postgate and Widdel's media. Recovery of *Desulfovibrio* populations was consistently higher with Postgate's medium, while Widdel's medium always yielded higher viable number of *Desulfobacter* and *Desulfobulbus.*

The addition of cetyl trimethylammonium bromide (CTAB) to the treated and untreated sediment samples significantly increased the viable numbers of SRB-genera (Gibson *et al.*, 1987). The presence of Ca^{2+} in growth medium causes cells of *D. vulgaris* to aggregate, leading to a decrease in plating efficiency. When the Ca^{2+} concentration in the medium was reduced 20-fold, cell aggregation did not occur and the plating efficiency increased from an initial value of 34% to a final value of 56% (Singleton *et al.*, 1988). The standard API medium (API Recommended Practice RP-38) for the enumeration of SRB in oil field waters was modified by the addition of an ammonium salt, a calcium salt, vitamins, trace metals and an additional reductant, cysteine. The modified medium was compared with the API-medium, Postgate's medium B and a modified Baar's medium. While the enumeration of *D. desulfuricans* and *D. vulgaris* was not influenced by the medium used, the enumeration of SRB from environmental samples did depend on the choice of medium. Higher counts of SRB were obtained from environmental samples in a shorter time with the modified compared to the standard API-medium. Enumeration of SRB was also generally more rapid with the modified medium compared to Postgate's medium B and the modified Baar's medium (Tanner, 1989). Enrichment studies for SRB in the littoral sediment of a lake, indicated that most types of SRB grew best in media with low salt concentrations consistent with the low salinity of the freshwater habitat. Enumerations were based on a medium with the following electron donors: H_2, lactate, acetate, propionate, butyrate, caprylate, succinate, benzoate or $S_2O_3^{2-}$. A maximum cell density of 6.3×10^6 cells per ml sediment was estimated, which is the highest number of SRB ever reported for anoxic sediments. A comparison with measured sulphate reduction rates showed that the enumeration techniques for SRB were about 10 to 100-fold more efficient than those previously used (Back and Pfennig, 1991).

According to Gaylarde and Morton (1988) the sensitivity of culture methods could be improved if the basal medium was complemented with another carbon source in addition to the commonly used sodium lactate. The reducing agent used in the medium was sulphur free, since sulphur may inhibit the growth of some SRB strains. They compared a new medium, SEBR-medium (Gaylarde and Morton, 1988), with different culture media test-kits available for the detection of SRB. The test kits included anaerobic broth bottles of modified postgate B-medium and API-medium as well as agar tubes and aerobic broth tubes. SEBRmedium contained two carbon sources (lactate and acetate) and a sulphur free reducing agent (titanium citrate). Using the SEBR-medium, detection of SRB was more sensitive and more rapid than the detection kits.

Hamilton (1985) concluded that viable count procedures for enumerating SRB underestimated the *in situ* populations by a factor of approximately 1000, compared to *in situ* sulphatereduction activity. This may have been due to poor recovery of these bacteria from sediment, an inappropriate choice of culture medium or the fact that only lactate types were enumerated (Gibson *et al.*, 1987). Since growth is possible on many carbon sources other than lactate, enrichment and culture media based solely on this carbon source might give rise to a biased and incomplete analysis of the natural population being sampled (Hamilton, 1985). This demonstrates the importance of careful medium selection.

In addition to the suitability of the chosen medium, the inoculum size affects the rate at which a black precipitate develops. It was recommended that the media should be incubated for up to 28 days (Herbert and Gilbert, 1984). Incubation at only one temperature is furthermore selective for strains that will grow at that temperature (Tatnall *et al.*, 1988).

The inclusion of ferrous ions in the media is convenient as an indicator of sulphide production. However, this technique can give erroneous results. Growth and sulphide generation may occur without a black precipitate, or a black precipitate may develop without sulphide generation. Another potential disadvantage of the inclusion of iron in the medium occurs when the water bearing samples contains sulphide. The medium will turn black immediately upon inoculation (Herbert and Gilbert, 1984).

Many organisms such as coliform bacteria, *Proteus, Citrobacter, Salmonella, Pseudomonas* and *Clostridia* (Atlas & Bartha, 1987, Laishley *et al.*, 1984, McMeekin & Patterson, 1975, Oltmann *et al.*, 1975) are capable of sulphide production. The presence of these organisms in an environmental sample will give rise to false positive results.

8.1.3 Serological techniques

Culture media for the detection of bacteria are designed to enumerate the culturable population. A count of culturable bacteria is obtained after growth on a suitable medium containing carbon and/or other energy sources. Since all media are selective to a lesser or greater extent, and not all bacteria are recoverable, viable counts are rarely quantitative. The use of either polyclonal or monoclonal antibodies offers a potentially sensitive and specific means for identifying environmentally important bacteria. Antibodies of either type can be used to identify specific marker gene products or even intact microorganisms that express an appropriate antigen (Pickup, 1991). Enzyme-linked immunosorbent assay (ELISA) has been used for the detection of specific *Rhizobium strains* (Martensson *et al.*, 1984). Immunofluorescence microscopy has been widely applied for the detection and

enumeration of particular microorganisms when conventional techniques have proved difficult (Pickup, 1991). Immunofluorescence detection has been used to detect, for example, methanogenic bacteria (Conway de Macario *et al.*, 1982).

Successful application of fluorescent antibodies (FA) can be affected by a range of factors; (a) the specificity of the antibody to be used and the problems with nonspecific staining, (b) the interference from autofluorescence or nonspecific absorption of FA to the background, (c) the stability of the antigen under different growth conditions and environments, (d) the inability to distinguish between live and dead cells and (e) the efficiency of recovery of the desired cells from natural samples (Bohlool & Schmidt, 1980). The degree of specificity desired, varies with the ecological studies performed. FA can be made more specific by absorption with cross reacting organisms or may be pooled to obtain a more species-specific FA reagent. Various studies indicated that FA-staining reactions were highly strain specific (Diem *et al.*, 1977, Schank *et al.*, 1979, Schmidt *et al.*, 1968). Antisera prepared for methylotrophs proved quite specific in that they only reacted with their homologous bacteria and not with any of 25 other bacteria which included natural isolates (Reed and Dugan, 1978). Cross reactivity was found among related and unrelated organisms. Cross reaction of pneumococcal antisera with exopolysaccharides of *Rhizobium* and *Xanthomonas* were reported (Ford and Olson, 1988). The titre of the antisera play a critical role in the specificity of the antibody (Cloete, 1984). Autofluorescence of material in the sample and nonspecific attachment of FA to the background may mask the specifically stained cells and limit immuno fluorescence (IF) observations (Bohlool and Schmidt, 1980). Several techniques have been developed to reduce autofluorescence and nonspecific staining (Ford and Olson, 1988). Strayer and Tiedje (1978) found that prestaining of samples with 2% bovine serum albumin (BSA) was effective in blocking out non-specific absorption. Hobbie *et al.* (1977) found that the type of membrane filter as well as the pore size were important when using FI-techniques for enumerating bacteria. Many more bacteria were visible on nucleopore filters than on cellulose filters. Only a few antigens have been tested for their stability in the environment and have appeared to be relatively stable (Ford and Olson, 1988). For example, rhizobia have been extensively tested on different media, in different soils and as bacteroides in nodules (Bohlool and Schmidt, 1970). A distinct limitation of FA procedures is their inability to distinguish between viable and nonviable cells. Quantification of cell numbers by FA-techniques can be difficult in environmental samples (Ford and Olson, 1988).

8.1.3.1 Serological relationships of sulphate-reducing bacteria

8.1.3.1.1 Comparative immunological studies of somatic antigens of sulphate-reducing bacteria

Serological work on SRB has yielded conflicting results. Pronounced cross-reactions between strains of *D. vulgaris* and *D. desulfuricans* have been reported by Baker *et al.* (1962) and Postgate and Campbell (1963). Antisera have been developed against the whole-cell antigens of *D. africanus* strains Benghazi

and Walvis Bay, *D. vulgaris* strain Hildenborough, *D. salexigens* strain British Guiana, *Desulfovibrio gigas* and *D. desulfuricans* strain Essex 6. An ELISA was developed to measure the reaction of these antisera with the homologous and heterologous antigens. Pre-immune sera cross-reacted with cells of *D. africanus, D. gigas* and *D. desulfuricans*, suggesting the presence of a lectin-like substance on the cell surfaces of these bacteria. Extensive cross-reactions were observed between the antisera and heterologous cells, suggesting the sharing of a number of surface antigens amongst *Desulfovibrio* (Singleton *et al.*, 1985).

Antisera were prepared against a *D. desulfuricans* strain, a *D. vulgaris* strain and a *D. salexigens* strain. The antisera were tested for cross-reactivity against 36 heterologous *Desulfovibrio* strains by both agglutination titration and by double immunodiffusion precipitin plates. No cross-reaction was demonstrated by agglutination even between heterologous strains of the same species, suggesting that the surface antigens of *Desulfovibrio* are highly specific. In immunodiffusion plates a single apparently genus-specific surface antigen was present in all but two of the strains tested. Although other common precipitin bands showed the presence of some antigens common between heterologous strains, these appeared to be randomly distributed among the strains tested, with the exception of one band shown to be generally specific to strains of *D. salexigens* (Abdollahi and Nedwell, 1980). Agglutination tests with antisera prepared against somatic antigens of three strains of *Desulfovibrio* MK, Hildenborough and MF, revealed that cross-reaction among strains was restricted. The three strains, MK, Hildenborough and NCR 49001 shared common somatic antigens and the two strains, MF and MY, had other antigens in common, but the cells of the other strains were not agglutinated with these antisera. *D. vulgaris* and *D. desulfuricans* are heterogeneous on the basis of antigenic diversities of its cell-surfacer antigens (Aketagawa *et al.*, 1985).

Immunofluorescence was found to be mainly strain specific in the genera *Desulfovibrio* and *Desulfotomaculum*, although weak fluorescence was seen both within and between recognized groups. A polyvalent antiserum was successfully used to detect SRB (Smith, 1982).

Antisera were prepared against various SRB-type strains and used as a polyvalent antiserum mixture in ELISA for the detection of SRB. The polyvalent antiserum mixture was compared with the MPN-technique for the detection of SRB in natural samples. The counts by ELISA was at least an order of magnitude greater than that from the MPN counts (Bobowski and Nedwell, 1987, Gaylarde and Cook, 1987). The antisera prepared by Bobowski and Nedwell (1987) were prepared against cell extracts and the antisera prepared by Gaylarde and Cook (1987) against whole cells. The direct ELISA technique would be suitable for the detection and enumeration of specific strains of SRB, whilst the indirect techniques are applicable for total SRB enumeration (Gaylarde and Cook, 1987).

To succeed with immunological assays it is important to increase the knowledge of the antigenic properties of the bacteria. Norqvist and Roffey (1985) studied the envelope proteins of 5 strains of the genus *Desulfotomaculum* and 12 strains of the genus *Desulfovibrio* with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. A close relationship between strains of *Desulfotomaculum nigrificans* was observed. A comparison between different species *Desulfotomaculum* revealed some degree of similarity between *Desulfotomaculum nigrificans* and *Desulfotomaculum ruminis* whereas *Desulfotomaculum orientis* seemed unique. The strains of *Desulfovibrio salixigens* were quite different from the strains of the other species of *Desulfovibrio*. In two of the strains of *D. desulfuricans*, species-specific antigen was observed. The strains of *D. vulgaris*, *D. africanus*, and *D. desulfuricans* exhibited a similar outer membrane protein profile and also showed very similar antigenic reactions.

The use of bacterial cultures that have been extensively subcultured in the laboratory for the production of antibodies often result in antibodies that fail to recognize bacteria present in field samples (Pope and Zintel, 1989).

Antisera were prepared against whole cells of different SRB-strains by Lillibaek (1992). It was intended to identify quantitavely dominant strains in specific sediments and to describe the spatial distribution of SRB relating to redox conditions and sulphate reduction rates. They concluded that antibodies against culturable SRB react with bacteria present in natural systems. Immunoprobes are therefore important tools for the study/identification of bacteria in natural habitats (Lillebaek, 1992).

8.1.3.1.2 Comparative immunological study of hydrogenases of sulphate-reducing bacteria

Like many other anaerobic bacteria, some SRB possess hydrogenase, an enzyme which reversibly catalyzes the formation of hydrogen gas from hydrogen ions. Periplasmic hydrogenase from *D. vulgaris* MK was purified and immunological properties were examined and compared with those of other *Desulfovibrio* hydrogenases. Ouchterlony double diffusion and immunotitration tests of crude extracts from several strains of *Desulfovibrio* revealed that the enzyme from MK cells was immunologically identical with those from *D. vulgaris* Hildenborough and *D. desulfuricans* NRC 49001, but different from those from *D. vulgaris* MF and MY and *D. desulfuricans* Essex 6 strains (Aketagawa *et al.*, 1983). Antiserum against hydrogenase of *D. vulgaris* MK cross-reacted with hydrogenase from strains Hildenborough and *D. desulfuricans* NRC 49001, but not with those from other strains tested (Aketagawa *et al.*, 1985).

The influence of inactivating factors on the immunological activity of *D. desulfuricans* hydrogenase was investigated by Ziomek *et al.* (1984). Affinity-purified antibodies specific for the purified, active periplasmic hydrogenase of *D. desulfuricans* were prepared. Immunodiffusion and ELISA methods

showed distinct differences between the native form of hydrogenase and the enzyme modified by heat, acid, active-site and group-specific chemical treatments. They also found that the hydrogenase of two strains of *Escherichia coli* and several other bacteria cross-reacted with the hydrogenase of *D. desulfuricans* and that the periplasmic and membrane-bound hydrogenase of *D. desulfuricans* differed in immunological properties.

8.1.3.1.3 Comparative immunological study of sulphite reductase and cytochrome C3 of sulphate-reducing bacteria

The structure of sulphite reductase as well as cytochrome C_3 may have been better conserved during evolution than other cellular components because these two proteins are essential for sulphate respiration (Postgate, 1984). Cytochromes C_3 are multiple-heme proteins found in the nonspore-forming SRB, where the protein serves as an electron carrier in sulphate reduction (Postgate, 1984). Sulphite reductase catalyzes sulphite reduction to sulphide (Widdel, 1988).

Antisera were prepared against purified sulphite reductase of *Desulfovibrio vulgaris* MK and MF. Both the antisera cross reacted with the extracts from all the strains tested. This indicates that the sulphite reductases from *Desulfovibrio* share common antigenic determinants (Aketagawa *et al.*, 1985). The bisulphite reductase, desulfoviridin, present in *D. vulgaris* do occur in most of the classical lactate- or hydrogen-utilizing *Desulfovibrio* species. However, desulfoviridin is absent from *Desulfovibrio baculatis, D. thermophilis, D. sapovorans, D. baarsii, Desulfotomaculum, Desulfobulbus, Desulfobacter, Desulfococcus niacini, Desulfosarcina, Desulfobacterium* and *Desulfonema* (Widdel, 1988).

The cytochrome C_3 of *D. desulfuricans* and that of *D. vulgaris* did not share a common precipitating antigenic determinant (Drucker and Campbell, 1969). According to Singleton *et al.* (1984) the cytochromes C_3 of *D. africanus, D. vulgaris* and *D. salexigens* exhibited some degree of cross-reaction when using ELISA, in contrast to previous experiments using the Ouchterlony technique. Cytochrome C_3 is not present in all the described SRB genus and species (Widdel, 1988).

8.1.3.1.4 Comparative imunological study of APS-reductase of sulphate-reducing bacteria

All sulphate-reducing bacteria investigated to date contain a soluble adenosine 5'- phosphosulphate (APS) reductase which catalyzes the reduction of APS to sulphite and adenosine mono-phosphate (AMP) (Peck, 1968).

The objective of the study of Odom *et al.* (1991) was to investigate the immunological relatedness of APS reductase to determine whether the enzyme from SRB is sufficiently conserved to serve as an antigen for the rapid immunoassay for these organisms and whether antibodies to the enzyme of SRB

are cross-reactive with the enzyme from sulphide oxidizers. Crude extracts from 14 species of SRB comprising the genera *Desulfovibrio, Desulfotomaculum, Desulfobulbus* and *Desulfosarcina* and from three species of sulphideoxidizing bacteria were tested in an ELISA with polyclonal antisera to APS reductase from *D. desulfuricans.* The results showed that extracts from *Desulfovibrio* species were all highly cross-reactive, whereas extracts from other SRB-genera showed significantly less crossreaction. Extracts from colourless photosynthetic sulphur bacteria were either unreactive or exhibited very low levels of reactivity with the antibodies to the enzyme from SRB. Two types of monoclonal antibodies to APS reductase were also isolated. One type reacted more variably with the enzyme of the sulphate-reducers and poorly with *Thiobacillus* (Odom *et al.* 1991).

Immunoassay of APS reductase using polyclonal antisera has the potential for use as a detection method for *Desulfovibrio* species. However, distantly related *Desulfobulbus* and *Desulfosarcina* species show significant cross-reaction as well (Odom *et al.*, 1991).

8.1.4 Alternative detection methods

8.1.4.1 Detection of marker genes using nucleic acid probes

Nucleic acid probes that are used to detect marker genes, can be designed to detect a particular genotype or to detect unique sequences in the genome of the target organism. Nucleic acid probes can be double stranded DNA from genomic or plasmid origin. Similarly, oligonucleotide probes constructed *in vitro* have been used successfully to detect specific 16S rRNA (Pickup, 1991).

Microbial species or subspecies can be distinguished by oligonucleotides complementary to the most variable regions of the 16S rRNA molecule (Stahl *et al.* 1988). By targeting regions of increasing conservation, probes can be made to encompass specific genera or higher taxons (Woes *et al.*, 1985). Furthermore, based on the extensive 16S rRNA sequence data base, probes can be designed for organisms which have not yet been cultured (Olse, *et al.* 1986). Data regarding the phylogeny of SRB on the base of 16S rRNA can be used to design hybridization probes for the detection of SRB.

Fowler *et al.* (1986) explored the phylogeny of sulphate- and sulphur-reducing bacteria on the basis of 16S rRNA analysis. Their results suggested that SRB as a group are not closely related to one another. The genus *Desulfotomaculum* appeared to be more closely related to the gram-positive bacteria, whereas the genus *Desulfovibrio* was not related to the other genera of SRB. *D. gigas* and *D. desulfuricans* were, however, closely related to one another on the basis of 16S rRNA sequence analysis.

Phylogenetic relationships among 20 nonsporeforming and two endosporeforming species of sulphate-reducing eubacteria were deduced from comparative 16S rRNA sequencing. All genera of mesophilic SRB except the new genus *Desulfomicrobium* and the gliding *Desulfonema* species were included. The sporeforming species *Desulfotomaculum ruminus* and *D. orientis* were gram-positive organisms sharing 83% 16s rRNA sequences similarity. The gram-negative nonsporing species could be divided into seven natural groups: group 1, *D. desulfuricans* and other species of this genus that do not degrade fatty acids (this group also included *Desulfomonas pigra*); group 2, the fatty acid-degrading *Desulfovibrio sapovorans*; group 3, *Desulfobulbus* species. group 4, *Desulfobacter* species; group 5, *Desulfobacterium* species and *Desulfococcus niacini*; group 6, *Desulfococcus multivorans* and *Desulfosarcina variabilis*; and group 7, the fatty acid-oxidizing *Desulfovibrio baarsii* (Devereux *et al.*, 1989). A relationship between DNA relatedness and level of similarity of 16S rRNA sequences was defined and indicated that many pairs of *Desulfovibrio* species shared less than 10% sequence homology (Devereux *et al.*, 1990).

Probes complementary to short sequence elements within the 16S rRNA of SRB were labelled with tetramethylrhodamine (Amann *et al.*, 1990). By using these probes in flow cytometry, Amann *et al.* (1990) were able to detect *D. gigas* in mixed cultures when the target cells composed no more than 3% of the total suspension (Amann *et al.*, 1990). A region of the 16S rRNA common to SRB was selectively amplified by the polymerase chain reaction. Sequences of amplification products, with reference to a collection of 16S rRNA sequences representing most characterized SRB were used to design both general and specific hybridization probes. Fluorescent versions of these probes were used in combination with fluorescence microscopy to visualize specific SRB-populations within developing and established biofilms (Amann *et al.*, 1992). Fluorescent-dye-conjugated oligonucleotides were used as phylogenetic probes to identify single cells in complex microbial environments. The oligonucleotide sequence of the probes was selected to be complementary to short sequence elements within the 16S rRNA. Five different probes including a sulphate-reducing bacterial probe and a probe for *Desulfobacter* were synthesized. The probes have been tested with positive results in mixtures of cells from well characterized pure cultures and subsequently to stain slices of a photosynthetic biofilm (Ramsing *et al.*, 1992).

Cloned genes encoding hydrogenase from *D. vulgaris* can be used to detect the presence of members of this genus following growth of samples derived from oil fields (Voordouw *et al.*, 1990).

Total DNA extracted from environmental samples can be labelled and used to probe filters on which denatured chromosomal DNA from relevant standards have been spotted. The latter technique is referred to as sample genome probing, since it is the reverse of the usual practice of deriving probes from reference bacteria for analyzing a DNA sample. Reverse sample genome probing allows

identification of bacteria in a sample in a single step, once master filters with suitable standards have been developed. Reverse sample genome probing has been applied to identify SRB in oil field samples (Voordouw, *et al.*, 1991).

8.1.4.2 The use of biochemical methods for the detection of sulphate-reducers

Biochemical methods fall into two basic groups: those designed to detect molecules that are a part of the microbial cells themselves and those designed to detect metabolic products. The former can again be subdivided into two groups: those techniques which give an estimate of the total amount of bacteria and those techniques that are specific for a particular group of microbes (Pope and Zintel, 1989).

Fatty acid fingerprinting is a good method to detect specific bacteria. The identification of unique fatty acid profiles was partly successful during a study on the distribution of different genera of SRB in sediment and other natural environment (Taylor and Parkes, 1983).

A relatively easy method is based on the presence of the pigment, desulfoviridin, in *Desulfovibrio*. *Desulfovibrio* cells appeared red after the addition of NaOH (Sharma *et al.*, 1987).

Other techniques include the detection or analysis of metabolic products including gases. Metabolic pathways that are being used in the bacterial community may also be an indication of the organisms present (Pope and Zintel, 1989). Sulphates, hydrogen or hydrogen sulphide can be identified using radiotracer materials. A field method is described for the assay of [^{35}S]sulphate reduction by SRB in biofilms on metal surfaces (Maxwell and Hamilton, 1986).

8.1.5 Commercially available test kits for sulphate-reducers

The Rapidchek SRB test (Conoco Speciality Products, Inc.) uses specific antibodies (purified antibodies to the APS reductase enzyme) to detect the presence of SRB in environmental samples. The SRB assay is based on the fact that all SRB possess the enzyme APS reductase.

The GEN-PROBE Chemilluminescent DNA probe (Gen-Probe Incorporated) and detection system is a non-isotopic hybridization system for detection of specific DNA or RNA sequences. These detection kits can be used for the detection of all SRB, members of the genus *Desulfobacter* or members of the genus *Desulfotomaculum*.

The TEST-KIT SRB LABEGE (GRAM S.A. Laboratory) is a test kit to detect and enumerate SRB by the use of culture medium. The medium is absolutely oxygen-free. An oxygen presence indicator

and a non-sulphur, non-toxic strong reducing agent is added. It is also comprises two carbon and energy sources.

Chapter 2

Evaluation of Nutrient Agars for the Enumeration of Viable Aerobic Heterotrophs in Cooling Water

V.S. BRÖZEL and T.E. CLOETE

Abstract

The agar plate method is still the method of choice for quantifying viable bacteria in water, especially in routine laboratories. Eleven nutrient agar media were compared for colony producing ability by incubating at 25°C and at 30°C. Appearance of colonies was recorded against time and colony forming curves were constructed. The highest counts after five days (*ca* 75% of maximum count) were obtained on R3A agar incubated at 30°C. R3A agar at 30°C also allowed the most rapid colony development, with most of the colonies appearing between two and five days. Both Plate Count Agar (PCA) and Standard I Agar (StdI) performed poorly. PCA yielded 30% of the highest count after five days at 30°C and at 25 °C. StdI yielded 27% at 30°C and 23% at 25°C. The standard procedure of 48 hours at 30°C on PCA yielded 14% of the final highest count, *i.e.* one log less.

In contrast to PCA and Std I, R3A agar contained magnesium and pyruvate, both of which on their own were shown to increase the plate count. R3A also had a lower nitrogen content and nitrogen to carbon ratio. The highest count after 14 days was achieved on double strength R3A (R4A) at 20°C. R4A also showed the highest colony diversity. It was therefore seen to be the most suitable for population studies. However, colony development was slower. This makes it unsuitable for plate counts, whereas R3A lets most cells develop into colonies within the first five days of incubation.

1 INTRODUCTION

The determination of bacterial numbers is a basic prerequisite in microbiology and yet the study of microorganisms in nature remains one of the most poorly quantitated areas of microbiological research (Karl, 1986). To date our attempts at understanding microbial ecology are limited by the lack of suitable methods (Karl, 1986). Quantification of live bacterial cells in medical or food samples can be performed using reliable counting techniques such as the agar plate count (VanDemark and Batzing, 1987). However bacteria from aquatic and soil environments cannot be accurately enumerated (Roszak, Grimes and Colwell, 1984; Atlas and Bartha, 1987). Non-critical use of so-called standard methods may yield data of questionable accuracy as microorganisms live in microenvironments having physicochemical properties which may be distinctly different from those of the surrounding environment (Karl, 1986).

The agar plate count is based on the assumption that a viable bacterial cell is capable of multiplying to form two progeny and so on in conditions that are "optimal" for the cell concerned (Postgate, 1963). Continued growth under such conditions on agar would result in a visible colony (Hattori, 1988). The count therefore reflects the number of cells capable of dividing under the given conditions, and not necessarily the total viable number. Due to the physiological diversity of microbial groups and the physicochemical diversity of their microenvironments, no single medium has ever been able to provide the growth requirements of more than a small percentage of the total number of bacteria in a natural habitat (Karl, 1986). Direct microscopic counts of aquatic samples are always much higher than plate counts (Jannasch and Jones, 1959; Kogure, Simidu and Taga, 1978). Kogure *et al.* (1978) reported the agar plate count of open sea samples to be 0.1% of the direct count. As living and dead particles could not be distinguished directly, this ratio could however have been too low (Kogure *et al*, 1978). Various methods have been reported which can be employed to distinguish between viable and non-viable bacteria (Herbert, 1990; Roszak and Colwell, 1987)

The bacterial culture media commonly used in laboratories are mostly designed for the cultivation of human pathogens or other fastidious bacteria and contain mainly proteinaceous substrates such as peptones and meat- and yeast extract (Stolp and Starr, 1981). Agars commonly used to determine viable bacterial numbers in waters, *e.g.* Plate Count Agar (Reasoner and Geldreich, 1985), Yeast Extract Agar (Gibbs and Hayes, 1988) and Standard I nutrient agar (Brözel, 1990) fall in this category. Yet planktonic bacteria in water systems have different nutrient requirements to the fastidious bacteria (Pointdexter, 1981). Consequentially many viable bacteria do not form visible colonies on certain agars, resulting in a false low count (Roszak *et.al.*, 1984; Sørheim, Torsvik and Goksøyr, 1989). This may be due either to substrate induced death, (Postgate and Hunter, 1964), where starved cells die upon exposure to excess nutrients, or to the induction of a nonculturable state of the cells (Roth, Leckie and Dietzler, 1988). Furthermore, if a specific nutrient is withheld from a cell where all others are abundantly available, unbalanced growth occurs; the cell grows but does not divide and subse-

quently dies (Dawes, 1989). The composition of a nutrient agar for enumeration of viable bacterial cells in a specific environment should therefore be tailored to the specific nutritional requirements of the relevant bacteria or of the majority thereof. Cooling waters are nutritionally and minerally enriched. Neither media for the cultivation of oligotrophic or fastidious bacteria are suited for their cultivation.

The number of colonies formed in the standard plate count are normally counted after 48 or 72 hours (Gibbs and Hayes, 1988 ;Greenberg, Trusell and Clesceri, 1985). Yet water contains many slow-growing bacteria which take longer to form a visible colony (Hattori, 1988; Reasoner and Geldreich, 1985). There is also a time lag before initiation of cell division takes place on agar surfaces, similar but longer than in liquid culture (Hattori, 1988). This time lag is not identical for every cell of a strain, but is distributed around a mean value (Hattori, 1988). Plots of the number of visible colonies formed over time are termed colony forming curves (Hattori, 1988). From these the kinetics of colony formation can be calculated and the optimal incubation time determined.

The authors constructed the colony forming curves of three cooling water samples on a series of eleven different nutrient agars and at two different temperatures, and report the results below. As pour plates are repeatedly reported to give consistently lower counts from water samples (Gibbs and Hayes, 1988; Klein and Wu, 1974; Reasoner and Geldreich, 1985), only spread plates were used in this study.

2 MATERIALS AND METHODS

2.1 Media used

The media used were two agars formulated for semi-oligotrophic water samples by Henrici (1933) and Taylor (1940); R2A agar formulated by Reasoner and Geldreich (1985) for drinking water counts, and three derivatives of higher strength (R3A, R4A and R5A); Standard I (StdI) nutrient agar and half and tenth strength StdI, and Plate Count agar (PCA) and half strength PCA. The compositions of these agars are given in Table 8.

The range of Standard I (Std I) agars were prepared from Merck Std I nutrient agar. Std I was prepared as specified. Half strength StdI was prepared using 18.5g Std I agar and adding 7.5g agar. Tenth strength Std I was prepared using 3.7g StdI agar and adding 13.5g agar. Plate count agar (PCA) was prepared as specified. Half strength PCA was prepared using 11.75g PCA and adding 7.5g agar. All other agars were prepared according to the formulations listed in Table 8. $FeCl_2$ was added in filter sterilized form to the media after autoclaving at. All media were dissolved in deionized water and pH was adjusted to 7.2 before autoclaving at 121 ^{o}C for 15 min. Agar, meat extract, proteose peptone and yeast extract were from Biolab Diagnostics, $MgSO_4$ was from BDH Laboratory Reagents, glycerol and K_2HPO_4 were from PAL Chemicals, D(+) Glucose was from May & Baker Ltd., soluble starch

was from Riedel - De Haen AG., $FeCl_2$, peptone, Plate Count Agar, Standard I Nutrient Agar and sodium pyruvate were from Merck and Casamino acids and sodium caseinate were from Difco Laboratories.

Table 8: Composition of agars used in the study (data given as $g.l^{-1}$ of final medium)

Agar:	Henr. *	Taylor**	R_2A ø	R_3Aø	R_4Aøøø	R_5Aøøø	Std 1#	1/2Std1# #	1/10Std1# ##	PCA$	1/2PCA$ $
Component:											
Proteose peptone			0.5	1.0	2.0	5.0					
Peptone (meat)	0.5	0.5					7.8	3.9	0.78	5.0	2.5
Yeast extract			0.5	1.0	2.0	5.0	2.8	1.4	0.28	2.5	1.25
Meat extract							7.8	3.9	0.78		
Casamino acids			0.5	1.0	2.0	5.0					
Na caseinate	0.5	0.5									
Glucose			0.5	1.0	2.0	5.0	1.0	0.5	0.1	1.0	0.5
Glycerol	0.5	1.0									
Starch (soluble)	0.5	0.5	0.5	1.0	2.0	5.0					
Na pyruvate			0.3	0.5	1.0	2.0					
NaCl							5.6	2.8	0.56		
K_2HPO_4		0.2	0.3	0.6	1.2	3.0					
$MgSO_4.H_2O$		0.05	0.05	0.1	0.2	0.5					
$FeCl_2$		trace									
Total	2.0	2.75	3.15	6.2	12.4	30.5	25.0	12.5	2.5	8.5	4.25
Agar	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0

* = Henrici (1933)
** = Taylor (1940)
ø = Reasoner and Geldreich (1985)
øø = Double strength R2A
øøø = Four strength R2A
øøøø = Ten strength R2A
= Standard I agar (Merck)
= Half strength Standard I agar.
= Tenth strength Standard I agar.
$ = Plate count agar (Merck).
$$ = Half strength plate count agar.

2.2 Colony forming curves

Samples were taken from three different water cooling systems. Sample 1 was from an untreated steel works cooling system. Sample 2 was from an untreated gold mine compressor pond. Sample 3 was from a pilot plant treated every seven days with 20 $mg.l^{-1}$ Na dimethyl dithiocarbamate. Serial dilutions were prepared in quarter strength Ringer's solution (Merck) directly after sampling. Aliquots of 0.1 ml were spread onto the series of nutrient agars, using a Drigalski spatula flamed with 70% ethanol.

Each dilution was spread onto six plates of each agar. Then three plates of each dilution were incubated at 25°C and 30°C respectively. Colonies visible to the naked eye were counted every 24 hours for six days and again after fourteen days. Colony forming curves were constructed by recording the daily means of three total counts on semi-log paper.

3 RESULTS

3.1 Incubation time

Sample 1: A variety of colony forming curves were obtained on the various agars. Some approached the maximum value after a short incubation period, whereas others were much slower, with new colonies appearing continually, even after 14 days of incubation, *e.g.* Std I and Plate Count Agar at 25 °C (Fig. 13). The highest final values were however obtained under those conditions where most colonies appeared after 120 hours, *e.g.* R2A at 25 °C and 30 °C which yielded 90% and 89%, respectively, of the final count. Even after 48 hours, R2A at 30 °C yielded 60% of the final count, but at 25 °C it was only 18%.

Sample 2: This sample also yielded a variety of counts under the various medium temperature combinations (Fig. 14). R3A at 30 °C yielded 4.4 X 10^5 $cfu.ml^{-1}$ or 69% of the highest value after 48 h and R2A yielded 3.2 X 10^5 $cfu.ml^{-1}$ or 50% of the highest value. After 96 hours R3A at 30 °C yielded 95% of the maximal value, and at 25 °C it yielded 92% after 144 h. All other combinations showed slower colony forming rates. Std I at 25 and 30 °C yielded all colonies after 144 h, but the total was 2.7 X 10^5 $cfu.ml^{-1}$ whereas the highest total was 6.4 X 10^5 $cfu.ml^{-1}$ on R2A at 25 °C. PCA gave 3.1 X 10^5 $cfu.ml^{-1}$ at 25 °C and 2.5 X 10^5 $cfu.ml^{-1}$ at 30 °C after six days (Fig. 14)

Sample 3: In this sample colonies developed faster at 30 °C than at 25 °C (Fig. 15). R2A at 30 °C yielded 3.7 X 10^5 cfu.ml-1 after 72 h but 3.8 X 10^5 cfu.ml-1 after 96 h at 25 °C. 1/10 Std I yielded 4.7 X 10^5 cfu.ml-1 after 96 h at 30 °C and 5 X 10^5 cfu.ml-1 after 120 h at 25 °C. The same trend holds for PCA, R2A, and 1/2 Std I. The colony forming rate was higher during the first 72h at 30 °C than it was at 25 °C on all the agars giving higher yields. At 25 °C the minimum incubation time to achieve the maximum number of colonies was 144 h, and at 30 °C it was 120 h (Fig. 15).

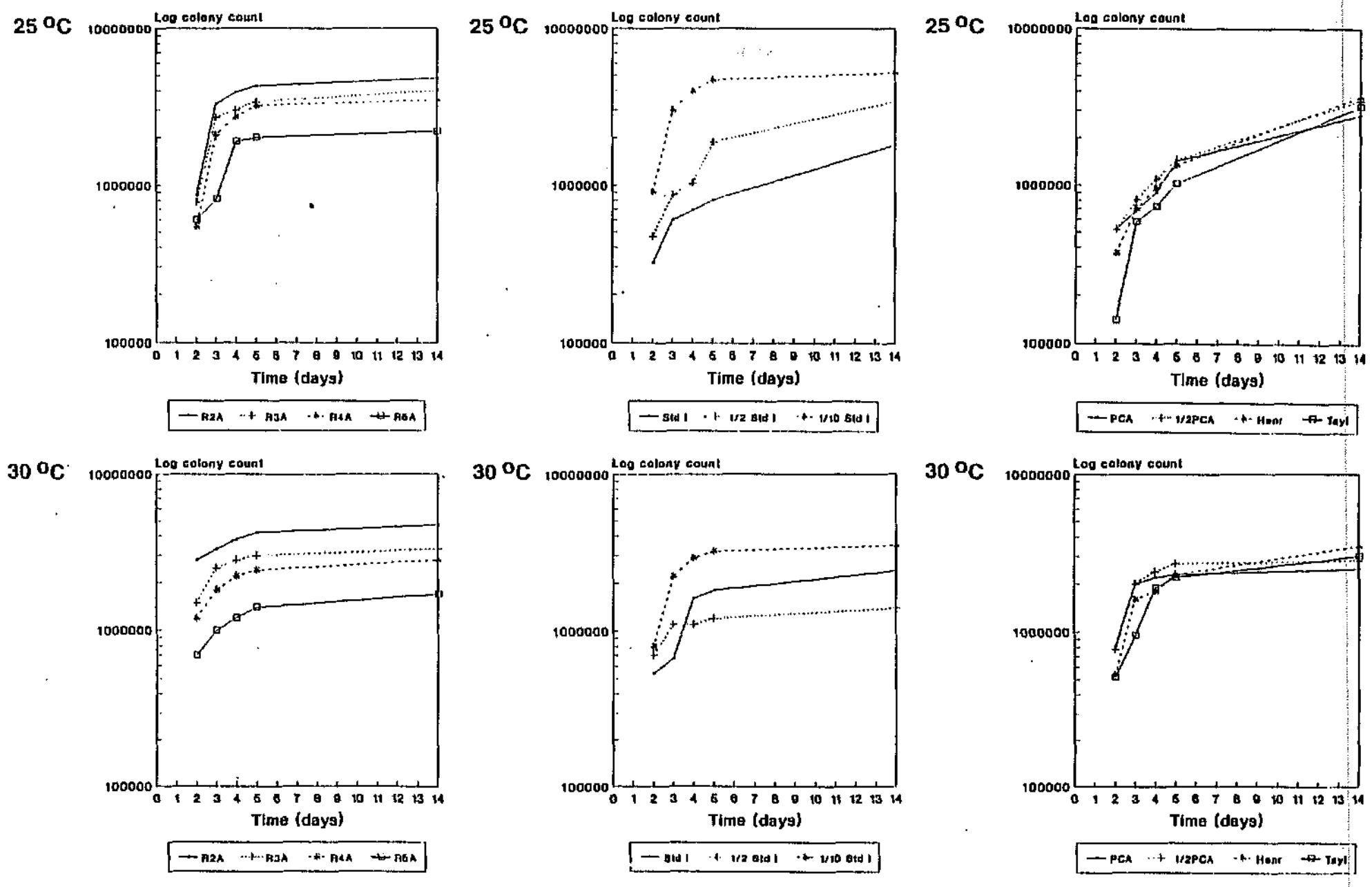


Figure 13: Colony forming curves of sample 1 incubated on various agars at 25 °C and 30 °C.

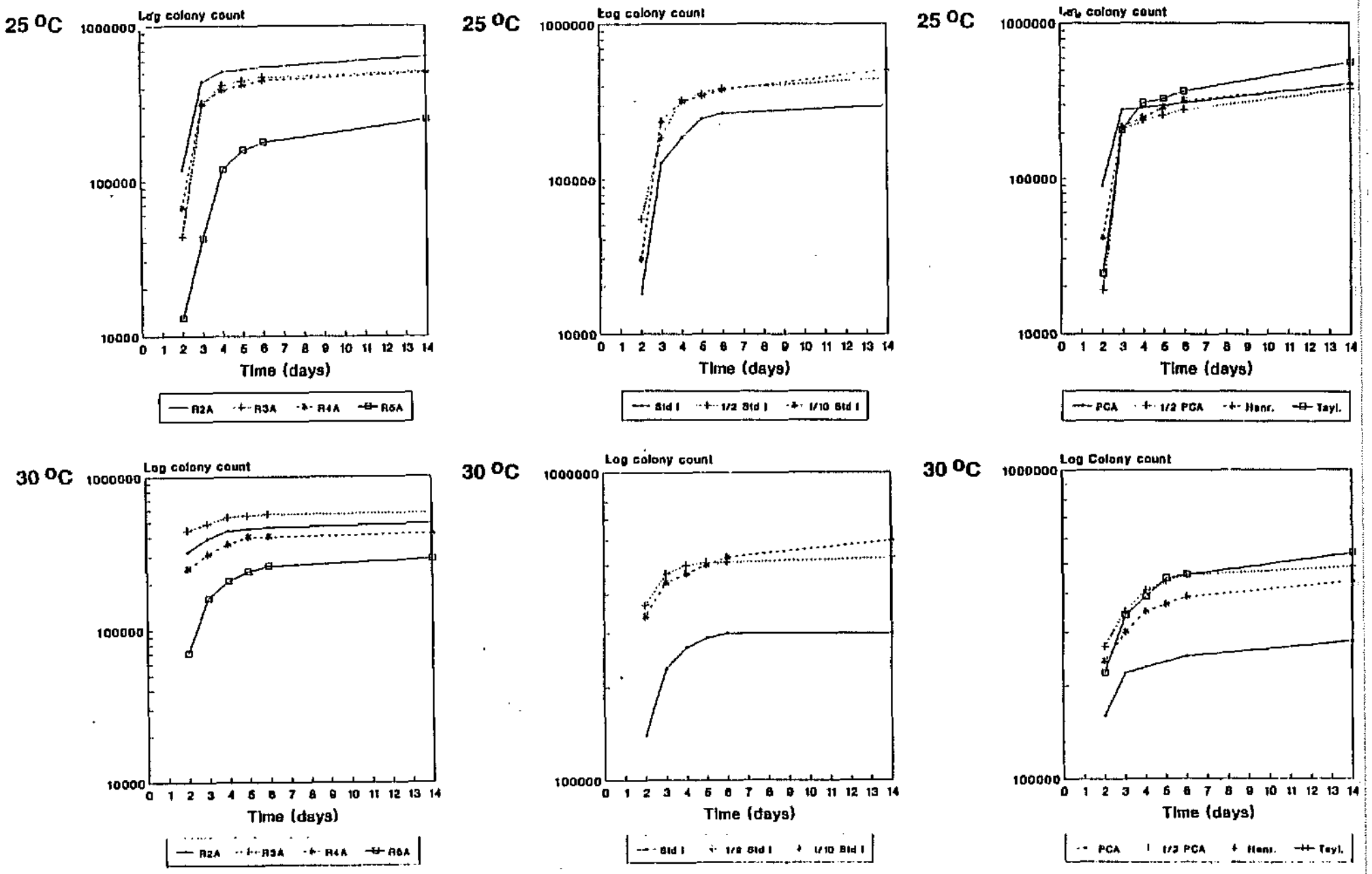


Figure 14: Colony forming curves of sample 2 incubated on various agars at 25 °C and 30 °C.

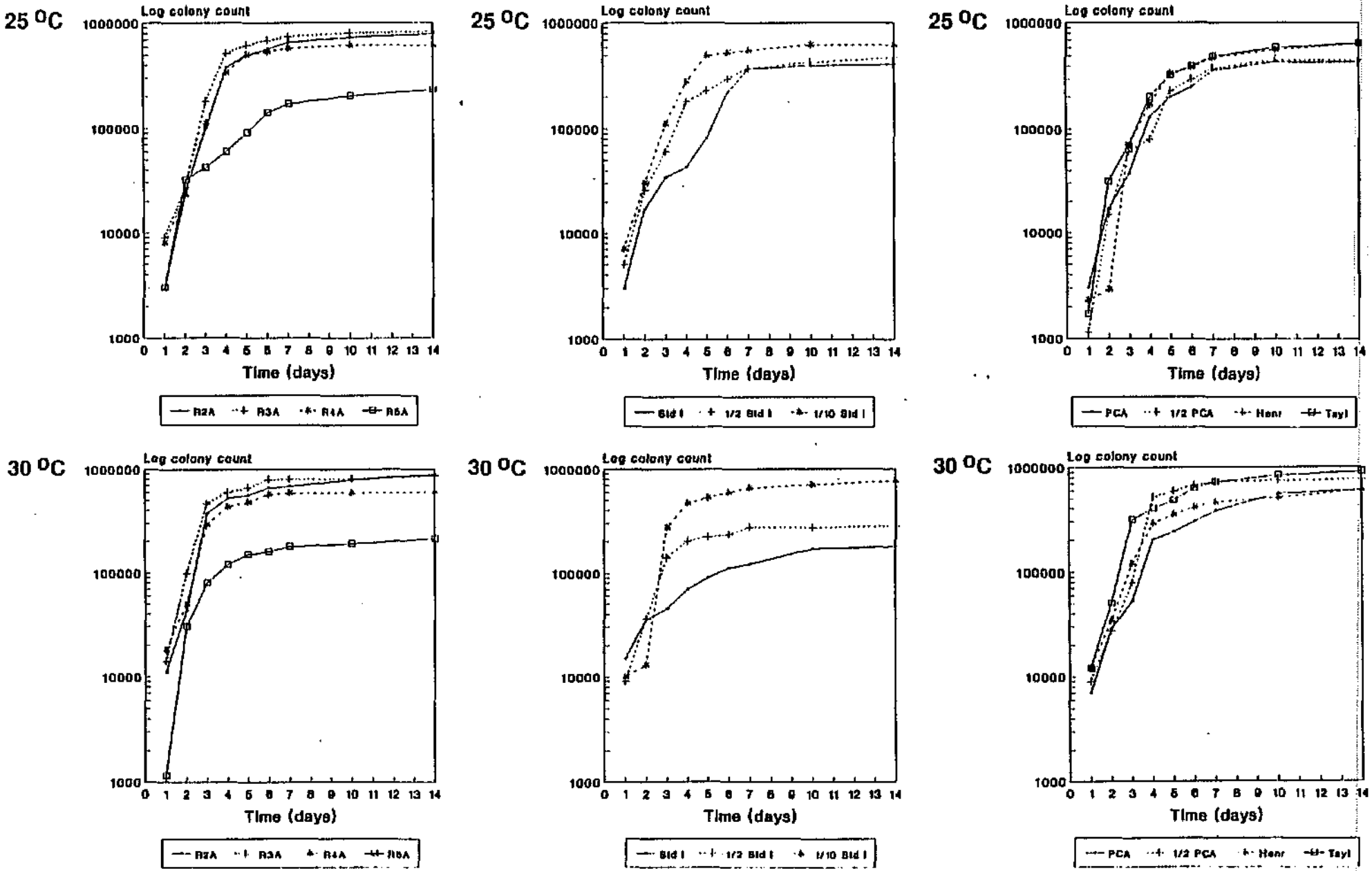


Figure 15: Colony forming curves of sample 3 incubated on various agars at 25 °C and 30 °C.

3.2 Incubation temperature

Sample 1: After 48 h the count on R2A at 30 °C was much higher than at 25 °C (1.5 X 10^6 *vs* 8.6 X 10^5 cfu.ml^{-1}) (Fig. 13). All RxA agars showed this tendency although the more concentrated ones gave more colonies at the lower temperature after 120h and 14 d (*e.g.* 3.4 X 10^6 cfu.ml^{-1} at 25 °C *vs* 3.0 X 10^6 cfu.ml^{-1} at 30 °C after 120 h on R3A). The Std I range gave similar counts between the two temperatures after 48 h, but with time the three agars reacted differently at the two temperatures. 1/10 Std I gave a higher count after 120 h at 25 °C (4.7 X 10^6 cfu.ml^{-1}) than at 30 °C (3.2 X 10^6 cfu.ml^{-1}), but Std I gave a higher count after 120 h at 30 °C (1.8 X 10^6 cfu.ml^{-1}) than at 25 °C (8.1 X 10^5 cfu.ml^{-1}) (Fig. 13).

Sample 2: The bacteria in this sample also produced more colonies after 48 hours at 30 °C than at 25 °C (*e.g.* 4.4 X 10^5 cfu.ml^{-1} *vs* 4.3 X 10^4 cfu.ml^{-1} on R3A) (Fig. 14). After 120 h R4A gave similar counts at both temperatures, but the poorer R3A gave more colonies at 30 °C than at 25 °C (5.6 X 10^5 cfu.ml^{-1} at 30 °C *vs.* 4.5 X 10^5 cfu.ml^{-1} at 25 °C), whereas the nutrient poorer R2A gave more at 25 °C than at 30 °C (5.3 X 10^5 cfu.ml^{-1} at 25 °C *vs.* 4.5 X 10^5 cfu.ml^{-1} at 30 °C). 1/2 PCA also gave a higher count at 30 °C (4.4 X 10^5 cfu.ml^{-1}) than at 25 °C (2.6 X 10^5 cfu.ml^{-1}), whereas PCA gave a higher count at 25 °C (3.0 X 10^5 cfu.ml^{-1}) than at 30 °C (2.4 X 10^5 cfu.ml^{-1}). This suggests a slight inverse proportional relationship between incubation temperature and nutrient concentration. The Std I range gave higher counts at 30 °C than at 25 °C (Fig. 14).

Sample 3: After 48 hours at 25 °C the counts on the RxA range were similar (Fig. 15). However at 30 °C R3A gave the highest count (1.0 X 10^5 cfu.ml^{-1}), followed by R4A (5.0 X 10^4 cfu.ml^{-1}) and R2A (4.3 X 10^4 cfu.ml^{-1}). At 25 °C the colony forming rate decreased after 96 h, whereas it decreased after 72 h at 30 °C. Irrespective of incubation temperature, R3A after 96 h gave the highest count, followed by R2A, R4A and R5A. In the period 72 to 168 h, 30 °C gave more colonies on R3A, R2A and R4A than did 25 °C. The Std I agars also yielded more colonies at 30 °C than at 25 °C. 1/10 Std I yielded 1.1 X 10^5 cfu.ml^{-1} at 25 °C and 2.7 X 10^5 cfu.ml^{-1} at 30 °C after 72 hours. PCA and even more so 1/2 PCA also yielded more colonies at 30 °C than at 25 °C. 1/2 PCA after 96 h gave 8.0 X 10^4 cfu.ml^{-1} at 25 °C and 5.2 X 10^5 cfu.ml^{-1} at 30 °C (Fig. 15).

3.3 Nutrient composition

Sample 1: There was a clear relationship between number of colonies and nutrient concentration in the RxA range; the higher the concentration, the less colonies were formed (Fig. 13). The same held for Std I at 25 °C. However at 30 °C Std I gave higher counts than 1/2 Std I, although lower than 1/10 Std I. PCA and 1/2 PCA gave similar counts. The number of colonies was not as temperature dependant on the RxA range as it was on the Std I and PCA ranges and with the agars of Taylor and of Henrici. However on R2A at 30 °C 60% of the colonies had already developed after 48 h. The other agars did not show this trend, suggesting that R2A has some property which induced colony

formation much faster than the other agars. PCA generated more colonies after 72 hours at 30 °C than at 25 °C although the counts after 14 days were similar. With 1/10 Std I the converse holds, but Std I reacted better at 30 °C. Again this suggests that nutrient composition and concentration have a marked effect on initiation of colony formation (Fig. 13).

Sample 2: Here too, there was an inverse relationship between number of colonies and nutrient concentration at 25 °C, but at 30 °C R3A gave a higher recovery than R2A Fig. 14). Although Std I gave lower counts at both temperatures, 1/2 and 1/10 Std I gave very similar results, suggesting that there was a maximum nutrient tollerance level in some of the cells, above which they would not form colonies. PCA gave much lower recovery than did 1/2 PCA (54% as 1/2 PCA after 120 h at 30 °C). The number of colonies after 96 h and later was again not as temperature dependant on the RxA range as it was on the other agars. After 48 h at 30 °C on R3A, 77% of the colonies had already developed. At 25 °C it was only 7.5%. At 25 °C on R2A the count after 48 h was 18% of the final count, whereas it was 65% at 30 °C. Again the nutrient composition and concentration and incubation temperature seemed to have an effect on colony formation. On Std I at 30 °C no more colonies formed after 144 h. On 1/2 and on 1/10 Std I colonies kept being formed after 144 h. The lower nutrient concentration seems to have left more cells viable and allowed them to divide after a rest or recovery period. Although these same cells were present on Std I, they could not form colonies on this culture medium (Fig. 14).

Sample 3: Thes bacterial population of this sample seemed more tollerant to various nutrient surroundings (Fig. 15). The Std I range did however show an inverse relationship between nutrient concentration and colony formation. Also R5A showed a lower colony forming rate after 48 hours than did the other RxA agars (Fig. 15).

