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MICROBIAL ECOLOGY OF SULPHIDE-PRODUCING BACTERIA IN WATER COOLING SYSTEMS

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MICROBIAL ECOLOGY OF SULPHIDE-PRODUCING BACTERIA IN WATER COOLING SYSTEMS

by

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DECLARATION

I certify that the thesis hereby submitted to the University of Pretoria for the degree Philosophiae Doctor has not been previously submitted by me in respect of a degree at any other University.

Signature: .....

Date: .....

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DEPARTMENT: Microbiology and Plant Pathology

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Summary

Microbiological induced corrosion (MIC) can result in damage and economic losses in various industries. Although the importance of sulphate-reducing bacteria (SRB) in MIC has been widely recognized for many years, there has been increasing evidence that other organisms in addition to SRB have been involved in the corrosion process.

There is no information available regarding the ecology of SRB in industrial cooling water systems. The problem has been detecting and quantifying these organisms. The potential of using species specific fluorescent antibodies (FA) prepared against authentic SRB-strains for studying the ecology of SRB in industrial cooling water systems was investigated. 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. Caution should be exercised when using FA for ecological studies.

Different isolation media and carbon sources for the isolation and detection of SRB were evaluated. Iron sulphite (IS)-medium yielded the highest numbers when used to enumerate SRB from pure culture and industrial water samples. H_2S-producing bacteria that utilized lactate, acetate, formate or palmitic acid as different carbon sources were also isolated from industrial water samples. Using IS-medium *Shewanella putrefaciens* was the dominant sulphide producing bacteria isolated from industrial cooling water systems. Corrosion studies indicated that *S. putrefaciens* could play an important role in microbially induced corrosion

Since enumerating SRB by conventional methods is difficult, laborious and time consuming, the Malthus system, using conductance measurements, can be used for counting SRB cells in pure culture and thus for laboratory biocide evaluations.

MIKROBE EKOLOGIE VAN SULFIED-PRODUSERENDE BAKTERIEë IN INDUSTRIëLE WATERSISTEME

deur

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DEPARTEMENT: Mikrobiologie en Plantpatologie

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Opsomming

Mikrobiologies geïnduseerde korrosie is die oorsaak van skade en ekonomiese verliese in verskeïe industrieë. Alhoewel die belangrikheid van sulfaat-reduserende bakteriïee (SRB) bekend is, is daar toenemende bewyse dat ander organismes, behalwe SRB ook berokke is by die korrosie proses.

Daar is geen inligting beskikbaar oor die ekologie van SRB in industriële water sisteme nie. Die probleem is die opsporing en kwantifisering van hierdie organismes. Die moontlikheid om fluoreserende spesie spesifieke teenliggame (FA), berei teen autentieke SRB-rasse, te gebruik om die ekologie van SRB in industrïele verkoelingsisteme te bestudeer, is ondersoek. Kweekings toestande het die uitdrukking van oppervlakte antigene beïnvloed en veroorsaak dat die sera baie spesifiek was en nie gebruik kon word vir die identifikasie van SRB geïsoleer vanuit industriële verkoelingsisteme nie. Versigtiheid sal aan die dag gel moet word wanneer FA gebruik word vir ekologiese studies.

Verskillende isolasie media en koolstof bronne was geëvalueer vir die opsporing van SRB. Yster sulfiet (IS)-medium het die hoogste tellings gelewer vir die opsporing van SRB in rein kulture en in industriële water monsters. H_2S-produserende bakterieë wat laktaat, asetaat, formaat of palmitiensuur as verskillende koolstof bronne kon gebruik was geïsoleer vanuit industriële water monsters. Deur gebruik te maak van IS-medium was *Shewanella putrefaciens* die dominante sulfied produserende bakterieë geïsoleer vanuit industriële verkoelings water sisteme. Na aanleiding van korrosie studies kan *S. putrefaciens* 'n belangrike rol speel in MIC.

Aangesien die tel van SRB deur middel van konvensionele metodes moeilik en tydrowend is, kan die bepaling van geleiding (Malthus sisteem) gebruik word vir die tel van SRB in reinkulture en dus vir biosied analises onder laboratorium toestande.

CONTENT

CHAPTER I. INTRODUCTION

Microbiological induced corrosion (MIC) is an important cause of corrosion of submerged structures, aircraft fuel tanks, ships, marine structures, cooling systems and heat exchangers. This can result in damage and economical 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 economical 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 other organisms, in addition to SRB, have been involved in the corrosion process (Ford and Mitchell, 1990; Iverson, 1987; Videla, 1991).