4 DISCUSSION

Bacteria from oligotrophic systems are enumerated by low-nutrient agars incorporating a range of suitable nutrients. Examples are Henrici's medium (Henrici, 1933) and Taylor's medium (Taylor, 1940) containing 2.0g and 2.75g of nutrients per litre respectively. Conversely bacteria in food samples are enumerated by high nutrient agars such as PCA and Std I, which contain between 8.5g and 25g of nutrients per litre. Reasoner and Geldreich (1985) devised their R2A agar for enumeration of heterotrophs in drinking water. However water used in cooling systems has a much higher nutrient content than drinking water as it is not pretreated and systems are open to the atmosphere. The nutrient status of such waters varies considerably, but are in the order of 2700 $mg.l^{-1}$ dissolved solids and 190 $mg.l^{-1}$ suspended solids (Howarth and McEwan, 1989). Further they contain calcium (6 - 12 mM), chloride (1 - 2 mM) sulphate (14 - 20 mM) and nitrate (0.3 - 0.8 mM) in small quantities (Howarth and McEwan, 1989). Therefore the dominant bacteria present in cooling water would have

higher average nutrient requirements than the semi-oligotrophic bacteria in drinking water. Consequently agars employed for enumeration of such heterotrophs should have a higher nutrient content than those used for enumeration of bacteria in drinking water.

Although no single medium can be expected to recover all viable cells from a sample, it would be appropriate to use the medium yielding the highest recovery (Reasoner and Geldreich, 1985). Various authors have reported R2A to yield higher counts than PCA of a variety of treated and untreated drinking waters and unchlorinated source waters (Greenberg *et al.*, 1985; Means, Hanami, Ridgway and Olson, 1981; Taylor and Geldreich, 1979). The results obtained by us show that total nutrient concentration *per se* does not dictate colony formation. R4A and 1/2 Std I contain $12.4 g.l^{-1}$ and $12.5 g.l^{-1}$ of nutrients respectively. Yet R4A yielded 2.4×10^6 cfu.ml^{-1} and 5.7×10^5 cfu.ml^{-1} respectively from samples 1 and 3 after 120 and 144 hours at 30 ^{o}C whereas 1/2 Std I yielded 1.2×10^6 cfu.ml^{-1} and 2.3×10^5 cfu.ml^{-1}. PCA contains only $8.5 g.l^{-1}$ of nutrients, and also gave lower recovery in above cases (2.3×10^6 cfu.ml^{-1} and 3.0×10^5 cfu.ml^{-1}). PCA and Std I contain a high percentage of nitrogenous nutrients (peptones, meat extract and yeast extract), whereas R4A contains only 50% nitrogenous nutrients. This shows that a lower nitrogen content is beneficial to colony formation. PCA has 88.2% nitrogen-rich nutrients, Std I has 73.6% and R4A has 48.4%. This shows that a lower nitrogen to carbon ratio yields more colonies from water samples.

The agars of Henrici (1933) and of Taylor (1940) also have lower nitrogen to carbon ratios (1:1 and 1:2) and yet they gave similar counts to PCA and 1/2 PCA with its ratio of 4:1. This implies that the specific carbon source or combination thereof is the contributing factor. The RxA range contained pyruvate as only additional carbon source. The question is, whether pyruvate exerts some form of inducing action on cells in a dormant state (Roth *et al.*, 1988), or prevents unbalanced growth of starved or damaged cells (Dawes, 1989). RxA also contains magnesium and phosphate which could contribute to induction of colony formation.

Although 30 ^{o}C gave higher results, these were often not so different to those at 25 ^{o}C. However two samples did give within 90% of the final count within 48 h at 30 ^{o}C on R2A or R3A. It seemed the best temperature of incubation for the samples studied. Furthermore R3A gave the highest counts on average, although R2A performed slightly better on one occasion. This shows that cooling waters require agars of higher nutrient content than do drinking waters where R2A was found the best agar (Reasoner and Geldreich, 1985) but of lower nutrient content than culture media used for enumerating bacteria from food or medical samples.

From the figures it is clear that each cooling water did react differently on the various agars at the two temperatures. Only R3A gave consistently good results at 30 ^{o}C. This indicated that each cooling

water contained a different bacterial population with its own unique set of nutrient requirements. However R3A seems to have the optimal combination of nutrients at the correct concentrations to fascilitate recovery of the highest achievable number of colonies.

5 ACKNOWLEDGEMENTS

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Chapter 3

Effect of Storage Time and Temperature on the Aerobic Bacterial Count and on the Population Structure of Water Samples

V.S. BRÖZEL and T.E. CLOETE

Abstract

The reactions of a cooling water sample and a tap water sample to storage at various temperatures were determined. Samples were stored at 4, 10, 20, and 30 °C for 24, 48, 72, and 216h and the total culturable count and population structure of each were determined. The culturable count in both samples varied over time, even at 4 °C after 24h. In the cooling water sample, the dominant isolates throughout were *Pseudomonas stutzeri* and an unidentified pink isolate although the other isolates were present randomly. No direct tendencies of rate of decrease or increase could be detected in any of the samples, either in the culturable count or in population structure. Therefore results of analysis after storage cannot be adapted by a known factor. They must be interpreted with extreme caution, as they do not of necessity reflect the bacterial composition of the sample as drawn, both in terms of total numbers and in terms of population structure. Only counts performed on fresh samples yield reliable results on the total culturable count, and only population structures performed immediately, reflect the state of the population in the system from which the sample was drawn.

1 INTRODUCTION

The enumeration of live bacteria from water samples is still performed mostly by the agar plate count technique (Brözel and Cloete, 1990[a]). As this technique requires specialized laboratory equipment, samples usually have to be transported to laboratories at locations distant from the sampling site. Lower temperatures are usually taken to imply lower enzymatic reaction rates (Laidler and Meiser, 1982; Stanier, Ingraham, Wheelis and Painter, 1986) and therefore to decrease the rate of bacterial cell division and death. Stanier *et. al.* (1986) state: "From the effect of temperature on the rate of a chemical reaction one would predict that all bacteria would continue to grow (although at progressively lower rates) as the temperature is reduced, until the system freezes. However, most bacteria stop growing at a temperature well above the freezing point of water." Therefore samples are shipped cold when bacterial analysis has to be performed away from the sampling site (Harrigan and McCance, 1976). This practice has often led to the assumption that samples retain their bacterial composition when refrigerated. The authors have often received samples for analysis after these have been kept in a refrigerated state for a day or more. As some intensive studies are based on results obtained from samples drawn at far-away sites (Brözel and Cloete, 1990[b]; Brözel and Cloete, 1990[c]), a change in the initial composition of a sample used, could yield results of reduced value.

Various factors influence the result obtained when performing a plate count. These include the ability of cells to form colonies on nutrient agar; cells are either in the so-called culturable state where they do form colonies (Roszak and Colwell, 1987) whereas many cells are unable to divide even on a nutrient agar optimal for the species, the viable but non-culturable state (Roszak, Grimes and Colwell, 1983). A further factor is the treatment of cells during analysis. The brief higher temperature to which cells are exposed during the pour plate method has repeatedly been shown to give lower recovery of cells (Klein and Wu, 1974). Lastly the nutrient composition of the agar used plays a decisive role on colony development by viable cells (Brözel and Cloete, 1990[a]) and on the species recovered (Gustafsson and Mården, 1989; Sørheim, Torsvik and Goksøyr, 1989).

The authors studied the reactions of two water samples to storage at various temperatures and over various times to determine the effect on the culturable count and on the bacterial population structure of the sample.

2 MATERIALS AND METHODS

2.1 Samples

Two water samples were evaluated, one from an open water cooling system treated weekly with 40 ppm Na methyldithiocarbamate bactericide and one from a drinking water tap in the laboratory. Four samples of each were taken by drawing 10 ml aliquots into sterile glass tubes and covering with aluminium caps. These were then taken directly to the laboratory and incubated at 4, 10, 20, and 30 ^{o}C.

2.2 Aerobic plate count

The number of culturable aerobes was determined on the fresh sample within ten minutes of drawing. This was repeated after 24, 48, 72, and 216h on samples stored at each of the four temperatures (*i.e.* 4, 10, 20, and 30 ^{o}C). The count was performed by preparing a serial dilution series in sterile tap water. 0.1 ml aliquots of these were spread aseptically and in triplicate onto R2A agar for the tap water sample, and onto R3A agar for the cooling water sample using a Drigalski spatula, and incubated at 30 ^{o}C for 120h (Brözel and Cloete, 1990[a]).

2.3 Population structure of samples

The dominant bacterial species in each sample were determined by picking *ca.* ten colonies off one of the three plates of highest dilution yielding a count over 30. Colonies were picked at random using the Harrison's disk method (Harrigan and McCance, 1976). These were suspended and vortexed in quarter strength Ringer's solution (Merck, (Pty) Ltd.). A drop of the suspension was streaked out onto R3A agar (Reasoner and Geldreich, 1985) using a platinum wire loop, and incubated at 25 ^{o}C. This procedure was repeated a further two times, after which colonies were regarded as pure. Incubation was always for 120 hours as some isolates grew slowly, and slow-growing contaminants could be expected in faster growing isolates. Gram stains were performed to check for purity.

Isolates were grown for 18 hours and were then subjected to Gram stain, oxidase, catalase and O/F tests (medium of Hugh and Leifson containing glucose) (Hugh and Leifson, 1953). All isolates showing non-fermentative alkali producing metabolism, were retested on O/F medium containing only 10% of the prescribed quantity of peptone (0.2g.l^{-1}) as acid production from glucose by some Gram negative oxidative rods is masked by alkali production from metabolism of amino acids in the peptone (Snell and Lapage, 1971; Ward, Wolfe, Justice and Olson, 1986). Motility of Gram negative isolates was determined by streaking onto motility agar (R3A agar containing only 0.4% agar) (Krieg and Gerhard, 1981). Where isolates were immotile on motility agar, transmission electron microscopy was performed on negative stains of 4h old liquid cultures to show absence of flagella. Preliminary identification was by a key modified from that of Fischer, Jooste and Novello (1986), as shown in Fig. 16.

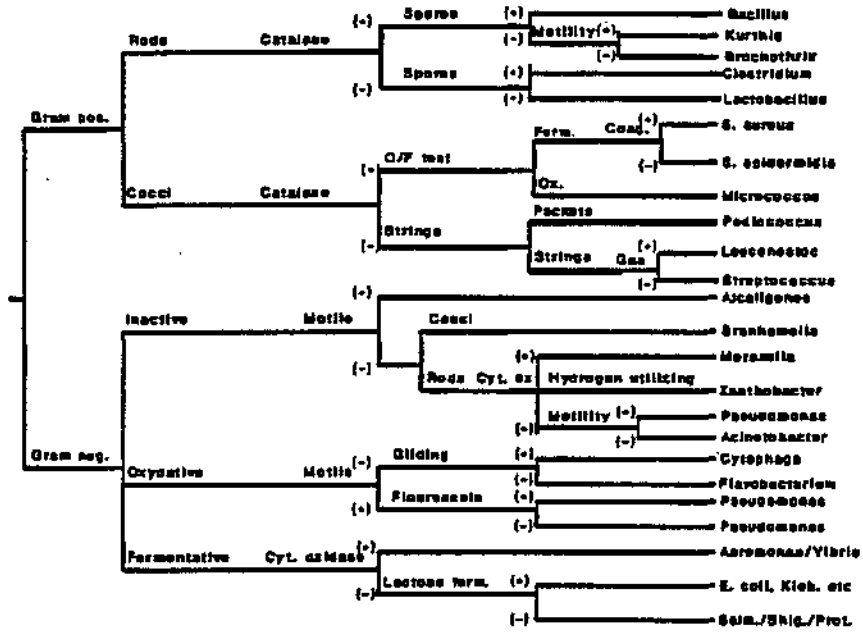


Figure 16: Flowchart used for the preliminary identification of bacterial isolates (as modified from Fischer *et al.*, 1986).

Gram-negative oxidative isolates were provisionally identified using the API 20 NE kit (API System, S.A.,-La Balme Les Grotes - 38390, Montalieu, Vercieu, France.), but were allocated to species by keys in Bergey's Manual of Systematic Bacteriology, vol. I (Krieg and Holt, 1984). Fermentative isolates were identified with the API 20 E kit. Gram-positive isolates were tested for spore-forming ability by pasteurising for ten minutes at 80 °C and plating out on R3A agar (Brözel and Cloete, 1990[a]). Spore-forming isolates were assigned to the genus *Bacillus*. Other Gram-positive isolates were only identified to the genus level by the keys given in Bergey's Manual of Systematic Bacteriology, vol. II (Sneath, Mair, Sharp and Holt, 1986).

3 RESULTS

3.1 Aerobic plate count

3.1.1 General trends

All samples underwent a continuous change in culturable count (Figs. 17 and 18; Tables 9 and 10). Although the two samples reacted differently, certain common trends were observed. Firstly, storage at the lower two temperatures (4 and 10 °C) resulted in an initial decrease in the count, followed by an increase after 72h. Storage at 30 °C initially stimulated an increase in numbers, followed by a decrease after 48h (sample 1) or 72h (sample 2). At 20 °C storage, a biphasic increase occurred. In sample 1 the count increased, decreased after 48h and increased to too numerous to count. In sample 2 the count remained constant, increased after 24h, and again after 72h. None of the storage tem-

peratures yielded the same count initially and after 24h. The cooling water sample (sample 1) yielded a similar count at 10 °C and less so at 4 °C. In contrast the tap water sample (sample 2) gave the same count after storage at 30 °C for 48h, and less so at 20 °C.

3.1.2 Cooling water

The cooling water sample culturable counts varied between 6.80 X 10^4 and 1.93 X 10^6 cfu.ml^{-1}, a factor of 28 times (Table 9 and Fig. 17). These two extremes were both achieved at 10 °C. The highest count was after 216h at 10 °C and was 10.16 times the original count. However after 24h the counts also varied considerably, *ie.* between 1.43 X 10^5 cfu.ml^{-1} or 75% of the original count at 4 °C and 1.23 X 10^6 cfu.ml^{-1} at 20 °C or a 6.47 fold increase.

Table 9. Total aerobic count of sample 1 (cooling water) stored at 4, 10, 20, and 30 °C for 24, 48, 72, and 216h (determined on R3A nutrient agar incubated at 30 °C for 120h). After 216h the sample stored at 20 °C was too numerous to count (TNTC). Values given are averages of three counts.

Storage temperature	0 h	24 h	48 h	72 h	216 h
4 °C	1.90 X 10^5	1.43 X 10^5	8.33 X 10^4	9.27 X 10^4	1.33 X 10^6
10 °C	1.90 X 10^5	1.56 X 10^5	7.73 X 10^4	6.80 X 10^4	1.93 X 10^6
20 °C	1.90 X 10^5	1.23 X 10^6	1.67 X 10^6	4.10 X 10^5	TNTC
30 °C	1.90 X 10^5	7.83 X 10^5	5.83 X 10^5	2.87 X 10^5	2.90 X 10^5

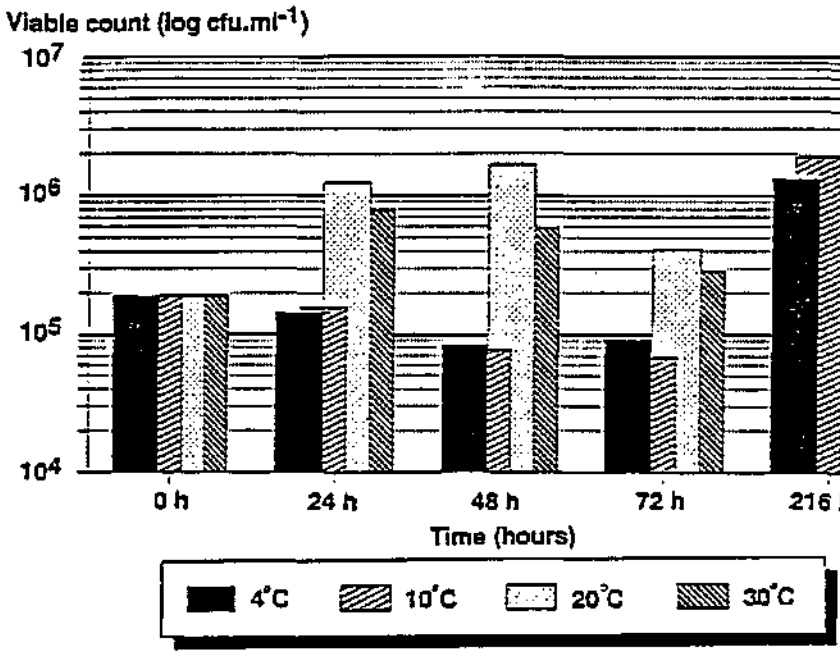


Figure 17: Total aerobic count of cooling water sample stored at 4, 10, 20, and 30 °C for 24, 48, 72, and 216h (determined on R3A nutrient agar incubated at 30 °C for 120h). After 216h the sample stored at 20 °C was too numerous to count (TNTC).

3.1.3 Tap water

The tap water sample showed more pronounced changes in culturable count than the cooling water sample (Table 10 and Figure 18). After 24h the sample count remained constant when stored at 30 ^{o}C. At 4 ^{o}C it decreased to 8.66 X 10^2 cfu.ml^{-1} or 30% of the initial value. At 4 and 10 ^{o}C the count did not vary much over the storage period and remained within $\log_{10}$ 0.82 below or $\log_{10}$ 0.23 above the initial value. Storage at 20 and 30 ^{o}C however resulted in marked increases in the count. After 48h at 20 ^{o}C it was $\log_{10}$ 2.09 greater or 122 times as much as the initial count. After 72h at 30 ^{o}C it was $\log_{10}$ 3.54 greater or 3448 times as much. This is a large increase in colony forming cell numbers.

Table 10. Total aerobic count of sample 2 (tap water) stored at 4, 10, 20, and 30 ^{o}C for 24, 48, 72, and 216h (determined on R2A nutrient agar incubated at 30 ^{o}C for 120h). After 216h the sample stored at 20 ^{o}C was too numerous to count (TNTC). Values given are averages of three counts.

Storage temperature	0 h	24 h	48 h	72 h	216 h
4 ^{o}C	2. 9 X 10^3	8.66 X 10^2	6.4 X 10^2	4.4 X 10^2	6.53 X 10^2
10 ^{o}C	2. 9 X 10^3	9.33 X 10^2	6.8 X 10^2	5.43 X 10^2	4.87 X 10^3
20 ^{o}C	2. 9 X 10^3	2.23 X 10^3	3.53 X 10^5	5.97 X 10^5	TNTC
30 ^{o}C	2. 9 X 10^3	2.87 X 10^3	1.87 X 10^6	1.00 X 10^7	1.67 X 10^5

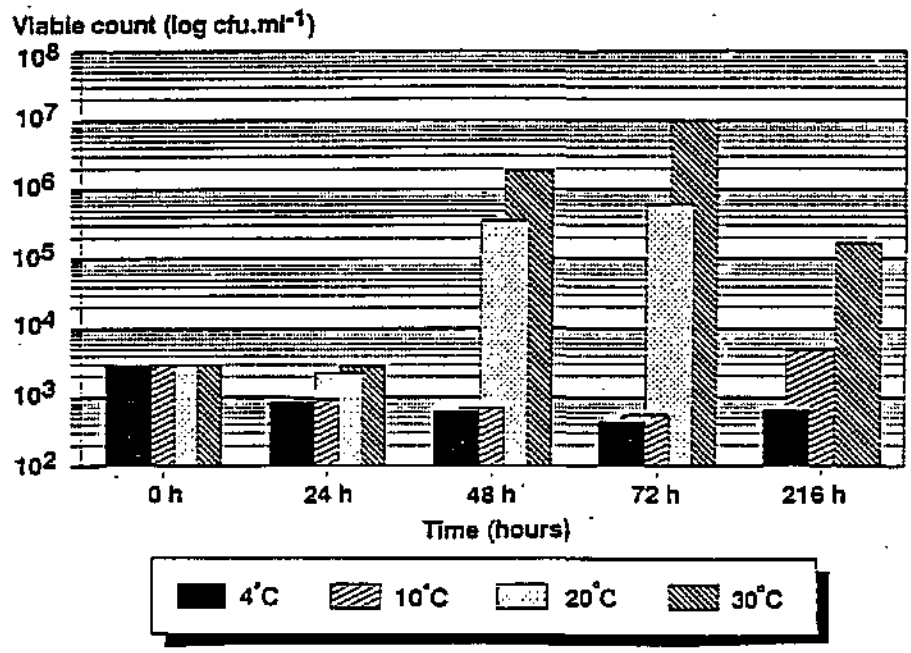


Figure 18: Total aerobic count of tap water sample stored at 4, 10, 20, and 30 ^{o}C for 24, 48, 72, and 216h (determined on R2A nutrient agar incubated at 30 ^{o}C for 120h). After 216h the sample stored at 20 ^{o}C was too numerous to count (TNTC).

3.2 Population structure

As opposed to the aerobic culturable counts, no general trends were apparent in the species compositions of samples stored at various temperatures (Figs. 19 - 26). Certain species not detected at first appeared dominant after time, whereas others lost their dominant position. This does not of necessity imply death of members of a species which lost dominance, as the total count increased in certain cases and other species could have multiplied and thus masked the presence of those dominant originally.

3.2.1 Cooling water

The two isolates present under most of the conditions were *Pseudomonas stutzeri* and a pink rod-shaped isolate which stained Gram negative and could not be identified to date. They were all immotile, catalase positive, cytochrome oxidase positive and did not produce acid or alkali from glucose. They were mainly coccobacilli, but a few thin long rods were present, and in older cultures rods of medium length were also seen. Although there were signs of a rod - coccus cycle, this was not as clear as in *Arthrobacter*.

The results are tabulated in Table 11 and are depicted in Figs. 19 - 22. Certain species occurred only once, *eg. Pseudomonas diminuta* and *Vibrio damsela*. However most species occurred sporadically at various storage temperatures and after various storage times. *P. maltophilia* occurred after 24h at 4 ^{o}C, after 216h at 10 ^{o}C and after 72h at 30 ^{o}C. *Alcaligenes latus* occurred only at 4 and 20 ^{o}C, but continuously at the latter temperature.

3.2.2 Tap water

The tap water sample yielded a variety of species after the various storage conditions (Table 12 and Figs. 23 - 26). *Alcaligenes latus* was the only species which retained dominance for 24h at all temperatures whereas all others decreased or decreased and increased again in time. *Moraxella phenylpyruvica* retained a dominant position for 24h at 10 and at 20 ^{o}C, whereas it regained its dominance after 216h at 4 ^{o}C. *P. diminuta* at 20 ^{o}C lost its dominant position after 24h, but regained it again before 216h. *M. lacunata* kept its relative dominance at 10 ^{o}C, but lost it at 20 ^{o}C. However the total culturable count remained rather constant at 10 ^{o}C, whereas it increased 205 fold after 72h at 20 ^{o}C. Therefore *M. lacunata* probably maintained a constant population, but its presence was masked by other species which multiplied at the higher temperatures.

Table 11: Population structure of cooling water stored at 4, 10, 20, and 30 °C for 24, 72, and 216h. Proportions of species are given as percentage of the total population.

Species		4°C			10°C			20°C			30°C		
	0h	24h	72h	216h	24h	72h	216h	24h	72h	216h	24h	72h	216h
P. paucimobilis	23	25		17	33							17	
P. stutzeri	23	13	25	32		83	20	17	20	20	44	33	50
P. maltophilia		13					20					17	
P. diminuta							20						
P. alcaligenes								17	20				
P. vesicularis												17	
Fl. odoratum	15				33	17					11		
Fl. multivorum	8												25
Flavobacterium sp.		13			33					20			
M. phenylpyruvica	8		12.5										
Moraxella sp.									20	20			
Pink isolate	15	25	25	17			20	17	20		22	17	25
Aureobacterium sp.	8												
Alc. latus		13	25					17	20	40			
Alcaligenes sp.				17			20						
Hydrogenophaga sp.			12.5	17				17					
Vibrio damsela								17					
Bacillus sp.											22		

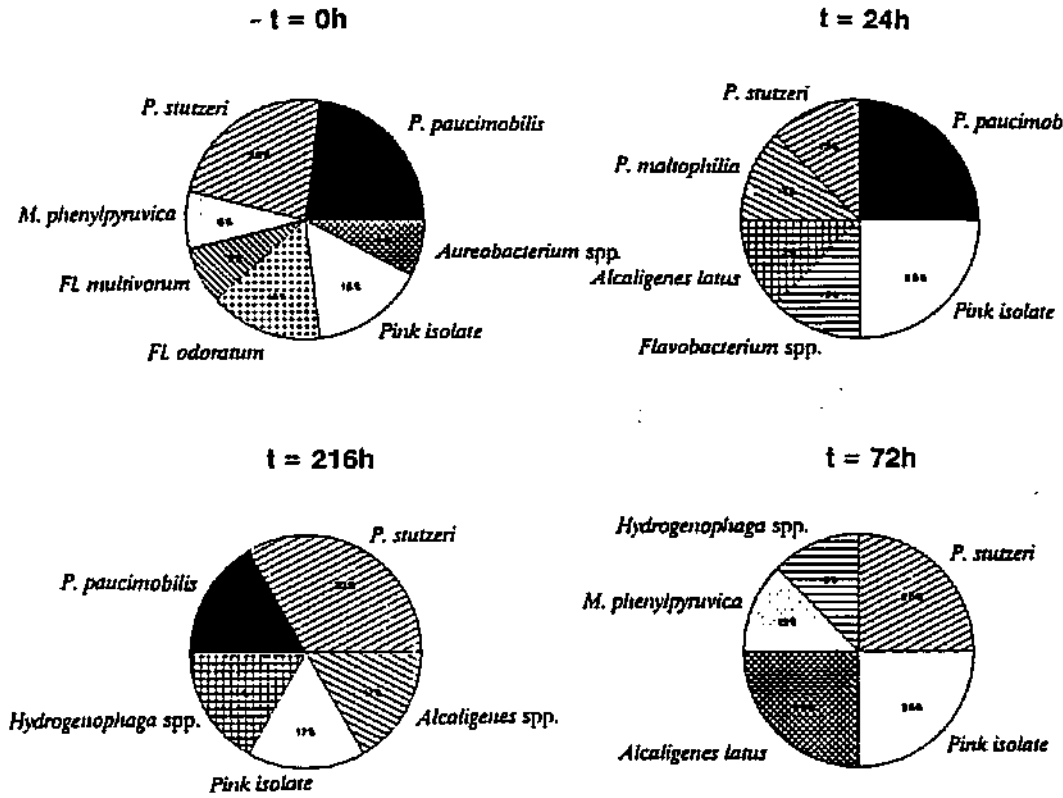


Figure 19: Population structure of cooling water sample as drawn and after 24, 72, and 216h storage at 4 °C.

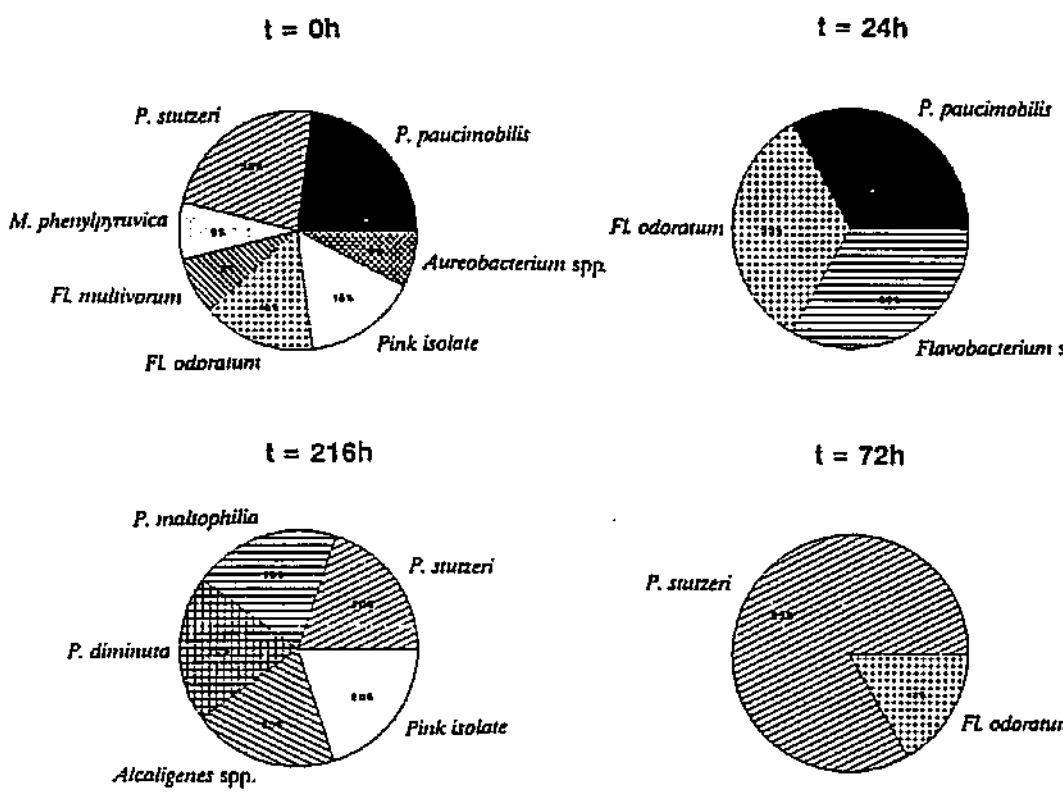


Figure 20: Population structure of cooling water sample as drawn and after 24, 72, and 216h storage at 10 °C.

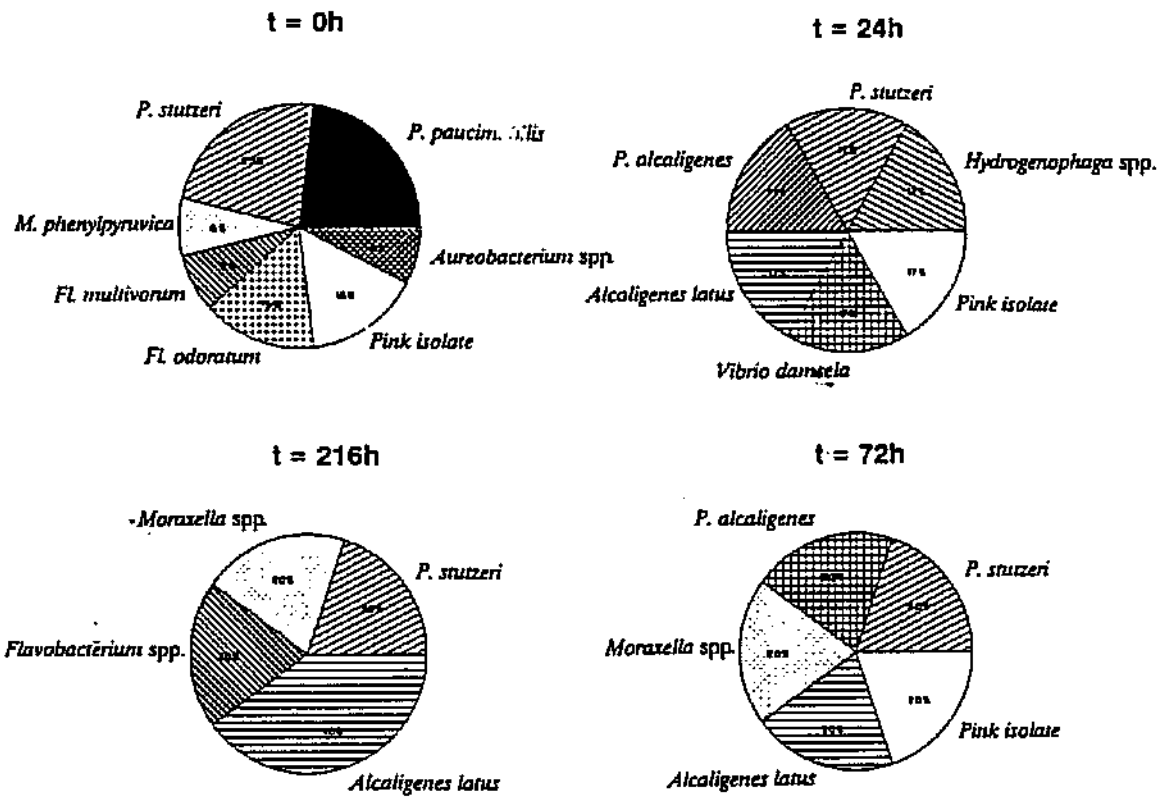


Figure 21: Population structure of cooling water sample as drawn and after 24, 72, and 216h storage at 20 °C.

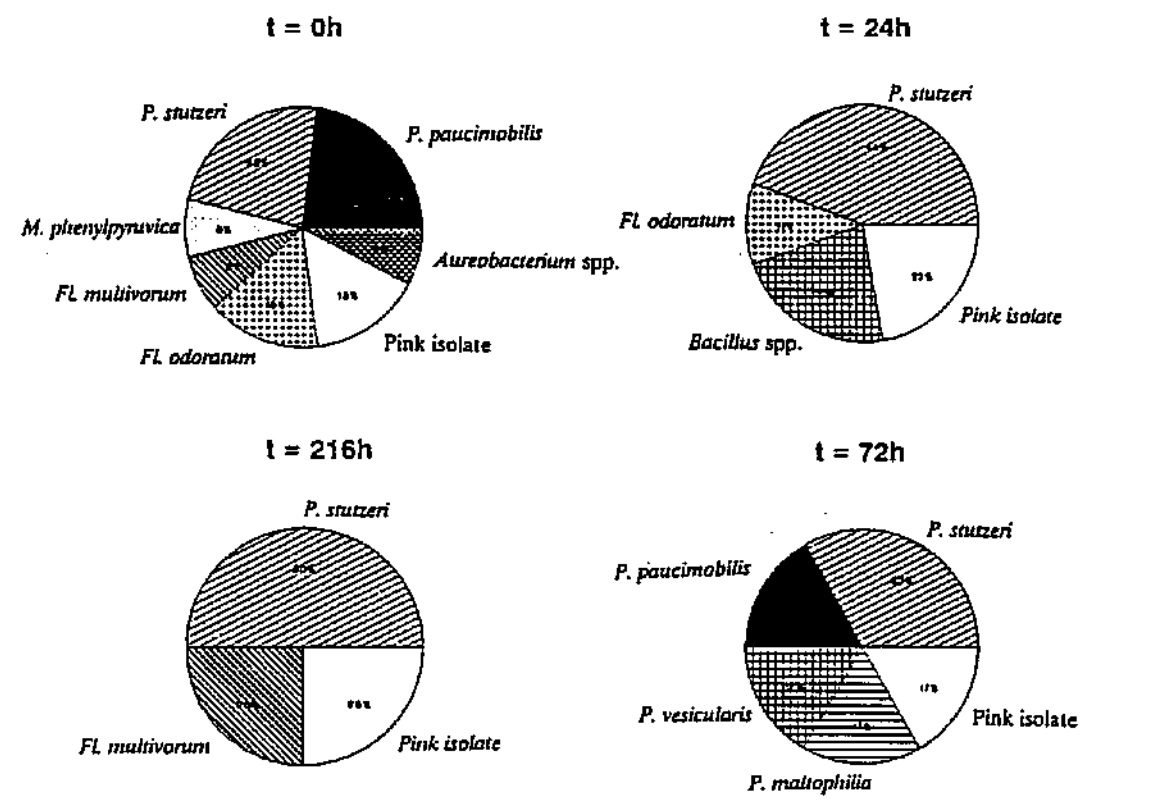


Figure 22: Population structure of cooling water sample as drawn and after 24, 72, and 216h storage at 30 °C.

Table 12: Population structure of tap water stored at 4, 10, 20, and 30 °C for 24, 72, and 216h. Proportions of species are given as percentage of the total population.

Species		4°C			10°C			20°C			30°C		
	0h	24h	72h	216h	24h	72h	216h	24h	72h	216h	24h	72h	216h
P. paucimobilis	9	29			17							9	
P. diminuta	18	14						17		33	14		
P. maltophilia		14	25				33					37	
P. vesicularis				33									
P. stutzeri			25						25				
Pseudomonas sp.	9				17				25				60
Alc. latus	37	29			17			17	25		14	18	20
Alc.denitr. subsp. *xyl.*											14		
Alcaligenes sp.	9		25						25			9	20
Agr. radiobacter	9												
M. phenylpyruvica	9			33	17			17					
M. lacunata					17	50	33	17					
Moraxella sp.						25		33		33		9	
Kingella sp.		29											
K. denitrificans				33									
Fl. odoratum			25										
Vibrio damsela							33						
Pink isolate										33			
Green isolate					17	25					29	18	
Gram positive rod											29		

4 DISCUSSION

4.1 Culturable count

Bacterial populations are dynamic by nature, with continuous cell division and death of cells (Atlas and Bartha, 1987). They can therefore be expected to occur in a constant state of flux, in terms of numbers, in terms of physiological state of the cells and in terms of their species composition and interaction. The results obtained prove that bacterial populations in water are dynamic. They react

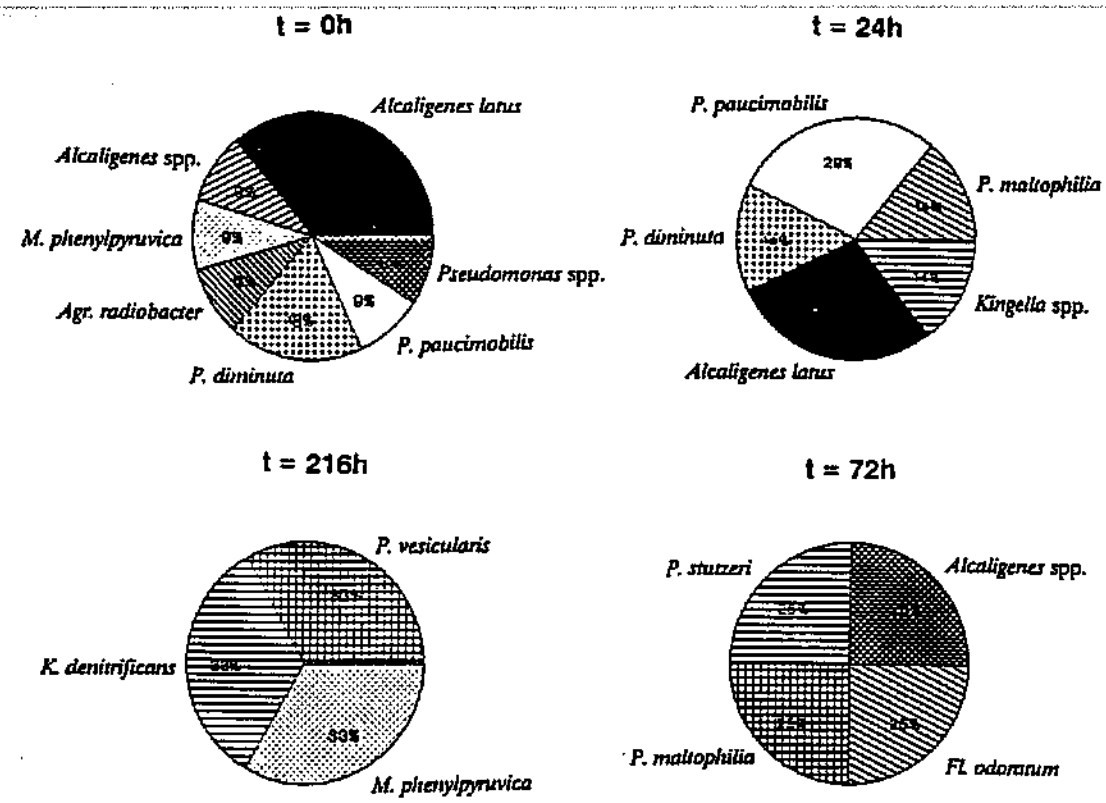


Figure 23: Population structure of tap water sample as drawn and after 24, 72, and 216h storage at 4 °C.

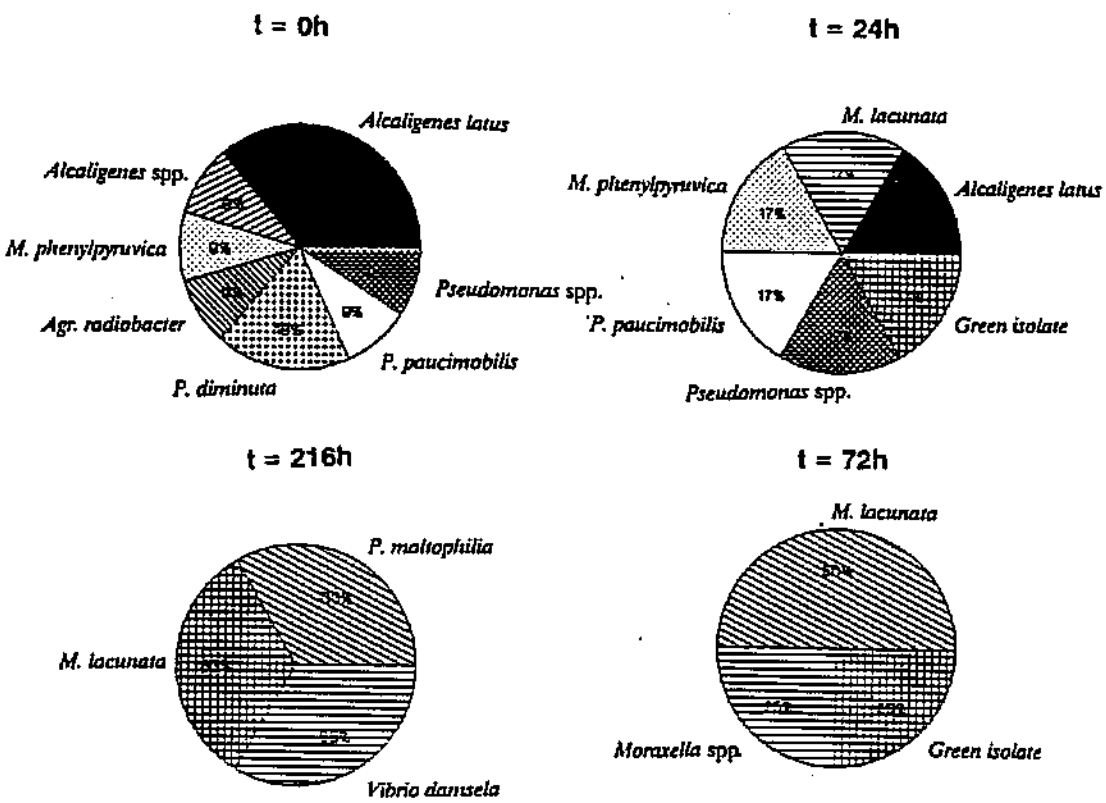


Figure 24: Population structure of tap water sample as drawn and after 24, 72, and 216h storage at 10 °C.

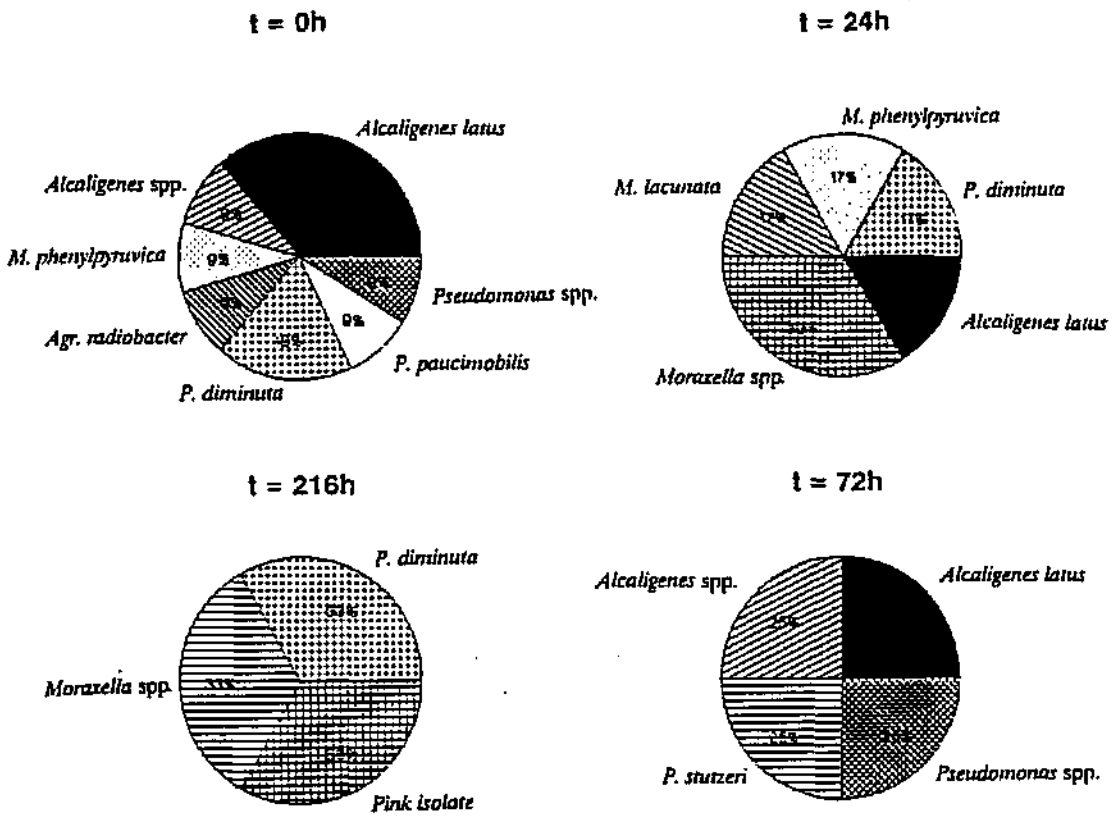


Figure 25: Population structure of tap water sample as drawn and after 24, 72, and 216h storage at 20 °C.

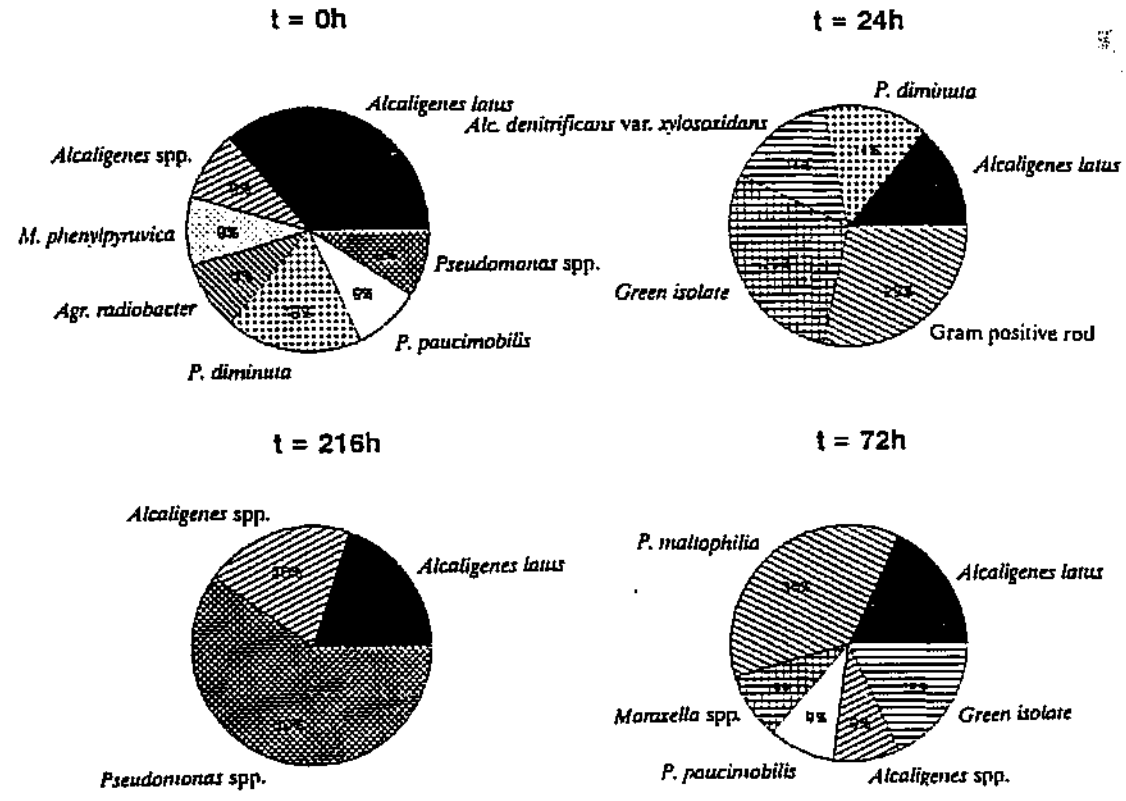


Figure 26: Population structure of tap water sample as drawn and after 24, 72, and 216h storage at 30 °C.

to external conditions such as temperature. As the various storage temperatures promoted either net decrease or net increase in the culturable count over time, total cell numbers and their physiological state changed over time during storage. Whereas 4 and 10 ^{o}C induced a net decrease followed by an increase, after 72h, 20 and 30 ^{o}C brought about an initial increase followed by either a decrease (30 ^{o}C) or a further increase (20 ^{o}C).

Drinking water is a low nurient environment, (Pointdexter, 1981) and the little nutrient available would be depleted by cellular respiration over time. Various authors have shown that bacteria attach to surfaces as a response to low nutrient conditions (Kjelleberg, Humphrey and Marshall, 1982; Zobell, 1943). The samples studied were stored in presterilized glass tubes and aliquots were drawn after vortexing. The decrease in the culturable count could possibly be partially ascribed to attachment of cells to the glass surface due to nutrient depletion in the water, the socalled bottle effect which can be decreased by reducing the surface to volume ratio of the container (Ferguson, Buckley and Palumbo, 1984). The bottle effect takes four hours before numbers are decreased significantly, and the count at t_0 would not have been affected. Where the nutrient concentration decreased even further, cells could have detached in search of possible nutrient sources. Delaquis, Caldwell, Lawrence and McCurdy (1989) showed that *Pseudomonas fluorescens* detaches from surfaces due to low nutrient stress.

The tap water sample showed a much larger increase in the culturable count after 24h storage at 20 and 30 ^{o}C (3448 fold) than did the cooling water sample (10.16 fold). This increase could not be ascribed to detachment, as the initial count was lower than subsequent ones. The sample would not have contained sufficient nutrients for such an increase in cell numbers. However sub-lethaly injured cells which survived chlorination, could have recovered after time and regained the culturable state (LeChevallier, Evans, Seidler, Daily, Merell, Rollins and Joseph, 1982; Roszak, Grimes and Colwell, 1983). This would explain the increase over time at the higher storage temperatures (20 and 30 ^{o}C), where residual chlorine would have dissipated more rapidly from the sample.

4.2 Population structure

No direct pattern of population development was detected in either of the samples, both samples showing a variety of resultant populations after storage. In both samples only one or two species attained any form of dominant position, while all others held temporary positions. In the cooling water sample it was *P. stutzeri* and the pink Gram negative isolate which maintained a dominant position. Brözel and Cloete (1990[b]) reported *P. stutzeri* to be the most dominant species in cooling water after sub lethal bactericide treatments. This organism appears to be very important in cooling water, as it appears omnipresent due to extreme fitness and its ability to adapt. The pink isolate has not been isolated from cooling water in this laboratory before, but previous isolations were always performed on Std I nutrient agar (Biolab). Reasoner, Blannon, Geldreich and Barnick (1989) reported

the isolation of pink Gram negative rods from drinking water using R2A agar, although these were not identified. R3A and R2A agars were used in this study. It is probably present in many cooling water systems, but has to date eluded detection as it does not grow on Std I nutrient agar. This isolate warants further investigation as to its role in cooling water and biofouling. All storage conditions resulted in population shifts, however, with the greatest change occuring at 10 ^{o}C storage temperature.

The only isolate holding any dominant position in the tap water sample, was *Alcaligenes latus*. However it only held its dominant position at 30 ^{o}C. At the lower temperatures various *Pseudomonas* and *Moraxella* species played the dominant role whereas at the higher temperatures it was the *Alcaligenes* species. This reflects the high degree of species diversity in tap water. As mentioned above, many dominant species may have eluded detection as they were sub-lethaly injured. Conversely the results could show random selection, where many more species were present in more or less equal numbers but only some were chosen. However clear shifts occurred upon storage at all four temperatures, even up to 24h.

4.3 Conclusion

The results do show that a culturable count performed any period after drawing of the sample does give different results to those obtained upon immediate analysis. The count obtained after sample storage is sometimes lower and sometimes higher than the initial count, depending on the storage time, temperature and initial state of the cells. Therefore results of analysis after storage cannot be adapted by a known factor. They must be interpreted with extreme caution, as they do not of necessity reflect the bacterial composition of the sample as drawn, both in terms of total numbers and in terms of population structure. Only counts performed on fresh samples yield reliable results on the total culturable count, and only population structures performed immediately, reflect the state of the population in the system from which the sample was drawn.

5 ACKNOWLEDGEMENTS

We thank Elizabeth da Silva for excellent technical assistance.

Chapter 4

Application of Sterikon(R) Bioindicators for the Determination of Bactericide Concentrations

T. E. CLOETE, E. DA SILVA and V. S. BRÖZEL

Abstract

Biofouling in industrial water systems is normally prevented by the use of bactericides. However, bactericide programmes often fail owing to the lack of suitable techniques for determining the *in situ* bactericide concentration and this usually results in either inadequate or excessive bactericide concentrations. In this study, the Sterikon(R) bioindicator was evaluated for determining the minimum inhibitory concentrations of 5 industrial bactericides (dichlorophen, sulphone, thiocarbamate, isothiazolone and a quaternary ammonium compound) for the monitoring of the concentrations of these compounds in industrial water systems. The results indicated that the Sterikon(R) bioindicator can be used for the determination of bactericide concentrations.

1 INTRODUCTION

Water cooling systems contain a variety of bacteria which colonize surfaces. This leads to biofilm formation and subsequent biofouling and microbially induced corrosion (Cloete *et al.*, 1992). The efficacy of bactericide programmes for biofouling control in industrial water systems relies not only on the spectrum of antibacterial activity of the bactericide, but also on the available concentration (Cloete *et al.*, 1989, Brözel and Cloete, 1991a). In many cases the correct available concentration is not attained due to a lack of knowledge on the size of the system or the difficulty to determine the residual concentration of the bactericide. In recirculating water systems, bactericides concentrations decrease after addition due to system blow-down and interaction with bacteria (Cloete *et al.*, 1992; Warner, 1985). Normal practice would be to add bactericide periodically to maintain the required concentration. It is emphasized that the concentration of a bactericide is not linearly related to its activity; a concentration exponent is involved in the relationship (Hugo and Denyer, 1987). In the case of most bactericides, a small decrease in concentration will result in a large decrease in activity. For bactericide programmes to be effective one would ideally want sufficient available bactericide and an adequate exposure time. This would prevent the depletion of the bactericide to sub-lethal concentrations and minimize the risk of bacteria becoming resistant to a specific product (Brözel and Cloete, 1991b). Due to the difficulty in determining available *in situ* bactericide concentration, rates of depletion due to inactivation are unknown and this has led to the mismanagement and failure of many biofouling control programmes.

The concentrations of non-oxidising bactericides can be determined by conventional analytical means. Most of these involve extraction followed by instrumental analysis. These techniques are sophisticated and cumbersome, and too lengthy and expensive for routine use. Rapid convenient tests are available for some oxidising bactericides, eg Merckoquant(R) peroxide for hydrogen peroxide and Merckoquant(R) chlorine for chlorine determinations.

In practice most bactericides react with substances contained in the water, decreasing the available concentration. Furthermore, even if the residual concentration could be determined accurately, it would not reflect the antimicrobial activity of the product. Techniques for the determination of antimicrobial activity of bactericides are available (Payne, 1988; Cloete *et al.*, 1989; Hill *et al.*, 1989). Results obtained using laboratory methods of bactericide evaluation cannot generally be related to the practical situation (Payne, 1988; Cloete *et al.*, 1989; Brözel and Cloete, 1992). Nevertheless, these tests do provide useful information during the development of bactericides.

A more serious problem is the lack of suitable techniques for the *in situ* determination of available bactericide concentrations. In this regard one bioindicator has been developed (Hill *et al.*, 1989). Bioindicators are considered to be biological preparations that usually contain spores of a single

bacterial strain with a known susceptibility towards an antimicrobial agent. Sterikon(R) is used as a bioindicator in heat sterilisation. It is a glass vial containing spores of the apathogenic *Bacillus stearothermophilus* ATCC 7953 suspended in a broth containing glucose and a pH indicator. After heat exposure the vial is incubated at 45 °C and viable spores germinate, produce acid and render the indicator yellow. A yellow vial is indicative of insufficient heat treatment. Sterikon(R) has not been evaluated before for the determination of bactericide concentrations. The objective of this study was, therefore, to determine whether Sterikon(R) could be used as a bioindicator for the *in situ* determination of available bactericide concentrations.

2 MATERIALS AND METHODS

2.1 Bactericides Used

1. Dichlorophen (2,2'-methylenebis(4-chlorophenol), 40% (m/v) solution) (BDH Chemicals)
2. Sulphone (bis-trichloro-methyl sulphone, commercial solution) (Chemserve Systems).
3. Quaternary ammonium compound (Tetradecylbenzyldimethylammonium chloride syrup) (Merck)
4. Thiocarbamate (Sodium dimethyldithiocarbamate) (Fluka)
5. Isothiazolone (10,1% 5-chloro-2-methyl-4-isothiazolin-3-one and 3,8% 2-methyl-4-isothiazolin-3-one) (Thor chemicals)

2.2 Standardisation of Bactericide Concentrations

A range of concentrations of each bactericide was added directly to the ampoules. These were then sealed and incubated at 45 °C for 24 h. All determinations were performed in triplicate. A clear violet colour after 24 h was taken as sufficient bactericide to inhibit spore germination, or alternatively to kill the spores. Yellow ampoules were a sign of acid production due to germination and growth, indicating insufficient bactericide.

2.3 Interaction of Bactericides with Sterikon(R)

The possibility existed that the bactericides used in this study could react with the indicator , or with other components in the ampoule, bringing about a colour change not due to bacterial acid production. Various bactericide concentrations were added to sterile aqueous solutions of the indicator bromocresol purple, and to Sterikon(R) and incubated at 25 °C for 24 h. Incubation was at 25 °C since

spores of *B. stearothermophilus* germinate only at *ca.* 45 °C. The metabolic activity of the bacteria was thus prevented without tampering with the contents of the Sterikon(R) vial. No colour changes to yellow were recorded, indicating that none of the bactericides evaluated interacted with the indicator to produce false positives.

3 RESULTS

The results are given in Tables 13 to 17. To use Sterikon(R) as a method for determining unknown bactericide concentrations, it was necessary to determine the minimum concentration of each bactericide required to inhibit outgrowth of the spores. Note that this minimum inhibitory concentration (MIC) should not be interpreted as the efficacy of a product in general as it is specific for spores of *B. stearothermophilus*. The results for isothiazolone indicated that after 18 h the MIC was 15 $\mu l.l^{-1}$, after 24 h the MIC was 20 $\mu l.l^{-1}$ and after 28 h the MIC was 30 $\mu l.l^{-1}$ (Table 13). No growth occurred at the latter concentration during the 48 h period of incubation and therefore this concentration (30 $\mu l.l^{-1}$) was taken as the standard MIC for this product.

The MIC of thiocarbamate after 18 h incubation was 10 $mg.l^{-1}$, after 24 h incubation 10 $mg.l^{-1}$, after 28 h incubation 20 $mg.l^{-1}$, and after 36 h incubation the MIC was 30 $mg.l^{-1}$ (Table 14). No further growth was detected at the latter concentration after a 48 h period of incubation. Therefore, 30 $mg.l^{-1}$ was taken as the MIC for the thiocarbamate bactericide. The results of the sulphone bactericide indicated that 3 $\mu l.l^{-1}$ was sufficient to prevent growth (Table 15). No growth occurred after 48 h incubation at 3 $\mu l.l^{-1}$; therefore this was taken as the standard MIC for the sulphone bactericide used. For dichlorophen the MIC after 18 h was 0,6 $\mu l.l^{-1}$ (Table 16). After 48 h it was 0,8 $\mu l.l^{-1}$. This indicated that 0,8 $\mu l.l^{-1}$ was the standard MIC of dichlorophen in Sterikon(R). The standard MIC for the quaternary ammonium compound used was 0,3 $\mu l.l^{-1}$ after 18 h (Table 17). After 36 h it was 0,4 $\mu l.l^{-1}$, and after 48 h it was 0,5 $\mu l.l^{-1}$. Therefore, the standard MIC of the quaternary ammonium compound used was taken as 0,5 $\mu l.l^{-1}$.

It is not surprising that the MIC value varied among the bactericides evaluated, as a given test organism would be more susceptible to certain bactericides than to others (Brözel and Cloete, 1991a). This is, however, not important in terms of using Sterikon(R) as a method of bactericide concentration determination. What is important is the fact that the MIC determined by Sterikon(R) should always be the same for the same bactericide.

Table 13: MIC for the isothiazolone bactericide using Sterikon(R)

Bactericide concentration	**Incubation Time (h)**					
(μl.l^{-1})	**8**	**18**	**24**	**28**	**36**	**48**
Control	-	+++	+++	+++	+++	+++
5	-	+++	+++	+++	+++	+++
10	-	--+	+++	+++	+++	+++
15	-	---	-++	-++	+++	+++
20	-	---	---	--+	--+	+++
30	-	---	---	---	---	---
40	-	---	---	---	---	---
50	-	---	---	---	---	---

\+ = growth (yellow)

\- = no growth (purple)

The standard MIC was taken as 30 μl.l^{-1} due to the lack of growth after 48 h incubation.

Table 14: MIC for the thiocarbamate bactericide using Sterikon(R)

Bactericide concentration	**Incubation Time (h)**					
(mg.l^{-1})	**8**	**18**	**24**	**28**	**36**	**48**
Control	-	+++	+++	+++	+++	+++
5	-	--+	+++	+++	+++	+++
10	-	---	---	--+	+++	+++
15	-	---	---	--+	+++	+++
20	-	---	---	---	+++	+++
30	-	---	---	---	---	--+
40	-	---	---	---	---	---
50	-	---	---	---	---	---

\+ = growth (yellow)

\- = no growth (purple)

The standard MIC was taken as 30 mg.l^{-1} due to the lack of growth after 48 h incubation.

Table 15: MIC for the sulphone bactericide using Sterikon(R)

Bactericide concentration	**Incubation Time (h)**					
(μl.l^{-1})	**8**	**18**	**24**	**28**	**36**	**48**
Control	-	+++	+++	+++	+++	+++
1	-	+++	+++	+++	+++	+++
2	-	---	---	---	---	+++
3	-	---	---	---	---	---
4	-	---	---	---	---	---
5	-	---	---	---	---	---
10	-	---	---	---	---	---
20	-	---	---	---	---	---
30	-	---	---	---	---	---
40	-	---	---	---	---	---
50	-	---	---	---	---	---

\+ = growth (yellow)

\- = no growth (purple)

The standard MIC was taken as 3 μl.l^{-1} due to the lack of growth after 48 h incubation.

Table 16: MIC for the dichlorophen bactericide using Sterikon(R)

Bactericide concentration	**Incubation Time (h)**					
(μl.l^{-1})	**8**	**18**	**24**	**28**	**36**	**48**
Control	-	+++	+++	+++	+++	+++
0,1	-	+++	+++	+++	+++	+++
0,2	-	+++	+++	+++	+++	+++
0,3	-	+++	+++	+++	+++	+++
0,4	-	+++	+++	+++	+++	+++
0,5	-	+++	+++	+++	+++	+++
0,6	-	---	---	---	---	-++
0,7	-	---	---	---	---	--+
0,8	-	---	---	---	---	---

\+ = growth (yellow)

\- = no growth (purple)

The standard MIC was taken as 0,8 μl.l^{-1} due to the lack of growth after 48 h incubation.

Table 17: MIC for the quaternary ammonium bactericide using Sterikon(R)

Bactericide concentration	**Incubation Time (h)**					
(μl.l^{-1})	**8**	**18**	**24**	**28**	**36**	**48**
Control	-	+++	+++	+++	+++	+++
0,1	-	+++	+++	+++	+++	+++
0,2	-	+++	+++	+++	+++	+++
0,3	-	---	---	---	+++	+++
0,4	-	---	---	---	---	+++
0,5	-	---	---	---	---	---

\+ = growth (yellow)

\- = no growth (purple)

The standard MIC was taken as 0,5 μl.l^{-1} due to the lack of growth after 48 h incubation.

4 DISCUSSION

The results in this study indicate that the Sterikon(R) bioindicator can be used successfully for the determination of the concentration of bactericides in water samples. Since *B. stearothermophilus* spores germinate only at temperatures above 45 °C, the Sterikon(R) ampoule must be incubated at this temperature. The bacterial flora of the water samples tested will, therefore, not interfere with the determination, as these bacteria are mesophilic. They cannot grow at 45 °C, or only at a very slow rate, and will not cause a colour-change to indicate an incorrect bactericide concentration. Water samples can, therefore, be used directly and do not have to be filter-sterilised as would be the case if the indicator organism itself was a mesophile.

This procedure has been evaluated in the laboratory situation by giving samples of water containing an unknown bactericide concentration to laboratory technicians. They were able to determine the bactericide concentration correctly.

5 ACKNOWLEDGEMENTS

We thank Fatima Da Silva for technical assistance, Chemserve Systems and Merck for donating bactericide samples.

Chapter 5

Resistance of Bacteria from Cooling Waters to Bactericides

V. S. BRÖZEL and T. E. CLOETE

Abstract

Bacteria from cooling water systems developed resistance to three different bactericides, i.e. quarternary ammonium compounds (QAC), isothiazolone and thiocarbamate. Resistance was induced by exposing isolates to increasing sublethal concentrations for a period of 10 weeks. *Bacillus subtilis* became resistant to 1999 mg.l^{-1} QAC. Cross resistance was also detected, e.g. isothiazolone induced resistance to QAC and thiocarbamate.

1 INTRODUCTION

Bacteria in aqueous environments attach to surfaces where subsequent growth leads to formation of biofilms (McCoy *et al.*, 1981; Van Loosdrecht *et al.*, 1990). These biofilms promote corrosion of metal surfaces by a variety of mechanisms, *eg.* by sulphate-reducing bacteria which grow in the anaerobic section of the biofilm (Ford & Mitchell, 1990). This phenomenon is termed microbially induced corrosion. Industrial water systems (*eg.* cooling water systems in power plants and mines) are treated with bactericides to eliminate or reduce corrosion. The various bacteria present differ in their susceptibility to bactericides (Brözel & Cloete, 1991).

The development of bacterial resistance to antibiotics is a well established fact (Franklin & Snow, 1981). Many authors have also reported that bacteria acquire resistance to antiseptics such as quarternary ammonium compounds (QACs) (Heinzel, 1988; Jones *et al.*, 1989; Sakagami *et al.*, 1989; Soprey & Maxey, 1968) and biguanides (Heinzel, 1988; Jones *et al.*, 1989). Development of resistance to aldehyde-releasing bactericides (hexahydro -1,3,5-triethyl -s-triazine) is also documented (Eagon & Barnes, 1986). Whether or not bacteria acquire resistance to water treatment bactericides is not really known. Biofilm bacteria have been reported to be up to one hundred times more resistant to chlorine dioxide than are free floating ones (LeChevallier *et al.*, 1988). Costerton and Lashen (1983) reported inherent resistance of biofilm bacteria to various bactericides due to impermeability of bactericide into the extracellular polysaccharide layer surrounding cells.

Water cooling systems are often treated with isothiazolone or thiocarbamate - based bactericides (Brözel & Cloete, 1991). Isothiazolones are non-oxidizing, do not release formaldehyde and are not membrane-active such as biguanides or QAC's (Woodcock, 1988). They react oxidatively with thiols to form disulphides (Collier *et al.*, 1990). The antimicrobial mechanism of thiocarbamates has not been reported to date. The objective of this study was to determine whether bacteria resident in water cooling systems become resistant to isothiazolone and thiocarbamate bactericides.

2 MATERIALS AND METHODS

2.1 Test cultures

The strains studied were from our collection of isolates found dominant in cooling waters (Cloete *et al.*, 1989), and from later isolations. These were maintained on R2A nutrient agar slants (Reasoner & Geldreich, 1985) and subcultured monthly. Five strains were evaluated, *i.e. Pseudomonas cepacia, P. stutzeri, Bacillus cereus, B. subtilis,* and an *Aureobacterium* species.

2.2 Resistance studies

The five strains were challenged with isothiazolone (a proprietary stabilized mixture of N-methyl isothiazolone and 5-Chloro-N-methyl isothiazolone), with Na-di-ethyl dithiocarbamate and with benzalkonium chloride. As it has repeatedly been reported that bacteria develop resistance to benzalkonium chloride (Jones *et al.*, 1989), cells were also challenged with tetradecyl-benzyl-dimethyl ammonium chloride to serve as a control. Minimum inhibitory concentrations (MIC) for the various isolates were initially determined as described previously (Brözel & Cloete, 1991). Cultures were grown in 100 ml volumes of R2A broth (Reasoner & Geldreich, 1985) in a shaking waterbath at 28 ^{o}C. The pH was adjusted to 7.5 as water cooling systems are operated under slightly alkaline conditions. Bactericides were added at half the relevant MIC, and cultures were left to grow for seven days before determining the new MIC as described below. Thereupon cultures were exposed to bactericide at half the new MIC.

2.3 Determination of minimum inhibitory concentration.

MIC was determined weekly. Suspensions of challenged cells were inoculated onto three batteries of R2A agar containing various concentrations of the three bactericides. Cell suspensions were inoculated onto the surface of the agar media by a 19 point inoculator. All combinations of isolate and bactericide were inoculated onto every bactericide-containing agar in triplicate. Plates were incubated at 25 ^{o}C for 48 h. Growth on the surface of the bactericide-containing agar indicated resistance to the relevant bactericide at the specific concentration, and no growth indicated inhibition of growth. MIC was taken to be the lowest concentration of bactericide where no surface growth occurred.

3 RESULTS

3.1 Resistance

The results obtained are given in Table 18. Isolates acquired a three- to six- fold increase in resistance to the isothiazolone. Resistance to the thiocarbamate increased four- to twenty- fold, and to the QAC twelve- to one hundred- fold. *Pseudomonas stutzeri* maintained its already high resistance to thiocarbamate. The one hundred fold increase in resistance of *Bacillus* to the QAC is remarkable.

3.2 Cross resistance

Table 18: Initial minimum inhibitory concentration (MIC) ($mg.l^{-1}$ bactericide) of selected bacteria as isolated and MIC after 10 weeks of exposure to sublethal concentrations of three bactericides.

Exposure to	Initial	Isothiazolone[1]	Thiocarbamate[2]	QAC[3]
Isothiazolone[1]		**(10 weeks)**	**(10 weeks)**	**(10 weeks)**
Bacillus cereus	10	40	40	200
B. subtilis	20	40	120	500
Aureobacterium species	20	60	300	100
Pseudomonas cepacia	10	60	500	200
P. stutzeri	10	60	500	1000
Thiocarbamate[2]		**(10 weeks)**	**(10 weeks)**	**(10 weeks)**
B. cereus	10	70	40	100
B. subtilis	10	80	200	250
Aureobacterium species	50	80	500	800
P. cepacia	30	80	500	250
P. stutzeri	500	80	500	1000
QAC[3]		**(10 weeks)**	**(10 weeks)**	**(10 weeks)**
B. cereus	10	50	500	950
B. subtilis	10	70	500	1000
Aureobacterium species	20	80	500	600
P. cepacia	40	80	500	1000
P. stutzeri	80	60	500	1000

1 Proprietary stabilized mixture of N-methyl isothiazolone and 5-Chloro-N-methyl isothiazolone

2 Na-di-ethyl dithiocarbamate

3 Tetradecyl-benzyl-dimethyl ammonium chloride

Strains became more resistant to the isothiazolone when challenged with the thiocarbamate or the QAC, than when challenged with the isothiazolone. Resistance of *Bacillus* to the thiocarbamate was highest when strains were challenged with the QAC. *B. cereus* became twelve times more resistant to the thiocarbamate when challenged with the QAC. The isothiazolone did induce resistance to the thiocarbamate in *Bacillus*, but to a lesser degree than did thiocarbamate itself. Resistance of *P. cepacia* to the thiocarbamate was induced equally strongly by all three bactericides. Similarly *P. stutzeri* became equally resistant to the QAC, independent of the challenging bactericide. Although the isothiazolone and the thiocarbamate induced resistance to QAC in *Bacillus*, *Aureobacterium* and in *P. cepacia*, cells exposed to the QAC were most resistant.

4 DISCUSSION

Gram positive bacteria are more susceptible to QAC than are gram negative bacteria. This susceptibility is due to the nature of the gram positive cell wall which is mainly peptidoglycan (Köhler & Mochmann, 1980). QAC blocks oxygen uptake and alters permeability of membrane proteins (Köhler & Mochmann, 1980). No reports of QAC-resistant *Bacillus* species have appeared to date. However the gram positive isolates studied became very resistant to the QAC. *B. cereus* became resistant to 950 $mg.l^{-1}$ and *B. subtilis* to 1000 $mg.l^{-1}$.

The development of cross-resistance as encountered is interesting as bacterial resistance to antibiotics or antiseptics is usually specific (Franklin & Snow, 1981; Heinzel, 1988). Water treatment regimes are often alternated with the intention of avoiding development of a resistant population. Resistance of *P. cepacia* to thiocarbamate was the same for all three pre-exposures. Also resistance of *P. stutzeri* to QAC was the same for all three pre-exposures. This suggests the development of a broad mechanism of resistance. QAC made *B. cereus* twelve times more resistant to thiocarbamate than did isothiazolone or thiocarbamate itself. *B. subtilis* pre-treated with QAC also made it more resistant to thiocarbamate than did thiocarbamate or isothiazolone. The selection pressure for *Bacillus* in QAC seems to have been higher than in thiocarbamate, resulting in a culture with a higher degree of protective properties towards QAC and thiocarbamate. However *Bacillus* challenged with thiocarbamate was more resistant to isothiazolone than when challenged with QAC. Therefore the mechanism of resistance cannot be the same. However it must be related in some way. The bactericides evaluated appear to promote development of a resistance factor. However the nature of this resistance factor may differ somewhat from bactericide to bactericide. This could explain the differing cross resistance found in many cases.

Bactericides are often alternated in water treatment programmes to prevent the development of resistant populations. The results presented here indicate that a bacterial population could become more resistant to any given bactericide after treatment with any other, than it would have been before. The mechanism of resistance, especially of gram positive bacteria will be studied in more detail.

5 ACKNOWLEDGEMENTS

We thank Elizabeth Da Silva for technical assistance

Chapter 6

Further Studies into the Development of Bacterial Resistance to Water Treatment Bactericides

V.S. BRÖZEL and T.E. CLOETE

Abstract

We aimed to investigate the development of resistance of various bacterial isolates to water treatment bactericides in order to determine the effect of sublethal dosage in cooling water systems. Pure cultures were cultured in the presence of sub-inhibitory concentrations of bactericides. Wild and resistant strains were then compared in order to ascertain factors conferring resistance. The relationship between culture age and resistance was investigated, as well as the resistance of attached cells. Resistance of *Pseudomonas stutzeri* and of *Bacillus cereus* doubled to all water treatment bactericides evaluated. Mucoid mutants did not exhibit increased tollerance to bactericides, indicating that extracellular polysaccharide does not confer increased resistance to bacteria in biofilms. However, attached cells were more resistant than free-living cells within 15 min following attachment. Cell age also had a marked influence on resistance, where actively growing cells were most resistant and late stationary phase cells were least resistant.

1 INTRODUCTION

Resistance to antibacterial agents can be defined in various ways, depending on the intention of treatment. Biofouling control programmes are designed to prevent the growth of bacteria on surfaces, and concentrate on the prevention or inhibition of growth. This is achieved at much lower concentrations than is the killing off of the bacterial community. In this report resistance will always indicate growth in the presence of a set concentration of bactericide, and not survival as is the case in the literature. Strictly speaking this should be termed "non-inhibitedness", but we refer to this as resistance.

The authors aimed to increase the resistance of various bacterial isolates by growing these in the presence of sub-inhibitory concentrations of various bactericides as to determine the effect of sublethal dosage in cooling water systems. The initial and resistant strains were compared as to their ability to survive a high dose of bactericide. The role played by LPS in resistance was investigated by treating with and without EDTA.

It is documented in the literature that cells in biofilms are more resistant to bactericides than are single cells (Brözel, 1994; LeChevallier, Cawthon and Lee, 1988; Nickel, Ruseska, Wright and Costerton, 1985). As the authors found that EPS does not play the primary role in resistance, the question arose as to what makes cells in biofilms resistant. The authors set out to determine the minimum time required for attached cells to attain the resistant state. The results have, however, raised new questions.

2 METHODS

2.1 Induction of Resistance

Two bacterial strains isolated from cooling water systems in a previous study, were used (Brözel and Cloete, 1992a). These were *P. stutzeri* and *Bacillus cereus*. *P. stutzeri* and *B. cereus* were dominant survivors in most cases after the systems were treated with various bactericides. They were therefore expected to be able to adapt to growth in the presence of bactericide. These original strains were termed "Initial". Two mutants of *P. aeruginosa* strain PAO were used to study the effect of EPS on resistance. One was PAO 579, a mucoid strain producing alginate constitutively (Fyfe and Govan, 1980). The other was PAO 381, a non-mucoid strain not being able to produce alginate. Two further non-mucoid strains, revertants of PAO 579, were used to determine the effect of cell age and of pre-growth on resistance. These were PAO 552 and 553. All PAO strains were *Leu*$^-$ and were grown on the minimal medium of Vogel and Bonner containing 5g.l^{-1} glucose and 1mM leucine (Vogel and Bonner, 1956).

Resistance was induced to four bactericides. These were Na dimethyl dithiocarbamate (Fluka), 2,2'-methylenebis(4-chlorophenol)(Panacide D, Merck), a stabilised blend of N-methyl isothiazolone and 5-chloro-N-methyl isothiazolone and a quaternary ammonium compound (Tetradecyl-benzyl-dimethyl ammonium chloride).

Strains were grown in 100 ml R2A broth (Reasoner and Geldreich, 1985) under shaking for 24 h at 28 °C. One ml of culture was drawn, centrifuged at 10 000 x g for 5 min and resuspended in 1ml 0.066M phosphate buffer (PBS)(pH = 7.00). This suspension was centrifuged at 10 000 x g for 5 min and resuspended in 500 µl PBS. Two loops full were inoculated into half-strength tryptic soy broth (TSB) (Biolab) containing various concentrations of the bactericides employed. Tubes were incubated at 28 °C for 24 h. The lowest concentration of bactericide showing absence of growth, was taken to be the minimum inhibitory concentration (MIC). One ml of the previous culture was transferred to 100 ml R2A broth containing bactericide at one quarter the concentration of the previous MIC. This procedure was repeated 10 times. A summary of the protocol is depicted in Fig. 27. The final cultures (termed "Resistant") were harvested, washed in PBS and frozen for later analysis.

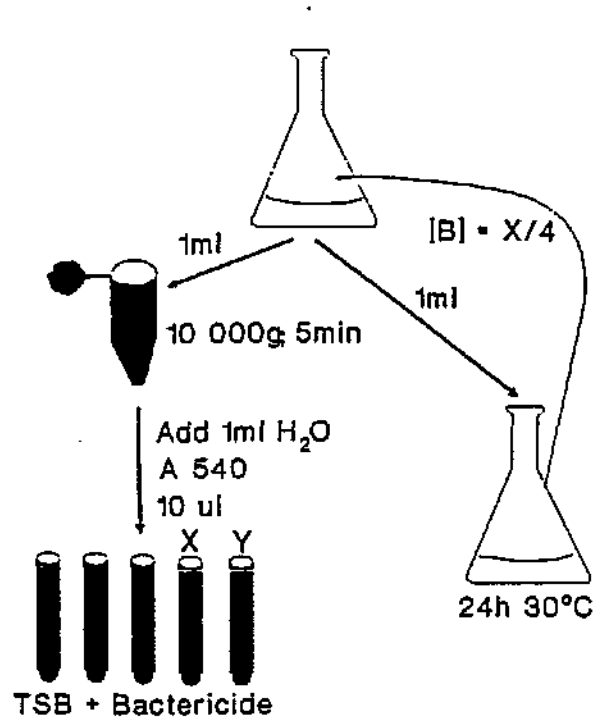


Figure 27: Diagrammatic representation of the protocol for the induction of resistance in bacterial isolates.

For the induction of resistance to isothiazolone, the protocol was modified slightly. All work was performed in triplicate so as to determine whether the development of resistance followed a set pattern, or whether it was random. The inoculum was also standardised by determining the cell concentration spectrophotometrically and diluting accordingly to a cell concentration of 2 X 10^7.ml^{-1}. 10 μl of this cell suspension were then inoculated into each tube.

2.2 Comparison of Initial and Resistant Strains

Cells were grown for 24 h in R2A broth, harvested and resuspended in phosphate buffer (pH 7.0). In the case of resistant cultures, one quarter the previous MIC of bactericide was included in the broth. Cultures were exposed to four times the highest MIC of bactericide for 60 min., with or without 50mM EDTA, after which a spread plate count was performed. EDTA is known to release 50% of the LPS out of the outer membrane, opening it to passage of molecules which would be excluded in its presence (Leive, 1965). It was added to determine the protective role played by LPS. Plating was onto R2A agar and in triplicate. Incubation was for 48 h at 30 °C. Plate counts were also performed on the culture before bactericide addition. The percentage survival was calculated, and results are given in Figures 35 to 37.

2.3 The Effect of Cell Age on Growth Inhibition

P. aeruginosa and PAO 579, 381, and 552 were grown on minimal agar (Vogel and Bonner, 1956) containing 1 mM leucine and 5 $g.l^{-1}$ glucose (MGLA). Serial dilutions of these cultures were prepared every 24h for five consecutive days, and plated in triplicate onto MGLA and onto MGLA containing 80 $\mu g.l^{-1}$ 2,2'-methylenebis(4-chlorophenol). MGLA plates were incubated for 48h, and plates containing bactericide were incubated until colonies appeared (*ca.* 96h) and then counted. The proportion of cells forming visible colonies on MGLA containing bactericide were taken to be resistant (uninhibited). The proportion of resistant cells in colonies growing on MGLA containing 80 $\mu g.l^{-1}$ 2,2'-methylenebis(4-chlorophenol) (as described in 3.4.1) was also determined.

2.4 Resistance of Mature Cells

P. aeruginosa and PAO 579, 381, and 552 were grown on MGLA. Suspensions of logarithmic cultures of these strains were prepared by suspending 12 hour old cultures in sterile tap water to a density of *ca.* 3 X 10^6 $cfu.ml^{-1}$. The cell density was determined according the McFarland nephelometer (Cloete, 1984). 2 μl drops were placed onto sterile nitrocellulose filters (0.22μm pore size) on MGLA. Filters were incubated for 24 h, and then transferred onto MGLA containing various concentrations of 2,2'-methylenebis(4-chlorophenol), Na-di-ethyl dithiocarbamate and isothiazolone. 2 μl drops of suspensions of logarithmically growing cells were also placed onto the agar, and the same culture was streaked onto the agar surface. Plates were incubated at 30 °C and inspected daily for 120h.

2.5 Resistance of Attached Cells

0.2 µl drops of *P. aeruginosa*, PAO 579 and PAO 381 were suspended onto sterile wedges of nitrocellulose filter on MGLA as described in 3.4.1. The cell suspension was diluted to a point where the drop would inoculate 10% of the surface area of the average drop size. One wedge was placed onto MGLA containing 100µg.ml^{-1} 2,2'-methylenebis(4-chlorophenol). Further wedges were placed onto this agar after after 10 minute intervals up to one hour, and then hourly up to 24 h. Incubation was for 48 h at 30 °C. Fresh culture was streaked and dropped onto the same agar to serve as control.

3 RESULTS

3.1 Induction of Resistance

All initial strains did acquire some degree of resistance over and above the initial value. The results are shown in Figs. 28 to 34. In the case of *Bacillus cereus* cultures, contamination by *P. aeruginosa* was often encountered. This phenomenon was due to the high degree of resistance of *P. aeruginosa*, and to the fact that it produces pyocins, antibacterial agents which aid it in out-competing other bacteria. As the *Bacillus* culture was grown under sub-inhibitory conditions, a single contaminant could take over the culture.

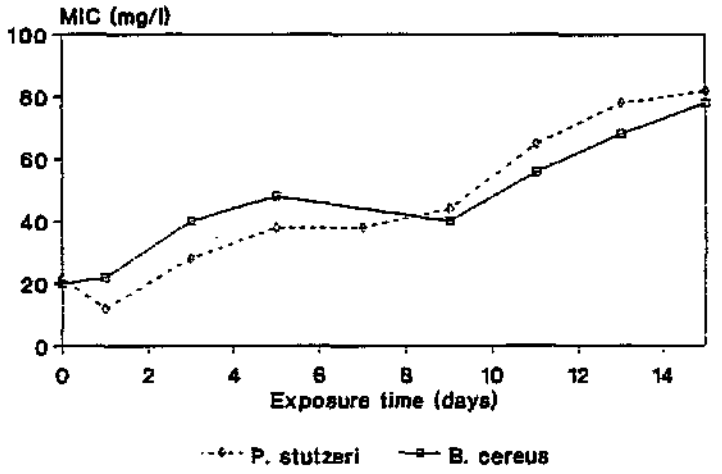


Figure 28: MIC of 2,2'methylenebis(4-chlorophenol) to P. stutzeri and B. cereus after growth in R2A broth containing one quarter the previous MIC of bactericide.

Both isolates attained a twofold resistance to 2,2'methylenebis(4-chlorophenol) after 18 days exposure to the bactericide.

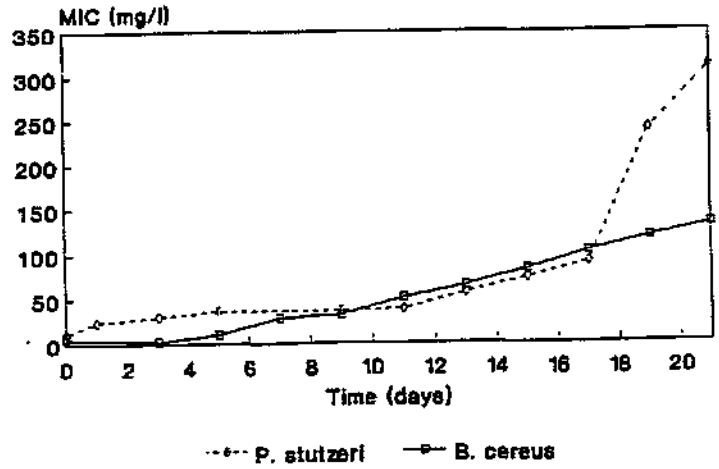


Figure 29: MIC of Na-di-ethyl dithiocarbamate to P. stutzeri and B. cereus after growth in R2A broth containing one quarter the previous MIC of bactericide.

B. cereus increased its resistance thirty-fold from 4 to 116 $\mu g.ml^{-1}$. *P. stutzeri* increased thirty-fold in resistance, from 10 to 300 $\mu g.ml^{-1}$.

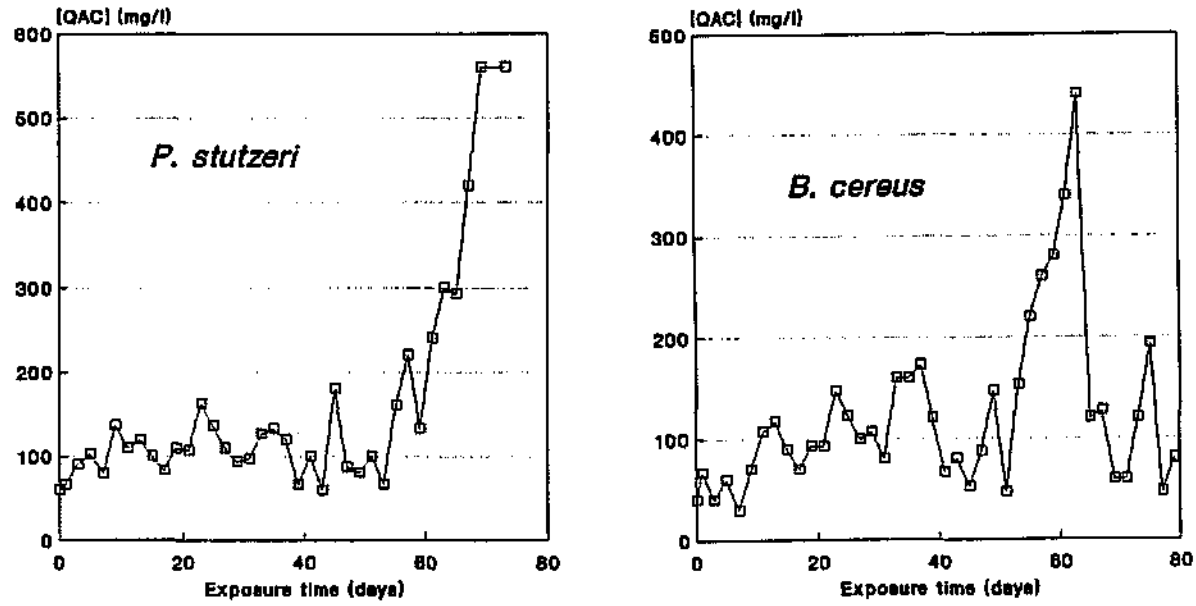


Figure 30: MIC of quaternary ammonium compound cultures of *P. stutzeri* and *B.cereus* after growth in R2A broth containing one quarter the previous MIC of bactericide.

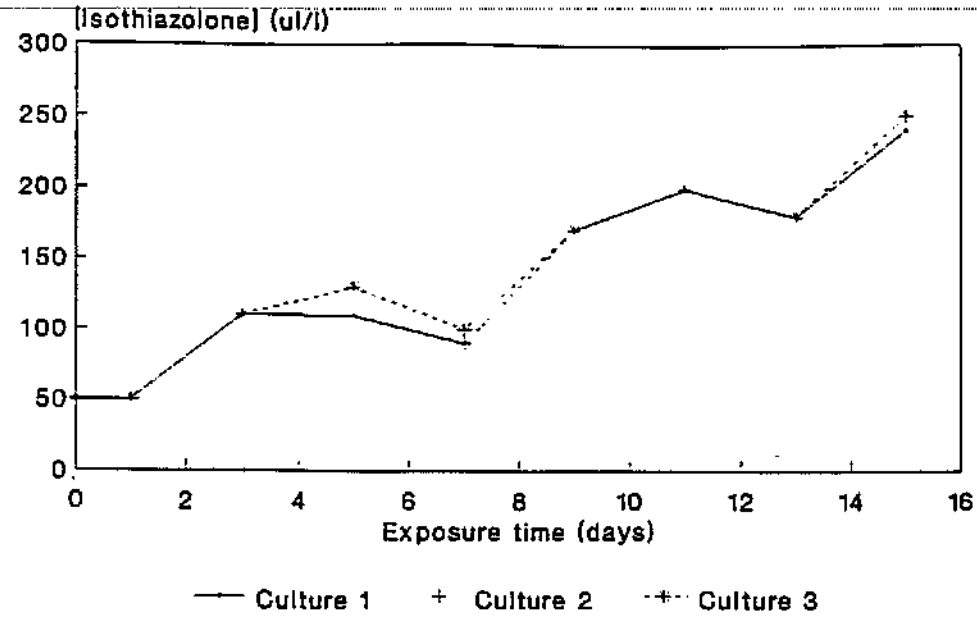


Figure 31: MIC of isothiazolone to three replicate cultures of *P. stutzeri* after growth in R2A broth containing one quarter the previous MIC of bactericide.

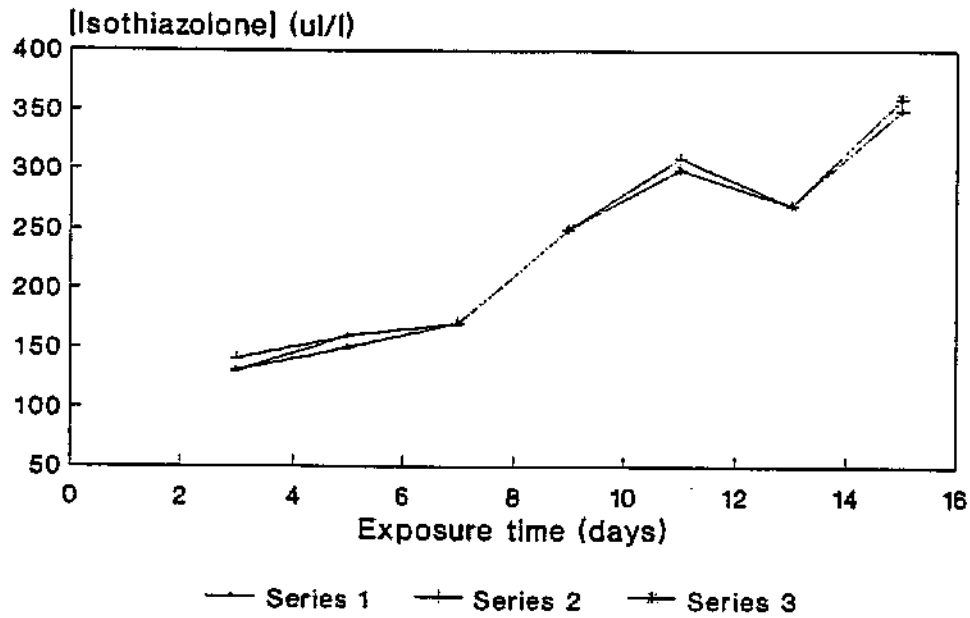
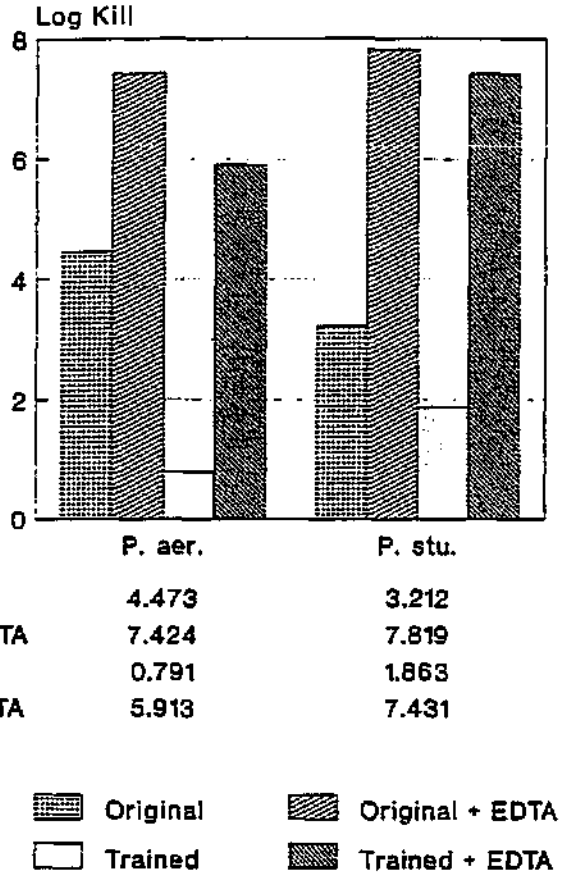


Figure 32: MIC of isothiazolone to three replicate cultures of *B. cereus* after growth in R2A broth containing one quarter the previous MIC of bactericide.

The three different cultures yielded similar results. The development of resistance to isothiazolone is, therefore, not a random process, but rather a constant one. The results indicate that the cultures studied would always develop resistance to a similar degree, and at a similar rate. In all three cultures, resistance increased, decreased slightly and increased again over time.

P. stutzeri resistance increased five-fold from 50 to 250 $\mu l.ml^{-1}$. This shows the ability of this species to adapt to grow in the presence of high concentrations of bactericide, despite its low initial MIC (see also Fig. 29). This would serve to explain the observation by Brözel and Cloete (1992a) that *P. stutzeri* was the dominant planktonic survivor after bactericide treatment of systems, despite its low initial MLC.

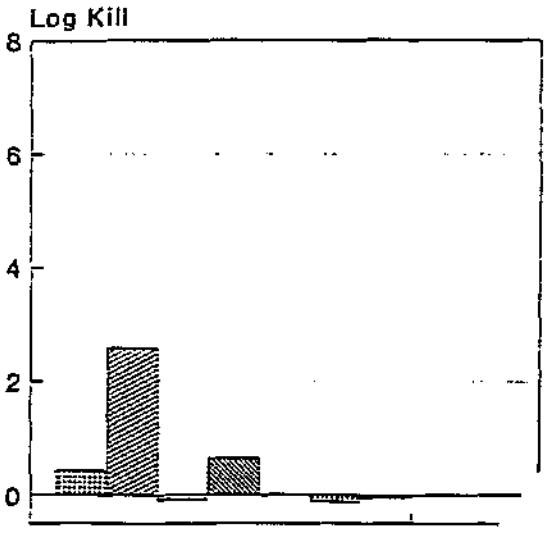
3.2 Comparison of Initial and Resistant Strains



	P. aer.	P. stu.
Original	4.473	3.212
Original + EDTA	7.424	7.819
Trained	0.791	1.863
Trained + EDTA	5.913	7.431

Figure 33: Log kill of *P. aeruginosa* and *P. stutzeri* treated for 60 min with 2,2'methylenebis(4-chlorophenol). Hatched bars show the added effect of 50mM EDTA.

In the case of the resistant *P. stutzeri*, LPS appears to play the major role in resistance as the percentage kill of the initial and resistant cultures in the presence of EDTA was similar.



	P. aer.	P. stu.
Original	0.44	-0.11
Original + EDTA	2.573	-0.0
Trained	-0.074	
Trained + EDTA	0.653	

Figure 34: Log kill of *P. stutzeri* treated for 60 min with Na-di-ethyl dithiocarbamate. Hatched bars show the added effect of 50mM EDTA.

LPS did not appear to play much of a protective role in the resistant culture.

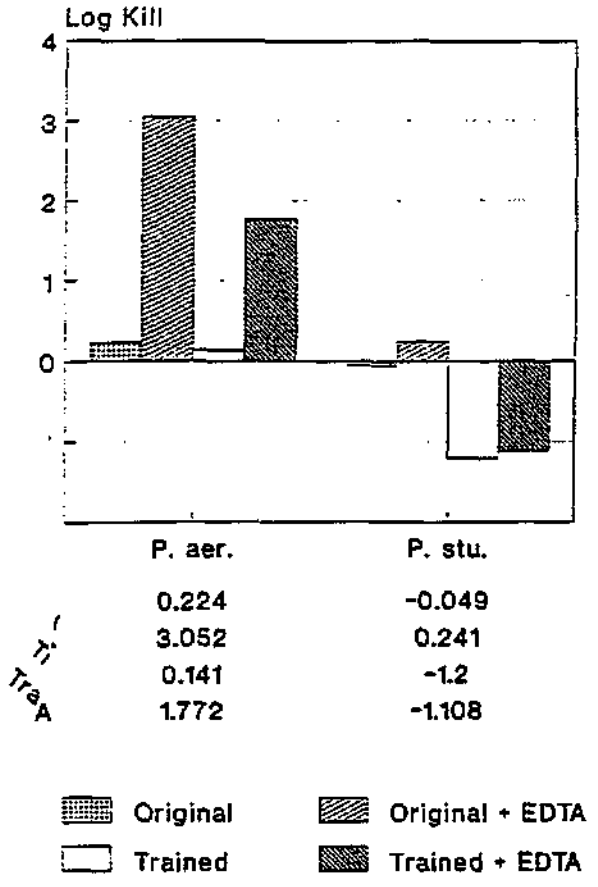


Figure 35: Log kill of *P. stutzeri* treated for 60 min with Isothiazolone. Hatched bars show the added effect of 50mM EDTA.

In *P. stutzeri* LPS does not appear to play an important role in the exclusion of isothiazolone. This is signified by the similarity in kill percentage with and without EDTA. The resistant culture grew well in the presence of the bactericide, and LPS did not appear to play any protective role in this.

3.3 The Effect of Cell Age on Growth Inhibition

The average culture age had a definite influence on the proportion of cells able to form colonies on MGLA containing 80 $\mu g.l^{-1}$ 2,2'-methylenebis(4-chlorophenol) (Fig. 29). No clear relationship could be determined between age and percentage resistant cells although analysis be SAS showed an eighth order polynomial to give the best fit (data not shown). PAO 579 showed little fluctuation in the percentage of resistant cells.

The proportion of resistant cells in colonies growing on MGLA containing 80 $\mu g.l^{-1}$ 2,2'-methylenebis(4-chlorophenol) was much higher than that of initial cultures (Fig. 30). The wild strain exhibited the greatest ability to adapt, whereas PAO 579 showed only a 50 fold increase in resistant cells.

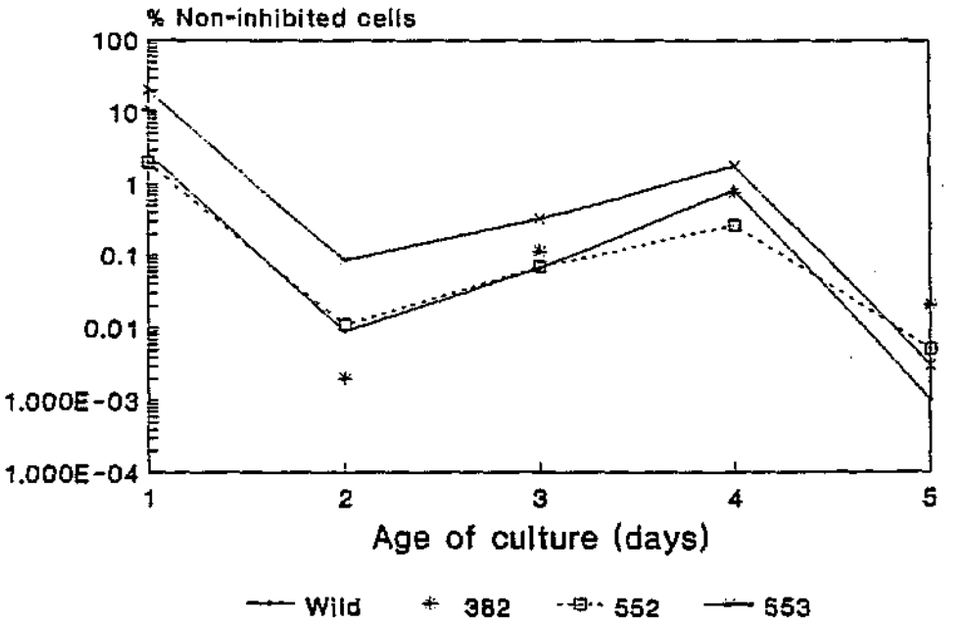


Figure 36: Relationship between culture age of *P. aeruginosa* strains and the percentage of cells able to grow on MGLA containing 80 $\mu g.l^{-1}$ 2,2'-methylenebis(4-chlorophenol).

3.4 Resistance of Mature Cells

Stationary cultures of *P. aeruginosa* and the three mutants grew on all 2,2'-methylenebis(4-chlorophenol) and isothiazolone-containing MGLA surfaces (Table 19). All four cultures grew off the filter directly onto the agar surface up to a certain bactericide concentration. Cultures streaked out, formed colonies up to a certain bactericide concentration, and grew in the area of the primary streak up to a considerably higher concentration. No growth was detected from single cells. Alginate synthesis in *P. aeruginosa* is under environmental control (Deretic, Dikshit, Konyecsni, Chakrabarty and Misra, 1989). Colonies of *P. aeruginosa* became mucoid within 12 h of placing filters onto bactericide agar. PAO 579 is a mucoid mutant producing alginate constitutively, but did not grow at higher bactericide concentrations than PAO 381 and 552 (non-mucoid). This indicates that the bactericides induce production of extracellular polysaccharide (EPS), but that this does not play an important roll in protecting cells.

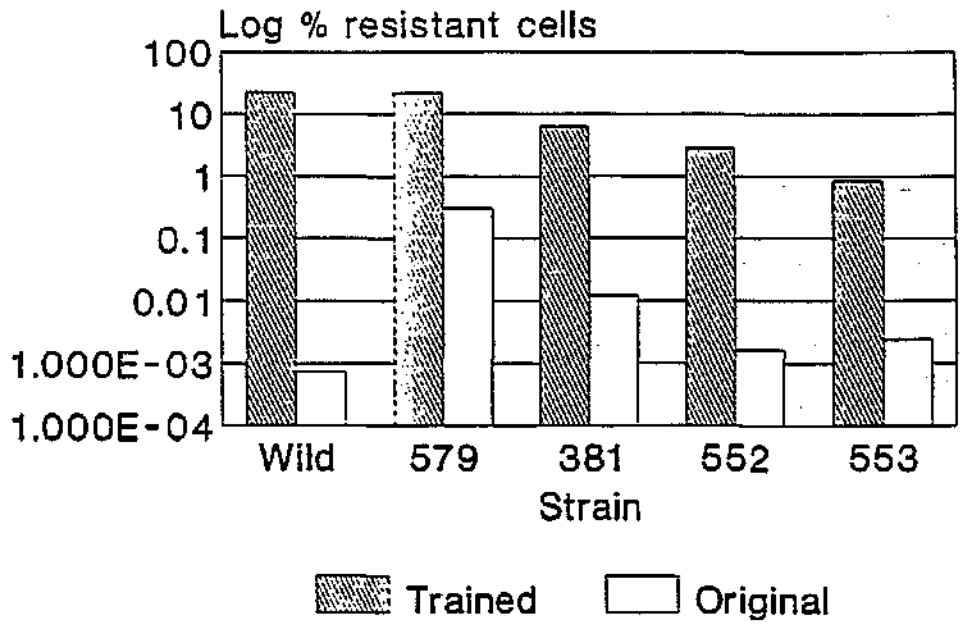


Figure 37: Proportion of cells of original and resistant cultures of *P. aeruginosa* strains able to grow on MGLA containing 80 $\mu g.l^{-1}$ 2,2'-methylenebis(4-chlorophenol).

Table 19: Highest bactericide concentration in MGLA permitting growth of four *P.aeruginosa* strains exposed to bactericide and nutrients in stationary phase. See text for details.

Strain	Filter	No spreading	Streak	Colonies
2,2'-methylenebis(4-chlorophenol) ($\mu g.l^{-1}$)				
Wild	> 800	> 800	640	360
PAO 579	> 800	400	400	320
PAO 381	> 800	560	> 800	480
PAO 552	> 800	480	> 800	480
Isothiazolone ($\mu l.l^{-1}$)				
Wild	> 2000	1400	500	100
PAO 579	> 2000	1400	500	300
PAO 381	> 2000	1600	300	300
PAO 552	> 2000	> 2000	900	400

3.5 Resistance of Attached Cells

Although the drop cultures did not grow, even the 10 minute old attached culture grew on the agar containing 100μg.ml^{-1} 2,2'-methylenebis(4-chlorophenol). All other pre-growth times yielded growth in the presence of the bactericide. The division time for rapidly growing bacteria is 15 min., so not more than one division could have taken place, excluding the possibility for the the formation of a biofilm, as one division would result in an average surface coverage of 20%. The attachment process must innitiate some changes in the cell envelope other than EPS production. This change must be brought about rapidly (within 10 min.), as the resistance factor starts functioning within 10 min.

4 DISCUSSION

4.1 Cellular Resistance

4.1.1 Resistance to 2,2'-methylenebis(4-chlorophenol)

The two strains adapted gradually to 2,2'-Methylenebis(4-chlorophenol); resistance was not acquired directly, as is the case in antibiotic resistance, or resistance to formaldehyde where formaldehyde dehydrogenase is constitutively encoded by a conjugable plasmid (Eagon and Barnes, 1986). The pattern of increasing tolerance suggests adaptation to a resistant physiological state (Jones *et al.*, 1989). The production of O-antigen by *P. stutzeri* after exposure to the bactericide suggests an environmental response, as cultures exposed for 24 h also produced O-antigen. A change in LPS production from a deep rough to a smooth state appears to be the mechanism of exclusion employed by *P. stutzeri* to adapt to growth in the presence of 2,2'-Methylenebis(4-chlorophenol).

4.1.2 Resistance to Na-di-ethyl dithiocarbamate

The resistance of *B. cereus* to Na-di-ethyl dithiocarbamate can be ascribed to adaptation, as it follows near straight line or first order kinetics. The initial increase in resistance of *P. stutzeri* also appears to be due to adaptation. However the increase of resistance of *P. stutzeri* after 19 days suggests some form of genomic change.

4.1.3 Resistance to isothiazolone

The similarity in the kinetics of resistance development to isothiazolone between the three replicate cultures indicates a mechanism of adaptation to the bactericide. Mutational change would be a more random process, and a greater difference between the three data sets would be expected. *P. stutzeri*'s inherent resistance was not due to LPS (Fig 37). O-antigen production was, however, under environmental control. It is possible that certain stabilising factors prevented loss of LPS after EDTA treatment, and that the strain is not susceptible to LPS loss due to a decrease in divalent cation concentration.

4.2 Extracellular Polysaccharide

The similarity in resistance of PAO 579and 381 to 2,2'-methylenebis(4-chlorophenol) (Fig 33) indicates the irrelevant role played by EPS as an exlusion agent of this bactericide. It appeared even to act as an attractant to thiocarbamate, as the alginate-producing PAO 579 was much more susceptible than was PAO 381 (Fig 34). It did, however, play a role in assisting growth on a surface (Fig 38). A higher percentage of EPS-producing cells formed colonies in the presence of bactericide than did the EPS-less PAO 381.

4.3 The Effect of Cell Age on Growth Inhibition

PAO 579 resistance to inhibition was influenced the least by cell age (Fig. 36). However, the strains not producing alginate (PAO 381 & 552) showed a high degree of fluctuation. This shows that EPS cannot play the sole protective roll. The surface hydrophobicity of Gram negative cells fluctuates with age, some becoming more hydrophobic in the stationary phase (Allison *et al.*, 1990), and others becoming less hydrophobic (Delaquis *et al.*, 1989). Detachment from biofilms is also regulated by surface hydrophobicity (Delaquis *et al.*, 1989). The fluctuating resistance to inhibition by the strains investigated is ascribed to altering surface hydrophobicity, and this relationship will be investigated further. The data do show that kill tests are dependant on the age of the test strains used up to a factor of $\log_{10} 2$.

4.4 Resistance of Mature Cells

Two facets of bacterial growth neglected until recently are their multicellular nature (Shapiro, 1991) and gene expression and mutation during stationary phase (Hall, 1990). Many bacteria, when grown on surfaces, display a high degree of cell-cell interaction (Shapiro, 1991). Mature cells in biofilms react very differently to bactericides than do single or exponentially growing cells (Costerton *et al.*, 1987; Shapiro, 1991). They can withstand and even multiply in the presence of otherwise lethal concentrations of bactericides (Table 19). The data obtained to date pose the question whether bactericides can be effective in controlling bacterial biofilm communities.

5 ACKNOWLEDGEMENTS

We would like to thank Edward P. Wallace and Belinda Pietersen for their excellent technical assistance and Prof. J. Petra Schumann and Drs. Bill McCoy and Annegret Schlemmer for helpful discussions. We thank Dr John Govan, Department of Bacteriology, University of Edinburgh Medical School, for kindly donating mutants of *P. aeruginosa* and Thor Chemicals SA (Pty) Ltd. for donating a sample of isothiazolone.

Chapter 7

Adaptation Mechanism of *Pseudomonas aeruginosa* to 2,2'-Methylenebis(4-chlorophenol)

V.S. BRÖZEL and T.E. CLOETE

Abstract

A culture of *Pseudomonas aeruginosa* isolated from a cooling water system was grown in the presence of sub-inhibitory concentrations of the water treatment bactericide 2,2'-Methylenebis(4-chlorophenol) (MBC). It grew in the presence of increasing concentrations by a mechanism of adaptation. The initial minimum inhibitory concentration was 36 μg.ml^{-1} and the highest attained was 80 μg.ml^{-1}. Resistant cultures exhibited a higher survival rate when exposed to 320 μg.ml^{-1} than did the original strain.

Lipopolysaccharide and outer membrane protein profiles were determined by SDS PAGE. No changes were detected in the LPS profile. The quantity of OprP, a phosphate uptake protein in the outer membrane decreased to a low level, correlating with decreased phosphate (P_i) uptake during growth. It is proposed that OprP is the place of entry for MBC through the outer membrane of *P. aeruginosa* and that the cell can adapt to growth in its presence by decreasing the level of OprP in the outer membrane.

1 INTRODUCTION

Many industrial water systems (*eg.* cooling water systems in power plants and mines) are treated with bactericides to eliminate or reduce microbial growth and concomitant microbially induced corrosion (Cloete *et al.*, 1992). Such systems contain a variety of bacteria, most of which are gram negative aerobic rods, eg *Pseudomonas stutzeri, P. fluorescens* and *P. aeruginosa* (Cloete *et al.*, 1989; Escher & Characklis 1990). The bacterial species present differ in their susceptibility to various bactericides, some having a higher degree of inherent resistance than others (Brözel & Cloete 1991a). Some species, showing a low degree of resistance under pure culture conditions, are the dominant planktonic survivors in systems 36 h after treatment with bactericide, indicating adaptation to the bactericide (Brözel & Cloete 1992). Examples encountered in this study were *P. stutzeri* and *P. aeruginosa.* A culture of *P. stutzeri* isolated from such a bactericide-treated system acquired a higher degree of resistance after sublethal exposure (Brözel & Cloete 1991b).

Bactericides attack specific components of bacterial cells. Direct action at the outermost surface is rare. Targets are either in the outer or inner membrane, or in the cytoplasm (Gilbert & Wright 1987). For such bactericides to be effective, they must be able to penetrate the cell envelope and attain a sufficiently high concentration at the target site in order to exert their antibacterial action. Bacteria have been reported to develop resistance to various antiseptics (Sakagami *et al.*, 1989; Heinzel 1988; Jones *et al.*, 1989; Kolawole 1984). Such resistance can be due to a mechanism of adaptation of the cell envelope (Heinzel 1988), or to plasmid transfer encoding a bactericide-degrading enzyme (Eagon & Barnes 1986) or an altered outer cell membrane (Battersby *et al.*, 1985). Exposure to non-oxidising bactericides induces cross resistance to certain other bactericides, indicating a broad mechanism of resistance such as improved barrier properties of the cell envelope (Brözel & Cloete 1991b). Hydrophobic antibacterial agents are prevented from entering through the outer membrane by the sugar chains of the O-antigen section of lipopolysaccharides (Nikaido & Vaara 1987). Rough and deep rough mutants are therefore more susceptible to the antimicrobial action of hydrophobic antibacterials as they lack O-antigen (Hancock 1991). Certain resistant bacterial strains have been found to either lack or overexpress certain outer membrane proteins. An example is the overexpression of Opr H in *P. aeruginosa* which renders it resistant to gentamicin and EDTA by replacing the function of stabilising divalent cations (Bell *et al.*, 1991).

2,2'-Methylenebis(4-chlorophenol)(MBC) is the active ingredient of an efficient water treatment bactericide found to kill a wide range of bacteria found dominant in water cooling systems (Brözel & Cloete 1991a). It belongs to the chlorinated phenol derivatives. These uncouple oxidative phosphorylation from respiration by increasing the permeability of the cell barrier (Gilbert & Brown 1978; Keweloh *et al.*, 1991). Gilbert and Brown (1978) ascribed resistance of *P. aeruginosa* to 3- and 4-chlorophenol to a decrease in the rate of penetration of the drug through the outer envelope.

They found this to be due to decreased affinity to the drug, due not to readily extractible lipids, but to an increase in phospholipid content, a decrease in fatty acid content and an increase in lipopolysaccharide content.

P. aeruginosa is not the most common bacteria in cooling water, but it is the model species in studies on biofilm formation (Escher & Characklis 1990) and its cell envelope is the best studied of the gram negative aerobic bacteria (Hancock *et al.*, 1990). We investigated the possible development of resistance of *P. aeruginosa* to MBC. We grew a strain of *P. aeruginosa* isolated from a cooling water system in increasing concentrations of MBC and investigated whether there were any changes in the lipopolysaccharide and outer membrane protein profiles by SDS PAGE.

2 MATERIALS AND METHODS

2.1 Test cultures

P. aeruginosa, isolated from a cooling water systems in a previous study, was used (Brözel & Cloete 1992). Two mutants of *P. aeruginosa* strain PAO were used to study the effect of extracellular polysaccharides (EPS) on growth inhibition. One was PAO 579, a constitutive alginate producer and the other was PAO 381, a non-mucoid strain not able to produce alginate. Both were a gift of Dr John Govan, University of Edinburgh Medical School. These three original strains were termed "O".

2.2 Resistance studies

Strains were grown in 100 ml R2A broth (Reasoner & Geldreich 1985) under shaking for 24 h at 28 °C. One ml of culture was withdrawn, centrifuged at 10 000 x g for 5 min and resuspended in 1ml sterile water. This suspension was centrifuged at 10 000 x g for 5 min and resuspended in 500 µl sterile water. Two loops full were inoculated into tubes containing 5 ml of half-strength tryptic soy broth (TSB) (Biolab) with a range of concentrations of 2,2'-Methylenebis(4-chlorophenol) (MBC) (Merck). Tubes were incubated at 30 °C for 24 h. The lowest concentration of bactericide showing absence of growth, was taken to be the minimum inhibitory concentration (MIC). One ml of the shake-culture was transferred to 100 ml fresh R2A broth containing MBC at one quarter of the concentration of the MIC as determined. After 24 h growth the new MIC was determined as above and a new shake-culture was inoculated. This procedure was repeated 10 times. The final cultures (termed "R") were harvested, washed in phosphate buffer (PB) and kept at - 20 °C for later analysis.

In order to determine whether MBC induced any immediate changes in the cell envelope, O strains were grown for 24 h in R2A broth containing MBC at one quarter the concentration of the original MIC. This induced state was termed "I". The cells were then harvested, washed in PB and kept at - 20 °C for later analysis. R cultures were grown for 24 h in R2A broth without bactericide in order

to determine whether the acquired changes in the cell envelope were of a permanent nature. These cells were harvested, washed in PB, termed "A" kept at - 20 °C for later analysis. Codes used for the various treatments are defined in Table 20.

Table 20: Notation of the various treatments of *Pseudomonas aeruginosa* cultures.

Notation:	Treatment:
O:	Original strain grown in R2A
R:	Resistant culture after 19 days exposure to MBC
I:	O Culture grown for 24 h in the presence of 20 $\mu g.ml^{-1}$ MBC
A:	R culture grown for 24 h in R2A without MBC
LO:	O culture with 50 mM EDTA
LR:	R culture with 50 mM EDTA

2.3 Survival of cultures after exposure to a lethal concentration of MBC

Cells were grown for 24 h in R2A broth, harvested and resuspended in phosphate buffer (pH 7.0). In the case of resistant cultures, one quarter of the highest MIC of bactericide was included in the broth. Cultures were exposed to four times the highest MIC of bactericide for 60 min., with or without 50mM EDTA, after which spread plate counts were performed. EDTA is known to release *ca.* 50% of the LPS out of the outer membrane, opening it to the passage of molecules which would otherwise be excluded (Leive, 1965). It was added to determine the protective role played by LPS. Plating was onto R2A agar and in triplicate. Incubation was for 48 h at 30 °C. Plate counts were also performed on the culture before bactericide addition to determine the initial inoculum. The log kill was calculated as shown in equation 1, and the results are given in Fig. 39.

$$\log kill = \log_{10} \frac{(initial count)}{(surviving count)} \quad \text{Eq 1}$$

2.4 Determination of outer membrane protein profiles

Outer membrane proteins (OMPs) of the various treatments (O, R, I and A) were prepared from 24 hour old cultures according to the method of Pugsley *et al.* (1986). Cells were harvested at 5000 x g for 10 min, washed in phosphate buffer (pH 7.2), suspended in 25 mM Tris (pH 7.4) containing 1mM Mg^{2+}, and disrupted by ultrasonication on ice using 4 bursts of 15 seconds each. Triton X-100 (BDH) was added to a final concentration of 2 % (v/v) and samples were kept on ice for 20 minutes. The insoluble outer membrane fractions were sedimented out at 13 000 x g for 30 min and the supernatants were removed. The pellets were washed with ice cold acetone to remove traces of Triton X-100, resedimented at 13 000 x g, and solubilised in the sample treatment buffer of Hancock and Carey (1979) but omitting 2-mercaptoethanol.

The protein content of extracts was determined by the method of Lowry *et al.* (1951) using ovalbumin (grade V) from Sigma Chemical Company, St Louis, Mo., USA as standard. Samples were supplemented with 5 % 2-mercaptoethanol and heat-treated at 90 °C for 10 min. Protein profiles were prepared by SDS polyacrylamide gel electrophoresis in a gel containing 14 % acrylamide and 0,25 % N,N'-methylenebisacrylamide according to the buffer system of Hancock and Carey (1979), with 0.07 % NaCl. The stacking gel was prepared as described by Hancock and Carey (1979). Protein molecular weight markers used for calibration were myosin (200 kD), phosphorylase b (97.4 kD), bovine serum albumin (69 kD), ovalbumin (46 kD), carbonic anhydrase (30 kD), trypsin inhibitor (21.5 kD), and lysozyme (14.3 kD), and were from Amersham. Gels were fixed and stained in an aqueous solution of 10 % acetic acid, 25 % isopropanol, and 0.1 % Coomasie brilliant blue R250 (Merck) at 30 °C for 1h. The positions and intensities of bands were determined using a Hoeffer Scientific Instruments GS 300 scanning densitometer.

2.5 Determination of lipopolysaccharide profiles

Lipopolysaccharides (LPS) of the various treatments (O, R, I and A) were extracted by boiling for 10 min in lysing buffer (pH 6,80) containing 20 % glycerol, 5 % mercaptoethanol, 4,6 % sodium dodecyl sulphate (SDS) and 2 % Tris by the method of Preston and Penner (1987). Proteins in the samples were digested by incubating for 10 min at 60 °C in lysing buffer containing 2,5 $mg.ml^{-1}$ proteinase K (Boehringer Mannheim) (Preston & Penner 1987). Profiles of the extracted LPS were prepared by SDS-PAGE using the buffer system devised by Laemmli and Favre (1973). The stacking gel contained 4,8 % acrylamide and 0,13 % N,N'-methylenebisacrylamide. The separating gel contained 12,05 % acrylamide and 0,32 % N,N'-methylenebisacrylamide and was run at 35 mA using 2 µl bromophenol blue (0,01 %) in 50 % glycerol as tracking dye. LPS components were visualised by the silver staining procedure of Tsai and Frasch (1982).

2.6 Phosphate uptake

Phosphate uptake was determined in triplicate cultures with and without Opr P. O cultures were grown in R2A broth, and R cultures were grown in R2A broth containing 20μg.ml^{-1} MBC for 24 hours under shaking at 30 °C. 100 ml broth was inoculated with 0.10 ml culture. Cells were removed after 24 h growth by centrifuging for 10 min at 10 000 x g. The phosphate content of the cell-free supernatant and of the original medium was determined by absorbance of the molibdate complex at 690 nm (Anon 1985) and the phosphate uptake was calculated.

3 RESULTS

3.1 Resistance

The resistance to inhibition by MBC increased over the period of exposure from 36 μg.ml^{-1} to 80 μg.ml^{-1} (Fig. 38). R cultures of *P. aeruginosa* were more resistant to killing by MBC than were O cultures, exhibiting a $\log_{10}$ kill of 0.791 as opposed to 4.473 (Fig. 39). Inherent resistance of *P. aeruginosa* to MBC is due mainly to LPS, indicated by the high log kill in LO cultures (with a decreased LPS content) (7.424 as opposed to 4.473). MBC is a hydrophobic molecule, and should be expected to be excluded from the cell by LPS (Hancock 1991; Nikaido & Vaara 1987). R cultures exposed to the bactericide in the presence of 50mM EDTA exhibited a higher survival than LO cultures ($\log_{10}$ kill = 5.913 as opposed to 7.424). This indicated that the protective factor in R cultures was not

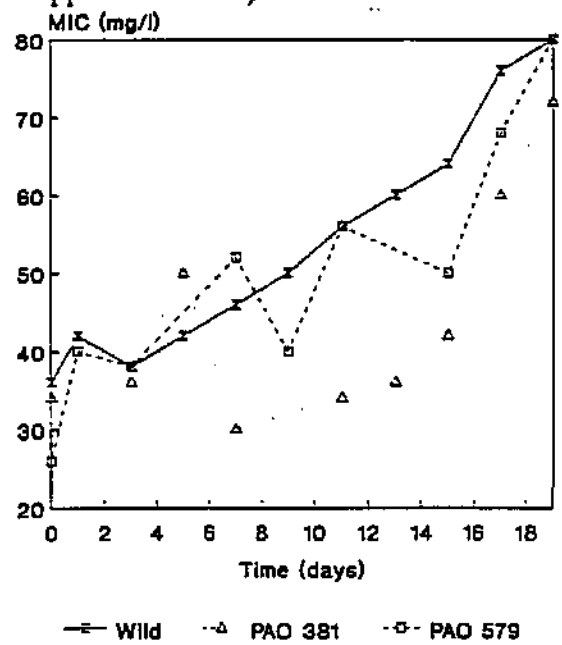


Figure 38: MIC of MBC to *Pseudomonas aeruginosa*, PAO 381 and PAO 579 after growth in R2A broth containing one quarter the previous MIC of bactericide.

only LPS as loss of LPS (LR cultures) still left an additional protective factor behind. Had LPS been the only protective factor, the $\log_{10}$ kill for the LR culture would be similar to that of the LO culture. No qualitative difference was detected by SDS PAGE between the LPS of the O and R cultures as the profiles were all the same.

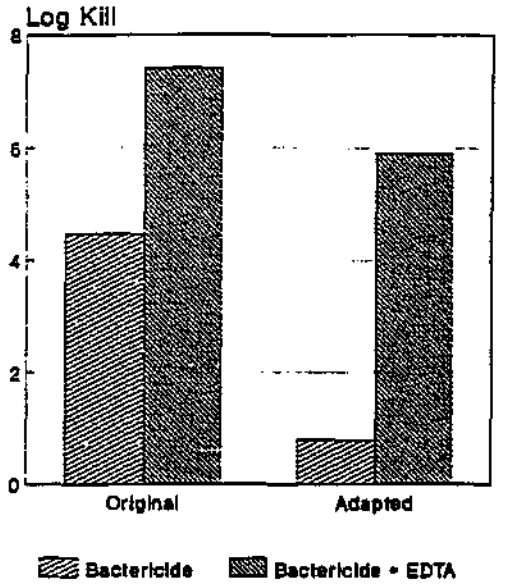


Figure 39: Log kill of *Pseudomonas aeruginosa* treated for 60 min with MBC. Hatched bars show effect on cells with a lower LPS content (treated with 50mM EDTA).

3.2 The effect of extracellular polysaccharide

The MIC of MBC to original cultures of PAO 579 was lower (26 $\mu g.ml^{-1}$) than that to PAO 381 (34 $\mu g.ml^{-1}$) or the wild strain (36 $\mu g.ml^{-1}$). PAO 579 did adapt better to growth in the presence of MBC than did PAO 381, but not as well as did the wild *P. aeruginosa* (Fig. 38). Extracellular polysaccharide can, therefore, not be the excluding agent as in the case of biguanides (Kolawole 1984) or the scavenging agent as is the case with oxidising bactericides (Learn *et al.*, 1987).

3.3 Outer membrane protein profiles

The profile of outer membrane proteins is clearly different in the O, R. I. and A cultures as shown in Fig. 40. OprP was detected in the resistant and induced cultures in very low quantities (Fig. 40 lanes 1 and 2). Its production appears to be suppressed by MBC. Opr P is the uptake protein for P_i in *P. aeruginosa* (Hancock *et al.*, 1990; Hancock *et al.*, 1992). This suggests that MBC relates to the gene system for the regulation of phosphate uptake in some way. Phosphate uptake (P_i) was lower in R but also in I cultures than in O or A cultures (Table 21). The P_i uptake observed probably

occurred via OprO which is the uptake protein for PP_i in *P. aeruginosa* (Hancock *et al.*, 1992). OprO was detected in higher levels in resistant, induced and relaxed cultures, probably due to P_i starvation (Fig. 40 lanes 1 - 3). A cultures exhibited a lower P_i uptake and less OprP than did O cultures. This indicates a delayed derepression of OprP production.

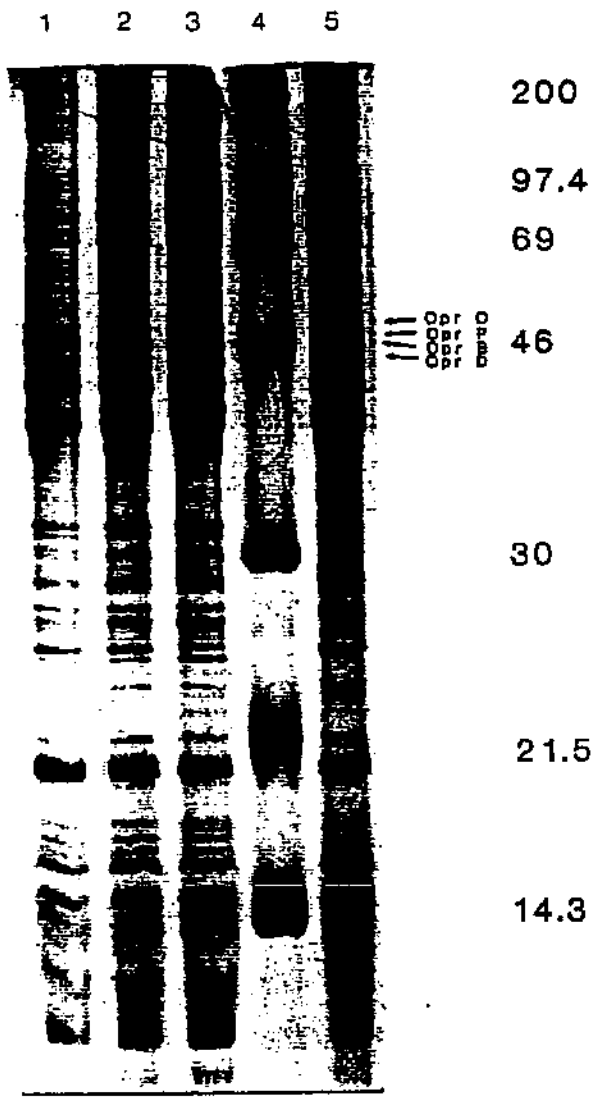


Figure 40: SDS-polyacrylamide gel electrophoresis of outer membrane proteins from *Pseudomonas aeruginosa* resistant to MBC (R) (lane 1), exposed to MBC for 24 h (I) (lane 2), resistant culture grown for 24 h in the absence of MBC (A) (lane 3), molecular weight markers (see materials and methods) (lane 4) and original culture (O) (lane 5). Values on the right side indicate the masses of the molecular mass markers.

Table 21: Phosphate uptake by initial, resistant, induced and relaxed cultures of *Pseudomonas aeruginosa*

Culture	Phosphate uptake ($mg.l^{-1}$)	% Phosphate uptake
Initial:	257	47.8
Resistant:	188	35.0
Induced:	224	41.7
Relaxed:	238	44.3

Production of OprB is favoured in the presence of glucose as carbon source and that of OprD in the presence of certain carbon sources (Hancock *et al.*, 1990). As all cultures were grown in the presence of glucose, starch and pyruvate as carbon sources, the increased levels of OprB and OprD in R, I and A cultures is not clear (Fig. 40 lanes 1 - 3).

4 DISCUSSION

The three strains adapted gradually to MBC; resistance was not acquired directly, as is the case in antibiotic resistance, or resistance to formaldehyde where formaldehyde dehydrogenase is constitutively encoded by a conjugable plasmid (Eagon & Barnes 1986). The pattern of increasing tolerance over time suggests adaptation to a resistant physiological state (Jones *et al.*, 1989). The high inherent resistance of *P. aeruginosa* (MIC = 36 $\mu g.ml^{-1}$) can be related to its outer layer of O-antigen as a culture of *P. stutzeri* revealed an MIC of 20 $\mu g.ml^{-1}$. Its LPS was devoid of O-antigen (Brözel and Cloete, unpublished). The O-antigen section of LPS is instrumental in excluding hydrophobic molecules from gram negative cells (Hancock 1991; Nikaido & Vaara 1987). Although no qualitative difference in LPS profiles of O and R cultures was detected by SDS PAGE, an increase in the concentration could have occured. This remains to be determined. The similarity in resistance of PAO 579 and 381 to MBC (Fig. 38) indicates the irrelevant role played by EPS as an exlusion agent of this bactericide.

The additional resistance factor of R cultures (other than LPS) can be explained by the changes in the outer membrane protein profile (Fig. 40). The low quantity of OprP seems to indicate that it serves as a port of entry for the bactericide (Hancock *et al.*, 1987). Its absence would prevent access

through the outer membrane and thereby to the target site. MBC is an anion when in solution, and could pass through the OprP pore. It is, however, not a spherical but an elipsoidal structure and could be expected be more similar to PP_i than to P_i. PP_i binds to OprP with a much lower affinity than does P_i (Hancock *et al.*, 1992). OprO binds PP_i and to a lesser degree PPP_i whereas its affinity for P_i is low (Hancock *et al.*, 1992). The increased level in OprO did not facilitate an increased entry of MBC into the cell, indicating that MBC cannot pass through this protein. The mechanism of selection for PP_i by OprO has not been clarified to date (Hancock *et al.*, 1992). The very low copy number of OprP in the presence of MBC indicates that it acts as a repressor of the *oprP* gene, mimicking P_i. Whereas repression occurs upon exposure, derepression of OprP production is slower as A cultures still had a low OprP content.

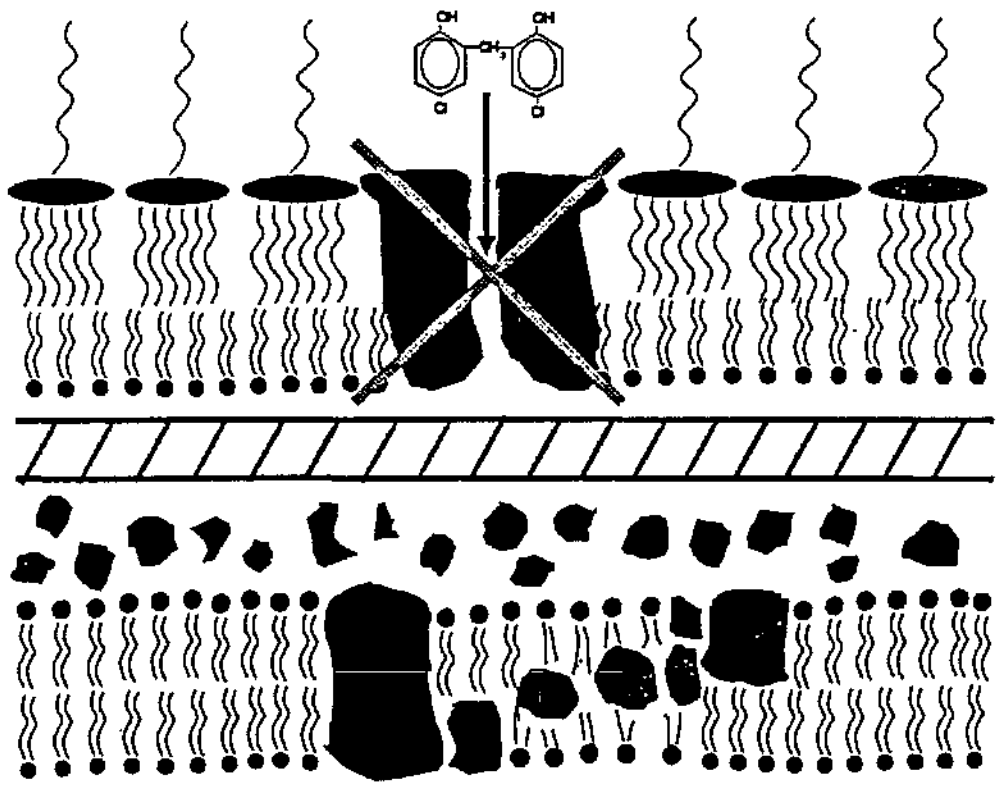


Figure 41: Proposed mechanism of resistance of *Pseudomonas aeruginosa* to 2,2'-methylenebis(4-chlorophenol). The P_i uptake porin Opr P, which is also the porin of entry for 2,2'-methylenebis(4-chlorophenol), is suppressed in resistant cells, thereby preventing entry of the bactericide.

Glucose-limited cells of *P. aeruginosa* are more susceptible to chlorinated phenols (Gilbert & Brown 1978). OprB production is induced by glucose (Hancock *et al.*, 1990), so glucose starvation would result in a low copy number of the protein. The higher level of OprB in the resistant culture as opposed to the initial strain suggests that this protein plays a role in decreasing the lethality of MBC.

P. aeruginosa was found to adapt to growth in an increasing concentration of MBC by adaptation. The LPS profile as determined by SDS PAGE did not change. The quantity of OprP in the outer membrane decreased to a low level, correlating with decreased phosphate (P_i) uptake during growth. It is proposed that OprP is the place of entry for MBC through the outer membrane of *P. aeruginosa* and that the cell can adapt to growth in its presence by decreasing the level of OprP in the outer membrane.

5 ACKNOWLEDGEMENTS

We thank Dr John Govan, Department of Microbiology, University of Edinburgh Medical School, for donating mutants of *Pseudomonas aeruginosa* PAO and Belinda Pietersen for technical assistance.

Chapter 8

Resistance of *Pseudomonas aeruginosa* to Sodium Dimethyldithiocarbamate by Adaptation

V. S. BRÖZEL and T. E. CLOETE

Abstract

Resistance and the development thereof in *Pseudomonas aeruginosa* to the bactericide sodium dimethyl dithiocarbamate (SMT) was investigated. *Ps. aeruginosa* was cultured in nutrient-poor broth in the presence of sub-inhibitory concentrations of SMT. It adapted over 21 days of exposure from 250 $\mu g.ml^{-1}$ to 490 $\mu g.ml^{-1}$. The initial high MIC was ascribed to exclusion of SMT by the lipopolysaccharide layer since removal thereof by EDTA rendered cells highly susceptible. The alginate-producing mutant PAO 579 was much more susceptible to SMT than was its parent PAO 381, indicating that extracellular polysaccharide does not act as an exclusion barrier to SMT. Following 24 h exposure to SMT, *Ps. aeruginosa* had an altered profile of outer membrane proteins as determined by SDS PAGE. Resistant cells had a further altered profile. Resistance of *Ps. aeruginosa* is ascribed to a change in the outer membrane protein profile, leading to improved exclusion of SMT.

1 INTRODUCTION

Many industrial water systems (*eg.* cooling water systems in power plants and mines) are treated with bactericides to eliminate or reduce microbial growth and concomitant microbially induced corrosion (Cloete *et al.*, 1992). Such systems contain a variety of bacteria, most of which are gram negative aerobic rods, *eg. Pseudomonas stutzeri* and *P. aeruginosa* (Cloete *et al.*, 1989; Escher & Characklis, 1990). The bacterial species present differ in their susceptibility to various bactericides, some having a higher degree of inherent resistance than others (Brözel & Cloete, 1991a). We have previously found that some species, showing a low degree of resistance under pure culture conditions, are dominant planktonic survivors in systems 36 h after treatment with bactericide, indicating adaptation of members of the population to the bactericide (Brözel & Cloete, 1992). Various cultures isolated from such a bactericide-treated system acquired a higher degree of resistance to a range of bactericides after sublethal exposure (Brözel & Cloete, 1991b).

Bacteria develop resistance to various antiseptics (Heinzel, 1989; Jones *et al.*, 1989; Kolawole, 1984; Sakagami *et al.*, 1989) but reports on resistance to water treatment bactericides are rare. Resistance to antimicrobial agents can be due to a mechanism of adaptation of the cell envelope (Heinzel, 1989), or to plasmid transfer encoding a bactericide-degrading enzyme (Eagon & Barnes, 1986). Bactericides attack specific components of bacterial cells. Direct action at the outermost surface is rare and targets are either in the outer or inner membrane, or in the cytoplasm (Gilbert & Wright, 1987). For bactericides to be effective, they must be able to penetrate the cell envelope and attain a sufficiently high concentration at the target site in order to exert their antibacterial action. Hydrophilic antibacterial agents are primarily prevented from entering through the outer membrane by the lipopolysaccharide layer and the underlying phospholipids, whereas hydrophobic agents are excluded by outer membrane proteins (Nikaido & Vaara, 1987). Certain antibiotic-resistant bacterial strains either lack or overexpress certain outer membrane proteins. An example is a strain of *P. aeruginosa* resistant to the antibiotic imipenem (Quinn *et al.*, 1986). This strain lacks Opr D, a porin selective for certain carbon sources (Hancock *et al.*, 1990).

SMT is the active ingredient of a water treatment bactericide used to control bacteria and algae in cooling water systems. It is also included in fungistatic paints and in certain cosmetic products. We have previously reported that *P. stutzeri* and *P. cepacia* acquire resistance to SMT (Brözel & Cloete, 1991b). The aim of this study was to determine the rate at which *P. aeruginosa* develops resistance to thiocarbamate, and what the differences between resistant and wild cells would be.

2 MATERIALS AND METHODS

2.1 Test cultures

A wild strain of *Pseudomonas aeruginosa* isolated from a cooling water systems in a previous study (Brözel & Cloete, 1992) was used. To study the effect of extracellular polysaccharide (EPS) on inhibition to growth, two mutants of *P. aeruginosa* strain PAO were used. One was PAO 579, a constitutive alginate producer and the other was its parent strain PAO 381, a non-mucoid strain not able to produce alginate. Both mutants were donated to us by Dr John Govan, University of Edinburgh Medical School.

2.2 Resistance studies

Strains were cultured in 100 ml R2A broth (Reasoner & Geldreich 1985) under shaking for 24 h at 28 °C. One ml of culture was centrifuged at 10 000 x g for 5 min and resuspended in 1ml sterile water. This suspension was centrifuged at 10 000 x g for 5 min and resuspended in 500 µl sterile water. Two inoculation loop fulls were inoculated into tubes containing 5 ml of half-strength tryptic soy broth (TSB) (Biolab) with a range of concentrations of Na dimethyl dithiocarbamate (SMT) (Mr = 179.24) (Fluka). Tubes were incubated at 30 °C for 24 h. The lowest concentration of bactericide inhibiting growth, was taken to be the minimum inhibitory concentration (MIC). One ml of the shake-culture was transferred to 100 ml fresh R2A broth containing SMT at one quarter of the concentration of the MIC as determined. After incubation for 24 h the new MIC was determined as above and a new shake-culture was inoculated. This procedure was repeated 10 times. The final cultures (termed resistant) were harvested, washed in phosphate buffer (PB) and kept at - 20 °C for later analysis.

In order to determine whether SMT induced any immediate changes in the cell envelope, wild strains were cultured for 24 h in R2A broth containing SMT at one quarter the concentration of the original MIC. These cultures were termed "induced". The cells were then harvested, washed in PB and kept at - 20 °C for later analysis. Resistant cultures were cultured and transferred three consecutive times for 24 h in R2A broth without bactericide in order to determine whether the acquired changes in the cell envelope were of a permanent nature. These cells were harvested, washed in PB, termed "relaxed" and kept at - 20 °C for later analysis.

2.3 Investigation of the role of lipopolysaccharides in resistance

Cells were cultured for 24 h in R2A broth, harvested and resuspended in phosphate buffer (pH 7.0). With resistant cultures, one quarter of the highest MIC of bactericide was included in the broth. Cultures were exposed to 2000 $mg.l^{-1}$ SMT for 60 min with or without 50 mM EDTA to determine

the protective role played by LPS. Spread plate counts were prepared in triplicate on R2A agar and incubated at 30 °C for 48 h. The log kill was determined by the negative logarithm of the ratio of surviving over initial cell number.

Lipopolysaccharides (LPS) of the four culture types (wild, resistant, induced and relaxed) were extracted by the method of Preston and Penner (Preston & Penner, 1987). Profiles of the extracted LPS were prepared by SDS-PAGE using the buffer system devised by Laemmli and Favre (1973). LPS components were visualised by the silver staining procedure of Tsai and Frasch (Tsai & Frasch, 1982).

2.4 Determination of outer membrane protein profiles

Outer membrane proteins (OMPs) of the four culture types (wild, resistant, induced and relaxed) were prepared from 24 h old cultures according to the method of Pugsley *et al.* (1986). Briefly, cells were harvested at 5000 x g for 10 min, washed in phosphate buffer (pH 7.2), suspended in 25 mM Tris (pH 7.4) containing 1mM Mg^{2+}, and disrupted by sonication on ice using 4 bursts of 15 s each. Lysates were supplemented with 2 % (v/v) Triton X-100 and kept on ice for 20 min. The insoluble outer membrane fractions were sedimented at 13 000 x g for 30 min and the supernatants were removed. The pellets were solubilised in the sample treatment buffer of Hancock and Carey (1979) but omitting 2-mercaptoethanol.

The protein content of extracts was determined by the method of Lowry *et al.* (1951) using ovalbumin (grade V) from Sigma Chemical Company, St Louis, Mo, USA as standard. Samples were supplemented with 5 % 2-mercaptoethanol and heat-treated at 95 °C for 10 min. Protein profiles were prepared by SDS polyacrylamide gel electrophoresis in a gel containing 14 % acrylamide and 0.25 % bis acrylamide according to the buffer system of Hancock and Carey (1979), with 0.07 % NaCl. Protein molecular mass markers used for calibration were myosin 200 kD, phosphorylase b (97.4 kD), bovine serum albumin (69 kD), ovalbumin (46 kD), carbonic anhydrase (30 kD), trypsin inhibitor (21.5 kD), and lysozyme (14.3 kD) (Amersham). Gels were fixed and stained with 10 % acetic acid, 25 % isopropanol, and 0.1 % Coomasie brilliant blue R250. The positions and intensities of bands were determined using a Hoeffer Scientific Instruments GS 300 scanning densitometer and Gel-Compar version 1.3 software (Helix C.V., Belgium).

3 RESULTS

3.1 Resistance

The wild strain of *Pseudomonas aeruginosa* was initially resistant to 250 mg.l^{-1} SMT. After 24 h growth in the presence of 65 mg.l^{-1} SMT, its resistance increased to 310 mg.l^{-1} SMT. 24 h Later its resistance decreased to 270 mg.l^{-1}, but increased following subsequent transfers to 490 mg.l^{-1} after 21 d exposure. PAO 381 was tolerant of much less SMT than was the wild strain. After 9 d exposure, resistance to SMT decreased to 70 mg.l^{-1}. It did, however, acquire resistance to 170 mg.l^{-1} after 21 d growth in the presence of sub-inhibitory concentrations. Strain PAO 579 was initially extremely susceptible to SMT, being resistant to 30 mg.l^{-1}. It became increasingly resistant during exposure, and after 21 d exposure grew in the presence of 165 mg.l^{-1} SMT. The production of the extracellular polysaccharide alginate did not afford PAO 579 greater resistance to SMT, indicating that extracellular polysaccharide does not decrease the rate of access of SMT into the cell but rather enhances it.

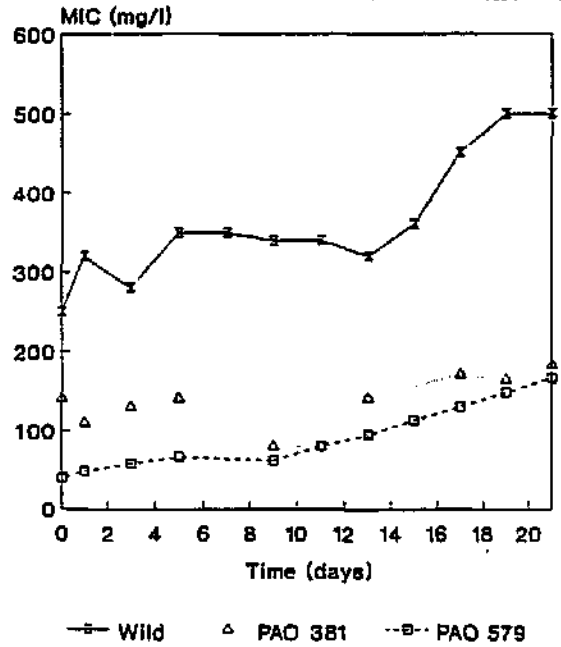


Figure 42: MIC of SMT to *P. aeruginosa*, PAO 381 and PAO 579 after growth in R2A broth containing one quarter the previous MIC of bactericide.

3.2 Lipopolyasaccharides

Removal of LPS in wild cells rendered these susceptible to SMT. After 60 min exposure to 2000 mg.l^{-1}, the log kill was 0.44. Where 50 mM EDTA was added the log kill was 2.6. The increased susceptibility of cells with decreased LPS content indicated that SMT was prevented from entering through the outer membrane by the LPS layer. The resistant culture was so resistant to SMT that it grew slightly in the presence of 2000 mg.l^{-1} (depicted by a negative log kill, Fig. 43). Treatment of

resistant cells with EDTA lead to a reduced log kill (0.65). This could indicate better binding of LPS in the outer membrane or the replacement of the protective role of LPS by another membrane component. LPS profiles as determined by SDS PAGE did not reveal any differences between the four culture types (results not shown).

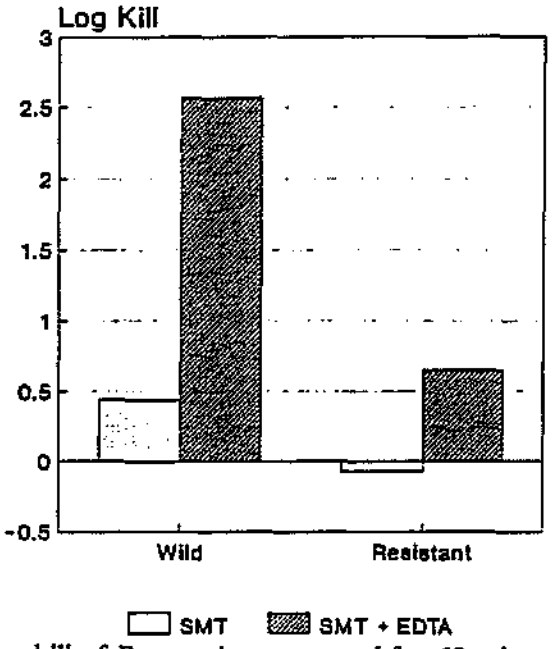


Figure 43: Log kill of *P. aeruginosa* treated for 60 min with 2000 mg.l^{-1} SMT. Hatched bars show effect on cells with a lower LPS content (treated with 50mM EDTA).

3.3 Outer membrane proteins

The outer membrane protein profiles of the 4 different treatments differed considerably (Fig. 44). Notable differences are listed in Table 22. Altogether 5 outer membrane proteins were absent in resistant cells, four of which have been reported to be porins (Hancock *et al.*, 1990). Opr B, the port for uptake of glucose, was not present in detectable levels in the resistant strain although it was over-produced in the induced culture. It was also not expressed in the relaxed strain, raising the question of a mutation. The inducible suppression of Opr D production is significant as *P. aeruginosa* resistant to the antibiotic imipenem also lacks this outer membrane protein (Quinn *et al.*, 1986). Two proteins, one 35 kD and one 36 kD, were present in wild cells and were produced in large quantities in resistant and relaxed cultures. The 35 kD protein was present in low quantities in induced cells whereas the 36 kD protein was not detected. Three proteins not detected in the wild strain were present in resistant cultures. One, produced in large quantities, was a ferripyochelin binding protein (FBP). The other two are of unknown function.

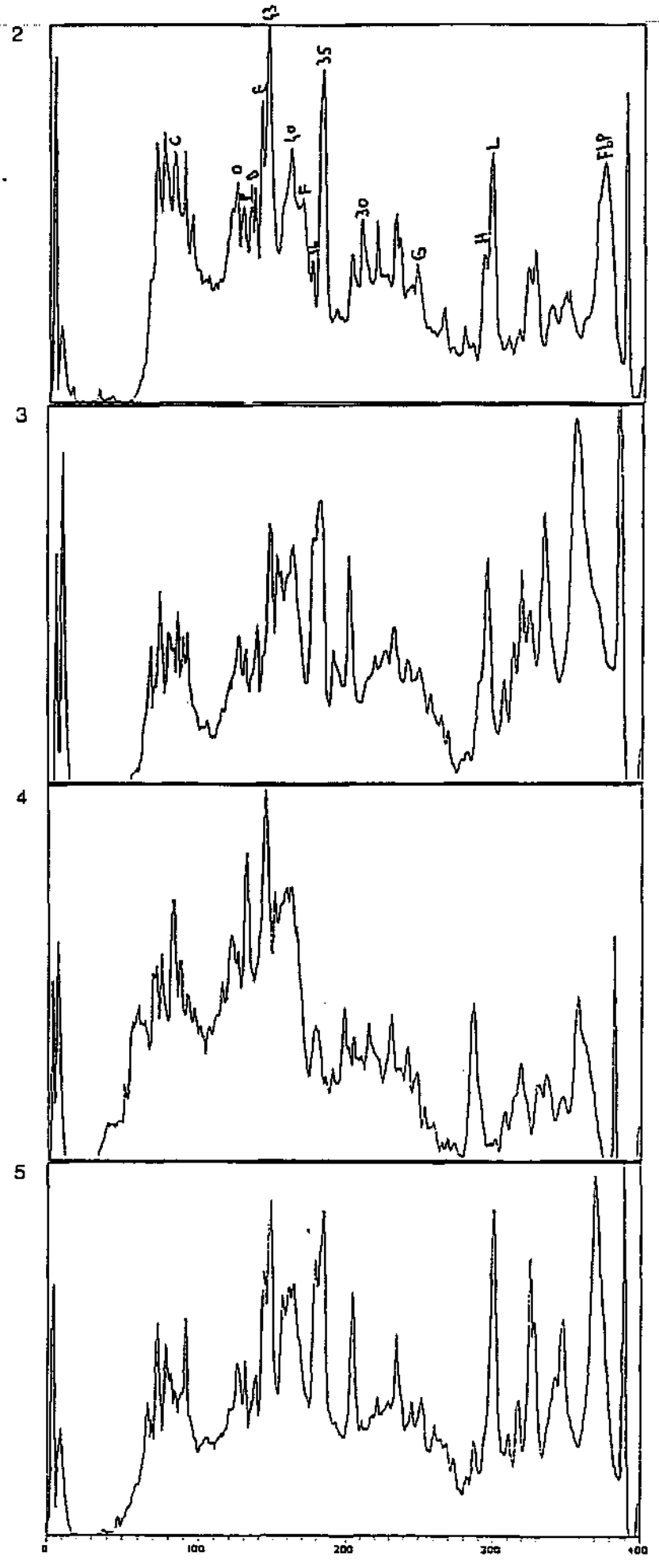


Figure 44: a.) SDS-polyacrylamide gel electrophoresis of outer membrane proteins of *P. aeruginosa* wild culture (lane 2), resistant to SMT (lane 3), exposed to SMT for 24 h (lane 4), resistant culture grown for 72 h in the absence of SMT (lane 5) and molecular weight markers (lane 1) (see materials and methods for details). b.) Densitometric scans of outer membrane proteins of wild (i), resistant (ii), induced (iii) and relaxed (iv) cultures.

Table 22: Notable differences in the outer membrane protein profiles of wild, resistant, induced and relaxed cultures of *Pseudomonas aeruginosa* (see text for details).

Protein	Wild	Resistant	Induced	Relaxed
Opr B	+	-	++	-
Opr D	+	-	-	-
Opr E	+	-	-	+
Opr F	+	+	++	+
36 kD	(+)	+	-	+
35 kD	+	++	-	+
30 kD	+	-	-	-
Opr G	-	+	-	-
Opr H	+	+	+	-
Opr L	+	+	-	+
FbP	-	+	-	+

Legend: + = present, ++ = present in large quantity, (+) = present in small quantity, - = absent

The general porins Opr C and Opr F were present in large quantities in the induced cultures but barely detectable in relaxed cultures. This could indicate a stress reaction to facilitate nutrient uptake prevented by suppression of other porins (Opr D and Opr E). Opr F was also present in larger quantities in induced than in other cells. Induced cells did not possess Opr L, a lipoprotein strongly associated with peptidoglycan (Hancock *et al.*, 1990). It did, however, produce an increased amount of the divalent cation replacing Opr H. Relaxed cells produced a high amount of Opr L and very little Opr H whereas both wild and resistant cells produced both in similar quantities.

4 DISCUSSION

The aim of bactericide addition to cooling water is to eliminate or reduce bacterial growth. The available concentration of bactericide in the water must be high enough to kill or inhibit those bacteria present. Where concentrations are too low, the bactericide is ineffective and bacterial growth proceeds. Application concentrations for SMT treatment of cooling water recommended by suppliers are between 12 and 24 $mg.l^{-1}$. We have previously determined that 70 $mg.l^{-1}$ are required to kill a spectrum of gram negative isolates from cooling systems under laboratory conditions (Brözel & Cloete, 1991a). 28 h After application of 70 $mg.l^{-1}$ SMT to an experimental system, bacterial numbers increased (Brözel & Cloete, 1992). This indicates that certain members of the bacterial community survived treatment, and that the concentration of SMT (70 $mg.l^{-1}$) was sub-inhibitory to these cells. If the system were to be treated repeatedly with the same concentration of SMT, some members of the bacterial community would continue to be exposed to sub-inhibitory concentrations. Some of these bacteria would acquire increased resistance and would grow in the presence of the SMT added. The treatment programme would decrease in effectivity by selecting for resistant strains, *eg P. aeruginosa* which has a high inherent resistance to most antimicrobial agents (Nikaido & Vaara, 1987), also to SMT. This situation often occurs in practice where application concentrations are too low due to economic reasons.

The initial increase in resistance of *P. aeruginosa* appears to be due to adaptation. The increase in resistance after 15 d exposure suggests some form of genomic change. This is supported by the observation that resistant cultures grown for 72 h in the absence of SMT still did not produce the outer membrane proteins Opr B, Opr D, and the 30 kD protein. The permanent loss of Opr B in cells cultured in the presence of glucose raises the question of a mutation as Opr B was not inducibly suppressed (Table 22). The increased level of Opr B detected in induced cultures indicates some form of stress response which was later abandoned by surviving cells. This could indicate either, that SMT caused an increased glucose detection by the cells, inducing production of the porin, or over-production due to the suppression of other porins also responsible for glucose uptake. Repression of production of Opr D was inducible, but production was not derepressed after removal of the agent of repression. The continued low expression in the absence of the bactericide suggests a long-term repression effect, similar to the continued production of alginate by *P. aeruginosa* PAO long after removal of induction factors (Deretic *et al.*, 1989). Opr D is known to act as a port of entry for the antibiotic imipenem and resistant *P. aeruginosa* have been found to lack Opr D (Quinn *et al.*, 1986). Imipenem has a Mr of 317.4 (Reynolds, 1989), and Na-di-ethyl dithiocarbamate an Mr of 179,24 so it should not be excluded by virtue of size. The inducible decrease in the production of Opr D must relate to an adaptative response to the bactericide. Quinn *et al.* (1986) reported that the imipenem resistant cells were isolated from a patient not responding to imipenem treatment. Our finding that production of Opr D is suppressed upon exposure of wild cells to SMT poses the

question whether the reported imipenem "resistance" was inducible and not due to selection for resistant strains. The repression of the general porin Opr E appeared linked to the presence of SMT as both induced and resistant cells did not reveal any whereas relaxed cultures did.

We have previously detected the inducible repression of the 35 kD outer membrane protein of *P. aeruginosa* in cultures exposed to the bactericide isothiazolone. The repression was, however, maintained in resistant cells (data not shown). The high levels of this protein present in resistant cultures indicated that it does not contribute to susceptibility to SMT but rather to resistance to this bactericide. Overall, two proteins (Opr B and Opr F) were over-produced and two (Opr L and a 35 kD protein) were not produced following exposure of cells to SMT, whereas resistant cells showed the opposite. This phenomenon indicated that *P. aeruginosa* reacts to chemical stress by a broad stress response. Some changes in the protein profile aid the cell in survival and are maintained whereas others do not and are abandoned.

The LPS did not appear to change structurally as a response to SMT, although resistant cells were less affected by EDTA treatment than were wild ones. Induced cells did possess increased levels of Opr H, a known LPS - stabilising factor (Bell *et al.*, 1991). Densitometry revealed that the level of Opr H in resistant cultures was only slightly higher than in wild ones so that LPS stabilisation was not due primarily to an increase in the level of Opr H. The low initial resistance of the alginate-producing mutant PAO 579, as well as its low final resistance indicated that extracellular polysaccharide did not play a protective role as it does in the case of chlorine or hypochlorite (LeChevallier *et al.*, 1988). Rather, it appeared to act as an attractant for SMT, rendering cells more susceptible.

The results obtained show that *P. aeruginosa* can adapt to grow in increasing concentrations of the bactericide SMT. The increased resistance to SMT is ascribed to changes in the outer membrane protein profile, resulting in improved exclusion of the bactericide. Conversely the results indicate the ability of water treatment bactericide SMT to act as an environmental modulator, inducing bacteria to adapt to the changed conditions. Those cells of *P. aeruginosa* acquiring resistance during treatment of systems with SMT would survive and attain a dominant position. This would lead to a bacterial community resistant to the bactericide concentration, and would necessitate revision of the bactericide treatment programme.

5 ACKNOWLEDGEMENTS

We thank Dr John Govan, University of Edinburgh Medical School for donating mutants of *Ps. aeruginosa* PAO, Professor P. L. Steyn of the Department of Microbiology and Plant Pathology for the use of the scanning densitometer and Belinda Pietersen for technical assistance.

Chapter 9

Resistance of *Pseudomonas aeruginosa* to Isothiazolone

V. S. BRÖZEL and T. E. CLOETE

Abstract

Bacteria acquire resistance to a number of antimicrobial agents. We wanted to determine whether *Pseudomonas aeruginosa* could acquire resistance to the bactericide isothiazolone, and what the nature of such a resistance mechanism would be. *P. aeruginosa* was cultured in nutrient limited broth in the presence of sub-inhibitory concentrations of isothiazolone (a mixture of 1.15 % 5-chloro-N-methylisothiazolone (CMIT) and 0.35 % N-methylisothiazolone (MIT)). Three cultures tested in parallel adapted gradually over 15 days of exposure from an initial minimum inhibitory concentration (MIC) of 300 $\mu l.l^{-1}$ to 607 $\mu l.l^{-1}$. The three parallel cultures adapted at similar rates, therefore the adaptation was not ascribed to mutation but to a specific mechanism. Resistant cells did not produce any extracellular isothiazolone-quenching compounds nor undergo detectable alterations in their lipopolysaccharide layer. In wild cells a 35 kD outer membrane protein (protein S) was detectable, whereas resistant cells lacked this protein. Production of protein S was suppressed within 24 h of exposure to isothiazolone. It was still suppressed after 72 h of growth in isothiazolone-free medium. We propose that *P. aeruginosa* acquires resistance to isothiazolone by a process of adaptation where the outer membrane protein S is suppressed.

1 INTRODUCTION

Industrial water systems (*eg.* cooling water systems in power plants and mines) contain a variety of bacteria, most of which are gram negative aerobic rods, *eg Pseudomonas stutzeri, P. fluorescens* and *P. aeruginosa* (Cloete *et al.* 1992; Escher & Characklis 1990). Many such systems are treated with bactericides to eliminate or reduce microbial growth and concomitant microbially induced corrosion (Cloete *et al.* 1992). The bacterial species present differ in their susceptibility to various bactericides, some having a higher degree of inherent resistance than others (Brözel and Cloete, 1991a). We have observed that some species (*eg P. stutzeri* and *B. cereus*) show a low degree of resistance under pure culture conditions (Brözel and Cloete, 1991a) but are the dominant planktonic survivors in systems 36 h after treatment with bactericide, indicating adaptation to the bactericide (Brözel and Cloete, 1992). Various isolates from such a bactericide-treated system acquired a higher degree of resistance to a range of bactericides after extended subinhibitory exposure (Brözel and Cloete, 1991b).

Bacteria develop resistance to various antiseptics (Heinzel, 1988; Jones, Herd and Christie, 1989; Kolawole, 1984; Sakagami, Yokoyama, Nishimura, Ose and Tashima, 1989). Such resistance can be due to a mechanism of adaptation of the cell envelope (Heinzel, 1988), or to plasmid transfer encoding a bactericide-degrading enzyme (Eagon & Barnes 1986). Bactericides attack specific components of bacterial cells, usually at the cytoplasmic membrane or in the cytosol (Gilbert and Wright 1987). Direct action at the outermost surface is rare. For bactericides to be effective, they must be able to penetrate the cell envelope and attain a sufficiently high concentration at the target site in order to exert their antibacterial action. Hydrophilic antibacterial agents enter through the outer membrane via membrane proteins (Nikaido Vaara 1987). In *Escherichia coli* the uptake of hydrophilic drugs of Mr less than 600 is by solute diffusion through general porins. *P. aeruginosa* has a wide array of specific permeation channels and only one porin (Opr F) which permits a low rate of solute diffusion (Nikaido 1992). This property affords *P. aeruginosa* its high degree of inherent drug resistance. Altered cell envelope structure can contribute decisively towards accessibility of antimicrobial agents to their periplasmic or cytosolic targets (Nikaido 1992). Certain resistant isolates either lack or overexpress certain outer membrane proteins. An example is an isolate of *P. aeruginosa* resistant to the antibiotic imipenem. It lacks Opr D, a porin selective for certain carbon sources (Hancock *et al.* 1990). *Escherichia coli* produces a variety of "stress proteins" after exposure to sub-lethal concentrations of pollutants, among them being various antimicrobial agents (Blom *et al.* 1992).

Isothiazolones such as 5-chloro-N-methylisothiazolone (CMIT) and N-methylisothiazolone (MIT) are widely used as industrial bactericides to control bacteria and algae in cooling water systems (Collier *et al.* 1990a; Wallhäußer 1988). Examples of commercially available isothiazolones are Kathon CG, Euxyl K 100 and Mergal C 20 (Wallhäußer 1988). Isothiazolones react oxidatively with accessible thiols such as cysteine and glutathione (Collier *et al.* 1990 b). These thiols are reduced to their

disulphide adjuncts which, in the case of cysteine, leads to an alteration of protein conformation and functionality. Isothiazolone is hereby oxidised to mercaptoacrylamide, which in the case of CMIT tautomerises to thioacyl chloride, the latter reacting with amines such as histidine and valine (Collier *et al.* 1990a). *P. aeruginosa* grows in the presence of high concentrations of a wide spectrum of antimicrobial agents, both antibiotics and antiseptics due to the low general permeability of its cell envelope (Nikaido 1992). In the case of isothiazolones, however, it is inhibited by concentrations similar to those inhibiting *E. coli* and *Staphylococcus aureus* (Wallhäußer 1988). We have previously reported that *P. stutzeri* and *P. cepacia* acquire resistance to isothiazolones (Brözel and Cloete, 1991b). The aim of this study was to determine whether *P. aeruginosa* develops resistance to isothiazolones, and what the mechanism of such resistance would be.

2 MATERIALS AND METHODS

2.1 Test culture

Pseudomonas aeruginosa, isolated from a cooling water system in a previous study (Brözel and Cloete, 1992) was used, maintained on R2A agar slants (Reasoner and Geldreich, 1985) containing 1 % glycerol, and subcultured monthly.

2.2 Isothiazolone used

Isothiazolone containing 1.15 % 5-chloro-N-methylisothiazolone (CMIT) and 0.35 % N-methylisothiazolone (MIT) stabilised with $MgCl_2$ and $MgNO_3$ (Thor chemicals) was used in this study.

2.3 Resistance studies

The strain was cultured in triplicate 100 ml R2A broths (Reasoner and Geldreich, 1985) under shaking for 24 h at 30 °C. One ml of culture was centrifuged at 10 000 g for 5 min, washed in 1 ml sterile phosphate buffer (pH 7.0) (PB) and resuspended in 500 µl PB. Cell concentrations were adjusted spectrophotometrically (540 nm) to 2×10^7 cfu.ml^{-1} with PB. 10 µl aliquots were inoculated into tubes containing 5 ml of half-strength Tryptic Soy Broth (TSB; Biolab) with a range of concentrations of isothiazolone in increments of 10 µl.l^{-1}. The tubes were incubated at 30 °C for 24 h. The tube with the lowest concentration of bactericide showing absence of growth was taken to represent the minimum inhibitory concentration (MIC). One ml of the shake-culture was transferred to 100 ml of fresh R2A broth containing isothiazolone at one quarter of the concentration of the MIC as determined. After incubation for 24 h the new MIC was determined as above and a new shake-culture was inoculated. This procedure was repeated 10 times. The final cultures (termed "resistant") were harvested, washed in PB and kept at - 20 °C for later analysis.

In order to determine whether isothiazolone induced any immediate changes, wild strains were cultured for 24 h in R2A broth containing isothiazolone at one quarter the concentration of the original MIC. These cultures were termed "induced". The cells were then harvested, washed in PB and kept at - 20 °C for later examination.

Resistant cultures were cultured and transferred three consecutive times for 24 h in R2A broth without isothiazolone in order to determine whether the acquired changes were of a permanent nature. These cells were harvested, washed in PB, termed "relaxed", and kept at - 20 °C for later examination.

2.4 Survival of cultures after exposure to a lethal concentration of isothiazolone

Wild and resistant cells were cultured for 24 h in R2A broth, harvested, and resuspended in PB. With resistant cultures, one quarter of the highest MIC of bactericide was included in the broth. Cultures were exposed to four times the highest MIC of bactericide (2400 $\mu l.l^{-1}$) for 60 min, with or without 50 mmol/l EDTA (pH 7.0), after which spread plate counts were prepared. EDTA was added to determine the protective role played by lipopolysaccharide (LPS) (Leive, 1965). Spread plate counts were prepared in triplicate on R2A agar and incubated at 30 °C for 48 h. The log kill was determined by the negative logarithm of the ratio of surviving over initial cell number.

2.5 Isothiazolone-quenching exudates

Supernatants of 24 h old wild and resistant cultures were filter-sterilised and 20 ml added to 180 ml half-strength TSB. The effect of these on the MIC of isothiazolone to wild cells was determined as described above.

2.6 Determination of lipopolysaccharide profiles

LPS of the various treatments were extracted by boiling for 10 min in lysing buffer (pH 6,80) containing 20 % glycerol, 5 % 2-mercaptoethanol, 4,6 % sodium dodecyl sulphate (SDS) and 2 % Tris by the method of Preston & Penner (1987). Proteins in the samples were digested by incubating for 10 min at 60 °C in lysing buffer containing 2,5 $mg.ml^{-1}$ proteinase K (Boehringer Mannheim) (Preston and Penner, 1987). Profiles of the extracted LPS were prepared by SDS-PAGE with the buffer system of Laemmli & Favre (1973). The stacking gel contained 4.8 % acrylamide and 0.13 % N,N'-methylenebisacrylamide and the separating gel contained 12.05 % acrylamide and 0.32 % N,N'-methylenebisacrylamide. The samples were separated at 35 mA using 2 μl bromophenol blue (0,01 %) in 50 % glycerol per well as tracking dye. LPS components were visualised by the silver staining procedure of Tsai & Frasch (1982).

2.7 Determination of outer membrane protein profiles

Outer membrane proteins (Opr's) of the various treatments were prepared from 24 h cultures by the method of Pugsley *et al.* (1986). Cells were harvested at 5000 g for 10 min, washed in phosphate buffer (pH 7.2), suspended in 25 mmol.l^{-1} Tris buffer (pH 7.4) containing 1 mmol.l^{-1} Mg^{2+}, and disrupted by ultrasonication on ice by four 4 bursts of 15 s each. Triton X-100 (BDH) was added to give a final concentration of 2 % (v/v) and samples were kept on ice for 20 min. The insoluble outer membrane fractions were sedimented out at 13 000 g for 30 min and the supernatant fluids removed. The pellets were washed with ice cold acetone, resedimented at 13 000 g, and solubilised in the sample treatment buffer of Hancock & Carey (1979) but omitting 2-mercaptoethanol.

The protein content of extracts was determined by the method of Lowry *et al.* (1951) with ovalbumin (grade V) (Sigma) as standard. Samples were supplemented with 5 % 2-mercaptoethanol and heated at 90 °C for 10 min. Protein profiles were prepared by SDS polyacrylamide gel electrophoresis in a gel containing 14 % acrylamide and 0.25 % N,N'-methylenebisacrylamide according to the buffer system of Hancock & Carey (1979), with 0.07 % NaCl. The stacking gel was prepared as described by Hancock & Carey (1979). Protein molecular mass markers used for calibration were myosin (200 kD), phosphorylase b (97.4 kD), bovine serum albumin (69 kD), ovalbumin (46 kD), carbonic anhydrase (30 kD), trypsin inhibitor (21.5 kD), and lysozyme (14.3 kD) (Amersham). Gels were fixed and stained in an aqueous solution of 10 % acetic acid, 25 % isopropanol, and 0.1 % Coomasie brilliant blue R250 (Merck) at 30 °C for 1 h. The positions and intensities of bands were determined with a Hoeffer Scientific Instruments GS 300 scanning densitometer at 580 nm using GelCompar version 1.3 software (Helix C.V., Belgium).

2.8 Isolation of protein samples for the determination of amino acid composition

Outer membrane proteins were separated on PAGE gels as described above. The pH of gels was adapted to pH 9.0 by placing in 10 mM CAPS (3-(cyclohexylamino)-1-propanesulfonic acid) buffer for 5 min. Proteins were then blotted onto PVDF (polyvinylidine difluoride) membranes by the semi-dry method at a constant voltage of 10.5 V using 10 mM CAPS buffer (pH = 9.0).

For amino acids other than cysteine, membranes were dyed for 20 min with 0.1 % Coomasie brilliant blue R250 as above. The relevant protein bands were cut out of destained membranes and subjected to hydrolysis in O_2 free HCl vapour. For determination of cysteine, membranes were dyed for 30 min with 1 % amido black in water. After destaining, bands were removed while submersed, transferred into extraction buffer (1 % Triton X-100 & 2 % SDS in 50 mM Tris HCl; pH = 9.0) (Szewczyk & Summers, 1988) and kept at 4 °C for 18 h. Samples were centrifuged at 3000 x g for 30 min to remove residual protein from the membranes. Supernatants were again subjected to centrifugation

at 3000 x g for 30 min to remove insoluble material. Acetone (4 volumes) was added, proteins were precipitated at -70 °C for 60 min and collected by centrifugation at 3000 x g for 30 min. Pellets were dried under a stream of nitrogen gas and hydrolysed with performic acid.

2.9 Determination of amino acid composition

The amino acid hydrolysates were derivatised to phenylthiocarbamyl (PTC) amino acids by the PICO.TAG method (Bidlingmeyer *et al.* 1984). PTC amino acids were separated by reverse phase HPLC on a Waters Pico.Tag column using Na acetate (pH 6.45) and acetonitrile: water: methanol and quantified by absorbance at 254 nM using system Gold software (Beckmann).

3 RESULTS

3.1 Induction of resistance

The minimum inhibitory concentration of isothiazolone to the wild *Pseudomonas aeruginosa* was 300 $\mu l.l^{-1}$. This value corresponds with that reported by Wallhäußer (1988), indicating that our strain was similar to other test strains employed for bactericide evaluation. *P. aeruginosa* adapted to growth in the presence of increasing concentrations of isothiazolone following sub-inhibitory exposure. The triplicate cultures followed the same pattern of increase in resistance (Fig. 45). This suggests that the development of resistance to isothiazolone follows a set pattern and is not due to a random process.

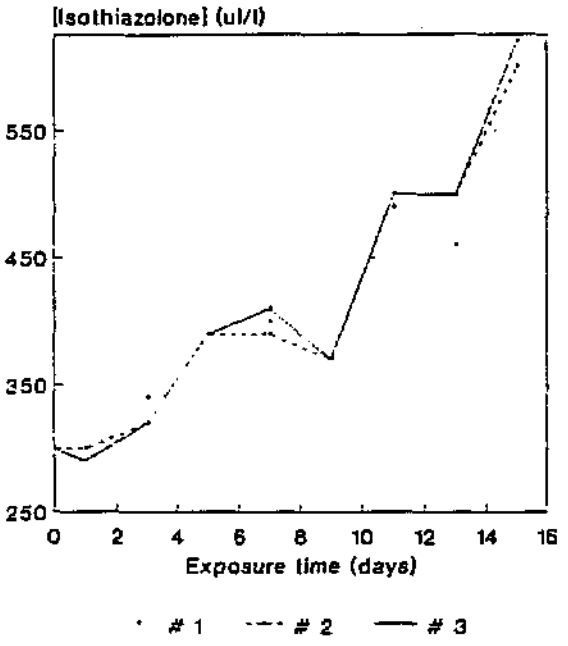


Figure 45: MIC of isothiazolone (proprietary mixture of 5-chloro-N-methyl isothiazolone and N-methyl isothiazolone) to *Pseudomonas aeruginosa* after 24 h growth in R2A broth containing one quarter the previous MIC.

3.2 Lipopolysaccharides

Exposure to 2400 $\mu l.l^{-1}$ isothiazolone for 60 min lead to the death of 40.3 % of wild cells, and 27.7 % of resistant cells. This confirmed the finding of Collier *et al.* (1990b) that isothiazolones are bacteriostatic rather than bactericidal in their action, only being bactericidal at high concentrations. No changes were detected in the LPS profile of resistant cells as determined by SDS PAGE. Removal of LPS by treatment with 50 mM EDTA appeared to render cells susceptible as 99.9 % of wild cells were killed by 2400 $\mu l.l^{-1}$ of isothiazolone. This showed that isothiazolone was bactericidal to *P. aeruginosa* once it entered into the cell. The LPS layer, however, prevented it from entering in sufficient quantity to attain a lethal intracellular level. Most resistant cells also lost their viability upon isothiazolone / EDTA treatment as depicted by a kill percentage of 98. 31 %. This indicated that the mechanism facilitating increased resistance was not due to LPS, but only functioned due to the barrier properties of the LPS layer as removal of LPS lead to inactivation of the resistance mechanism to a large extent.

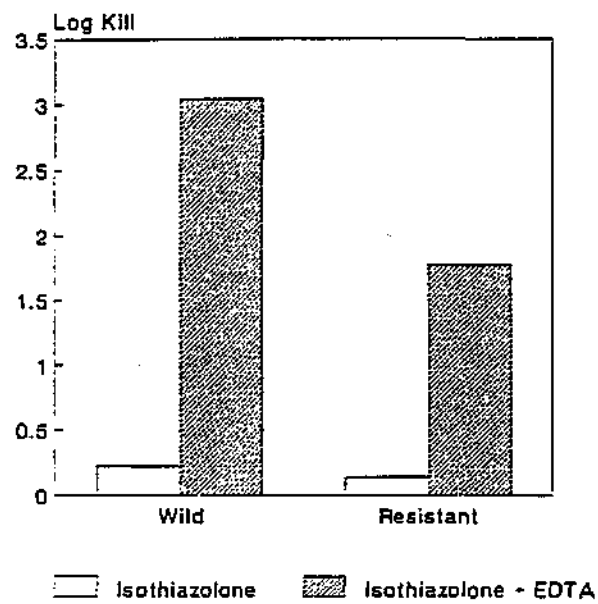


Figure 46: Log kill of wild and resistant *Pseudomonas aeruginosa* treated for 60 min with 2.4 $\mu l.ml^{-1}$ isothiazolone. Hatched bars show the added effect of 50 mM EDTA.

3.3 Isothiazolone-quenching exudates

The supernatants of both wild and resistant cultures quenched the bacteriostatic activity of isothiazolone to the same degree (Table 23). The MIC in controls was 300 $\mu l.l^{-1}$ whereas it was 350 $\mu l.l^{-1}$

where supernatants of wild and resistant cultures were added. This indicated that cells of *P. aeruginosa* did release isothiazolone-quenching agents into the culture medium, but that acquired resistance was not due to an increased degree of release.

Table 23: Effect of culture supernatants on the minimum inhibitory concentrations ($\mu l.l^{-1}$) of isothiazolone to wild cells of *Pseudomonas aeruginosa*.

Control	Wild supernatant	Resistant supernatant
300	350	350

3.4 Outer membrane proteins

SDS PAGE analysis of outer membrane proteins revealed two differences suggesting adaptive mechanisms upon exposure to isothiazolone (Fig 47). Cultures of wild *P. aeruginosa* exposed to 300 $\mu l.l^{-1}$ of isothiazolone for 24 h produced copious amounts of extracellular polysaccharide (EPS). A 54 kD outer membrane protein was detected in these cells (Fig 47, lane 3), correlating with the EPS production (Grabert et al 1990). Both wild and resistant strains did not produce EPS, and lacked the protein in detectable levels.

The wild culture of *P. aeruginosa* produced a specific outer membrane protein with a molecular mass of 35 kD as determined by mobility in SDS PAGE. We termed it protein S. This protein was not detected in resistant or induced cultures. Production of protein S was, therefore, suppressed upon 24 h exposure to isothiazolone. It was also not detected in resistant cells cultured for three consecutive times in the absence of isothiazolone (Fig. 47), indicating a more permanent repression or a mutation. The three parallel cultures reacted in the same way, indicating that the absence of protein S was due to a specific mechanism. Subsequent analysis of protein S revealed that it contained 7 cysteine residues per molecule (Table 24).

Figure 47: SDS-polyacrylamide gel electrophoresis of outer membrane proteins from *Pseudomonas aeruginosa* (lane 2), resistant to isothiazolone (lane 3), exposed to isothiazolone for 24 h (lane 4), resistant culture grown and transferred three x 24 h (lane 5) and molecular mass markers (lane 1). The repressible protein is marked S.

Table 24: *Amino acid composition of protein S*

Amino acid	Number of residues
Aspartic acid	22
Glutamic acid	41
Serine	32
Glycine	50
Histidine	5
Arginine	13
Threonine	13
Alanine	25
Proline	4
Tyrosine	10
Valine	10
Isoleucine	9
Leucine	13
Phenylalanine	9
Lysine	22
Methionine	3
Cysteine	7

4 DISCUSSION

4.1 Resistance development

The gradual adaptation of *P. aeruginosa* to tolerate an increasing concentration of isothiazolone followed a set pattern, so that adaptation was due to a set mechanism. Firstly, resistance in the cultures was not acquired at a certain fixed point in time, but increased gradually as the pressure was increased. Secondly, the three parallel cultures adapted to similar values of tolerance at the same rate (Fig. 45). The EPS production of cultures exposed for 24 h to isothiazolone appears to have been a stress response which was not maintained following extended exposure. The induced culture further produced minor outer membrane proteins not detected in the other three states (Fig. 47), indicating

a wider stress response. This phenomenon appears similar to the stress response of *E. coli* upon exposure to various micropollutants (Blom *et al.* 1992). Since not any one of these minor proteins was detected in resistant cultures, they appear not to have contributed to improved resistance and production was subsequently abandoned.

4.2 Mechanism of resistance

Isothiazolone is regarded primarily as bacteriostatic in action (Collier *et al.* 1990 b), explaining the high degree of survival of both wild and resistant cultures following exposure to a 2400 $\mu l.ml^{-1}$. The large number of EDTA-treated cells losing viability following isothiazolone exposure indicated that LPS plays an important role in excluding the bactericide from the cell. LPS must decrease the rate of entry of isothiazolone into the cytosol where most of the isothiazolone-interactive proteins are situated (Collier *et al.* 1990a). Collier *et al.* (Collier *et al.* 1990a) found that most of the isothiazolone entering cells of *E. coli* was located within the cytosol within 10 min, indicating a readily accessible mechanism of entry. In that study isothiazolone was found to be bacteriostatic. However, we have shown here that, upon removal of LPS from the outer membrane of *P. aeruginosa*, isothiazolone does become bactericidal. This bacteriocidal action could be due to some other mechanism than entry into the cytosol via porins. It is possible that isothiazolone enters through the outer membrane of wild cells via porins, but that the rate of entry is limiting. Upon removal of LPS, the rate of entry would increase, permitting a large percentage of the isothiazolone to enter the cell.

Resistant *P. aeruginosa* did not produce detectable isothiazolone-quenching agents, as was also found previously for *E. coli* where no specific isothiazolone-binding proteins were detected (Collier *et al.* 1990a).

Porin-deficient mutants of *E. coli* showed similar rates of isothiazolone uptake to porin-sufficient strains (Collier *et al.* 1990a). Yet the uptake isotherms of radiolabelled MIT and CMIT into *E. coli* indicated that these two isothiazolones interacted initially with cell envelope targets such as membrane proteins. Whereas in *E. coli* a large number of the outer membrane proteins are general porins, *P. aeruginosa* has only one general uptake porin, Opr F exhibiting a low rate of solute transport (Nikaido 1992). Most outer membrane proteins of *P. aeruginosa* are specific for certain substrates. Many, found to be specific for certain substrates, *eg* for basic amino acids (Opr D) or for phosphate (Opr P), do permit entry of structural analogues (Hancock *et al.* 1990). An example is Opr D which is the protein by which the antibiotic imipenem enters through the outer membrane of *P. aeruginosa* (Nikaido 1992).

There are two possible explanations for the increased resistance due to absence of protein S. The first would be that protein S itself is more susceptible to reaction with isothiazolone than are the

other outer membrane proteins, resulting in the loss of a membrane function. This would be supported by its content not only of cysteine (7 residues) but also of amino acids with accessible amines such as lysine (22), arginine (13) and histidine (5). Reaction of CMIT and its derivative thioacyl chloride with more accessible amino acids could lead to conformational alterations of the protein, and to either increased uptake of isothiazolone or to decreased uptake of an essential substrate.

Alternatively, isothiazolone could normally enter through the outer membrane via protein S. In this case isothiazolone would be a structural analogue to a protein S substrate. Most outer membrane proteins of *P. aeruginosa* have limited rates of substrate uptake (Nikaido 1992). As a protein S substrate analogue, isothiazolone's rate of uptake would be limited. Specific uptake of isothiazolone would therefore not lead to a high degree of inherent susceptibility. The continued suppression of protein S production in the absence of isothiazolone could be due to a genotypic switch to the suppressive state. This would be similar to continued alginate production by *P. aeruginosa* isolates growing in the absence of *algD* activators (Maharaj et al. 1992).

We propose that protein S is the protein through which isothiazolone enters through the outer membrane of wild cells because removal of the penetration barrier (LPS) rendered resistant cells as susceptible as wild cells. If protein S would be more reactive with isothiazolone than the other outer membrane proteins, resistant cells treated with EDTA should be less susceptible than wild cells because they were lacking the "soft" target.

In wild cells a 35 kD outer membrane protein (protein S) was detectable, whereas resistant cells lacked this protein. Production of protein S was suppressed within 24 h of exposure to isothiazolone. It was still suppressed after 72 h of growth in isothiazolone-free medium. We propose that *P. aeruginosa* acquires resistance to isothiazolone by a process of adaptation where the outer membrane protein S is suppressed.

5 ACKNOWLEDGEMENTS

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Chapter 10

Response of *Pseudomonas aeruginosa* and *Escherichia coli* to Hypochlorous acid and Hydrogen Peroxide Oxidising Stress

B. PIETERSEN, V. S. BRÖZEL and T. E. CLOETE

Abstract

Hypochlorous acid and hydrogen peroxide are employed as biofouling control agents and as surface disinfectants. Whereas the bacterial development of resistance to non-oxidising bactericides is well established, the question whether bacteria develop resistance to oxidising agents has not been answered. The minimum inhibitory concentration (MIC) of HClO for *Pseudomonas aeruginosa* fluctuated during repeated culturing in presence of sub-inhibitory concentrations of HClO. The MIC did, however, not increase during the 49 d period. No detectable stress response to either HClO or H_2O_2 was found in *P. aeruginosa* following exposure to relevant oxidant stress. Rather, cultures became more susceptible to oxidant following such stress. *Pseudomonas aeruginosa* exhibited a degree of recovery to H_2O_2 60 min after exposure to stress, but this protective mechanism was not maintained. Inhibition of *de novo* protein synthesis by chloramphenicol prior to exposure to H_2O_2 stress lead to decreased susceptibility of *P. aeruginosa*. The converse was true in the case of HClO, with an intact protein-synthesising system leading to a lesser increase in susceptibility. *Escherichia coli* demonstrated a stress response to H_2O_2, and to a lesser degree to HClO. This response to HClO lead to increased protection to inhibition, but not to cell death.

1 INTRODUCTION

Hypochlorous acid and hydrogen peroxide are used in various applications to prevent, control, or decrease bacterial activity. Hypochlorous acid was first employed as wound disinfectant by Hueter in 1831, and its bactericidal activity was confirmed by Koch in 1881 (Wallhäußer, 1988). Hypochlorous acid is used amongst others in industrial water systems to control biofouling (Characklis, 1990), in swimming pools and in the sanitation of surfaces and pipelines in food and other industries. Hydrogen peroxide is also used as a disinfectant and water treatment bactericide (Cloete *et al.*, 1992).

Hydrogen peroxide penetrates cells, causing site-directed damage due to metal-dependant ·OH formation (Schraufstätter *et al.*, 1990; Storz *et al.*, 1990). It causes DNA strand breaks and hydroxylation of bases in intact DNA (Schraufstätter *et al.*, 1990). Guanine and thymine are the two main targets of peroxide-generated free radical attack. The resulting 7,8-dihydro-8-oxoguanine mispairs with adenine whereas thymine oxidation products stop DNA polymerase, halting replication (Ahern, 1993). Hydrogen peroxide also inhibits mitochondrial ADP-phosphorylation (Schraufstätter *et al.*, 1990). Superoxide inhibits synthesis of branched amino acids (Storz *et al.*, 1990).

The antibacterial mechanism of action of hypochlorous acid is not clear to date although much work on the mechanism of action in eukaryotic cells has been done (Schraufstätter *et al.*, 1990). HClO does not enter freely into eukaryotic cells but attacks surface and plasma membrane proteins, impairing transport of solutes and the salt balance (Schraufstätter *et al.*, 1990). It oxidises sulfhydral groups and inhibits plasma membrane ATPases. It does, however, not cause any damage to the genomic material of eukaryotic cells (Schraufstätter *et al.*, 1990).

The development of resistance to non-oxidising bactericides has been established repeatedly, and reviewed recently (Brözel and Cloete, 1993). The development of resistance to oxidising bactericides such as hypochlorous and hypobromous acid and to hydrogen peroxide has, however, not been reported to date. The degree of bacterial susceptibility to oxidants depends on cellular composition which is again determined by growth conditions and cell age (Brözel and Cloete, 1993; LeChevalier *et al.*, 1988). However, a variety of bacteria, all facultative anaerobes, exhibit oxidising stress response by producing oxidant-degrading as well as damage - repair enzymes (Storz *et al.*, 1990). These include *Escherichia coli*, *Salmonella typhimurium*, (Storz *et al.*, 1990) and *Bacillus subtilis* (Hartford and Dowds, 1992). A variety of defense genes to naturally occurring oxidising agents have been characterised in *Escherichia coli*. These defence genes encode various superoxide dismutases, catalases, alkyl hydroperoxide reductases and glutathione reductases, as well as DNA repair enzymes (Storz *et al.*,

1990). In addition various regulatory genes have been characterised, including *OxyR, RecA and SoxR* (Storz *et al.*, 1990). These regulators determine intracellular redox potential, and activate stress response when cells are exposed to oxidising agents.

The aim of the work reported here was, to investigate the response of *Pseudomonas aeruginosa* and *Escherichia coli* to Hypochlorous acid and Hydrogen Peroxide Oxidising Stress

2 MATERIALS AND METHODS

2.1 Bacterial strains used in this study

Pseudomonas aeruginosa EBL was isolated from a cooling water system during a previous study (Brözel and Cloete, 1992). *P. aeruginosa* PAO1 was obtained from the Deutsche Sammlung von Mikroorganismen, and *Escherichia coli* K12 was obtained from Prof. W.O.K. Grabow (Department of Medical Virology, University of Pretoria, Pretoria, 0002, South Africa). All cultures were maintained on R2A agar slants (Reasoner and Geldreich, 1985) with 1% glycerol, and were subcultured monthly.

2.2 Chemicals used

Casamino acids (Difco), soluble starch and glucose (BDH chemicals), peptone, yeast extract and bacteriological grade agar (Biolab), Na pyruvate, K_2HPO_4 and $MgSO_4$ (Merck) and chloramphenicol (Sigma) were used throughout this study. Oxidants used were H_2O_2 (30 %) (Saarchem), and $Ca(OCl)_2$ (Olin (Pty) Ltd.). HClO was prepared fresh by dissolving $Ca(OCl)_2$ in deionised water at pH 6.8, and concentrations given are in terms of $mg.l^{-1}$ $Ca(OCl)_2$.

2.3 Reaction of *P. aeruginosa* to sub-inhibitory HClO stress during 49 day exposure

P. aeruginosa EBL was cultured in R2A broth (pH 6.8) (Reasoner and Geldreich, 1985) while shaking at 28 °C. After 24 h the minimum inhibitory concentration (MIC) was determined in triplicate. This was done as described previously (Brözel *et al.*, 1993). Briefly, 10 µl volumes of a washed cell suspension were inoculated into tubes containing R2A broth (pH 6.8) with increasing concentrations of hypochlorous acid. The hypochlorous acid solution was freshly prepared in deionised water before addition to the tubes. R2A broth was then inoculated with 1ml of the previous culture together with one fourth of the previous MIC of hypochlorous acid. This procedure was repeated 25 times.

2.4 Reaction during 24 h following exposure to sub-inhibitory oxidising stress

P. aeruginosa PAO1 and *E. coli* K12 were cultured for 24 h in R2A broth at 28 °C. The MIC's of hypochlorous acid and of H_2O_2 were determined as described above. 24 h old cultures were exposed to sub-inhibitory oxidising stress by adding one fourth the determined relevant MIC. In the case of H_2O_2, Na pyruvate was omitted from the R2A broth. The MIC's and culturable counts were determined at 5, 10, 20, 30, 60 min, and 2, 3, 4, 6, 8, 12 and 24 h after exposure to stress. The culturable counts were determined by plating serial dilutions onto triplicate plates of R2A agar and incubating these at 28 °C for 48 h.

To determine the role of *de novo* protein synthesis, 24 h old cultures of *P. aeruginosa* were exposed to 5μg/ml of chloramphenicol which inhibits protein synthesis. Cells were exposed to oxidising stress (HClO and of H_2O_2) 2 min after exposure to chloramphenicol as described above. The MIC's were determined at the times as given above.

2.5 Effect of oxidant treatment on viability of cultures

24 h old cultures of *P. aeruginosa* PAO1 and *E. coli* K12 (cultured in R2A broth for 24 h at 28 °C) were exposed to various concentrations of HClO (10 mg.l^{-1} and 20 mg.l^{-1} for *P. aeruginosa* and 15 mg.l^{-1} and 30 mg.l^{-1} for *E. coli.*) and to H_2O_2 (1000 μl.$^{-1}$ and 2000 μl.$^{-1}$ for *P. aeruginosa* and 400 μl.$^{-1}$ and 800 μl.$^{-1}$ for *E. coli.*) for 15 min. The culturable counts were then determined by plating serial dilutions onto R2A agar plates (Reasoner and Geldreich, 1985) in duplicate. The effect of pre-exposure to oxidant was studied by treating cultures with HClO (10 mg.l^{-1} for *P. aeruginosa* and 4 mg.l^{-1} for *E. coli.*) and H_2O_2 (40 μl.$^{-1}$ for *P. aeruginosa* and 10 μl.$^{-1}$ for *E. coli.*) for 60 min. These cultures were then exposed to oxidant as described above. The percentage of surviving cells was calculated as follows:

$$\%Survival = \left(\frac{CFU/ml\,survivor\,count}{CFU/ml\,inoculum\,count} \right) X\,100$$

3 RESULTS

3.1 Reaction of cultures during 49 day exposure to sub-inhibitory oxidising stress

The degree of susceptibility of *P. aeruginosa* to hypochlorous acid during growth in the presence of sub-inhibitory concentrations fluctuated during the period of exposure (Fig. 48). The initial MIC value (culture not exposed previously to HClO stress) was the same in various determinations, but varied after initial exposure (Fig 48). The common denominator was that the MIC value of cultures exposed to a sub-inhibitory concentration of hypochlorous acid for 24 h was always lower than that

of unexposed cultures. This phenomenon was also observed where the wild *P. aeruginosa* isolate was grown in the presence of sub-inhibitory concentrations of H_2O_2 and 3-Bromo -1-Chloro -5,5 -dimethylhydantoin (Pietersen, 1993). The MIC to HClO did increase after 11 d of exposure, but decreased again thereafter. Resistance did, however, not develop over time as is the case with non-oxidising bactericides (Brözel and Cloete, 1993). The MIC fluctuated around a mean value of 179 $mg.l^{-1}$.

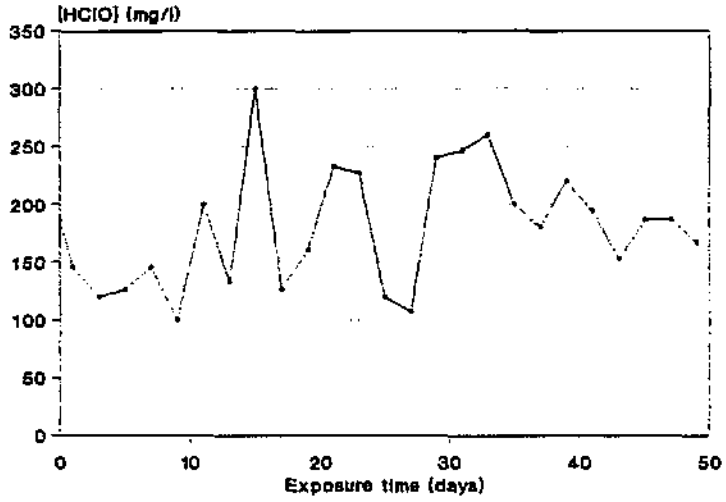

Figure 48: Minimum inhibitory concentration of HClO against *Pseudomonas aeruginosa* strain EBL during growth in the presence of sub-inhibitory concentrations (for details, see material and methods section).

3.2 Reaction of cultures during 24 h following exposure to sub-inhibitory oxidising stress

P. aeruginosa did not exhibit stress response to either of the two oxidising agents. Exposure to a sub-inhibitory concentration of H_2O_2 or HClO sensitised cells to these oxidants (Figs. 51 a & b). Although *P. aeruginosa* did recover to some extent to H_2O_2 stress, increasing its resistance between 30 and 60 min after exposure to stress, this was still less than the initial resistance of 340 ppm. The decline in resistance from 60 min after exposure to stress indicated that the mechanism of increased resistance could not be upheld. The increased resistance 60 min after exposure to stress was also demonstrated by the increase in the culturable count after 60 min (Fig 49a). This showed that cells either started to divide again, or at least recovered to the culturable state. Yet cells were again more susceptible to H_2O_2 upon re-exposure (Fig 49a). *P. aeruginosa* was slower to respond to HClO than to H_2O_2, and increased only marginally in resistance 60 min after exposure to stress (Fig 49b). This

increase was confirmed by an increase in the culturable count. In both cases the degree of resistance and the culturable count did not exceed the original values within 24 h after exposure to sub-inhibitory stress.

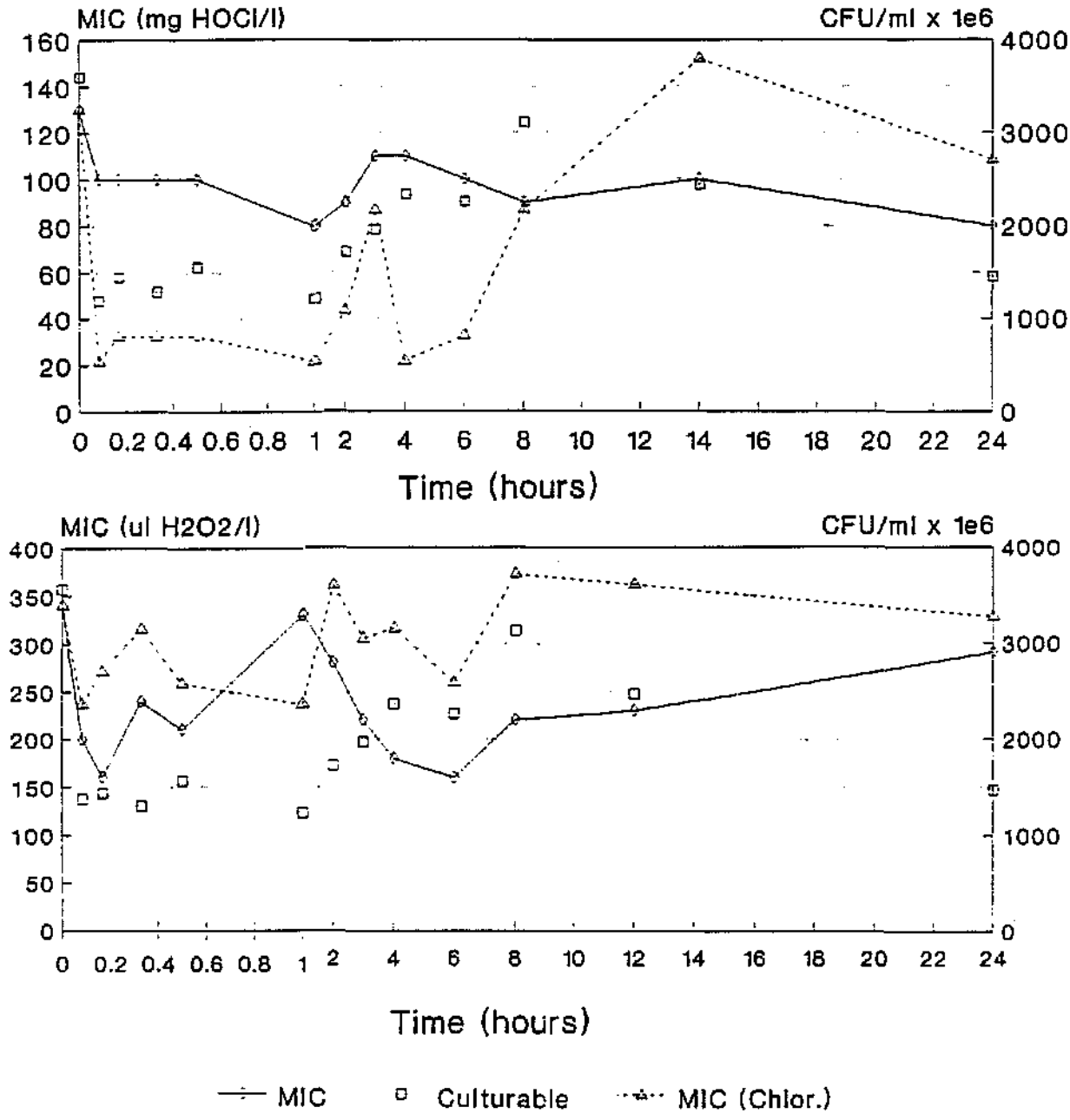

Figure 49: Minimum inhibitory concentration of HClO (a) and of H_2O_2 (b) as well as culturable count of *Pseudomonas aeruginosa* cultures during 24 h following exposure to a sub-inhibitory concentration of the relevant oxidant. The MIC was determined on cultures with and without (5 $\mu g.ml^{-1}$ chloramphenicol) an intact protein synthesizing system.

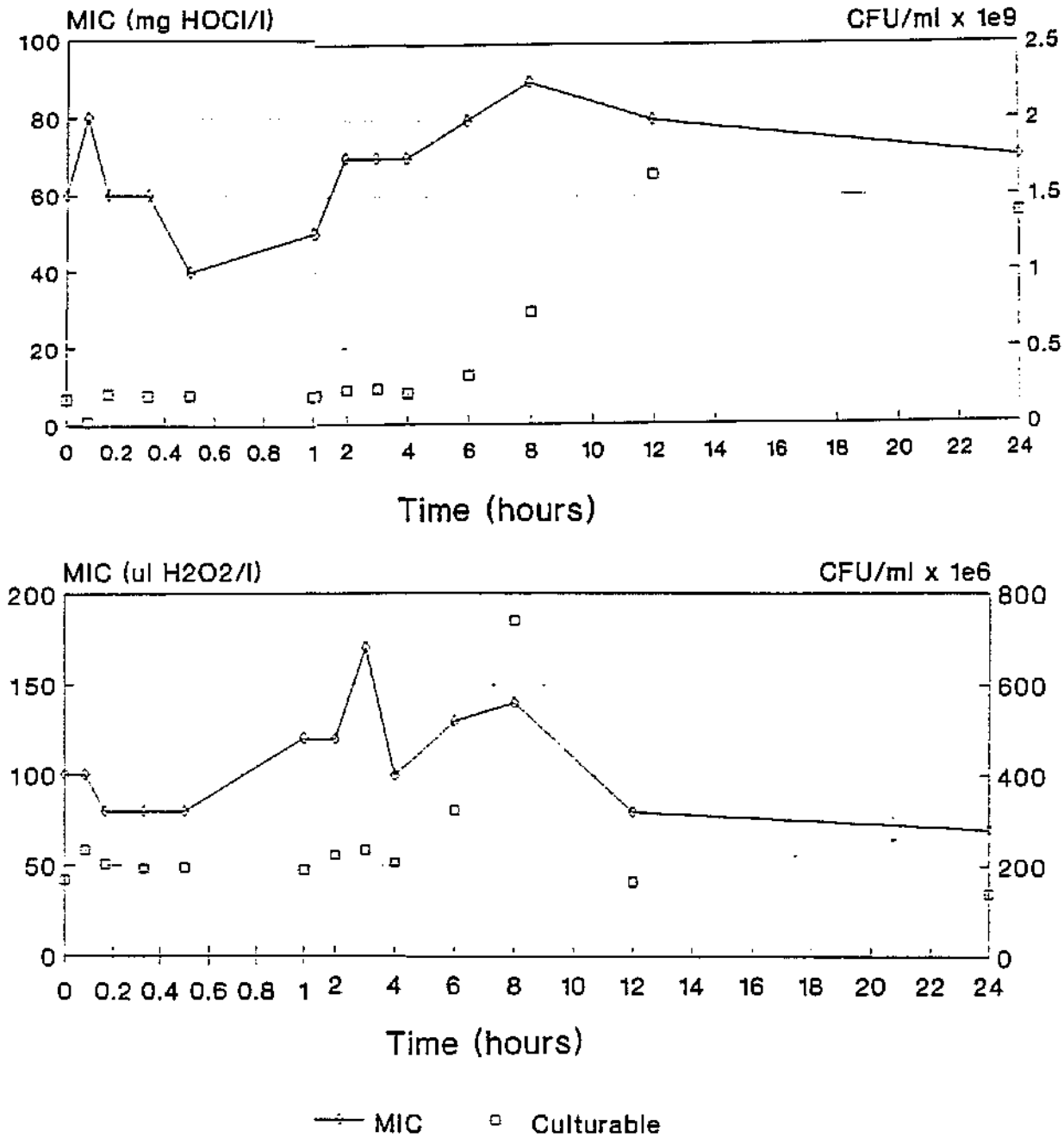

Figure 50: Minimum inhibitory concentration of HClO (a) and of H_2O_2 (b) as well as culturable count of *Escherichia coli* cultures during 24 h following exposure to a sub-inhibitory concentration of the relevant oxidant.

Exposure to sub-inhibitory H_2O_2 stress during inhibition of *de novo* protein synthesis by chloramphenicol caused *P. aeruginosa* to be more resistant than with an intact protein synthesis mechanism (Fig 49a). The MIC of un-stressed cultures in the presence of chloramphenicol was not found to be meaningfully lower than in its absence, so that chloramphenicol itself did not affect the resistance of *P. aeruginosa* to H_2O_2. This indicated that the reaction to H_2O_2 stress elicited a response which rendered cells more susceptible, and that this response was linked to *de novo* protein synthesis. 8 h after H_2O_2 stress, cells defective in protein synthesis were more resistant than before stress, whereas cells with an intact protein synthesis mechanism were not (Fig 49).

Exposure to HClO stress during inhibition of *de novo* protein synthesis by chloramphenicol caused *P. aeruginosa* to be more susceptible than with an intact protein synthesis mechanism (Fig 49b). The MIC of un-stressed cultures in the presence of chloramphenicol was not lower than in its absence, so that chloramphenicol did not render *P. aeruginosa* more susceptible to HClO. This demonstrated that *P. aeruginosa* did produce some protective substances after exposure to HClO stress. The degree of protection afforded was, however, not noteworthy. The protective mechanism was not as effective after sub-inhibitory exposure than it was before, even with the *de novo* synthesis of protective factors.

The strain of *E. coli* used demonstrated stress response to H_2O_2 as does *E. coli* in general (Storz *et al.*, 1990) (Fig 49a). However, the increased protection to H_2O_2 was short-lived. It was only detected between 45 and 180 min, and between 240 and 480 min after exposure to stress. There was no decrease in the culturable count until 12 h after exposure to sub-inhibitory stress. Even then the count did not decrease to below the initial value. Therefore the sub-inhibitory concentration applied did not cause any detectable death of cells.

Escherichia coli further responded to sub-inhibitory HClO stress within 60 min after exposure (Fig 50b). This was supported by the culturable count which increased 6 h after exposure (Fig 50b). The sub-inhibitory concentration of HClO applied did not cause any detectable cell-death. The protective effect of the stress response was active from 60 min after exposure, and did not deplete completely within the 24 h period.

3.3 Effect of oxidant treatment on viability of cultures

The suspension kill tests confirmed that *P. aeruginosa* did not respond to oxidising stress by producing a protective mechanism (Table 25). *Escherichia coli* did respond to H_2O_2 stress by exhibiting increased resistance after 60 min. The response of *E. coli* to HClO detected in the MIC experiment was, however, not confirmed by the suspension kill tests as the stressed culture was found more susceptible to HClO after exposure to stress than it was before (Table 25)

Table 25: Effect of H_2O_2 and HClO treatment on the culturability of *Pseudomonas aeruginosa* PAO1 and of *Escherichia coli* K12. Cells (20 X 10^8 CFU.ml^{-1}) were treated with two concentrations of oxidant as described in Materials and Methods. Cells were grown for 24 h in R2A broth. Stress response cells were exposed to 1/10 the relevant MIC of oxidant for 60 min. The percentage of surviving cells was calculated as described in Materials and Methods.

Isolate	H_2O_2		HClO	
	Non-stressed	Stressed	Non-stressed	Stressed
Pseudomonas aeruginosa PAO1	111.9	82.5	80.9	94.9
"2MIC"	96.6	79.2	28.1	4.0
Escherichia coli K12	66.9	123.0	48.0	47.3
"2MIC"	9.4	99.3	21.55	8.8

4 DISCUSSION

4.1 Reaction of *P. aeruginosa* to Oxidising Stress

Pseudomonas aeruginosa PAO1 did not exhibit an active oxidising-stress response to either H_2O_2 or HClO. Rather, it appeared to have an inverse stress response, becoming more susceptible after exposure to sub-inhibitory stress. The increased susceptibility towards both agents was not due to cell-damage caused by the sub-inhibitory stress as the culturable count decreased only slightly. The inoculum was also standardised spectrophotometrically, so that the tubes used to determine MIC were not inoculated with less cells. *Pseudomonas aeruginosa* PAO1 did, however, exhibit a high level of constitutive protective mechanism towards both agents, much higher than that of *E. coli* K12.

Pseudomonas aeruginosa PAO1 did exhibit some form of recovery towards H_2O_2 30 min after exposure to stress. This indicated that some form of protective mechanism was induced or repaired within 20 min following stress exposure. This mechanism could not be maintained despite the fact that cells were growing as the MIC decreased again after 60 min. Because inhibition of *de novo* synthesis decreased the susceptibility to H_2O_2, it appears that *P. aeruginosa* does have an oxidising-stress response, but that this response is deleterious to the cell. *Pseudomonas aeruginosa* has at least three UV-inducible stress-genes (Warner-Bartnicki and Miller, 1992). However, none of these genes lead to increased protection to damage by UV light (Simonson *et al.*, 1990). This is probably due to

lack or poor expression of one or more of the UV-damage repair enzymes (Warner-Bartnicki and Miller, 1992). Similarly, it is possible that enzymes for the repair of H_2O_2 damage are poorly expressed or lacking, so that response to oxidising stress does not lead to increased protection. Alternatively, enzymes expressed upon H_2O_2 stress could have rendered cells more susceptible.

Root surface peroxidases of many plants produce superoxide anions and hydrogen peroxide (Katsuwon and Anderson, 1989). Pseudomonads colonising the rhizosphere, such as *P. fluorescens* and *P. putida*, express high levels of catalase and superoxide dismutase. Additionally these pseudomonads have further oxidising-stress inducible catalases, affording them protection from the root defence system (Katsuwon and Anderson, 1989). *P. aeruginosa*, although being an autochtonous soil and water organism, is not a rhizosphere colonizer (Korsten and Lubbe, 1993). The reason for this could be that *P. aeruginosa* does not appear to have an oxidising-stress inducible response, so that it cannot survive in the rhizosphere. Similarly, *P. aeruginosa* does not have a functioning UV-inducible stress response (Simonson *et al.*, 1990). Although it encodes for a UV-inducible RecA protein, and this controls at least three *din* (damage-inducible) genes, the response does not lead to any degree of detectable protection to UV damage (Warner-Bartnicki and Miller, 1992). *P. stutzeri* is in the same DNA-homology group as *P. aeruginosa*, and also lacks an inducible oxidising-stress response (Pietersen, 1993). *Pseudomonas fluorescens* and *P. putida* are in a different DNA-homology group of the r-RNA homology group I of the genus *Pseudomonas* (Palleroni, 1984). It is possible that stress-response systems in *P. aeruginosa* (and in other members of this DNA-homology group) have either reverted to non-functioning forms, or are the primitive predecessors of functioning stress-response systems of the other pseudomonads.

HClO did not elicit any stress response in *P. aeruginosa* PAO1. It inhibits protein synthesis in eukaryotes (Schraufstätter *et al.*, 1990). The increased susceptibility of chloramphenicol-treated cells could be due to damage to the protein-synthesising mechanism by HClO. This will have to be investigated further.

4.2 Reaction of *E. coli* to Oxidising Stress

The stress response of *E. coli* K12 to H_2O_2 was not found to be as marked as reported in the literature (Storz *et al.*, 1990). It was not sustained for very long after the exertion of stress, and cells were more susceptible after the response than they were before exposure to stress. This indicated that the stress response was energy intensive, rendering cells more susceptible in the long term. This may be because the natural colonic habitat of *E. coli* is anaerobic (Storz *et al.*, 1990), and cells exposed to air must

survive against the damaging effects of H_2O_2. Once a microcolony has been formed, cells afford each other protection from H_2O_2, so that the high level of protection required by a single cell is no longer required. This could explain why the stress response depleted 10 h after exposure to stress.

The stress response of *E. coli* to HClO has not been reported previously. It was slower than that to H_2O_2 and lasted longer, so that at least some of the regulatory genes involved must be different. HClO has a different mode of action to H_2O_2 (Schraufstätter *et al.*, 1990), so that it is unlikely that the protective mechanism to HClO overlaps much with H_2O_2 stress response. Because macrophages do produce HClO as part of their antimicrobial activity (Schraufstätter *et al.*, 1990), it is conceivable that *E. coli*, being a member of the mammalian flora, has evolved a protective mechanism to this oxidant.

Pseudomonas aeruginosa did not exhibit any protective stress response to the oxidants HClO and H_2O_2, but rather was sensitized to a more susceptible state. *Escherichia coli* did exhibit a protective stress response to both oxidants, but over a limited time-period.

5 ACKNOWLEDGEMENTS

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Chapter 11

De Novo Protein Synthesis of *Pseudomonas aeruginosa* PAO after Attachment

V.S. BRÖZEL, G.M. STRYDOM and T.E. CLOETE

Abstract

The study of biofilms has indicated that bacteria behave fundamentally differently in the biofilm habitat than in suspension. The aim of the study reported here was to determine whether available carbon (COD) had an effect on the rate of attachment of bacteria to surfaces, and whether *P. aeruginosa* PAO underwent any changes during attachment. There was a relationship between available glucose and rate of attachment during the first 20 min of exposure. Glucose concentrations between 0.05 and 0.001 $g.l^{-1}$ yielded *ca.* 10 attached cells per field. Concentrations of between 0.5 and 10 $g.l^{-1}$ yielded *ca.* 100 attached cells per field. Within 1 h of exposure, however, there was little difference in the numbers of attached cells between various levels of available carbon. The level of available carbon has little effect on the rate of bacterial attachment to surfaces. A method for the study of *de novo* protein synthesis in attached bacteria has been developed. This was used to demonstrate that the synthesis of several proteins in *P. aeruginosa* PAO was influenced by attachment of cells. Attachment influenced the synthesis of at least 11 proteins during various stages after attachment. Attachment *per se* was demonstrated to exert some effect on the regulation of certain genes. It is not clear whether this is at the transcriptional or translational level, and what the nature of the attachment-mediated signal is.

1 INTRODUCTION

Bacterial biofilms, *ie.* conglomerates of attached bacteria embedded in extracellular polysaccharide, have been studied extensively (Costerton *et al.*, 1987; Escher & Characklis, 1990; Lawrence *et al.*, 1991). Bacterial biofilms, it is now accepted, play a decisive role, not only in microbially induced corrosion (Cloete *et al.*, 1992), but in many other fields of microbiology. These include medical, dental, biodegradation, water purification and industrial microbiology (Brözel, 1994). Bacteria in nature grow and survive mostly in biofilms, and seldom planktonically. The ratio between attached and planktonic bacteria in streams is in the order of 1000 to 1 (Costerton *et al.*, 1987).

The study of biofilms has indicated that bacteria behave fundamentally differently in this habitat than in suspension (Brözel, 1994; Kinniment & Wimpenny, 1992). Bacterial physiology, as it is widely accepted, is based on the study of suspended cells growing under controlled, mostly chemostat, conditions. A variety of phenotypic differences exist between planktonic and biofilm bacteria. For example, bacteria in biofilms are orders of magnitude more resistant to antimicrobial agents such as antibiotics and biocides (Costerton *et al.*, 1987; Heinzel, 1989; LeChevallier *et al.*, 1988). Biofilms catalyse the biodegradation of xenobiotics more rapidly and completely than do planktonic cultures (Wolfaardt *et al.*, 1993). Textbooks teach that bacterial cells grow actively until the adenylate energy charge (AEC) falls below 0.85 and that cells are dead when the AEC drops to below 0.6 (Dawes, 1986). Many bacterial enzymes are regulated by AEC (Dawes, 1986). *Pseudomonas aeruginosa* growing in a biofilm exhibit an AEC of between 0.6 and 0.2 (Kinniment & Wimpenny, 1992). This points to a different set of regulatory systems for bacteria situated in biofilms.

There are indications that attachment or the state of being attached is an environmental signal regulating certain bacterial genes (Dagostino *et al.*, 1991). *LacZ* gene fusions have been constructed, which are expressed directly upon attachment to a polystyrene surface (Dagostino *et al.*, 1991). At least some genes involved in synthesis of the extracellular polysaccharide alginate in *Pseudomonas aeruginosa* are regulated by attachment (Davies *et al.*, 1993; Vandevivere & Kirchman, 1993). The *algC* promoter fused to promoterless *lacZ* was activated in *P. aeruginosa* after cell growth on Teflon mesh (Davies *et al.*, 1993). It is, however, not clear what the extent of phenotypic differences between planktonic and attached bacteria is, and how these differences are regulated.

The aim of the study reported here was firstly, to develop a method for the study of *de novo* protein synthesis in attached bacteria and secondly, to determine whether attachment had any effect on the types of proteins synthesized in *P. aeruginosa* PAO1.

2 MATERIALS AND METHODS

2.1 Bacterial Cultures used in this Study

Pseudomonas aeruginosa PAO 1 (DSM 1707) was maintained on R2A agar slants containing 1 % glycerol (Reasoner & Geldreich, 1985), and transferred monthly. MGL minimal broth (Vogel & Bonner, 1956) containing 1 % glucose and 1 mM leucine was used in all experiments. Cultures were grown at 28 °C in an environmental shaker at 160 rpm. The growth curve of *P. aeruginosa* PAO in MGL broth at 28 °C was determined. Whole-cell protein profiles were determined at various stages of the growth curve, *ie.* at 1, 5, 9, 24 and 48 h after inoculation from a 24 h old broth culture. Whole-cell proteins were prepared by suspending washed cells in 50 mM Tris (pH 6.8), breaking by ultrasonication (4 x 15 s), treating with lysozyme (100 $\mu g.ml^{-1}$) at 25 °C for 30 min, and supplementing with 50 $\mu l.ml^{-1}$ mercaptoethanol and SDS to 0.01% (m/v). Proteins were heat-treated by boiling for 10 min, and were then separated by SDS PAGE as described below.

2.2 Investigation into the Effect of COD on the Rate of Bacterial Attachment

A culture of *P. aeruginosa* PAO was maintained in a chemostat vessel at 28 °C at a dilution rate of 40 $ml.h^{-1}$, the volume of the chemostat vessel being 200 ml. The medium used for maintenance of the culture was mineral basal medium of Davies *et al.* (1993), containing 10 $g.l^{-1}$ glucose. Clean sterile microscope slides were fitted into a Pedersen - type device affording linear flow past the slides. Sterile mineral basal medium of Davies *et al.* (1993) containing various concentrations of glucose (0.001, 0.01, 0.05, 0.1, 0.5, 1.0, 5.0 and 10.0 $g.l^{-1}$) was passed through the device at 4 $l.h^{-1}$. The inflowing medium was fed with cells from the chemostat at a rate of 40 $ml.h^{-1}$. Duplicate slides were withdrawn from the device at 10, 20, 30, 40, 50, 60, 90 and 120 min. Slides were washed by gentle irrigation with sterile deionised water and stained with safranine. The degree of attachment was determined by counting ten microscope fields per plate at 1000 x magnification under bright-field illumination.

2.3 Method for Studying Attached Bacteria

1 ml Aliquots of 24 h old cultures were transferred into 2.5 ml Eppendorf tubes containing glass wool (mean diameter = 15 μm). Samples of glass wool were removed after 5 and 30 min and after 8 and 24 h. Broth (containing non-attached cells) was removed by placing the glass wool samples into 0.4 ml Eppendorf tubes with a pin hole in the bottom, placing these into 1.5 ml Eppendorf tubes and centrifuging at 5 000 x *g* for 60 s. To check for attachment, glass wool samples were stained with toluidine blue for 60 s and studied by bright-field microscopy.

2.4 Labelling of Proteins to Study *De Novo* Protein Synthesis

To study *de novo* protein synthesis at various times after attachment (5 and 30 min and 8 and 24 h), 1 µl (15 µCi) of ^{35}S methionine (Amersham, UK) was added to each Eppendorf tube 5 min before removing the respective sample. The radioactive proteins were indicative of proteins synthesized during the 5 min preceding sampling time. A new sample was prepared for each time period analyzed. Incorporation of radioactive methionine was stopped after 5 min by addition of 160 µl of cold methionine (200 mM). Total cell proteins were prepared and separated as described below.

2.5 Characterisation of Whole-Cell Proteins of Attached Cells by SDS PAGE

Total cell proteins of the respective samples were prepared for SDS PAGE as follows. The broth-free glass wool was transferred into 1.5 ml Eppendorf tubes and supplemented with 400 µl of sample treatment buffer described previously (Brözel & Cloete, 1993) (5 % mercaptoethanol, 10 % glycerol, 82 mM Tris and 69 mM SDS) and 20 µl 0.25 % PMSF (phenylmethylsulfonyl fluoride) in 96 % ethanol to inhibit protein degradation by inhibition of protease activity (Colby & Chen, 1992). Samples were exposed to ultrasonication for 4 x 15 s bursts and then heat-treated for 10 min at 96 °C. Total cell proteins were separated at 60 mA in 12.5 % acrylamide gels using the discontinuous buffer system of Laemmli and Favre (Laemmli & Favre, 1973). Protein molecular mass markers used for calibration were in the range 200 - 14.3 kDa (Amersham, UK). Proteins were visualised with Coomassie Brilliant Blue R250 staining, or by the silver staining procedure as described by Kersters (Kersters, 1990). Labelled proteins were recorded by exposure of vacuum - dried gels to autoradiography films (Amersham Hyperfilm - ßmax; Amersham, UK) for 24 h. Autoradiography films were developed by the procedures as specified by the manufacturer.

3 RESULTS

There was a relationship between available glucose and rate of attachment during the first 20 min of exposure. Glucose concentrations between 0.05 and 0.001 $g.l^{-1}$ yielded *ca.* 10 attached cells per field (Fig 51). Concentrations of between 0.5 and 10 $g.l^{-1}$ yielded *ca.* 100 attached cells per field (Fig 52). Within 1 h of exposure, however, there was little difference in the numbers of attached cells between various levels of available carbon.

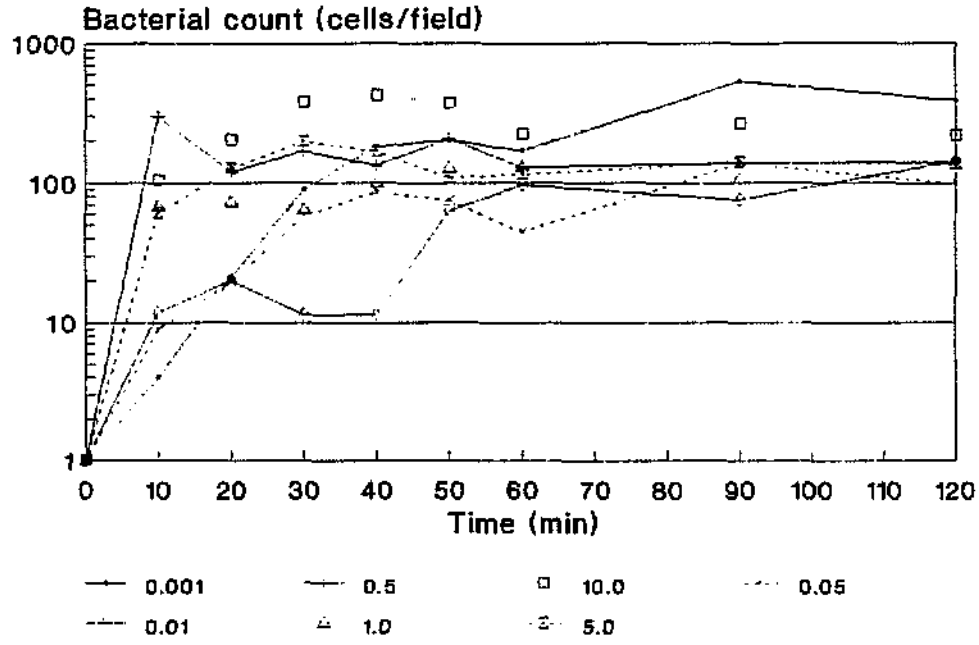

Figure 51: Rate of attachment of *P. aeruginosa* PAO to glass at various concentrations of available glucose.

The logarithmic growth phase of *P. aeruginosa* PAO1 in MGL broth at 28 °C was between 2 h and 9 h after inoculation from a 24 h culture. No differences were detected in the whole-cell protein profiles of the various stages of the growth cycle, excepting in late stationary phase (48 h) (Fig 52). Three new proteins were present (96, 50 & 23.5 kDa) which were not detected in logarithmic or early stationary phase, although they were present in lower concentration in the lag phase (Fig 52, lane 5). One protein (68 kDa) was not present in the late lag phase (Fig 52, lane 5).

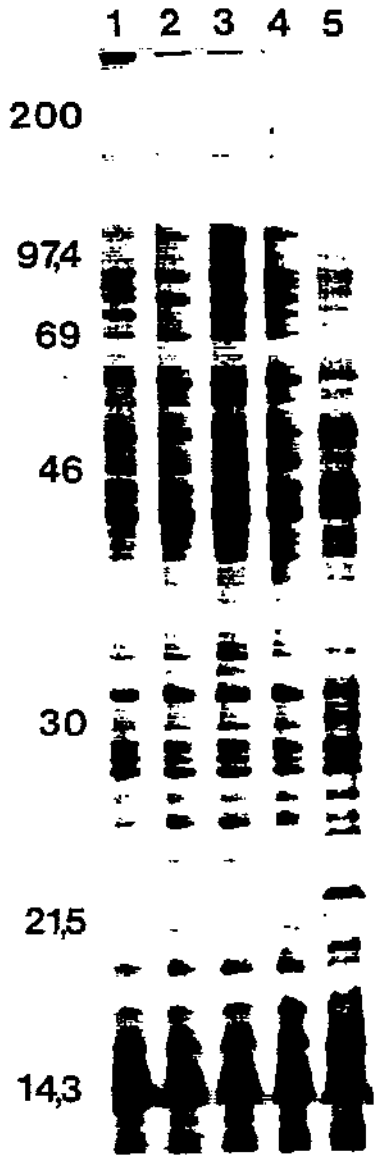

Figure 52: Whole-cell protein profile of *P. aeruginosa* PAO1 during various stages of growth. Lane 1: lag phase; lane 2: early logarithmic phase; lane 3: late logarithmic phase; lane 4: early stationary phase; lane 5: late stationary phase. Values at the left indicate molecular mass markers.

Attachment of cells to the glass wool occurred within five min of exposure (Fig 53). Attached cells were visible on the glass wool throughout the 24 h period. The whole cell protein profile of attached *P. aeruginosa* PAO1 changed within 5 min of exposure to the surface (Fig 54, lanes 1 - 5). Two new proteins (39 & 38 kDa) were detected within five min of exposure and remained as part of

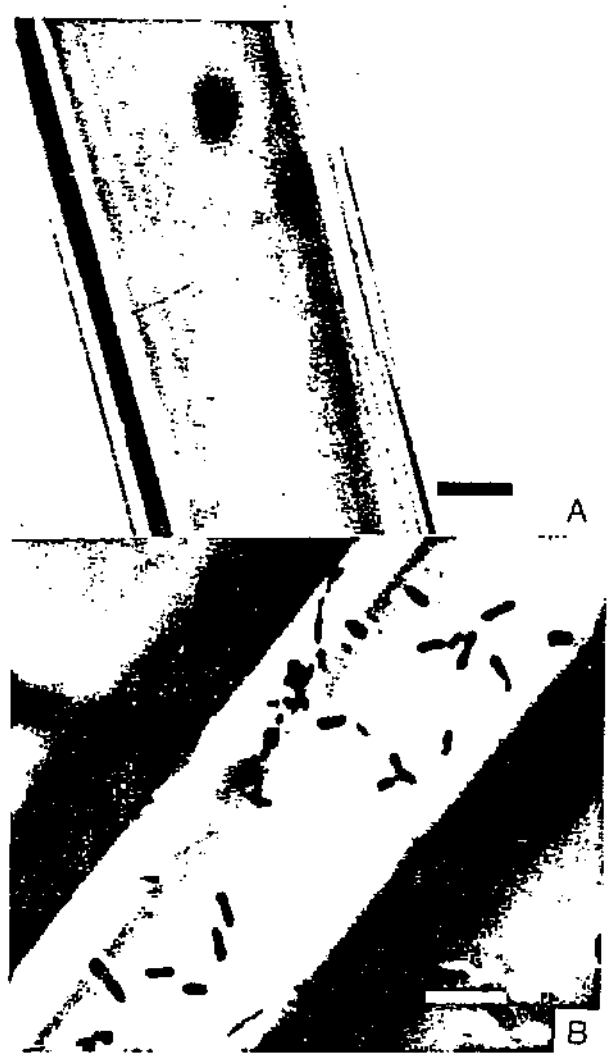

Figure 53: Photomicrographs of glass wool (a) and *P. aeruginosa* PAO1 attached to glass wool 5 min after exposure (b). (Marker = 5 μm, magnification = 2000 x).

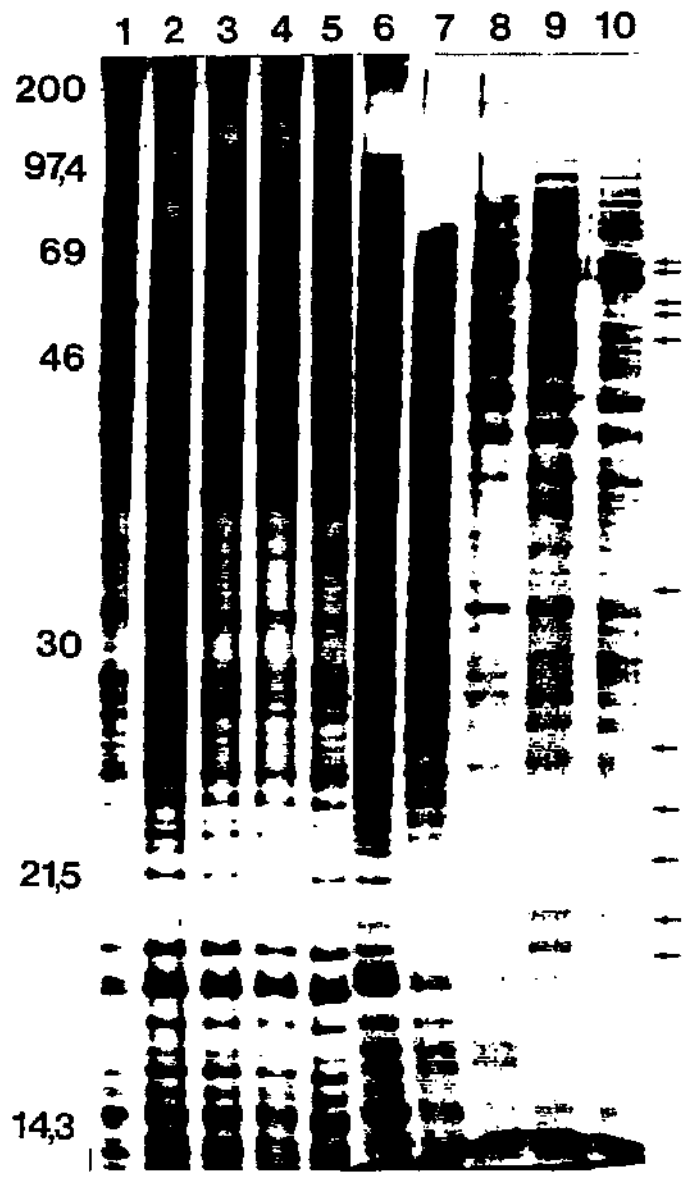

Figure 54: Protein profiles of *P. aeruginosa* PAO1 at various times following attachment to glass wool. Lanes 1 through 5 are of whole-cell proteins visualised by silver staining, and lanes 6 through 10 are of ^{35}S methionine pulse-labelled proteins visualised by exposure to autoradiography film. Lanes 1 & 6: suspended cells; lanes 2 & 7: 5 min after attachment; lanes 3 & 8: 30 min after attachment; lanes 4 & 9: 8h after attachment; lanes 5 & 10: 24 h after attachment. Values at the left indicate molecular mass markers.

the cells during the 24 h period (Fig 54, lanes 2 - 5). A further protein (26 kDa) normally present in planktonic cells disappeared upon attachment but appeared after 24 h (Fig 54, lane 5). The rate of synthesis of one protein (20 kDa) decreased after attachment (Fig 54, lanes 1 - 5).

Attachment influenced the synthesis of several proteins during various stages after attachment (Fig 54, lanes 6 - 10). One protein (61 kDa) was synthesized *de novo* immediatelly and up to 30 min following attachment. Two proteins (65 & 51 kDa) were synthesized immediately following attachment and synthesis was maintained throughout the 24 h period. The synthesis of eight proteins was terminated following attachment. Synthesis of a 23 kDa protein was terminated directly following attachment. Synthesis of five other proteins (56, 53, 34, 27 & 25 kDa) decreased upon attachment and terminated 30 min after attachment. The synthesis of a 20.5 and a 20 kDa protein was terminated following attachment, but was resumed in attached cells after 8 h.

4 DISCUSSION

The level of available carbon has little effect on the rate of bacterial attachment to surfaces. Although the initial rate of attachment is related to the conentration of available carbon, the rate of attachment becomes similar within 1 h. This is possibly due to the development of a conditioning film, where organic compounds from the surrounding liquid adsorb to the surface.

Attachment influenced the synthesis of several proteins during various stages after attachment, either by suppression of occurring synthesis, or by induction of *de novo* synthesis. This indicates that attachment of *P. aeruginosa* to glass *per se* does exert an influence on gene regulation, or at least on translation. The synthesis of at least 11 proteins is controlled by some or other factor or by multiple factors related to the attached state.

Various factors could be involved in these regulatory processes, but it is as yet unclear which factors do play a role. Altered surface free energy of the outer membrane during and / or after attachment could influence certain membrane functions locally. Membrane-embedded signal-transducing proteins could play a role by signalling attachment across the membrane. Bacteria attaching to a non-living surface do not have direct contact initially, as they are optimally adsorbed at a distance of *ca.* 5 nm from the surface where the Gibbs energy of interaction is at a minimum (Van Loosdrecht *et al.*, 1990). At this distance from the surface, the influence of the glass surface on the local Gibbs energy at the cell surface is negligible (Van Loosdrecht *et al.*, 1990). Transcription of the *algC* gene (encoding phosphomannomutase) in *P. aeruginosa* is necessary for production of the extracellular polysaccharide alginate, the extracellular matrix of biofilms. The *algC* gene is activated by both high osmolarity (Zielinski *et al.*, 1992) and by attachment to "Teflon" mesh (Davies *et al.*,

1993). It is not known whether transcription of the *algC* gene is regulated by more than one effector, or whether more than one inducer is involved. If only one regulatory system is involved in the regulation of *algC*, then attachment would simulate high osmolarity, indicating that attachment exerts a dehydrating effect on the membrane.

There was a considerable difference between the total cell protein profile (four proteins affected) and the *de novo* profile (11 proteins affected). Whereas many of the major proteins in the whole-cell and labelled protein profiles coincided, this was not true for all bands. This could be ascribed partially to the drying process, whereby the relative distances between proteins could change to yield a somewhat altered autoradiographic replica of the radiolabelled gel. It was only the 20 kD protein which showed an identical time-pattern in both whole-cell and *de novo* protein profiles. The proteins produced as detected by silver staining after attachment, but not detected on the radioactive gel (*ie.* not synthesized *de novo*), were probably modified post-translationally after attachment of cells. A more detailed investigation into the whole-cell and *de novo* protein profile by two-dimensional gel electrophoresis may yield more information on the question of different patterns.

A method for the study of *de novo* protein synthesis of attached bacteria has been developed. This was used to demonstrate that the synthesis of several proteins in *P. aeruginosa* PAO was influenced by attachment of cells. Attachment *per se* was demonstrated to exert some effect on the regulation of certain genes. It is not clear whether this is at the transcriptional or translational level, and what the nature of the attachment-mediated signal is.

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Chapter 12

Immunological techniques for the detection of Sulphate-reducing bacteria in industrial water systems

E.E. DE BRUYN and T.E. CLOETE

Abstract

The importance of sulphate-reducing bacteria (SRB) in microbial induced corrosion has been widely recognized for many years. However, little is known about the ecology of SRB in industrial cooling water systems. The problem has been in detecting and quantifying these organisms. There are many shortcomings in the use of culture media for this purpose. As an alternative, immunological techniques were evaluated as a method for detection and identification of SRB in industrial cooling water systems. Antisera were prepared against whole cells of different species of SRB and evaluated for detection and identification of these organisms in industrial cooling water systems. Antisera prepared against the surface antigens of SRB were species specific and the different species shared no antigenic determinants. In addition, culture conditions influenced the expression of surface antigens causing the antisera to be extremely specific and unsuitable for the identification of SRB enriched from industrial cooling water systems. These results were confirmed by the SDS PAGE profiles of membrane proteins.

1 INTRODUCTION

Sulphate-reducing bacteria (SRB) constitute a group of morphologically different anaerobic bacteria that have in common the capacity to reduce sulphate to hydrogen sulphide in dissimilatory reactions (Walker *et al.*, 1971). The importance of these bacteria in microbial induced corrosion (MIC) has been widely recognized for many years (Crombie *et al.*, 1980; Pope and Dziewulski, 1990; Pope and Zintel, 1989; Videla, 1990).

Although research has been performed regarding the incidence and species diversity of SRB (Antloga and Griffin, 1985; Back and Pfennig, 1991; Laanbroek and Pfennig, 1981; Pfennig, 1989; Taylor and Parkes, 1985) and utilizable carbon sources (Parkes *et al.*, 1989; Sørenson *et al.*, 1981) in freshwater and marine environments, little is known about the ecology of SRB in industrial freshwater environments, especially cooling water systems. An ecological study of SRB in industrial water systems would yield useful information regarding biocide programs to control corrosion.

The enumeration and classification of SRB by conventional methods are very time consuming (Gaylarde and Cook, 1987). Since growth is possible on many nutrients, enrichment and growth media based on only one carbon source might give rise to a biased and incomplete picture of the natural population being sampled (Hamilton, 1985). One way to overcome these problems would be to use immunological methods. Immunofluorescence microscopy has been widely applied for the detection and enumeration of particular microorganisms when conventional techniques have proved difficult (Conway de Macari *et al.*, 1985; Hobbie *et al.*, 1977; Strayer and Tiedje, 1978). Successful application of fluorescent antibodies can be affected by a range of factors, including specificity and cross reactivity (Pickup, 1991).

Serological work on SRB has yielded conflicting results. Cross reaction was found between *Desulfovibrio vulgaris* strains 8303 and 8305 as well as *Desulfovibrio desulfuricans* strains 8380 and 8393 (Baker *et al.*, 1962; Postgate and Campbell, 1963; Singleton *et al.*, 1985); whereas in other studies few cross reactions were found amongst different strains of *D. desulfuricans* and or among *D. desulfuricans*, *D. vulgaris* and *Desulfovibrio salexigens* (Abdollah and Nedwell, 1980). Immunofluorescence was found to be mainly strain specific with SRB, although weak fluorescence was seen both within and between recognized groups. A polyvalent cocktail comprising antisera prepared against different genera and species was successfully used to detect SRB (Smith, 1982). In natural samples, higher counts of SRB were obtained with a similar polyvalent cocktail compared to the MPN technique (Gaylarde and Cook, 1987; Bobowski and Nedwell, 1987). These studies suggested that whole cell and surface antigens of these organisms are different, at least for those organisms considered to be related at species level.

The objective of this study was therefore to investigate the potential of species specific fluorescent antibodies prepared using authentic SRB strains for studying the ecology of SRB in industrial cooling water systems.

2 MATERIALS AND METHODS

2.1 Organisms

Cultures of *Desulfovibrio desulfuricans* subsp. *desulfuricans* (DSM No 1924), *Desulfovibrio africanus* (DSM No 2603), *Desulfovibrio gigas* (DSM No 1382), *Desulfotomaculum nigrificans* (DSM No 574), *Desulfotomaculum orientis* (DSM No 765) and *Desulfotomaculum guttoideum* (DSM No 4024) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM).

For the isolation of SRB from industrial cooling water systems, samples were obtained from the Technical Division of a petroleum company in Sasolburg, South Africa. A total of 5 different samples were used, of which four were biofilm deposits and one, a water sample. Iron sulphite (IS)-medium (Mara and Williams. 1970) or synthetic medium (Pfennig *et al.*, 1981) containing sodium lactate (0,4 percent (%) w/v), sodium acetate (0,4 % w/v), sodium formate (0,4% w/v) or palmitic acid (0,1 % w/v) as carbon sources were used as isolation media. Agar roll tubes (Hungate, 1969) of each medium were inoculated by injecting 0,1 ml aliquots of sample dilutions into tubes kept at 45 ^{o}C. The tubes were filled with a gas phase of 20 % CO_2, 10 % H_2, balanced with N_2, sealed with neoprene rubber stoppers and screw caps and incubated at 30 ^{o}C. Titanium (III) citrate (Zehnder and Wuhrman, 1976) was used as reducing agent. All the black colonies that developed after 14 d were considered to be SRB. An agar roll tube of each of the different media with 10-20 black colonies was selected for each sample. Subcultures were prepared from all of these colonies. An aerobic bacterial count was also performed on these water samples on Standard 1 agar plates (BIOLAB). The plates were aerobically incubated at 28 ^{o}C for 48 h. Subcultures were prepared from all the single colonies on the agar plate of a dilution containing 10-20 colonies. These cultures together with the H_2S-producing bacterial cultures isolated from the industrial cooling water system samples were tested for cross reaction with the antisera prepared against different sulphate-reducing bacteria species obtained from DSM by the fluorescent antibody technique.

2.2 Preparation of antiserum

Antigens that were used for antisera preparation, were whole cells of *D. desulfuricans* subsp. *desulfuricans*, *D. gigas*, *D. orientis*, *D. guttoideum* and *D. nigrificans* grown in IS medium (Mara and Williams, 1970). Trisodium citrate (0.3 $g.l^{-1}$) was added to the medium and ferrous sulphate and iron(III)citrate omitted to prevent the formation of iron sulphide precipitates as the result of H_2S production by the bacteria. Cells were harvested by centrifugation (10 000 X g for 20 min), washed

in saline (8.5 % w/v NaCl) and suspended in saline. The suspensions were diluted to 10^9 cells ml^{-1} (McFarland, 1970). The cell suspensions were boiled for 5 min. Two white New Zealand rabbits per bacterial strain were immunized. Before immunization a serum control (10ml) was taken from each rabbit. Antigens were administered according to the following schedule: day 1, 10 and 30, 1ml of cell suspension in 1ml incomplete Freund's adjuvant was injected intramuscularly. Boosters (1ml of antigen) were given intravenously on day 37. Blood samples were obtained from the marginal ear of the rabbits at 7 d intervals after the injections and the samples tested for titre (Garvey *et al.*, 1977). Blood samples were left to clot overnight at 4 °C and the serum was then collected by centrifugation at 3 000 X g for 20 min and stored at -12 °C. The agglutination titre of the different antiserum all proved to be > 1024 (titres are reported as the reciprocal of the greatest dilution showing reaction).

2.3 Immunodiffusion

The prepared antisera were examined for immunological similarities as described by Garvey (Garvey *et al.*, 1977) using ouchterloney double diffusion. Each antiserum was compared against the following test antigens; *D. desulfuricans*, *D. gigas*, *D. nigrificans*, *D. guttoideum* and *D. orientis*. The cells of the different strains were cultured in modified IS-medium (Mara and Williams, 1970) without an iron source, collected by centrifugation (10 000 X g for 20 min), washed in phosphate buffered saline (PBS) and then suspended in PBS. Standard cell suspensions in PBS were boiled for 5 min and used as antigenic solutions. A concentration curve was performed to establish the proper ratio of antisera to antigen (Garvet *et al.*, 1977). The immunodiffusion plates were incubated at 4 °C and inspected every 24 h for precipitation bands and stained with Coomassie blue (Garvey *et al.*, 1977).

2.4 Preparation of fluorescent antibodies

The immunoglobulins were precipitated from the prepared antisera by using polyethyleneglycol 6000 (12% w/v) (Cloete, 1984). The final precipitates were dissolved in PBS and the protein concentration determined using spectrophotometry (Walker *et al.*, 1971).

The purified immunoglobulin fraction of the prepared antisera was conjugated with fluorescein isothiocyanate isomer I (FITC) (Cloete, 1984). After conjugation the unbound FITC was removed from the conjugate by gelfiltration through Sephadex G-25 (Cloete, 1984).

2.5 Direct fluorescent antibody (FA)-stains

The fluorescent antibody conjugates prepared against whole cells of *D. desulfuricans*, *D. gigas*, *D. guttoideum*, *D. orientis* and *D. nigrificans* were used. Air dried smears of cells, the same SRB-strains used for antiserum preparation, cultivated in IS-medium without an iron source, were fixed by gentle heat and used as antigens in FA-stains. The different FITC-antibody-conjugates were double diluted down to 1/2048. Each dilution of a conjugate was used in FA-stains with the homologous antigen smear. The bacteria were stained by placing a drop of a conjugate on a smear and incubating the slide

in a humidity chamber in the dark for 30 min. The slides were rinsed in PBS and mounted in sodium carborate buffered glycerol for optimum fluorescence (Pital and Janowitz, 1963). Stained preparations were examined for fluorescence under a Zeiss ultra-violet microscope using an HBO-200 mercury vapor lamp as the exciting light source. The highest dilutions of the various FITC-antibody conjugates at which fluorescence could clearly be observed were used to test for cross reactions between the different FITC-antibody conjugates and the following antigens; (a) Homologous SRB-cells cultivated in IS-medium (Mara and Williams, 1970), (b) Homologous SRB-cells cultivated in synthetic medium (Pfennig *et al.*, 1981) with lactate as carbon source, (c) Heterogeneous SRB-cells cultivated in IS-medium (Mara and Williams, 1970), (d) H_2S-producing bacteria isolated from industrial cooling water system samples using IS-medium and synthetic medium with various carbon sources, (e) H_2S-producing bacteria isolated from industrial water samples using synthetic medium with lactic acid as carbon source, that were recultured in IS-medium and (f) aerobic bacteria isolated from industrial cooling water systems.

2.6 Sodium dodecyl sulphate-polyacrylamide- gel electrophoresis (SDS-PAGE)

Cells of *D. gigas* and *D. nigrificans* were cultured in IS-medium (Mara and Williams, 1970) without an iron source (tryptone 10 g, sodium sulphite, 0.5 g, sodium(III) citrate 0.3 g, lactic acid 6 ml of a 60 % solution, $MgSO_4.7H_2O$ 2 g, ascorbic acid 0.75 g, dist. H_2O 1000 ml, pH 7,6). *D. desulfuricans*, *D. orientis* and *D. africanus* cells were cultured in both IS-medium and modified synthetic medium (Pfennig *et al.*, 1981) without iron. Cells were harvested by centrifugation (10 000 X g for 20 min), washed in saline and suspended in saline. For the extraction of total membrane proteins the pH of the bacterial suspensions was lowered to 1,5 by adding 10 M HCl in order to degrade extracellular polysaccharides. The cells were washed three times in saline and the membrane proteins extracted according to the method of De Maagd *et al.* (DeMaagd *et al.*, 1988). The membrane protein pellets were dissolved in 5% mercaptoethanol, 4,6 % SDS and 2 % Tris-HCl (pH 6,8).

SDS-PAGE was performed using the method of Laemmli (Laemmli, 1970), modified according to Kiredjan *et al.* (Kiredjan *et al.*, 1986). Electrophoresis was carried out using a HSI vertical slab gel unit SE-600 series (Hoefer Scientific Instruments, San Francisco) at a constant current of 15 mA and 25 mA per stacking and separation gel respectively, at 10 ^{o}C. Membrane protein gels were stained with Coomassie Brilliant Blue according to the method of Jackman (Jackman, 1985) and destained according to Anderson and Anderson (Anderson and Anderson, 1977). Gels were scanned on a Hoefer GS 300 Transmittance/ Reflectance Scanning Densitometer (Hoefer Scientific instruments, San Francisco). Numerical analysis, based on the correlation coefficient (r) which was determined using the unweighted average linkage cluster analysis, was done using the Gel Compar programme version 1.3 supplied by Helix C.V., Belgium.

3 RESULTS

Gel immunodiffusion was used to examine for immunological similarities between *D. desulfuricans*, *D. gigas*, *D. nigrificans*, *D. guttoideum* and *D. orientis*. The patterns of the immunoprecipitation bands formed during immunodiffusion showed that the different SRB-species shared no antigenic determinants. Precipitation bands formed only between the antisera and the homologous cells used for the preparation of the antisera (Fig. 55).

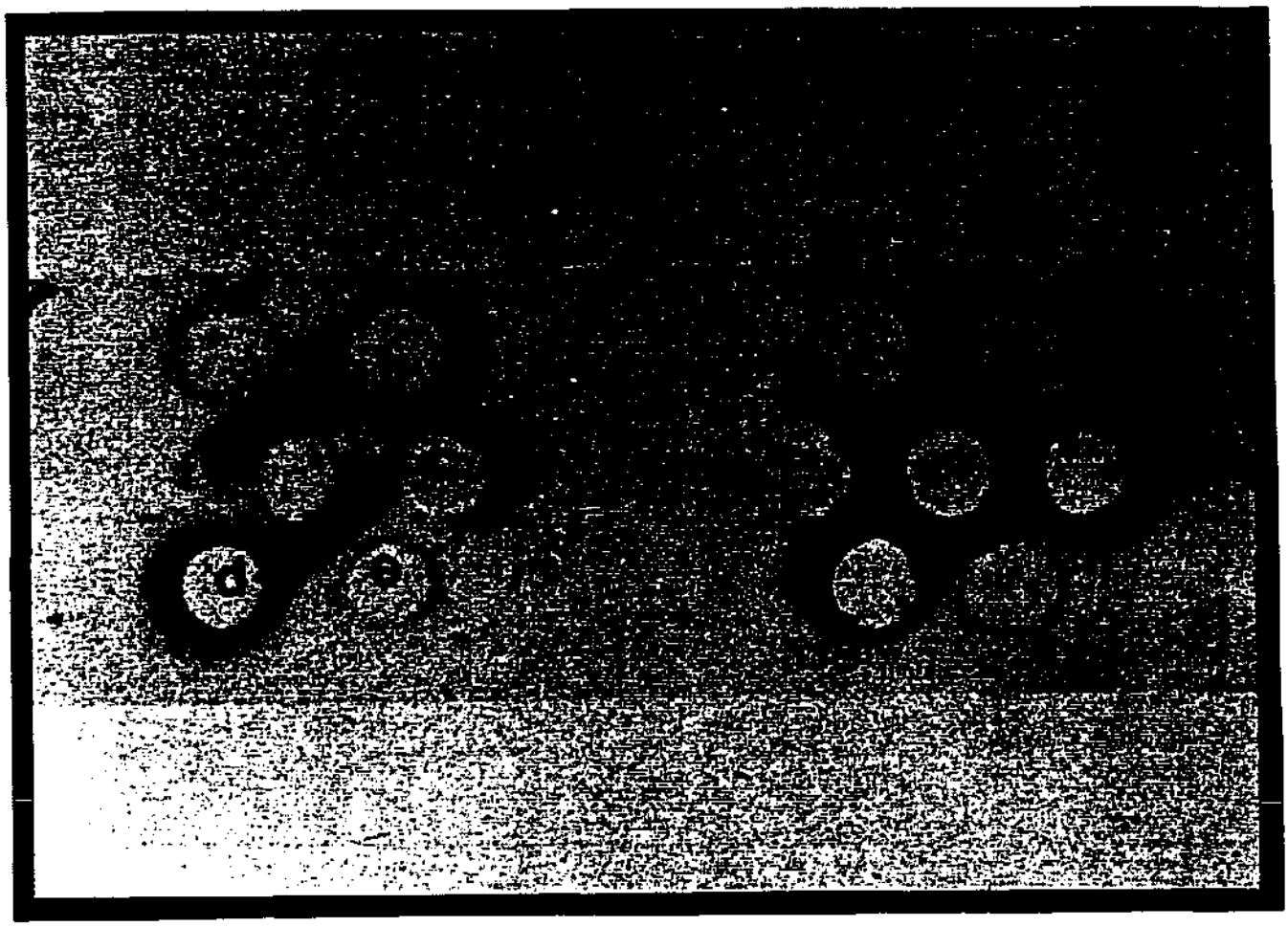

Figure 55: Double immunodiffusion agar plate with; a, antisera prepared against *D. desulfuricans* in the center well and the following antigens cultured in IS-medium (Diem *et al.*, 1977) placed in the outer wells; a, e, *D. desulfuricans*; b, *D. gigas*; c, *D. orientis*; d, *D. guttoideum* and f, *D. nigrificans*.

When using FITC-antibody conjugates prepared against the different SRB-species in direct FA-stains the following results were obtained. 1) Cross reactions only between the conjugates and cells of the homologous SRB-species cultured in the same medium (IS-medium) as used for the preparation of the antisera (Fig. 58). 2) No cross reactions were observed between the conjugates and both heterologous and homologous cells of SRB-species cultured under different conditions (synthetic medium) than the cells used for antiserum preparation. 3) The conjugates did not cross react with

the 120 H_2S-producing bacteria isolates enriched from industrial cooling water system samples in IS-medium or in synthetic medium with various carbon sources, neither enriched in medium with lactic acid as carbon source that were recultured in IS-medium nor finally aerobic bacteria isolated from industrial cooling water systems.

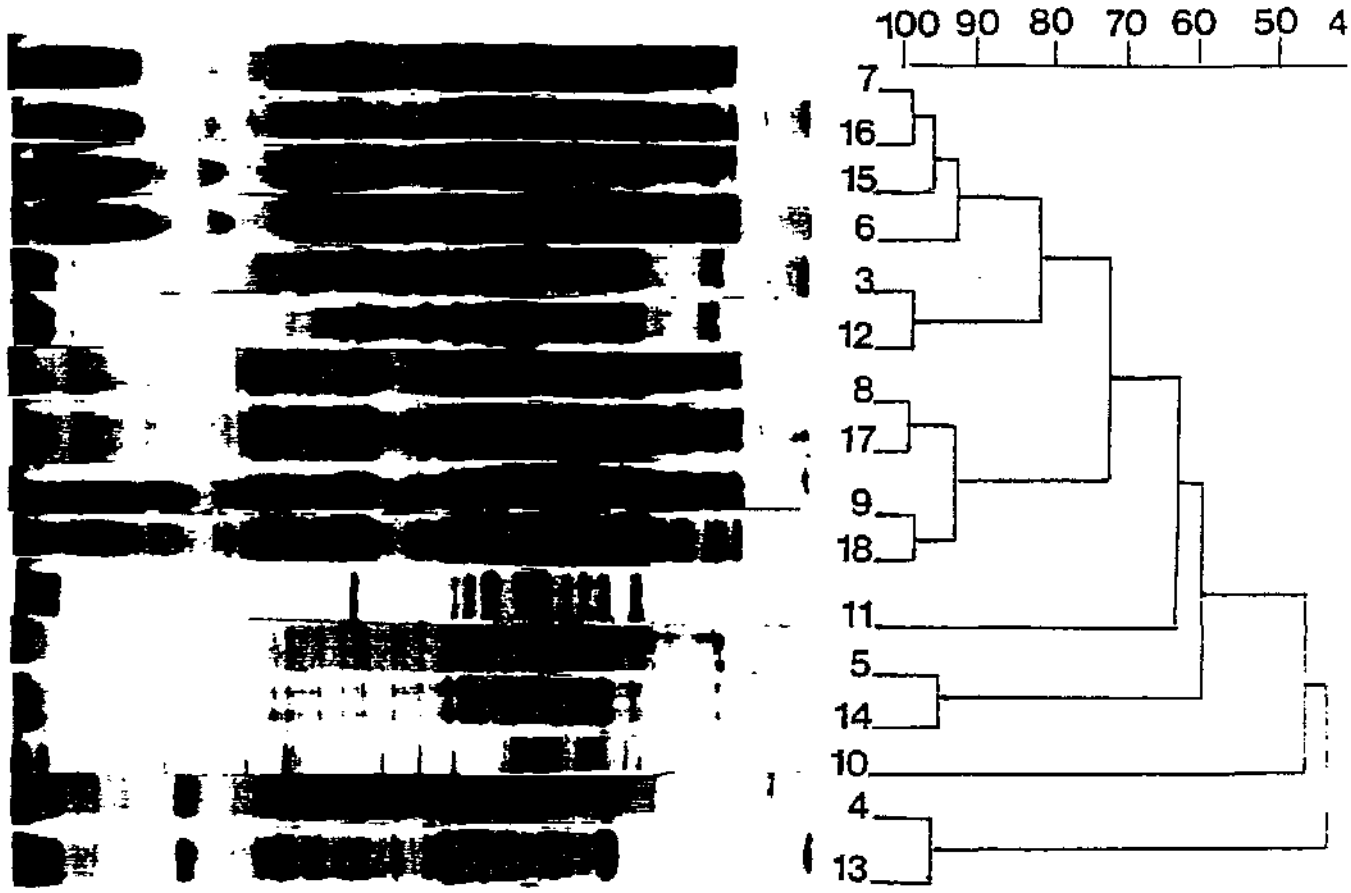

Figure 56: Dendrogram of the relationships of different SRB-strains cultivated in IS-medium (Diem *et al.*, 1977) and synthetic medium (Ford and Olson, 1988) based on r values, as calculated by the unweighted average pair group method using SDS-PAGE of total membrane proteins. Lanes 3, 12 represent the protein profile of *D. gigas*; 4, 13, *D. orientis* [a]; 5, 14, *D. orientis*; 6, 15, *D. desulfuricans*; 7, 16, *D. desulfuricans* [a]; 8, 17, *D. africanus* [a]; 9, 18, *D. africanus* and lane 11, *D. nigrificans*. All the cells were cultured in IS-medium except for the strains indicated with; [a], that were cultured in synthetic medium. The profile of the total soluble proteins of *Psychrobacter* was used as a standard (lane 10).

The membrane protein profiles of the different SRB-species cultivated in IS and synthetic medium are shown in Fig. 56. The SDS-PAGE of membrane protein profiles of different SRB-species showed prominent differences between the bacteria. The relationships between the various strains can be seen on the dendogram (Fig. 56). The three species of the genus, *Desulfovibrio*, i.e. *D. desulfuricans*, *D. gigas* and *D. africanus* clustered together at r = 0,73. *Desulfotomaculum nigrificans* clustered at r = 0,63 with the genus *Desulfovibrio*. Although *D. orientis* and *D. nigrificans* belong to the same genus they clustered at only r = 0,6. Differences could also be observed between the same SRB-species cultivated in IS-medium and synthetic medium. Scans of the different membrane protein bands expressed as peaks are shown in Fig. 57. The appearance of new protein bands, the disappearance of bands and a difference in the amount of the expression of certain proteins can be observed. *D. orientis* cultivated in synthetic medium clustered at less than r = 0.5 with the rest of the organisms.

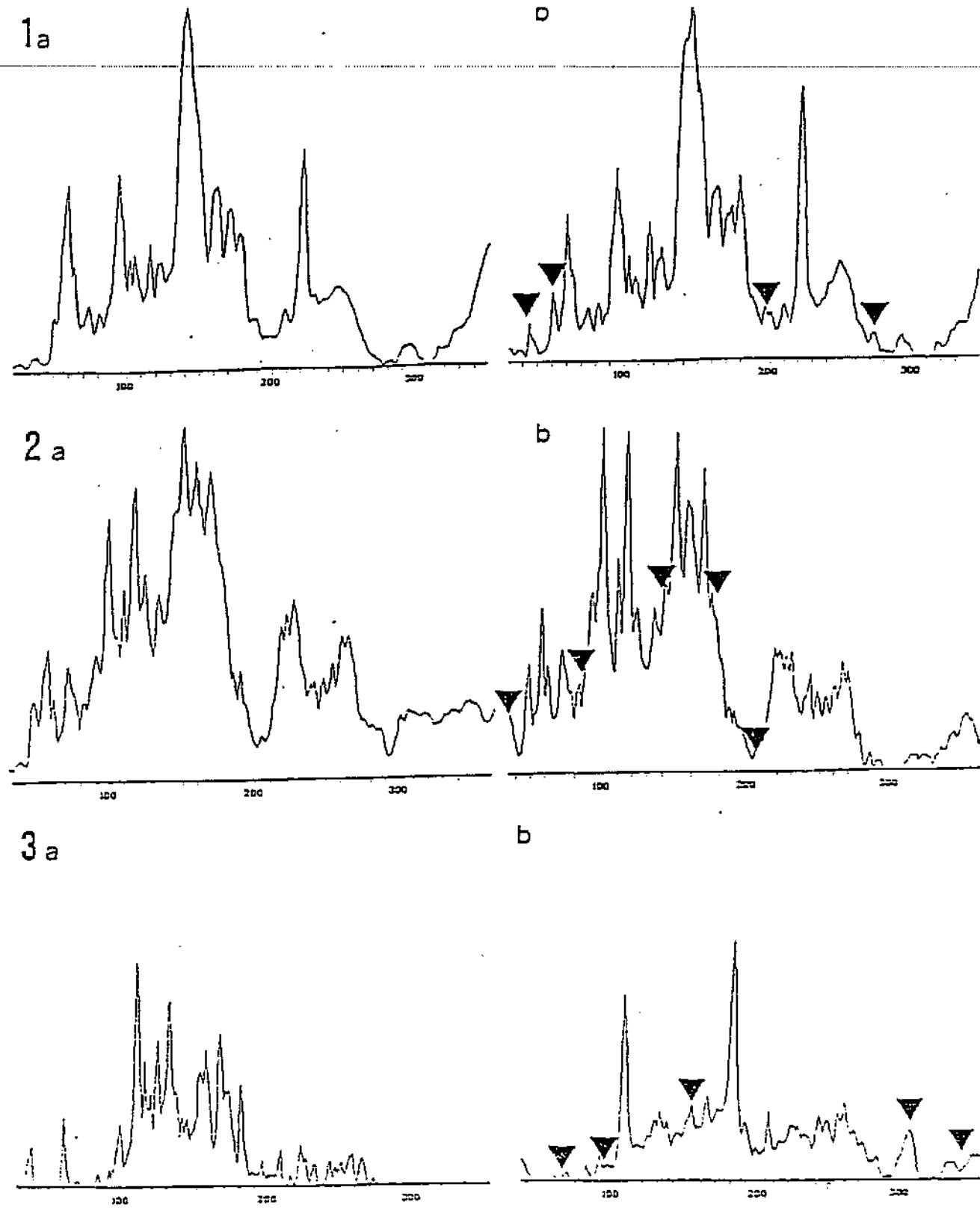

Figure 57: Scans of; 1, the membrane protein profile of *D. desulfuricans* cultured in; a, IS-medium (Diem *et al.*, 1977) and b, synthetic medium (Ford and Olson, 1988), 2, *D. africanus* cultured in; a, IS-medium and b, synthetic medium and 3, *D. orientis* cultured in; a, in IS-medium and b, in synthetic medium. Arrows indicate prominent differences in the profiles of the SRB-species cultured in synthetic medium from the species cultured in IS-medium.

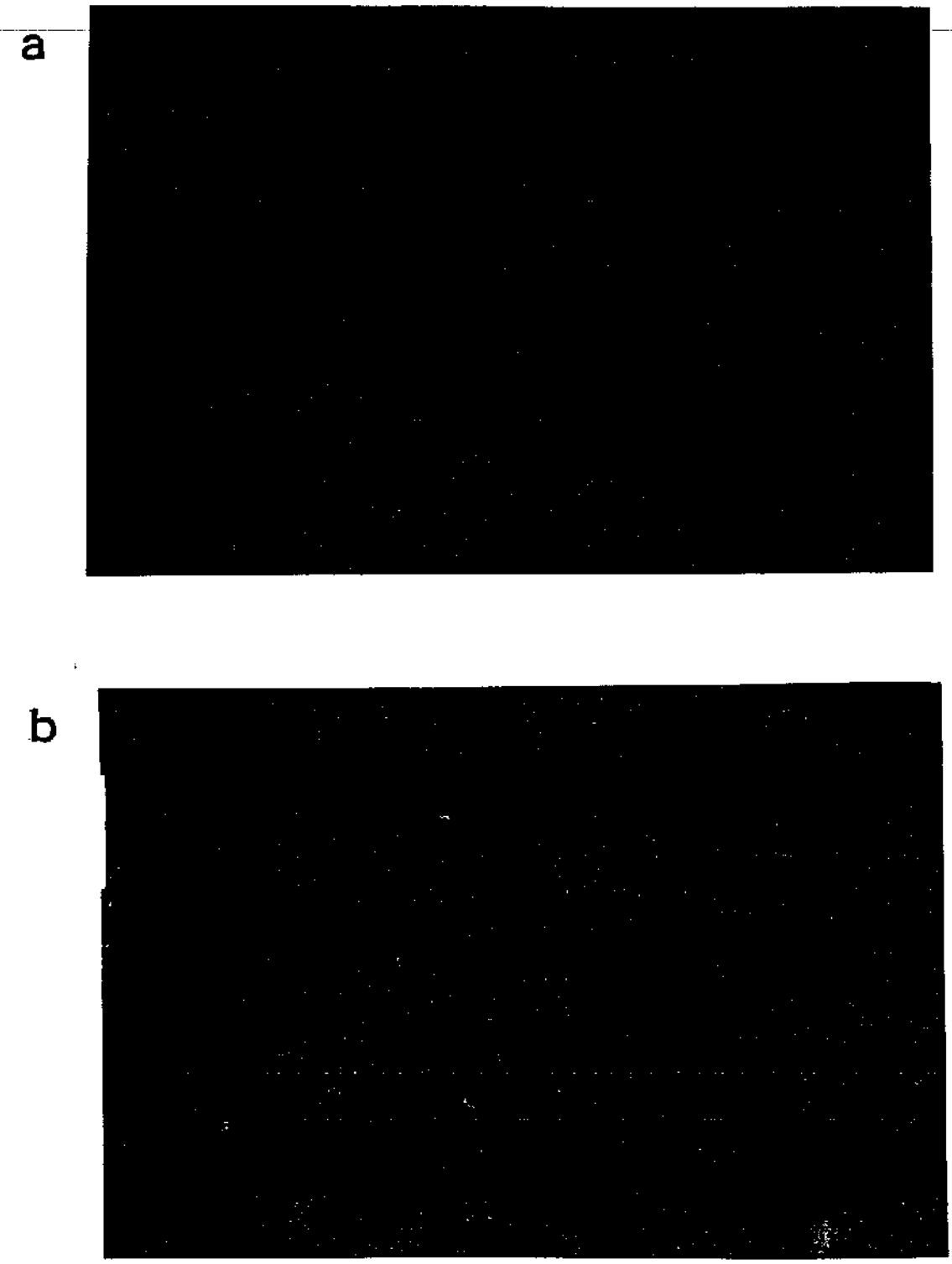

Figure 58a: Cross-reactions between the fluorescent-antibody conjugates and the homologous cells of a; *Desulfovibrio desulfuricans* and b; *Desulfotomaculum orientis* cultured in the same medium as used for the preparation of antisera.

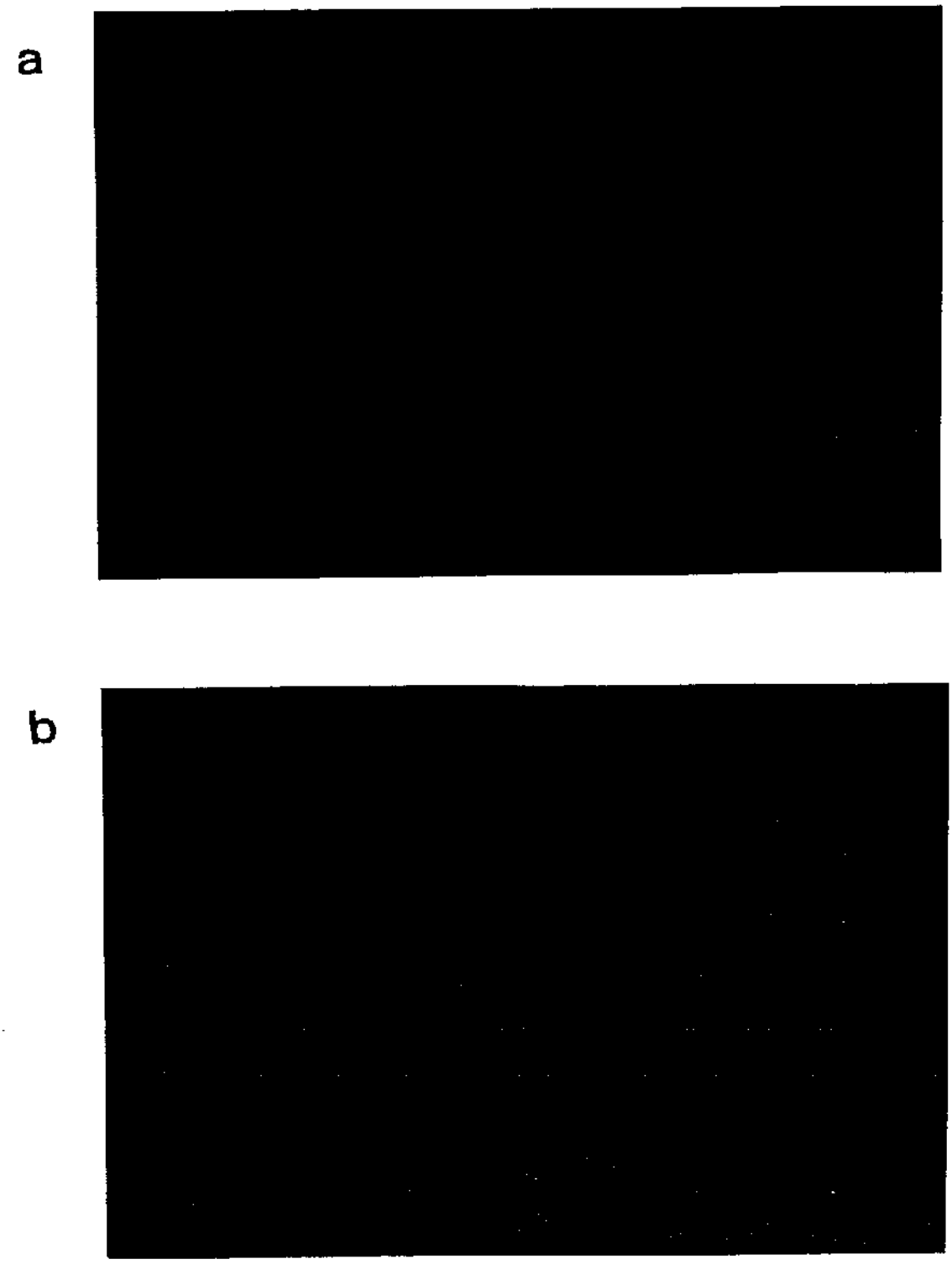

Figure 58b: Cross-reactions between the fluorescent-antibody conjugates and the homologous cells of a; *Desulfotomaculum guttoideum* and b; *Desulfovibrio gigas* cultured in the same medium as used for the preparation of antisera.

4 DISCUSSION

The antisera prepared against different SRB-species cross reacted only with the cells of homologous SRB-species cultivated in the same medium (IS-medium) that were used during the preparation of antigens for antisera production (Fig. 60). No cross reactions were observed between the antisera and nonhomologous species. The different SRB-species tested shared no antigenic determinants. H_2S-producing bacteria isolated from industrial cooling water systems using various media and carbon sources could not be detected with the antisera prepared against different SRB-species.

Smith (Smith, 1982) also found FA's prepared against *D. salexigens*, *D. desulfuricans*, *Desulfovibrio vulgaris* and *D. nigrificans* to be mainly strain specific. This antiserum was however not tested for the detection of SRB in nature (Smith, 1982). Polyvalent cocktails comprised of antisera prepared against various strains of *D. desulfuricans*, *D. gigas*, *D. salexigens*, *D. vulgaris*, *Desulfobacter postgatei* and *D. nigrificans* were successfully used to detect SRB in nature by using the ELISA-technique (Bobowski and Nedwell, 1987; Gaylarde and Cook, 1987). The antisera prepared by Bobowski and Nedwell (1987) were prepared against cell extracts and not whole cells. These authors all used Postgate's media (Postgate, 1984) for the preparation of antigens for antisera production. We chose IS-medium because of the high yield of SRB cells obtained with this medium in industrial water systems (DeBruyn and Cloete, 1992). Postgate media differs from IS-medium in that Postgate media contains yeast extract and IS medium tryptone. There is no sulphite present in Postgate medium. Iron was omitted from IS-medium, whereas iron sulphide precipitates were removed from the SRB-cells after culturing of SRB cells in Postgate media for antisera preparation.

Successful application of FA's can be affected by a range of factors; 1) the specificity of the antibody to be used and the problems with nonspecific staining, 2) the interference from autofluorescence or nonspecific absorption of FA to the background, 3) the stability of the antigen under different growth conditions and environments, 4) the inability to distinguish between live and dead cells and 5) the efficiency of recovery of the desired cells from natural samples (Bohlool and Schmidt, 1970). The use of FA stains has nevertheless been successfully applied for the detection and enumeration of many microorganisms in their natural habitat (Crombie *et al.*, 1980; Reed and Dugan, 1978). Various studies indicated that FA staining reactions were highly specific (Diem *et al.*, 1977; Schank *et al.*, 1979; Schmidt *et al.*, 1968).

Several techniques have been developed to reduce autofluorescence and nonspecific staining (Ford and Olson, 1988, Hobbie *et al.*, 1977; Stryer and Tiedje, 1978). A major concern in the application of any marker technique in ecological studies is the stability of the marker under different conditions and environments (Bohlool and Schmidt, 1988). Only a few antigens have been tested for their stability in the environment and have appeared to be relatively stable (Ford and Olson, 1988). For example, rhizobia have been extensively tested on different media, in different soils and as bacteroides in

nodules (Bohlool and Schmidt, 1970). However, when culturing the cells of SRB under different conditions (synthetic medium) no cross reaction was observed between the antisera prepared and its homologous SRB-species.

When SRB cells were cultivated in synthetic medium, different membrane proteins were expressed when compared to those from cells cultivated in IS-medium, a more nutritious medium (Fig. 58, 59). Iron did not influence the expression of the proteins, since cells cultured in both IS-medium and synthetic medium were starved for iron. The cultivation of cells in different media had an influence on *D. orientis*. *D. orientis* cultured in synthetic medium fell outside the group (r = 0,6) formed by the SRB-strains and clustered with the other SRB-strains and *D. orientis* cultured in IS-medium with r less than 0.5. *D. orientis* cultured in IS-medium clustered with *D. nigrificans* at r = 0,6. When a study of the cell envelope proteins in SRB was performed by Norqvist and Roffey (1985) they also concluded that *D. orientis* was unique. A relationship between DNA relatedness and level of similarity of 16S rRNA was defined and indicated that many pairs of *Desulfovibrio* species shared less than 10 % sequence homology (Devereux *et al.*, 1990). The results from our study are similar to those of Davies *et al.* (Davies *et al.*, 1992) who indicated that the outer-membrane protein (OMP) profiles in SDS PAGE of *Pasteurella haemolytica* demonstrated significant differences in the synthesis of certain *P. haemolytica* OMP under various growth conditions (Davies *et al.*, 1992).

Surface antigens of SRB are strain specific. Antisera prepared against the surface antigens of SRB, cultured in IS-medium, could not be used to identify SRB enriched from natural systems, since the expression of proteins on the surface of the cells are influenced by the culture medium used. SDS-PAGE profiles of membrane proteins confirmed the diversity of SRB-species and the influence of culturing conditions on the expression of membrane proteins. This emphasizes that caution should be exercised when using FA for ecological studies.

5 ACKNOWLEDGEMENTS

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Chapter 13

Media for the detection of Sulphide-producing bacteria in industrial water systems

E.E. DE BRUYN and T.E. CLOETE

Abstract

The importance of sulphate-reducing bacteria (SRB) in microbiological induced corrosion (MIC) has been widely recognized for many years [1]. There are many formulations of culture media used for enumerating SRB [2-3]. Previous studies indicated that viable count procedures underestimated the *in situ* population by a factor of approximately 1000 compared to *in situ* sulphate reduction activity [1, 4]. The aim of this study was to evaluate different isolation media and carbon sources for the isolation and detection of SRB in South African industrial water systems. Modified Iron sulphite (IS) medium yielded the highest numbers when used to enumerate SRB from pure cultures and industrial water samples. When comparing API, SABS, IS, Oxoid and modified synthetic medium using pure cultures of *Desulfovibrio desulfuricans* IS-medium gave a 12.1 %, 40 %, 53.3 % and 60.3 % higher recovery than SABS-, Postgate-, API- and synthetic medium, respectively (P @ 0.05). IS-medium gave a 20.1 %, 61.8 % and 100 % higher recovery than SABS-, API- and Oxoid medium, respectively, when using pure cultures of *Desulfotomaculum orientis* (P @ 0.05). The dominant sulphide-producing bacteria isolated from the industry using IS-medium were facultative aerobic gram-negative rods that were able to produce sulphide from sulphite under strictly anaerobic conditions. IS-medium was therefore not selective for SRB only. H_2S-producing bacteria that utilized lactate, acetate, formate or palmitic acid as different carbon sources were also isolated from industrial water samples when using synthetic medium.

1 INTRODUCTION

The importance of dissimilatory sulphate-reducing bacteria (SRB) in microbial induced corrosion (MIC) has been widely recognized for many years. Whilst their role in the sulphur cycle is fundamental in maintaining our environment, the adverse economic consequences of their activities can be devastating in industrial processes. These bacteria can result in health hazards and corrosion of equipment and pipe lines (Herbert and Gilbert, 1984). Although many organisms can play a part in the corrosion processes, the chief culprits are the SRB (Hamilton, 1985).

SRB are an ubiquitous group of microorganisms which share an ability to couple the reduction of sulphate to the oxidation of a variety of electron donors (Postgate, 1984). Despite this common metabolic feature, these organisms are exceedingly diversified from both morphological and biochemical perspectives. Growth is possible in CO_2, and on a range of organic compounds including benzoate and on fatty acids from acetate to stearate (Widdel, 1988).

There are many formulations of media used for enumerating SRB which include a carbon source, usually lactic acid, small amounts of yeast extract, inorganic salts and a reducing agent to poise the medium at a low potential. Several of these different media have been used to enumerate SRB from environmental samples (Pankhurst, 1971; Postgate, 1984; Gibson, *et al.*, 1987; Herbert and Gilbert, 1984; Hardy, 1988; Fedorak*et al.*, 1987).

Hamilton (1985) concluded that viable count procedures for enumerating SRB underestimated the *in situ* populations by a factor of approximately 1000, compared to *in situ* sulphate reduction activity. This may have been due to poor recovery of these bacteria from sediment, an inappropriate choice of growth medium or the fact that only lactate types were enumerated (Gibson, *et al.*, 1987). The isolation of a large number of new types of SRB which use carbon sources other than lactate (Widdel and Pfennig, 1984) demonstrate that acetate, propionate, butyrate and hydrogen are also important *in situ* substrates for sulphate reduction. This emphasizes the importance of careful medium selection. The aim of this study was to evaluate different isolation media and carbon sources for the isolation and detection of SRB.

2 MATERIALS AND METHODS

2.1 The evaluation of different isolation media using pure cultures of sulphate-reducing bacteria

Pure cultures of *Desulfovibrio desulfuricans* and *Desulfotomaculum orientis* were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM). A medium based on the formula of the American Petroleum Institute (API), which culminated in procedure No. 38 (American

Petrolium Institute, 1965), the modified medium of Postgate C with a lower calcium content (Singleton *et al.*, 1988), The modified iron sulphite (IS)-medium (Mara and Williams, 1970), SABS-medium prepared in accordance to method 553 of the South African Buro of Standards, the Oxoid medium for SRB and a modified synthetic medium of Pfennig *et al.* (Pfennig *et al.*, 1981) with lactate as carbon source were evaluated as media for the detection of SRB. The modified synthetic medium was prepared as follows: Solution 1 (mineral salts base), solution 2 (trace element stock solution), solution 3 (vitamin stock solution) and solution 4 (growth factors stock solution) were prepared as described by Pfennig *et al.* (Pfennig *et al.*, 1981). The pH of solution 1 was adjusted to 7,6, sterilized after the addition of the agar (1.65 % w/v) and kept at 45 ^{o}C to prevent the agar to solidify. Solutions 2 to 4 together with the carbon source solution were combined in the following proportions: solution 2 (1 ml) + solution 3 (1 ml) + solution 4 (1 ml) + carbon source (4 g sodium lactate in 10 ml H_2O)

Table 26: Medium composition of the various modifications of iron sulphite medium.

Medium composition	**Amounts in g.1000 ml^{-1} of the various modifications**						
Tryptone	1	10	10	1	1	0.1	1
Sodium sulphite	0.5	-	-	0.5	0.5	0.5	0.5
Iron (III) citrate	0.5	0.5	0.5	-	0.5	0.5	0.5
Sodium lactate	3.5	3.5	3.5	3.5	3.5	3.5	-
$MgSO_4.7H_2O$	2	2	-	2	-	-	-
$FeSO_4.7H_2O$	0.5	0.5	-	0.5	-	-	-
Ascorbic acid	0.75	0.75	0.75	0.75	0.75	0.75	0.75
$FeCl_2.4H_2O$	-	-	1.2	-	1.2	1.2	1.2
$MgCl_2.6H_2O$	-	-	-	-	0.8	0.8	0.8

sterilized by filtration and added to solution 1. Finally the iron solution (0,5 g Fe $SO_4.7H_2O$ in 17 ml H_2O) were sterilized by filtration and added to solution 1. Anaerobic tubes with 5 ml pre-reduced sterile medium with 1, 65 % agar were used during the evaluation of these different media. Resazurin (1ml of a 0,1 % w/v solution) was added to the media as a redox-potential indicator. The tubes were filled with a gas phase of 20 % CO_2, 10 % H_2, balance with N_2 and sealed with neoprene rubber stoppers and screw caps. Titanium(III)citrate (1 x 10^{-3} M) (Zehnder and Wuhrman, 1976) was used as reducing agent. The agar roll tube technique (Hungate, 1969) was applied. Three dilution series of each of the pure cultures of *D. desulfuricans* and *D. orientis* were prepared. Each medium was inoculated in duplicate by injecting 0,1 ml of each dilution with a sterile syringe and needle into the

anaerobic tube with molten agar kept at 45 °C. After the inoculation and preparation of agar roll tubes, the tubes were incubated at 30 °C. All the black colonies that developed after 14 d were counted as being SRB.

2.2 Statistical analysis

The results obtained when evaluating different isolation media using pure cultures of SRB were statistically analyzed by using Duncan's multiple range test for variables with P = 0.05.

2.3 The detection and isolation of sulphide-producing bacteria from industrial water systems using different media and carbon sources

Sample numbers D, E and F were water samples obtained from Anglo American Research Laboratories (AARC), samples 17 and 18 water samples from Vaalreef mine in Orkney, samples 51, 53, C1, X1, R1 and G2 sediment samples from AARC, sample AS, BS, CS, and DS biofilm deposits from Sasol Technical Division (SASTECH) and sample ES, a water sample from SASTECH.

IS-medium (Mara and Williams, 1970), SABS-medium and the modified synthetic medium (Pfennig *et al.*, 1981) with lactate as carbon source were used to count SRB in industrial water systems with the agar roll tube technique.

Modified synthetic medium (Pfennig *et al.*, 1981) with sodium lactate (0,4 %), sodium acetate (0,4 %), sodium formate (0,68 %) or palmitic acid (0,1 %) as different carbon sources were used for the isolation of SRB from industrial water system samples. The agar roll tube method was used. Subcultures were prepared from the black colonies that developed on a lactate containing agar roll tube with 10-20 colonies. Modified synthetic medium (Pfennig *et al.*, 1981) with lactate as carbon source was used for preparing the subcultures. All these H_2S-producing isolates were tested for growth on the same medium with acetate as carbon source. The gas phase with 10% H_2, 20% CO_2, balance with N_2 was excluded from the anaerobic tubes when these experiments were performed since incompletely oxidizing sulphate reducers using lactate are usually able to grow just as well with hydrogen as electron donor (Widdel, 1988).

2.4 Partial characterization of sulphide-producing organisms isolated from industrial water samples using IS-medium

The agar roll tube method and IS-medium were used for the isolation of SRB from sample No. AS and ES. Isolations were made from all the black colonies on a agar roll tube containing 10 to 20 colonies by transferring the single colonies to anaerobic tubes with liquid IS-medium. Dilution series were prepared from these cultures. Agar roll tubes were prepared from each dilution to obtain single colonies. A single colony of each culture was transferred to anaerobic tubes with liquid IS-medium. The whole process was repeated twice. The single colony subcultures were tested for aerobic growth

by streaking the culture onto IS-agar plates and incubating the plates aerobically at 28 °C. Single colonies obtained from the aerobic incubated IS-agar plates were reinoculated into anaerobic tubes with prereduced IS-medium. The single colony subcultures that turned IS-medium black under strictly anaerobic conditions were kept for examination. Colony and cell morphology, motility, gram stain reaction (Doetsch, 1981), oxidase and catalyze activity and fermentation on Hugh and Leifson medium (Smibert and Krieg, 1981) were performed on these cultures.

The cultures were tested for the production of sulphide under anaerobic and aerobic conditions by streaking the cultures in duplicate on IS-agar plates. One set of plates was aerobically incubated and the second set incubated under anaerobic conditions in a gas atmosphere of 10 % H_2, 20 % CO_2, balance with N_2. Sulphide production by the cultures was tested in the following media used in the industry for the detection of SRB; SABS-medium, IS-medium (Mara and Williams, 1970), Postgate mediums (Postgate, 1984) and API-medium. Different modifications of IS-medium (Table 26) were also tested for the induction of sulphide production under strictly anaerobic conditions by the various cultures. Anaerobic tubes with prereduced media containing 1,65 % agar were filled with a gas atmosphere of 10 % H_2, 20 % CO_2, balance with N_2 and sealed with neoprene rubber stoppers and screw caps. These tubes were inoculated with aerobic growth of the different cultures and incubated at 30 °C. Black discoloring of the media was noted as positive for sulphide production.

3 RESULTS

3.1 The evaluation of different isolation media using pure cultures of sulphate-reducing bacteria

The results of the evaluation of the different media for the isolation of SRB from pure culture, using the agar roll tube technique are shown in Fig. 59. The highest numbers of *D. desulfuricans* cfu ml^{-1} of pure culture were obtained when Oxoid-medium for SRB and IS-medium (Mara and Williams, 1970) were used. IS-medium (Mara and Williams, 1970) yielded the highest numbers when used for the isolation of *D. orientis* from pure culture. No visible growth was detected when Oxoid-medium was used for the isolation of *D. orientis* from pure culture. No explanation for this phenomena can be given at this stage.

3.2 Statistical analysis

The results of the statistical analysis of the results obtained when comparing different isolation media for the isolation of SRB from pure culture are shown in Table 27. The means of the counts obtained with Oxoid-medium and IS-medium were not significantly different when isolating *D. desulfuricans* from pure culture, but were significantly different from the means of the counts obtained with the other media. The mean of the counts obtained when IS-medium was used to isolate *D. orientis* from

pure culture differed significantly from the means of the counts obtained with SABS, API and Oxoid medium. This confirmed that IS-medium, when compared with the other media for isolation of *D. orientis* and *D. desulfuricans* from pure culture, yielded the highest numbers.

Table 27a: Analysis of variance by using the Duncan's multiple range test for variables when comparing different media for the isolation of *Desulfotomaculum orientis* from pure culture.

Duncan Grouping*		Mean	N	Medium
	A	3.1367	3	IS-medium
B	A	2.8900	3	Synthetic medium
B	A	2.7233	3	Postgate medium
B		2.5100	3	SABS medium
	C	1.2633	3	API medium
	C	1.0000	3	Oxoid medium

* Means with the same letter are not significantly different @ P = 0.05.

Table 27b: Analysis of variance by using the Duncan's multiple range test for variables when comparing dfferent media for the isolation of *Desulfovibrio desulfuricans* from pure culture.

Duncan Grouping*		Mean	N	Medium
	A	9.1933	3	Oxoid medium
	A	8.9000	3	IS-medium
	B	7.8300	3	SABS medium
	C	5.3867	3	Postgate medium
	D	4.1633	3	API medium
	E	3.5433	3	Synthetic medium

* Means with the same letter are not significantly different @ P = 0.05.

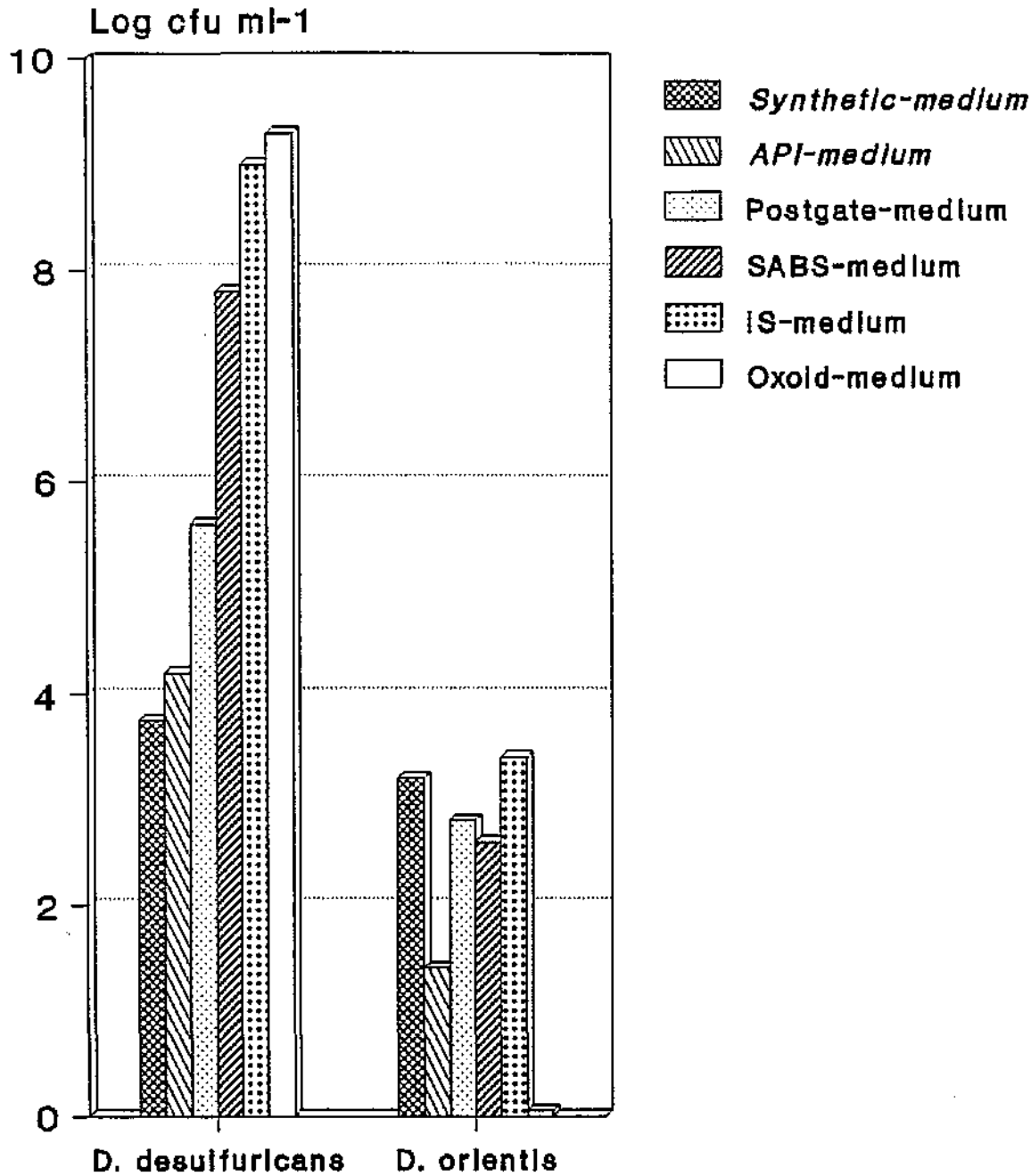
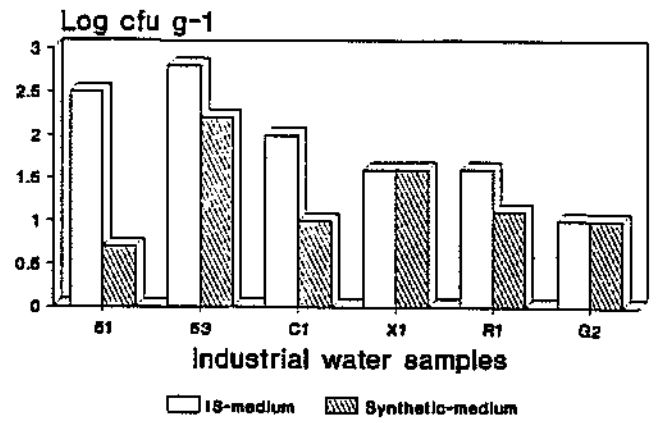
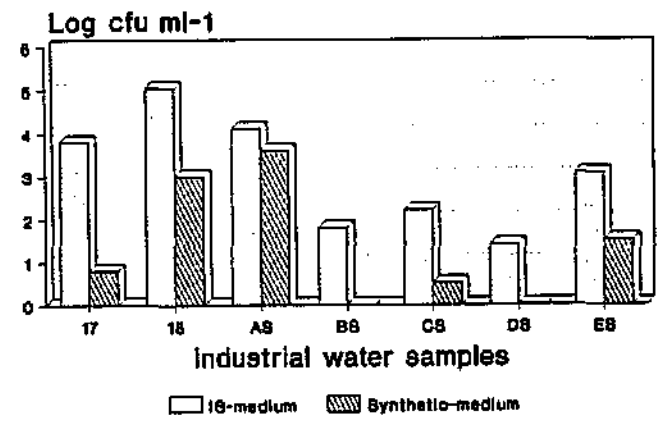


Figure 59: Comparison of different media for the isolation of sulphate-reducing bacteria from pure cultures.





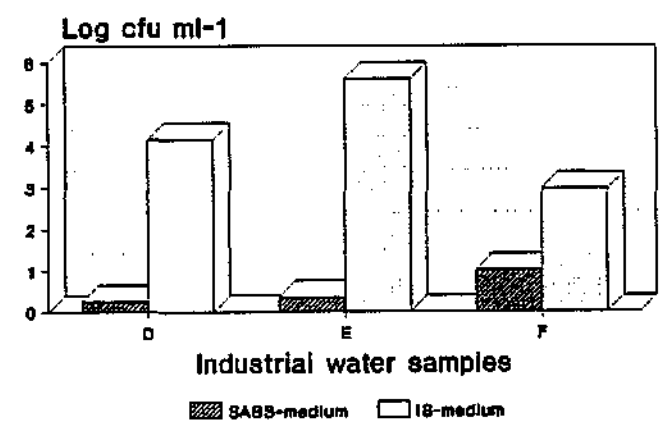


Figure 60: The detection of sulphide-producing bacteria in industrial water samples using different isolation media with lactate as carbon source.

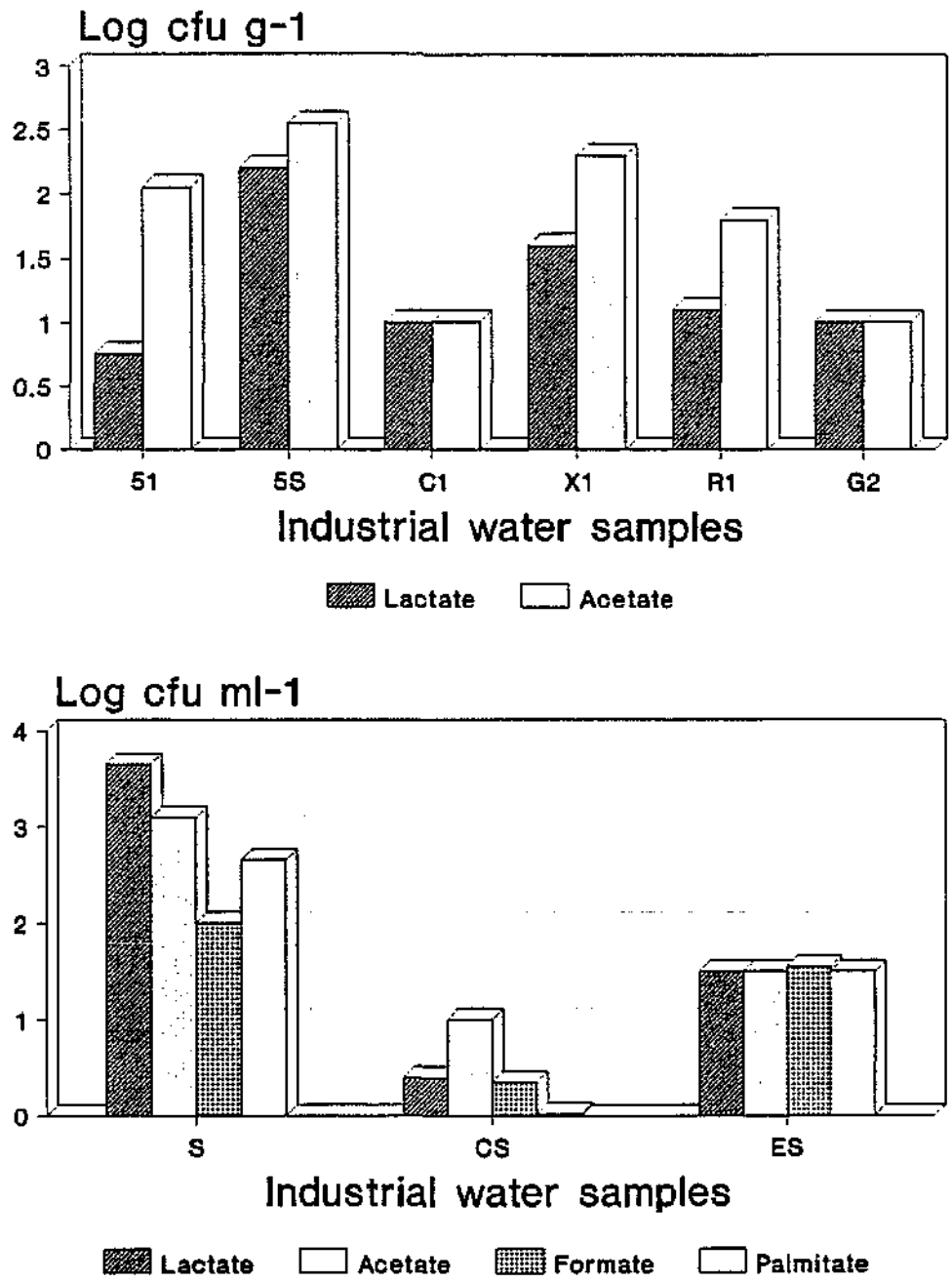


Figure 61: The detection of sulphide-producing bacteria in industrial water samples using the modified synthetic medium with different carbon sources.

3.3 The detection and isolation of sulphide-producing bacteria from industrial water systems using different media and carbon sources

The results of the isolation of sulphide producing organisms from the industry using different media with lactic acid as carbon source are presented in Fig. 60. The highest numbers of sulphide producing bacteria were obtained by using IS-medium.

When comparing the utilization of different carbon sources by SRB present in industrial water systems, H_2S-producing bacteria were isolated using the modified synthetic medium (Pfennig *et al.*, 1981) with lactic acid, acetate, formic acid or palmitic acid as different carbon sources (Fig. 61). The organisms that were isolated from industrial water samples using the modified medium (Pfennig *et al.*, 1981) with lactate as carbon source were not able to utilize acetate. The importance of the use of media with at least lactate and acetate as different carbon sources for the detection of SRB in industrial water samples were confirmed by these results.

3.4 Partial characterization of sulphide-producing organisms isolated from industrial water samples using IS-medium

All the black single colony subcultures isolated from industrial water samples using IS-agar roll tubes were able to grow under aerobic conditions on IS-agar plates. Sixty seven single colonies obtained from the aerobic growth on the IS-agar plates were reinoculated into anaerobic tubes with pre-reduced IS-medium. Twenty six of these colonies produced sulphide under strictly anaerobic conditions. These 26 organisms were oxidase positive, catalyze positive, non fermentative, gram negative, motile rods.

The facultative aerobic organisms produced sulphide under strictly anaerobic conditions when cultured on IS-medium. No sulphide was produced under anaerobic conditions when the organisms were cultured on SABS, Postgate or API-medium. When using different modifications of IS-medium (Table 26) iron sulphide production by these organisms occurred only when cultured on modifications 1, 4 and 5 of IS-medium (Table 26). These results showed that these organisms produced sulphide from sulphite under strictly anaerobic conditions using lactic acid as electron donor. They did not produce sulphide directly from sulphate.

4 DISCUSSION

When comparing API-medium, SABS-medium, IS-medium (Mara and Williams, 1970), Oxoid-medium and the modified synthetic medium (Pfennig *et al.*, 1981), using pure cultures of *D. desulfuricans* and *D. orientis*, the best results were obtained with IS-medium. Although Oxoid-medium and IS-medium yielded the highest counts when isolating *D. desulfuricans* from pure culture, no visible growth of *D. orientis* could be detected when Oxoid-medium was used for isolating this organism from pure culture (Fig. 59).

Sulphate-reducing bacteria are a diverse group of organisms that are able to utilize various carbon sources (Widdel, 1988). The use of isolation media with one carbon source, for example lactate, will detect only SRB that utilize lactate and thus represent only a fraction of the SRB present in the sample tested.

Modified synthetic medium (Pfennig *et al.*, 1981) with lactate, acetate, formate and palmitic acid as different carbon sources were used for the isolation of SRB from industrial water samples. H_2S-producing bacteria that utilized lactate, acetate, formate and/or palmitic acid were isolated from industrial water samples when these different carbon sources were used (Fig. 61). The sulphate-reducing bacteria present in South African industrial water systems are diverse in terms of the utilization of carbon sources and the use of isolation media based on only lactate as carbon source would not represent the whole population of SRB present in the samples tested. H_2S producers which did not grow on lactate based modified medium were isolated using the acetate based medium.

The highest numbers of sulphide producing bacteria were isolated from industrial water samples using IS-medium with lactate as electron donor/carbon source (Fig. 60). Aerobic, facultative aerobic and anaerobic bacteria were isolated from corrosion lesions on metal surfaces (Obuekwe *et al.*, 1981). Some of these organisms were facultative aerobes that reduced ferric to ferrous iron under anaerobic conditions and also reduced, together with SRB, sulphite, thiosulphate and sulphur to sulphide (Obuekwe *et al.*, 1981). According to Atlas and Bartha (Atlas and Bartha, 1987), not only SRB but some species of *Bacillus, Pseudomonas* and *Saccharomyces* as well, produce H_2S from sulphate and sulphite. Experiments performed by Laishley and Krouse (Laishley and Hugh, 1978) provided evidence for both a dissimilatory and a assimilatory pathway for sulphate reduction in *Clostridium pasteurianum* similar to those reported in the genus *Desulfovibrio*. Since such a high number of sulphide producing bacteria were isolated from the industry using IS-medium, which contains sulphate and sulphite as electron acceptors, compared to the modified synthetic medium and SABS-medium with lactate as carbon source, the question arose whether all the sulphide-producing organisms detected with IS-medium in industrial water samples were the classical dissimilatory sulphate-reducing bacteria. Sulphide-producing bacteria isolated from industrial water samples using IS-medium were therefore characterized. These organisms were facultative aerobic gram negative rods, able to produce sulphide from sulphite under strictly anaerobic conditions while utilizing lactic acid. Since these organisms were facultative and not able to produce sulphide from sulphate they were not dissimilatory sulphate reducing bacteria. IS-medium was therefore not selective for SRB only. Medium containing sulphite can therefore not be used in the industry for selectively isolating SRB.

According to Costello (Costello, 1974) and Hardy (Hardy, 1983) the generation of sulphide is of greater importance in corrosion than the cathodic removal of hydrogen. The precise mechanism and role of SRB and other organisms in MIC must still be determined and described. Corrosion is a complex process with many inter-related factors (Crombie *et al.*, 1980; Videla, 1991; Pope and

Dziewulski, 1990). A synergistic reaction between facultative aerobes, which were capable of reducing ferric to ferrous iron and reducing sulphite, thiosulphite and sulphur to sulphide, and SRB could result in an increase in ferrous iron and sulphide and thus in the formation of potentially corrosive ferrous sulphides (Obuekwe *et al.*, 1981).

The importance of sulphide producing organisms other than SRB in industrial water systems and in MIC must be investigated in more detail and determined. IS-medium would be recommended for use in the industry to detect sulphide production, if these facultative sulphide producing bacteria, besides SRB play an important role in MIC. A more selective method should be used for the detection of SRB as group and the diversity of this group of organisms and ability to utilize a wide range of carbon sources must be taken into account. Monitoring total counts of sulphide producing bacteria as well as SRB in industrial systems could render useful information regarding the organisms involved in MIC.

5 ACKNOWLEDGEMENTS

Chapter 14

Shewanella putrefaciens: Facultative anaerobic H_2S-producing bacteria, isolated from industrial cooling water systems

E.E. DE BRUYN and T.E. CLOETE

Abstract

Several gram negative facultative aerobic bacteria cultures that were capable of corroding mild steel were isolated from oil field water (Obuekwe *et al.*, 1981). These bacteria and other iron reducing bacteria, isolated from oil field water were classified as *Shewanella putrefaciens* (Semple and Westlake, 1987). Gram negative facultative aerobic rods capable of anaerobic growth and H_2S-production on lactate using sulphite as electron acceptors, were the dominant bacteria isolated by De Bruyn and Cloete (1993) from industrial cooling water systems using Iron Sulphite medium (Mara and Williams, 1970). In this study these bacteria were identified as *S. putrefaciens*. This is the first time that *S. putrefaciens*, a potentially corrosive bacterium, has been isolated from industrial cooling water systems.

1 INTRODUCTION

Microbial corrosion, primarily due to sulphate-reducing bacteria (SRB), is a significant cause of the corrosion of metal structures in a number of industries. This corrosion has been mainly in cooling systems and heat exchangers (Iverson, 1987). However, there has been increasing evidence that other organisms, in addition to SRB, have been involved in the corrosion process (Crombie *et al.*, 1980, Pope and Dziewulski, 1990 and Videla, 1991). Several gram negative bacteria cultures that were capable of corroding mild steel were isolated from oil field water and classified as members of the genus *Pseudomonas* (Obuekwe *et al.*, 1981). These bacteria and other iron reducing bacteria, also isolated from oil field water, were reclassified as *Shewanella putrefaciens* (Semple and Westlake, 1987). *S. putrefaciens*, also known as *Pseudomonas putrefaciens* or *Alteromonas putrefaciens*, have diverse habitats that include marine and clinical isolates and organisms responsible for the spoilage of cold-stored, protein-rich foods (Moule and Wilkinson, 1989).

Using Iron Sulphite (IS)-medium (Mara and Williams, 1970), De Bruyn and Cloete (1993) found that gram negative facultative anaerobic rods capable of anaerobic growth on lactate using sulphite as electron acceptor were the dominant bacteria isolated from industrial cooling water systems. There are many formulations of media used for enumerating SRB which usually include lactic acid as a carbon source. Several of these different media, including IS-medium, have been used to enumerate SRB from environmental samples (Herbert and Gilbert, 1984). According to Costello (1974) and Hardy (1983) the generation of sulphide is of greater importance in corrosion than the cathodic removal of hydrogen. A synergistic reaction between facultative aerobes, which were capable of reducing ferric to ferrous iron and reducing sulphite, thiosulphate and sulphur to sulphide, and SRB could result in an increase in ferrous iron and sulphide and thus in the formation of potentially corrosive ferrous sulphides (Obuekwe *et al.*, 1981). This is the first time that these facultative aerobic organisms have been isolated from cooling water systems. The objective of this study was therefore to identify these bacteria using Sodium Dodecyl Sulphate-polyacrylamide-gel electrophoresis (SDS-PAGE) of the total soluble cell proteins.

2 MATERIALS AND METHODS

2.1 Origin and isolation of bacteria used in SDS-PAGE

The *S. putrefaciens* strains were obtained from the LMG Culture Collection (University of Gent, Belgium) (Table 28). The isolation of the gram negative H_2S-producing aerobic facultative organisms from industrial cooling water systems is described by De Bruyn and Cloete (1993). A total of 26 organisms, that were oxidase positive, catalase positive, non fermentative, gram negative motile rods,

were isolated from the cooling towers of a petroleum company in Sasolburg, South Africa, using IS-medium (Mara and Williams, 1970). Of these isolates, six were used in this study for further identification.

2.2 Identification of isolates obtained from the cooling towers of a refinery in Sasolburg

The API 20 NE identification system for non-enteric gram negative bacteria were used to identify the industrial isolates. ii) SDS-PAGE of total soluble cell proteins: The organisms (Table 28) were cultured for 24 h at 28°C on Nutrient agar (BIOLAB) plates. Approximately 60 mg of the bacteria of each strain was weighed off in 2 ml Eppendorf tubes. These cells were washed in phosphate buffered solution (pH 6,88) (MERCK) and harvested by centrifugation (12 000 g for 3 min). Subsequently the cell pellets were suspended in sample buffer (Laemmli, 1970). The cell suspensions were sonicated with an Ultrasonic Homogenizer (4710 Series, Cole-Parmer Instruments Co., Chicago, Illinois). Cells were broken by applying 30 to 60 W output for three 15 s intervals with 15 to 20 s cooling in between. The samples were boiled for 10 min and centrifuged for 3 min at 12 000 g. Routinely 15 to 20 μl of a sample was used for electrophoresis.

SDS-PAGE gels were prepared using the method of Laemmli (1970), modified according to Kiredjan *et al.* (1986). Gels were 1,5 mm thick and 160 mm long. Electrophoresis was performed using a Protean II vertical electrophoresis unit from Bio-Rad. A constant current of 25mA/gel for the stacking gel and 35 mA/gel for the separating gel was used. Water at a constant temperature of 15 °C was circulated through the cooling core for the duration of the run. Gels were fixed, stained and destained according to the staining procedure of Anderson and Anderson (1977). *Psychrobacter* sp. was used as standard for the numerical analysis of the gels. Gels were scanned on a Hoefer GS 300 Transmittance/ Reflectance Scanning Densitometer (Hoefer Scientific instruments, San Francisco). Numerical analysis, based on the correlation coefficient (r) which was determined using the unweighted average linkage cluster analysis, was done using the Gel Compar programme version 1.3 supplied by Helix C.V., Belgium.

3 RESULTS

3.1 Identification of H2S-producing bacteria obtained from the cooling towers of a refinery in Sasolburg using the API 20 NE system

The industrial isolates were oxidase positive, catalase positive, non fermentative, gram negative motile rods that produced sulphide from sulphite under strictly anaerobic conditions (De Bruyn and Cloete, 1993). The isolates were identified by using the API 20 NE system as *S. putrefaciens* by 70, 3 %. Atypical test results for *S. putrefaciens* were glucose, maltose and arabinose assimilation.

3.2 Identification of H2S-producing isolates obtained from the cooling towers of a refinery in Sasolburg using SDS-PAGE of total soluble cell proteins

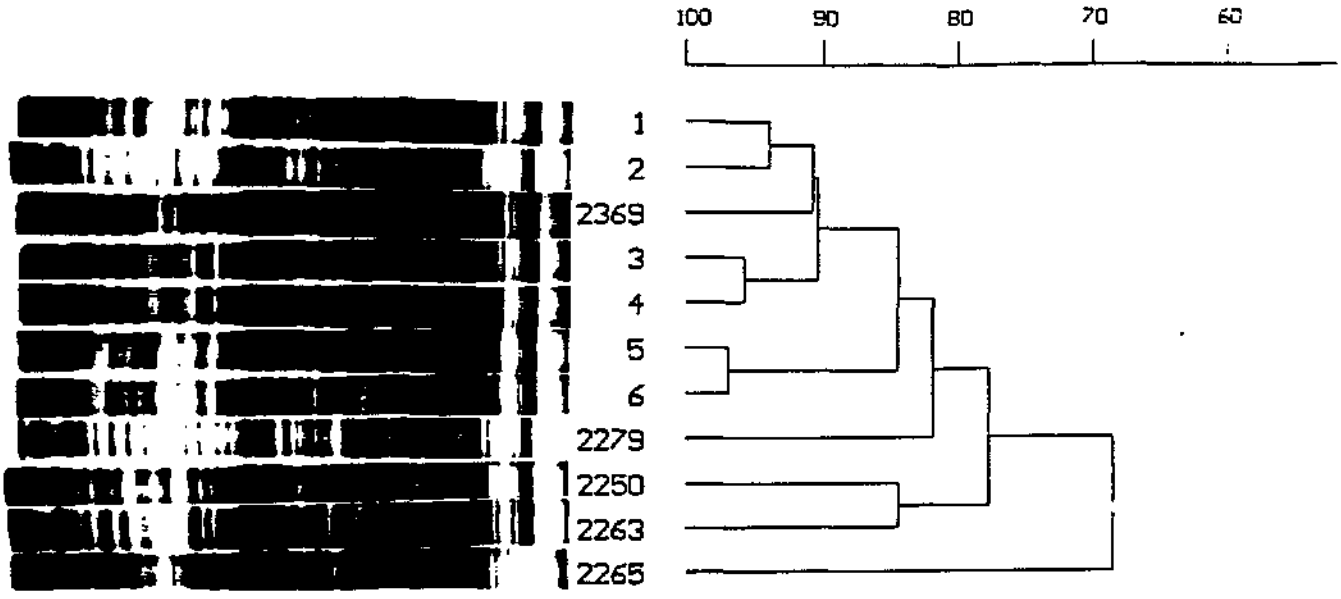


Figure 62: Dendogram of the relationship of different *Shewanella putrefaciens* strains and facultative aerobic H_2S-producing isolates obtained from industrial cooling water systems based on r values, as calculated by the average pair group method using SDS-PAGE of total soluble proteins. LMG numbers of *S. putrefaciens* strains and industrial isolates numbered from 1 to 6 are shown on the dendogram.

Table 28: *Shewanella putrefaciens* strains used for the SDS-PAGE of total soluble cell proteins

Culture collection number of strains	Origin
LMG 2250 or NCTC 10735	Japan, oil-brine
LMG 2263 or NCTC 10737	Cuttlefish
LMG 2265 or NCTC 10738	Faeces
LMG 2279 or NCIB 8768	Oil emulsion
LMG 2369 or NCIB 10473	Butter

The relationships between the industrial isolates and the *S. putrefaciens* strains obtained from the LMG culture collection can be seen on the dendogram (Fig. 62). According to the dendogram 5 groups can be distinguished, Group 1; *S. putrefaciens* (LMG 2369) and Isolates 1, 2 and 3 (r = 0,9), Group 2; Isolates 5 and 6 (r = 0,97), Group 3; *S. putrefaciens* (LMG 2279), Group 4; *S. putrefaciens* (LMG 2250) and *S. putrefaciens* (LMG 2263) (r = 0,85) and group 5; *S. putrefaciens* (LMG 2265).

The industrial isolates 1, 2, 3 and 4 clustered together with *S. putrefaciens* (LMG 2369) at r = 0,9. Isolates 5 and 6 clustered with isolates 1, 2, 3 and *S. putrefaciens* (LMG 2369) at r = 0,83. These isolates were therefore identified as *S. putrefaciens* and closely related to *S. putrefaciens* (LMG 2369) that originated from butter.

4 DISCUSSION

The dominant bacteria isolated from representing industrial cooling water systems (De Bruyn and Cloete, 1993) using IS-medium (Mara and Williams, 1970) were identified in this study as *S. putrefaciens* by using the API 20 NE identification system and SDS-PAGE of total soluble proteins (numerical taxonomy).

S. putrefaciens are a diverse group of bacteria. Strains of *S. putrefaciens* have been divided into four groups on the basis of DNA renaturation and binding studies and phenotypic characterizations (Owen *et al.*, 1978, Van Landschoot and De Ley, 1983). The isolates obtained from industrial cooling water systems were compared with *S. putrefaciens* from various origins (Table 28). The four DNA-DNA homology groups of *S. putrefaciens* (Owen *et al.*, 1978) were used as reference strains in the SDS-PAGE studies: LMG 2279 (group I), LMG 2263 (group II), LMG 2369 (group III) and LMG 2265 (group IV). The four DNA-DNA homology groups of *S. putrefaciens* formed four separate groups in the numerical analysis of the SDS-PAGE gel. The *S. putrefaciens* isolates obtained from industrial cooling water systems were closely related to LMG 2369 (Group III) that originated from butter.

S. putrefaciens has the ability to couple iron reduction to growth or carbon oxidation (Lovley *et al.*, 1989) and to reduce sulphite, thiosulphate and elemental sulphur to sulphide under anaerobic conditions (Semple and Westlake, 1987). An increase in the concentration of ferrous ions together with the production of sulphide would result in the formation of potentially corrosive ferrous sulphide (Costello, 1974, and Hardy, 1983). According to Obuekwe *et al.* (1981) *S. putrefaciens* isolates from oil fluids were capable of corroding mild steel. The importance of *S. putrefaciens* in industrial water systems and in microbial corrosion should therefore be investigated in more detail.

5 ACKNOWLEDGEMENTS

We would like to thenk Helga Dagutat and Prof. P.L. Steyn for assistance with the taxonomic analysis.

Chapter 15

Shewanella putrefaciens as Corrosion Causing Bacterium

E.E. DE BRUYN and T.E. CLOETE

Abstract

According to various authors the most widely distributed and economical important organisms associated with microbial induced corrosion (MIC) are the dissimilatory sulphate-reducing bacteria (SRB). However, there has been increasing evidence that organisms, other than SRB have been involved in the corrosion process. Using iron sulphide medium *Shewanella putrefaciens* was the dominant sulphide producing bacteria isolated from industrial cooling water systems. It was therefore considered necessary to determine the role of these organisms in MIC. Silicon plates coated with a thin layer of copper or nickel were used for rapid screening of the corrosivity of *S. putrefaciens* isolates, *Desulfovibrio desulfuricans*, *Pseudomonas fluorescens* and *Escherichia coli* in liquid medium under anaerobic conditions or on solid medium under aerobic conditions. The results obtained demonstrated the importance of iron sulphide production in the corrosion process. The degree of corrosion varied with the bacterial species and media used. The most severe corrosion obtained on solid media under aerobic conditions was with *S. putrefaciens* cultured on nutrient agar. When using benzyl viologen as evidence for cathodic depolarization it was demonstrated that *S. putrefaciens* has the ability to utilize cathodic hydrogen. Microscope studies were also performed to determine the role in MIC of *S. putrefaciens* and *D. desulfuricans* respectively, cultured in iron sulphide medium with simultaneous production of iron sulphide, and 3CR12 metal coupons. After exposure of the coupon to a *D. desulfuricans* culture, a thin layer of iron sulphide covered the metal surface, whereas exposure of the metal to a *S. putrefaciens* culture resulted in bulk iron sulphide-like deposits. This study indicated that *S. putrefaciens* could play an important role in MIC.

1 INTRODUCTION

Microbiological induced corrosion (MIC) can result in damage and economic losses in the petroleum industries, power generation stations and chemical and other process industries (Iverson, 1987). According to various authors the most widely distributed and economically important organisms associated with MIC are the dissimilatory sulphate-reducing bacteria (SRB) (Boivin and Costerton, 1991, Crombie *et al.*, 1980, Ford and Mitchell, 1990, Hamilton, 1985). However, there has been increasing evidence that organisms, other than SRB have been involved in the corrosion process (Ford and Mitchell, 1990, Iverson, 1987, Videla, 1991).

Two marine isolates, a *Pseudomonas* sp. and *Vibrio alginolyticus* were reported to corrode a 70:30 copper nickel alloy (Gomez *et al.*, 1989). Thin copper films were corroded by a rod shaped gram-variable facultative anaerobic bacteria isolated from copper coupons exposed to flowing municipal water (Bremer and Geesey, 1991). The colonies of both a *Pseudomonas* strain and *Serratia marcescens* on agar plates quickly corroded metal films that were brought into contact with the bacterial colonies (Pedersen *et al.*, 1988). A variety of aerobic, anaerobic and facultative aerobic bacteria could readily be isolated from oil, oilfield water and internal pipeline encrustations. Among such bacteria were a group of facultative aerobic, oxidase positive, non sporeforming motile rods with a polar flagellum. These organisms were capable of reducing ferric to ferrous iron and reducing sulphite, thiosulphate and elemental sulphur (but not sulphate) to sulphide under anaerobic conditions. Synergistic interactions between SRB and these bacteria would result in an increase in the concentration of ferrous sulphide (Obuekwe *et al.*, 1981). The corrosive isolates of Obuekwe *et al.* (1981) as well as other organisms isolated from oil field water were identified as *Shewanella putrefaciens*.

Using iron sulphite (IS)-medium (Mara and Williams, 1970), De Bruyn and Cloete (1992a) found that gram negative facultative anaerobic rods capable of anaerobic growth on lactate using sulphite as electron acceptor were the dominant bacteria isolated from industrial cooling water systems. These organisms were identified as *S. putrefaciens* by using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (De Bruyn and Cloete, 1992b). Since these organisms were the dominant sulphide producers in the water cooling systems sampled, and were detected using IS-medium (a medium commonly used for the enumeration of SRB from environmental samples) (Herbert and Gilbert, 1984) it was considered necessary to determine the role of these organisms in MIC.

2 MATERIALS AND METHODS

2.1 Cultures

Desulfovibrio desulfuricans subsp. *desulfuricans* (DSM No 1924), *Desulfovibrio africanus* (DSM No 2603), *Desulfotomaculum orientis* (DSM No 765) and *Desulfotomaculum guttoideum* (DSM No 4024) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen. *Shewanella putrefaciens* (LMG 2369) was obtained from the LMG culture collection (University of Gent, Belgium). H_2S-producing aerobic facultative organisms were isolated from industrial cooling water systems using IS-medium (Mara and Williams, 1970) as described by De Bruyn and Cloete (1992a). A total of 26 organisms, that were oxidase positive, catalase positive, non fermenting gram negative, motile rods, were isolated from the cooling towers of a petroleum company in Sasolburg, South Africa. Of these isolates, six were further identified by using SDS-PAGE as *S. putrefaciens* (De Bruyn and Cloete, 1992b). *Pseudomonas fluorescens* was isolated from water cooling systems and identified by Cloete *et al.* (1989). *Escherichia coli* K 12 was obtained from the culture collection of the Department of Microbiology and Plant Pathology, University of Pretoria, South Africa.

2.2 Screening for bacterial corrosion using silicon plates coated with a thin film of metal

Slides were supplied by the Council for Scientific and Industrial Research (CSIR), Pretoria, South Africa. Silicon 100 was coated with a 1000 Å thick layer of copper or nickel (Pedersen *et al.*, 1988). Corrosion tests were performed in liquid media and on agar plates. The following media were used; IS-medium (Mara and Williams, 1970), IS-medium with the ferrous sulphate and iron(III) citrate omitted and trisodium citrate (0,3 g/1000 ml) added (IS-Fe), synthetic medium (Pfennig *et al.*, 1981) and synthetic medium with filter sterilized $FeSO_4.7H_2O$ solution (5g/20 ml) added to the medium (20 ml solution/1000 ml) after sterilization of the medium (synthetic + Fe). The media all contained lactate as carbon source. Resazurin (1 ml of 0,1 % w/v solution) was added to the media as a redox potential indicator. For solid media, 1,5 % agar was added and for screening methods where nutrient agar (MERCK) was used, bromothymol blue indicator (0.1 g/1000 ml) was added.

Anaerobic tubes (Hungate, 1969) were filled with liquid medium. Copper coated plates were added to the different liquid media prior to autoclaving. Nickel coated plates were sterilized with alcohol, flamed and added aseptically to the different liquid media after sterilization of the media. SRB-strains were precultured under strictly anaerobic conditions using IS-Fe liquid medium and *S. putrefaciens*, *E. coli* and *P. fluorescens* strains aerobically, using nutrient broth (MERCK). Hungate tubes of each medium (IS, IS-Fe, synthetic, synthetic + Fe) with a copper coated plate were inoculated in duplicate using the different precultures as inoculum. Uninoculated tubes were included in the experiments as controls. After inoculation the liquid media were reduced by using titanium(III) citrate (Zehnder and Wuhrmann, 1976). The anaerobic tubes were sealed with neoprene rubber stoppers and screw

caps to exclude oxygen. The Hungate tubes were incubated at 30 °C for up to 7 days. The copper coated plates were periodically investigated for the removal of the metal film from the silicon. The experiments were repeated using the nickel coated silicon plates.

Alternatively, 2 agar plates of each medium (IS, IS-Fe, nutrient agar) were streaked with the following precultered organisms; *S. putrefaciens* 2369, three *S. putrefaciens* industrial isolates, *P. fluorescens* and *E. coli*. Silicon plates coated with nickel were sterilized with alcohol, flamed and placed on an agar plate with the metal side in contact with the agar surface streaked with the bacteria cultures. Controls where the agar surface were not streaked with bacteria cells were included. The plates were incubated aerobically at 28 °C for up to 7 d.

2.3 SEM studies of metal coupons exposed to Desulfovibrio and Shewanella in batch culture

Anaerobic containers were filled with IS-medium (Mara and Williams, 1970). A 3CR12 metal coupon was added to each container with media and autoclaved. *D. desulfuricans* were precultured under anaerobic conditions in IS-Fe medium. *S. putrefaciens* (LMG 2369) and a *S. putrefaciens* isolate were precultured in nutrient broth under aerobic conditions. Anaerobic containers were inoculated with *D. desulfuricans*, *S. putrefaciens* LMG 2369 and the *S. putrefaciens* isolate, respectively. This was done in triplicate. Three bottles were left uninoculated as controls. The media were reduced after inoculation with titanium(III) citrate (Zehnder and Wuhrmann, 1976) and sealed to exclude oxygen. After 4 weeks of incubation at 30 °C, the bottles were opened, the 3CR12 coupons removed and rinsed with sterile dist. H_2O.

The coupons were fixed by the following series of treatments: 2 % Gluteraldehyde in 0,1M Na cacodylate buffer; 3 X 15 min. 50 % ethanol; 1 X 15 min., 70 % ethanol; 1 X 15 min, 90 % ethanol; 1 X 15 min, 100 % ethanol; 3 X 15 min. Coupons were drained and freed from traces of ethanol by critical point drying with CO_2 until no ethanol could be traced in the outlet and coated with gold plasma and examined by using a Hitachi S-450 scanning electron microscope.

2.4 The use of benzyl viologen as evidence for cathodic depolarization

The method of Iverson (1966) was used to demonstrate cathodic depolarization with mild steel using Benzyl viologen. *D. desulfuricans* was precultured on trypticase soy broth with 2% agar in a hydrogen atmosphere. Iron(III) citrate (0,5 g/ 1000 ml) was added to the trypticase soy broth for preculturing of *S. putrefaciens* LMG 2369 and a *S. putrefaciens* industrial isolate. The precultured cells were placed on the surface of agar (2%) containing 0.01M tris buffer (pH 7.0) and 0.01 % benzyl viologen. Sterilized coupons of 1010 mild steel were placed on the surface of the agar plates with one end on the area

with bacteria cells. After incubation at 30 °C for 3 d under anaerobic conditions, the metals were removed from the agar. The iron in the agar was made visible by adding a 10% aqueous solution of potassium ferricyanate.

3 RESULTS

3.1 Screening for bacterial corrosion using silicon plates coated with a thin film of metal

Table 29a: The action of various bacteria in liquid media under anaerobic conditions on copper.

Bacterial cultures	**Corrosion of copper coated silicon in the following media**			
	Corrosion of IS	**IS - Fe**	**Synth**	**Synth + Fe**
Desulfovibrio desulfuricans	++++*	++	++	++++*
Desulfovibrio africanus	++++*	++	++	++++*
Desulfotomaculum orientis	++++*	++	++	++++*
Desulfotomaculum guttoideum	++++*	++	++	++++*
Shewanella putrefaciens 2369	++++*	+-	--	--
S. putrefaciens isolate 1	++++*	++	--	--
S. putrefaciens isolate 3	++++*	++	--	--
S. putrefaciens isolate 5	++++*	++	--	--
S. putrefaciens isolate 6	++++*	+-	--	--
Escherichia coli	++	--	--	--
Pseudomonas fluorescens	+-	--	--	--
Control	--	--	--	--

IS: Iron Sulphite medium (Mara and Williams, 1970)

IS - Fe: Iron sulphite medium with the ferrous sulphate and Iron(III) citrate omitted and trisodium citrate (0,3 g/1000 ml) added.

Synth: Synthetic medium of Pfennig *et al.*, (1981)

Synth + Fe: Synthetic medium with 0,5g/1000ml $FeSO_4.7H_2O$

*, Iron sulphide precipitate present, ++ Metal film degraded, + metal partially degraded and/or lifted from silicon, - Metal film intact.

The results obtained when corrosion tests were performed with copper and nickel coated silicon in inoculated liquid medium under anaerobic conditions are represented in Table 29a and 29b. The copper and nickel films were severely corroded by SRB- and *S. putrefaciens* strains when iron sulphide precipitates formed in the media by the reaction of iron(II) with the bacterially produced sulphide ion. In the absence of iron in the medium, the copper film was partially degraded and/or lifted from the silicon by the SRB-strains and *S. putrefaciens*. When iron was omitted from IS-medium, the nickel film was not corroded by *S. putrefaciens*, whereas the SRB-strains did corrode the nickel film in the absence of iron in the media. The action of *E. coli* and *P. fluorescens* corroded the copper film slightly

Table 29b: The action of various bacteria in liquid media under anaerobic conditions on nickel.

Bacterial cultures	**Corrosion of copper coated silicon in the following media**			
	Corrosion of IS	**IS - Fe**	**Synth**	**Synth + Fe**
Desulfovibrio desulfuricans	++++*	++	++	++++*
Desulfovibrio africanus	++++*	++	++	++++*
Desulfotomaculum orientis	++++*	++	++	++++*
Desulfotomaculum guttoideum	++++*	++	++	++++*
Shewanella putrefaciens 2369	++*	--	--	--
S. putrefaciens isolate 1	++*	--	--	--
S. putrefaciens isolate 3	++*	--	--	--
S. putrefaciens isolate 5	++*	--	--	--
S. putrefaciens isolate 6	++*	--	--	--
Escherichia coli	--	--	--	--
Pseudomonas fluorescens	--	--	--	--
Control	--	--	--	--

IS: Iron Sulphite medium (Mara and Williams, 1970)

IS - Fe: Iron sulphite medium with the ferrous sulphate and Iron(III) citrate omitted and trisodium citrate (0,3 g/1000 ml) added.

Synth: Synthetic medium of Pfennig *et al.*, (1981)

Synth + Fe: Synthetic medium with 0,5g/1000ml $FeSO_4.7H_2O$

*, Iron sulphide precipitate present, ++ Metal film degraded, + metal partially degraded and/or lifted from silicon, - Metal film intact.

in IS-medium. However, these two organisms did not corrode the copper film in the other media or the nickel film. *S. putrefaciens* did not corrode the copper or the nickel film in synthetic medium or synthetic+Fe medium. Since these media contained no sulphite or ferrous ions the *S. putrefaciens* strains were unable to grow on these media.

The results obtained when corrosion tests of nickel coated silicon plates were performed on a solid surface under aerobic conditions are shown in Table 30
. When *S. putrefaciens* cells were cultured on IS-medium 1 out of 9, 1 out of 10, 0 out of 6 and 0 out of 2 nickel plates were corroded for *S. putrefaciens* 2369 and *S. putrefaciens* isolates 1, 3 an 4, respectively. However when culturing these bacteria on IS-Fe agar, 5 out of 9, 6 out of 7, 3 out of 5 and 1 out of 2 coupons, respectively, corroded. All the coupons corroded when *S. putrefaciens* cells were cultured on nutrient agar. *P. fluorescens* corroded 4 out of 11 plates when cultured on IS-medium, 0 out of 9 when cultured on IS-Fe medium and 1 out of 3 when cultured on nutrient agar. *E. coli* corroded 2 out of 11 plates on IS-medium. No corrosion was observed when *E. coli* was cultured on IS-Fe or nutrient agar. Corrosion of nickel on nutrient agar by *P. fluorescens* was associated with acid production as indicated by the pH indicator Bromothymol blue that was incorporated into the agar.

Table 30: The action of various bacteria, cultured on solid media under aerobic conditions, on nickel.

Bacterial Cultures	Corrosion of nickel coated silicon plates indicated as the amount of plates corroded out of the total amount tested when placed on the following media		
Shewanella putrefaciens 2369	1/9	5/9	3/3
S. putrefaciens isolate 1	1/10	6/7	3/3
S. putrefaciens isolate 3	0/6	3/5	2/2
S. putrefaciens isolate 4	0/2	1/2	2/2
Escherichia coli	2/11	0/9	0/3[a]
Pseudomonas fluorescens	4/11	0/9	1/3[a]

IS: Iron Sulphite medium (Mara and Williams, 1970)

IS-Fe: Iron sulphite medium with the ferrous sulphate and Iron(III) citrate omitted and trisodium citrate (0,3 g/1000 ml added).

a: Acid production

3.2 SEM studies of metal coupons exposed to Desulfovibrio and Shewanella in batch culture

The metal surface of a 3CR12 coupon before exposure to culture medium is shown in Fig. 63a. In Fig. 63b areas of chemical attack were visible after 4 weeks of exposure in IS-medium under anaerobic conditions. After exposure of the coupon to IS-medium inoculated with *D. desulfuricans* a thin layer of iron sulphide covered the metal surface (Fig 64a and 64b). In Fig. 64b cracks in the iron sulphide film with *Desulfovibrio* cells are shown. After exposure of a 3CR12 coupons for 4 weeks to IS-medium inoculated with a *S. putrefaciens* industrial isolate, bulk iron sulphide-like crystals deposited on the metal surface. Areas of corrosion are visible underneath the deposits (Fig. 65a). In Fig. 65b the attachment of *S. putrefaciens* cells to the metal surface is visible. Bulk iron sulphide-like deposits with corrosion areas on the metal surface exposed to IS-medium inoculated with *S. putrefaciens* 2369 are shown in Fig. 66.

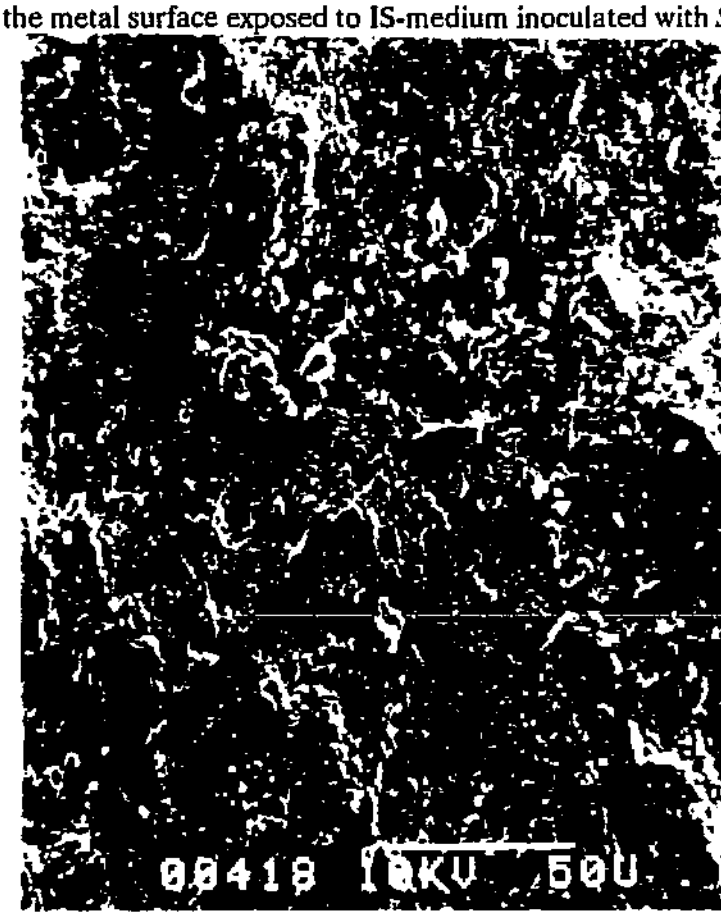


Figure 63a: SEM micrograph of the surface of a 3CR12 coupon before exposure to culture media (490 X)

Figure 63b: SEM micrograph of 3CR12 after 4 weeks' exposure to IS-medium under anaerobic conditions (580 X)

Figure 64a: SEM micrograph of 3CR12 exposed to IS-medium inoculated with *Desulfovibrio desulfuricans* (580 X)

Figure 64b: SEM micrograph of 3CR12 exposed to IS-medium inoculated with *Desulfovibrio desulfuricans* (4 500 X)

Figure 65a: SEM micrograph of 3CR12 exposed to IS-medium inoculated with a *Shewanella putrefaciens* isolate (130 X)

Figure 65b: SEM micrograph of 3CR12 exposed to IS-medium inoculated with a *Shewanella putrefaciens* isolate (5 000 X)

Figure 66: SEM micrograph of 3CR12 exposed to IS-medium inoculated with a *Shewanella putrefaciens* 2369 (1 300 X)

3.3 The use of benzyl viologen as evidence for cathodic depolarization

After removal of the metal coupons from the plates a dark purple area of reduced benzyl viologen was observed in the agar underneath the area covered with cells. Lighter areas of reduced benzyl viologen, probably due to the direct reduction of the dye by the metal, were observed in the agar underneath both ends of the coupon (Fig. 67a). These areas disappeared (oxidized) after a short time and left the heavily reduced area produced by cellular (Fig 67b) reduction. After the agar plates were developed with potasium ferricyanide a large concentration of Fe^{++} ions under the coupon not in contact with the cells (anode) and relatively few Fe++ ions at the cathode (coupon in contact with the cells) were visible (Fig. 67c). These results were obtained with *D. desulfuricans*, *S. putrefaciens* 2369 and a *S. putrefaciens* industrial isolate.

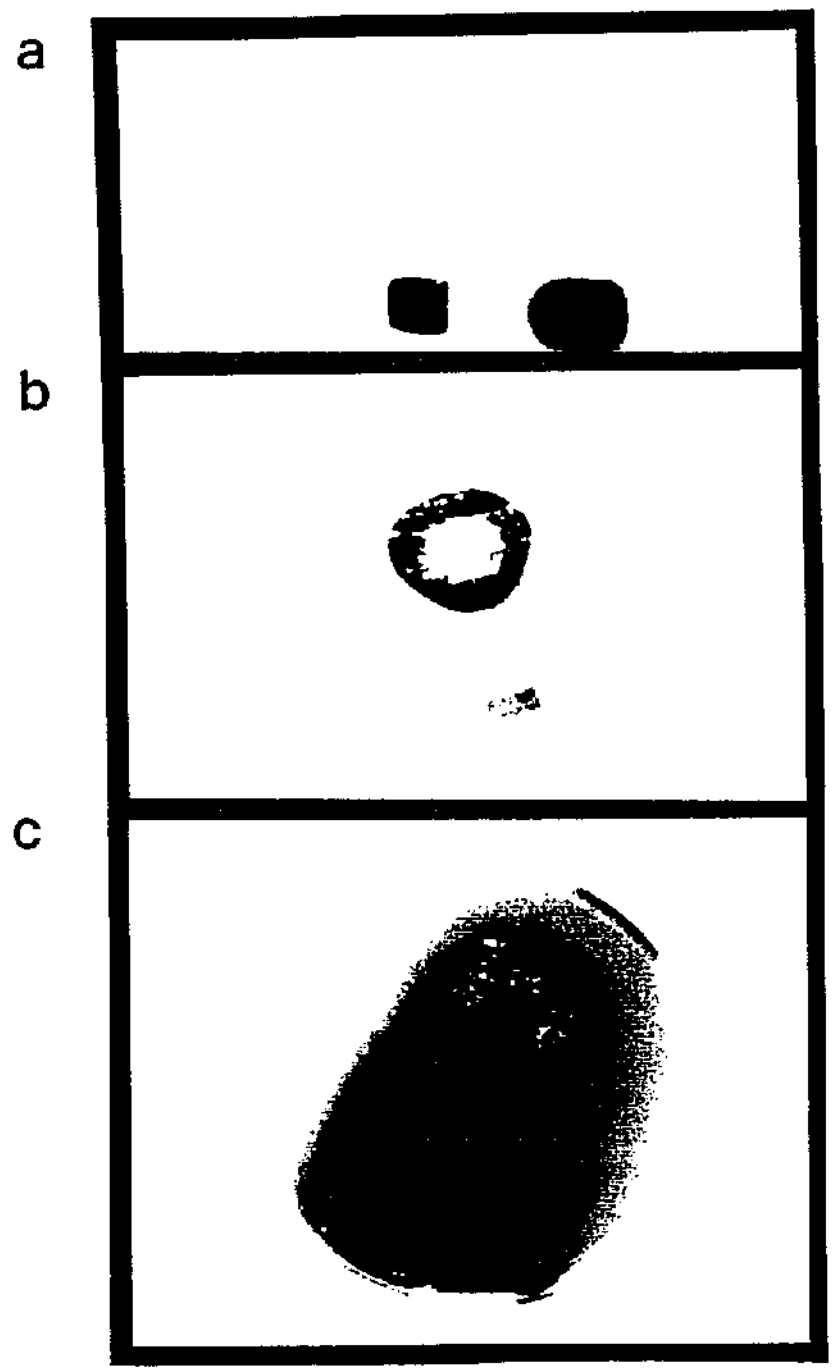


Figure 67: Areas in agar under mild steel coupon indicating locations of reduced benzyl viologen and ferrous ions. (a) Agar surface immediately after removal of steel coupon. (b) The same plate 10 minutes later. (c) Plate after development with potassium ferricyanide showing heavy Fe^{++} concentration at the anode

4 DISCUSSION

The use of silicon coated with a thin defined layer of metal for corrosion studies was based on the method of Pedersen *et al.*, (1988). The method is easy and may be used for rapid screening of the corrosivity of bacterial isolates (Pedersen *et al.*, 1988). Copper and nickel was chosen as test material mainly because of its resistance to corrosion and the frequent use of nickel in metal alloys (Iverson, 1987). The corrosive ability of *S. putrefaciens* was compared with *D. desulfuricans*, the most widely distributed and economically important organism associated with corrosion (Boivin and Costerton, 1991, Crombie et al., 1980, Ford and Mitchell, 1990, Hamilton, 1985), as well as two organisms frequently isolated from water; *E. coli* and *P. fluorescens*.

The results obtained demonstrated the importance of iron sulphide production in the corrosion process. Severe corrosion was obtained when both copper and nickel coated silicon were exposed to media where iron sulphide precipitates formed by the reaction of iron(II) present in the media with the sulphide ion, produced by both *D. desulfuricans* and *S. putrefaciens*. The role of iron sulphides in the corrosion process is well documented (Booth *et al.*, 1968, Hamilton, 1985, King and Miller, 1971). In the absence of iron in the media, the copper film was partially degraded and / or lifted from the silicon by the SRB-strain and *S. putrefaciens*, indicating that besides the production of iron sulphide, other mechanisms are involved in the corrosion process. The mere production of hydrogen sulphide can cause corrosion (Iverson, 1987).

The results obtained when corrosion tests of nickel coated plates were performed on solid surfaces under aerobic conditions showed that the degree of corrosion varied with the bacterial species and media used. The most severe corrosion obtained, was with *S. putrefaciens* cultured on nutrient agar.

Little corrosion was obtained with *P. fluorescence* and *E. coli*. Corrosion obtained when *P. fluorescens* cells were cultured on nutrient agar was accompanied by the production of acid which was not the case with *S. putrefaciens* indicating different corrosion mechanisms for *S. putrefaciens*. The action of *E. coli* and *P. fluorescens* corroded the copper film slightly in liquid IS-medium. A wide variety of organisms are capable of colonizing metals and thereby causing an oxygen differential and establish corrosive oxygen-differential cells (Iverson, 1987, Videla, 1991). Extracellular polymers produced by bacteria (Ford and Mitchell, 1990) as well as inorganic and organic acids can be corrosive (Iverson, 1987).

The most widely accepted theory for mechanisms of corrosion involves cathodic depolarization. This theory was first postulated by von Wolzogen Kuhr and Van der Vlught (1934). Results obtained by Daumas *et al.* (1988) showed that, although the influence of iron sulphide deposition on the surface

was not negligible, the major mechanism for corrosion was the oxidation of cathodically formed hydrogen (Daumas *et al.*, 1988). Although SRB were able to utilize cathodic hydrogen, the concomitant generation of sulphide was probably of more significance in terms of corrosion (Hardy, 1983).

The experiment of Iverson (1966) using benzyl viologen as evidence for cathodic depolarization was repeated with *S. putrefaciens* to determine whether this organism, besides the production of iron sulphide, was capable of the cathodic removal of hydrogen. Evidence is provided for the utilization of cathodic hydrogen by *S. putrefaciens*. The use of benzyl viologen was questioned by Costello (1974) who reported that the oxidized form of the alternative electron acceptor, benzyl viologen, would depolarize mild steel and that the additional depolarization seen when SRB were present was due to their ability to re-oxidize reduced benzyl viologen. This statement, however, was based on a described procedure for the assay of bacterial hydrogenase using methyl viologen, instead of benzyl viologen. Redox dyes, therefore, can be used as electron acceptor (Iverson, 1987). This experiment, using benzyl viologen, was performed to determine whether *S. putrefaciens* has the ability to reduce benzyl viologen by utilizing cathodic hydrogen.

Microscope studies of the interaction under laboratory conditions between some bacterial isolates from crude oil and oil field water, identified as *S. putrefaciens* by Semple and Westlake (1987), and mild steel coupons submerged in cultures of the organism were performed (Obuekwe *et al.*, 1981). Under microaerobic conditions and in the absence of the bacteria, a dense, crystalline amorphous coat formed on the surface of the steel coupon. In the presence of the bacteria, the surface coat was extensively removed, exposing the bare metal to the environment. Modified Butlin medium, used in the experiments performed by Obuekwe *et al.* (1981) contained no sulphide or iron(II) source and B10-medium no sulphide. *S. putrefaciens* are capable of reducing sulphite, thiosulphate and elemental sulphur to sulphide (Semple and Westlake, 1987). Microscope studies were performed to determine the interaction under laboratory conditions between *S. putrefaciens* cultured in IS-medium (containing sulphite and iron), with simultaneous production of iron sulphide, and 3CR12, a metal frequently used in the industry.

After exposure of the coupon to IS-medium inoculated with *D. desulfuricans* a thin layer of iron sulphide covered the metal surface (Fig. 64a and 64b). After exposure of the coupon to IS-medium inoculated with *S. putrefaciens* bulk iron sulphide-like deposits were present on the surface of the medium (Fig 65a, 4). The nature of adherent sulphide film that was formed in iron containing media was investigated (King *et al.*, 1973a, King *et al.*, 1973b). FeS may be formed as a film on the surface of the iron or as bulk FeS. In the former case the film usually inhibits corrosion, but may break down, with an increase in corrosion rate. In the case where bulk FeS formed, preventing film formation, the corrosion rates were very high (Iverson, 1987, King *et al.*, 1973a, King *et al.*, 1973b).

Microbial induced corrosion is rarely linked to a single mechanism or to a single micro-organism (Videla, 1991). *S. putrefaciens* can be considered as a potentially corrosive organism. Under anaerobic conditions these organisms are capable of iron sulphide production and attachment to metal surfaces as well as the removal of cathodic hydrogen. These bacteria reduce ferric to ferrous iron under anaerobic conditions. *S. putrefaciens* is also capable of corroding nickel under aerobic conditions. This study indicated that *S. putrefaciens* could play an important role in MIC.

5 ACKNOWLEDGEMENTS

We thank Fatima Da Silva for technical assistance.

Chapter 16

The Malthus system for biocide efficacy testing against *Desulfovibrio desulfuricans*

E.E. DE BRUYN, E. CROUKAMP and T.E. CLOETE

Abstract

Microbiological induced corrosion (MIC) makes an important contribution to corrosion in various industries. Considerable success has been achieved by the use of biocides. Little information for controlling MIC is however available on the effectivity of biocides against SRB due to the difficulties of culturing these organisms using conventional techniques. Conductance changes monitored using the Malthus system was evaluated as an alternative method of estimating numbers of *Desulfovibrio desulfuricans* for laboratory biocide evaluations. The correlation of $\log_{10}$ counts of *Desulfovibrio* cells in IS-medium using conventional techniques with detection times using the Malthus systems was highly significant ($r = 0.974$), indicating that the Malthus system can be used as a alternative method to conventional media for the enumeration of SRB. Growth studies of *Desulfovibrio* using the Malthus system were useful in the evaluation of biocides.

1 INTRODUCTION

The importance of dissimilatory sulphate-reducing bacteria (SRB) in microbial induced corrosion (MIC) has been widely recognized for many years. Whilst their role in the sulphur cycle is fundamental in maintaining our environment, the adverse economic consequences of their activities can be devastating in industrial processes. These bacteria can result in health hazards and corrosion of equipment and pipe lines (Boivin and Costerton, 1991, Crombie *et al.*, 1980, Ford and Mitchell, 1991, Hamilton, 1985). The detection and monitoring of SRB in industrial water systems as well as their control through the use of biocides are therefore important to the industry.

The use of biocides to control biofouling in industrial water systems is an accepted practice (Cloete *et al.*, 1992). However, incorrect use of biocides give rise to biofouling and resistance build - up in bacteria (Brözel and Cloete, 1991). It is therefore essential to select the correct biocide or combinations and their respective concentrations for the organisms to be killed. There are a variety of techniques for determining the effectivity of biocides (Hill *et al.*, 1989, Cloete *et al.*, 1990). Little published information is however available on the effectivity of biocides against SRB (Sharma *et al.*, 1987).

There are many culture media formulations available that can be used for enumerating SRB (Ferodak *et al.*, 1987, Pankhurst, 1971, Pfennig *et al.*, 1981, Postgate, 1984). The preparation of anaerobic media is difficult and laborious (Gaylarde and Cook, 1987). It was recommended that media should be incubated for up to 28 days (Herbert and Gilbert, 1984). The use of alternative methods on the other hand, such as antibodies (Bobowski and Nedwell, 1987, Gaylarde and Cook, 1987, Odom *et al.*, 1991), have a low sensitivity. The high cost involved in using nucleic acid probes (Amann *et al.*, 1990, Amann *et al.*, 1992) and antibodies limit their use in the industry as well as in routine evaluations of biocides in the laboratory. Because of the difficulties associated with the enumeration of SRB, biocide evaluations against SRB have been neglected in the past.

Electrical methods (conductance, impedance and capacitance) are established methods of monitoring microbial growth and estimating bacterial numbers (Richards *et al.*, 1978). One such system (Malthus) is based on the automated monitoring of electrical conductance in growing bacterial cultures. Conductance is measured by the introduction of platinum electrodes in the medium and the application of a low frequency voltage. When conductance values increase beyond a threshold value, these are recorded by the system and displayed graphically. The change detected in conductance is due to the metabolism of the constituents of the culture medium by the organisms. The time lapse between inoculation and a noticeable change in conductance are termed the detection time. Detection time is inversely proportional to the logarithm of the number of viable organisms inoculated into the medium assayed so that the instrument can be used for determining bacterial numbers (Gibson, 1985).

Gibson (1987) used conductance measurements (Malthus Instruments, LTD Stoke and Trent, UK) to detect the growth of *Clostridium botulinum* in selective medium. This indicated that the Malthus system had successfully been used for enumerating bacteria using selective media.

Therefore conductance changes monitored using the Malthus system was evaluated as an alternative method of estimating numbers of *Desulfovibrio desulfuricans* for laboratory biocide evaluations. Not all culture media may be appropriate for conductive measurements (Gibson, 1987). IS-medium (Mara and Williams, 1970) was chosen for these experiments, since this medium yielded the highest numbers when counting pure cultures of *D. desulfuricans*, when comparing this media with other generally used culture media for SRB (De Bruyn and Cloete, 1992).

2 MATERIALS AND METHODS

2.1 Test organism

Desulfovibrio desulfuricans subsp. *desulfuricans* (DSM 1924) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM).

2.2 Culture medium

Six ml of Iron sulphite (IS)-medium (Mara and Williams, 1970) was dispensed into Malthus tubes which were then autoclaved and used for conductance studies. Resazurin (1 ml of a 0,1 % w/v solution) was added to the media as a redox-potential indicator. Titanium(III) citrate (Zehnder and Wuhrmann, 1976) was used as reducing agent. Anaerobic tubes with 5 ml pre reduced sterile IS-medium (Mara and Williams, 1970) with 1,65 % w/v agar was used for the enumeration of *Desulfovibrio* cells. The tubes were filled with a gas phase of 20 % CO_2, 10% H_2, balanced with N_2 and sealed with neoprene rubber stoppers and screw caps.

2.3 Malthus calibration experiments

D. desulfuricans was precultured in IS-medium under anaerobic conditions at 30 °C for 3 d. A primary dilution series (10^{-1} to 10^{-9}) was prepared from the culture. A secondary dilution series (10^{-1} to 10^{-9}) was prepared from each primary dilution. A 0,1 ml aliquot of each of the latter dilutions were inoculated into anaerobic tubes containing molten (45°C) IS-agar. After inoculation and preparation of agar roll tubes (Hungate, 1969) these were incubated at 30 °C for 14 d. All the black colonies that developed after 14 d were counted as being *Desulfovibrio*. Subsequently, 23 Malthus tubes (the minimum required amount for statistical analysis) were inoculated with 1 ml of the primary and secondary dilution series. Conductance readings at 30 °C were recorded for up to 48 h, using the Malthus 2000 detection system (Swift Micro Laboratories (Pty) Ltd.). Detection times were defined as the time at which there was a significant change in conductance (Fig. 68). Detection times were

against the conventional *Desulfovibrio* counts in agar roll tubes containing IS-agar. Statistical analysis of the results were performed using the Malthus statistic software version H2.02.01. (Malthus 2000, Swift Micro Laboratories).

2.4 Biocide evaluations

All experimental work was carried out in triplicate. A *Desulfovibrio* culture was grown anaerobically in IS-medium (Mara and Williams, 1970). Initial numbers of this culture was determined by inoculating six Malthus tubes each, with 1 ml of the culture and monitoring detection time using the Malthus 2000 system. After determining the initial *Desulfovibrio* numbers a Quaternary Ammonium compound (QAC) was added to three of the respective tubes to give a final concentration of 20 ppm, 40 ppm and 200 ppm, respectively. The other three tubes were used as a control. The Malthus tubes with culture and bactericides as well as Malthus tubes with culture alone (control) were incubated at 25 ^{o}C for 6 h, after which the *Desulfovibrio* numbers were again determined. The initial *Desulfovibrio* numbers and the numbers after 6 h biocide exposure were used to calculate the % kill using the following equation: 100 - (survivor count/initial count x 100).

3 RESULTS

3.1 Malthus calibration experiment

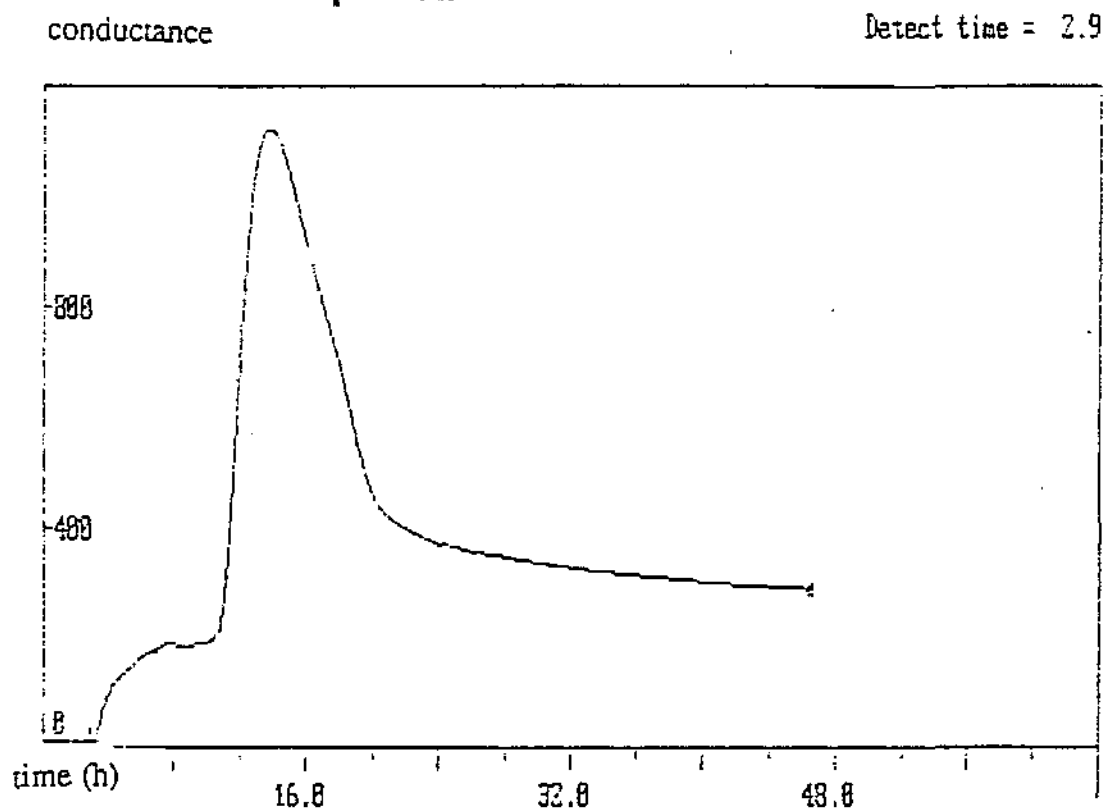


Figure 68: A Typical plot of the conductance change over time (h) of *Desulfovibrio desulfuricans* in Iron Sulphite broth

The relationship between detection time using the Malthus system and conventional enumeration (log_{10}/ml) of *Desulfovibrio* cells using IS-agar is shown in Fig. 69. Regression analysis of the number of viable cells (log_{10}/ml) against detection time using the Malthus system gave a regression line with a slope of -3.820 and a correlation coefficient of r = 0.974. This indicated a statistically significant correlation between detection time using the Malthus system and bacterial numbers in IS-agar using the roll tube method, indicating that the Malthus system could be used for the enumeration of pure cultures of *Desulfovibrio*.

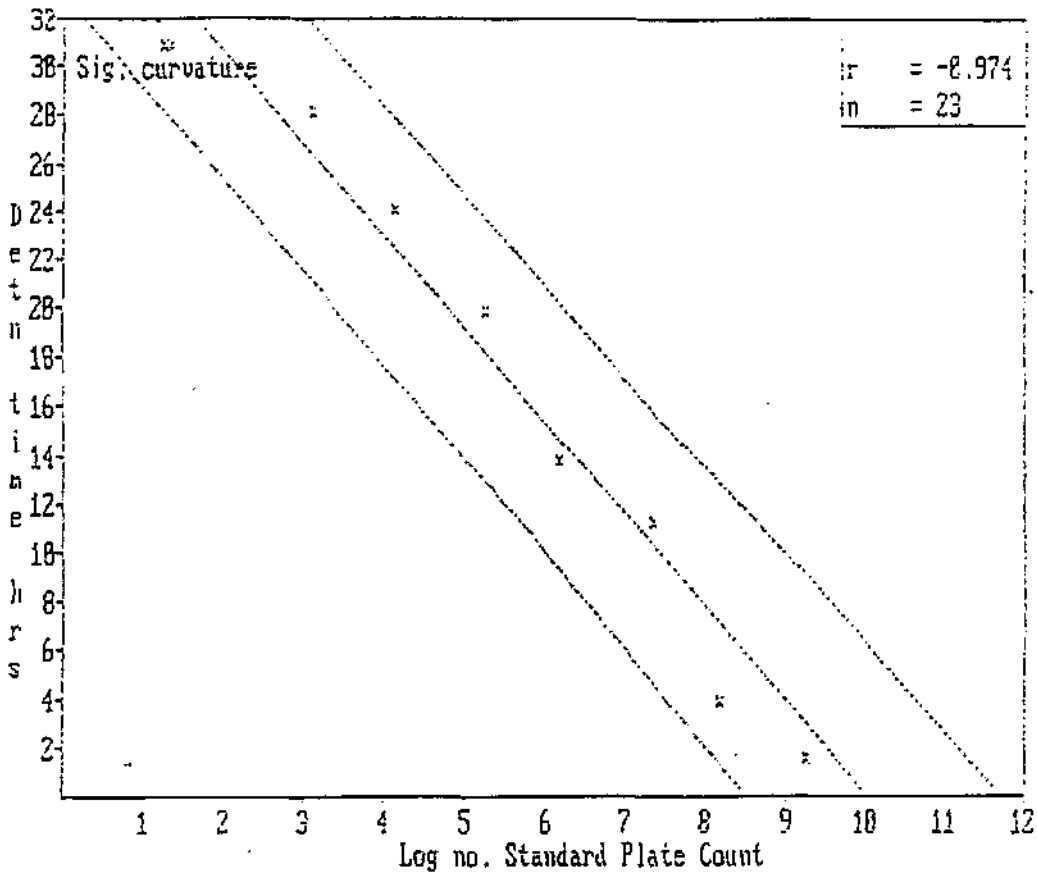


Figure 69: Correlation between detection time using the Malthus system and numbers (log_{10}/ml) of *Desulfovibrio desulfuricans* determined in agar roll tubes containing Iron Sulphite medium.

3.2 Biocide evaluations

The detection times and bacterial numbers (deducted from the regression line) of the 6 h kill test of the different biocide concentrations are shown in Table 31. Biocide concentrations of 20 ppm were not effective against *Desulfovibrio*, whereas a 56 % and a 100 % kill were obtained when using 60 and 200 ppm biocide, respectively.

Table 31: Biocide evaluations using the Malthus system

[Biocide]	Treatment	Detection time (h)	Numbers	% Kill
20 ppm	Initial numbers	3.8	2×10^9	
	Control after 6 h	1.7	6×10^9	
	6h after biocide addition	2.0	5×10^9	0
60 ppm	Initial numbers	0.4	9×10^9	
	Control after 6 h	0.4	9×10^9	
	6h after biocide addition	2.4	4×10^9	56
200 ppm	Initial numbers	3.1	3×10^9	
	Control after 6 h	2.0	5×10^9	
	6h after biocide addition	0	0	100

4 DISCUSSION

Distinct, easily measurable detection times using the Malthus system were obtained when using pure cultures of *D. desulfuricans* in IS-medium. There was a good correlation ($r = 0.974$) between *D. desulfuricans* numbers in IS-agar and detection time of *D. desulfuricans* which indicated that the Malthus system could be used to enumerate SRB.

Desulfovibrio cells were not killed when QAC was used at concentrations of 20 ppm, whereas a 56 % and a 100 % kill was obtained when using 60 and 200 ppm biocide, respectively. The Malthus system therefore proved useful in determining whether a particular biocide concentration would be effective against a SRB-strain or not. The procedures involved when using the Malthus system were less difficult and less laborious than when using anaerobic culture media for the enumeration of SRB because of the smaller volumes of media used than with standard methods. Samples could furthermore be inoculated directly into the system without the need for the preparation of serial dilutions. Biocide evaluations could be completed within 48h when using the Malthus system opposed to 14 d using conventional techniques.

5 ACKNOWLEDGEMENTS

We thank Fatima Da Silva for technical assistance and Swift Micro Laboratories for supplying the Malthus 2000 detection system.

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Contributions at international conferences

1. **Brözel, V. S. and Cloete, T. E.** The adaptation mechanism of *Pseudomonas aeruginosa* to growth in the presence of 2,2'-methylenebis(4-chlorophenol). Water Quality International '92. 16 th Congress of the IAWPRC, Washington, USA, May 1992.
2. **Brözel, V. S. and Cloete, T. E.** The adaptation mechanism of *Pseudomonas aeruginosa* to growth in the presence of 2,2'-methylenebis(4-chlorophenol). ISME-6, Barcelona, Spain, September 1992.
3. **Brözel, V. S. and Cloete, T. E.** The mechanism of resistance of *Pseudomonas aeruginosa* to isothiazolone is by inducible suppression of an outer membrane protein. 93rd General Meeting of the American Society for Microbiology. Atlanta, USA, May 1993.
4. **Brözel, V. S. and Cloete, T. E.** The antibacterial mechanism of action of thiocarbamates. 9th International Biodeterioration and Biodegradation Symposium, Leeds, UK, September 1993.
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8. **Cloete, T.E. and De Bruyn, E.E.** The role of *Shewanella putrefaciens* in microbially induced corrosion. 9th International Biodeterioration and Biodegradation Symposium, Leeds, UK, September 1993.
9. **De Bruyn, E.E. and Cloete, T.E.** Immunological techniques for the detection of sulphate-reducing bacteria in industrial water systems. Congress of the IAWPRC, Washington, USA, May 1992.
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Contributions at local conferences

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2. **Brözel, V.S.** Biofilm structure: An update on recent discoveries. *SAIWA 93* (The Southern African Industrial Water Association Symposium), October 1993, ESKOM Auditorium, Midrand.

3. **Brözel, V. S. and Cloete, T. E.** Evaluation of agar plating methods for the enumeration of viable heterotrophs in cooling water. Sixth biennial congress of the SASM, 28 March 1990.

4. **Brözel, V. S. and Cloete, T. E.** Effect of storage on the aerobic plate count and on the community structure of water samples. The Water Institute of South Africa Biennial Conference. 13 - 15 May 1991.

5. **Brözel, V. S. and Cloete, T. E.** Adaptation of *Pseudomonas aeruginosa*, *P. stutzeri* and *Bacillus cereus* to bactericides. Seventh biennial congress of the SASM, March 1992.

6. **Brözel, V. S. and Cloete, T. E.** Resistance of *Pseudomonas aeruginosa* to 2,2'methylene-bis(4-chlorophenol) is not alginate-mediated. Seventh biennial congress of the SASM, March 1992.

7. **Brözel, V.S. and Cloete, T.E.** Bacterial resistance to non-oxidising biocides. *SAIWA 93* (The Southern African Industrial Water Association Symposium), October 1993, ESKOM Auditorium, Midrand.

8. **Brözel, V.S. and Cloete, T.E.** The antibacterial mechanism of action of thiocarbamates. (Poster) *SAIWA 93* (The Southern African Industrial Water Association Symposium), October 1993, ESKOM Auditorium, Midrand.

9. **Brözel, V. S., Pietersen, B. and Cloete, T. E.** Development of Resistance to Water Treatment Bactericides by Bacteria from Cooling Waters. *SAIWA 91* (The Southern African Industrial Water Association Symposium), October 1991, CSIR Conference Center, Pretoria.

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14. **Pietersen, B., Brözel, V.S. and Cloete, T.E.** Response of *Pseudomonas aeruginosa* to hypochlorite and to oxidising stress. (Poster) *SAIWA 93* (The Southern African Industrial Water Association Symposium), October 1993, ESKOM Auditorium, Midrand.

15. **Pietersen, B., Cloete, T. E. and Brözel, V. S.** Adaptation of *Pseudomonas aeruginosa* to 2,2'-methylene bis (4-chlorophenol). Seventh biennial congress of the SASM, March 1992.

16. **Strydom, M., Brözel, V.S. and Cloete, T.E.** Response of *Pseudomonas aeruginosa* upon attachment to a surface. (Poster) *SAIWA 93* (The Southern African Industrial Water Association Symposium), October 1993, ESKOM Auditorium, Midrand.