SRB are an ubiquitous group of microorganisms which share the ability to couple the reduction of sulphate, sulphite thiosulphate and sulphur 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 with CO_2 as carbon source, a range of organic compounds, including benzoate and on a range of fatty acids i.e. acetate, stearate etc. (Widdel, 1988). There are many culture media formulations used for enumerating SRB (Fedorak *et al.*, 1987; Gibson *et al.*, 1987; Hardy, 1981; Herbert and Gilbert, 1984; Pankhurst, 1971; Postgate, 1984). Hamilton (1985) concluded that viable count procedures for enumerating SRB underestimated the *in situ* population by a factor of approximately 1000, compared to *in situ* sulphate reduction activity. This may have been due to an inappropriate choice of culture medium (Gibson *et al.*, 1987). Detection of SRB is based on the presence of a black precipitate, iron sulphide, formed by reaction of iron(II) with the bacterially

produced sulphide ion in an appropriate culture medium. Sulphide production is common amongst organisms such as *Proteus*, *Citrobacter*, *Salmonella*, *Pseudomonas* and *Clostridia* (Atlas and Bartha, 1987; Laishley *et al.*, 1984; McMeekin and Patterson, 1975; Oltmann *et al.*, 1975). This emphasizes the importance of careful medium selection since an inappropriate choice of culture media can underestimate *in situ* SRB-populations or give rise to false positive results.

The enumeration and classification of SRB by conventional methods is time consuming (Gaylarde and Cook, 1987) and might give rise to a biased and incomplete analysis of the natural SRB population being sampled (Hamilton, 1985). Counts might also include non-dissimilatory sulphate-reducers or sulphite, thiosulphate and sulphur reducing bacteria. One potentially possible way to overcome these problems would be to use immunological methods.

Serological work on SRB has yielded conflicting results regarding strain specificity of antibodies and cross reactions found amongst different strains of SRB (Abdollahi and Nedwell, 1980; Baker *et al.*, 1962; Postgate and Campbell, 1963; Singleton *et al.*, 1985). Some researchers proved to have prepared antisera that can successfully be used for the detection of SRB *in situ* (Bobowski and Nedwell, 1987; Gaylarde and Cook, 1987; Smith, 1982).

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; Sorensen *et al.*, 1981) in freshwater and marine environments, little is known about the ecology of SRB in industrial freshwater environments, especially cooling water systems.

The aim of this investigation was to do an ecological study of the SRB in industrial cooling water systems. An ecological study of the incidence and species diversity of SRB and other possible corrosive bacteria in industrial water systems would render useful information regarding the role of SRB in MIC and biocide addition programs to control corrosion and biofouling. In order to do this it was necessary to (a) evaluate different isolation media and carbon sources for the isolation and detection of SRB in industrial cooling water systems, (b) to determine the specificity of these media for dissimilatory sulphate-reducers, (c) to evaluate an alternative method (Malthus system) to the conventional techniques associated with the use of isolation media, (d) to evaluate the use of fluorescent antibodies for the detection and identification of SRB in industrial cooling water systems and (e) to determine the corrosiveness of sulphide producing isolates other than SRB.

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CHAPTER II. LITERATURE REVIEW

A. CORROSION

1. Electrochemical mechanisms

Corrosion is a surface electrochemical phenomenon common to all base metals in aqueous, or at least humid, environments. The occurrence of corrosion is favoured by the natural tendency of elemental metals to revert to a combined form. Corrosion involves the formation of electrolytic cells that cause a flow of electricity between certain areas of a metal (anode and cathode) through a solution which has the ability to conduct an electric current (Iverson, 1987; Videla, 1991) and is represented by the following equations:

$M = M^{2+} + 2e^-$	(metal dissolution at anode)	I
$1/2\ O_2 + H_2O + 2e^- = 2OH^-$	(cathode at neutral or alkaline pH)	II
$Fe^{2+} + 2(OH^-) = Fe(OH)_2$	(corrosion products)	III
$Fe(OH)_2 + 1/2H_2O + 1/4O_2 = Fe(OH_3)$	(corrosion products)	IV
$2H^+ + 2e^- = 2H^+ = H_2$	(cathode at acid pH)	V

Corrosion products may form loose films or very thin slightly adhesive protective films which decreases the rate of corrosion. In order for corrosion to proceed, two conditions have to be met: the anodic and cathodic reactions must remain in balance and the electrolytic cell must continue functioning over prolonged periods. Under aerobic conditions, these conditions are generally met by the continuous supply of

oxygen to the cathode, which results in the oxidation of the metal, and by the removal of insoluble iron oxides and hydroxides at the anode (Hamilton, 1985).

2. Microbial induced corrosion

Microbial induced corrosion (MIC) may be defined as the deterioration of a material, usually a metal, by processes which occur, either directly or indirectly, as a result of bacterial activity (Crombie *et al.*, 1980; Iverson, 1987). Corrosion reactions may be induced or enhanced by microbial activity (Ford and Mitchell, 1990). The electrochemical model of corrosion remains valid for MIC. From a two-component system (metal + electrolyte) we turn to a three-component system (metal + electrolyte + microorganism). Microbial activity at the metal/solution interface can affect both anodic and cathodic reactions. Anodic effects can be accomplished through; (a) the production of corrosive metabolites, (b) the production of metabolites that enhance the corrosive action of other chemical species already present in the medium and (c) the microbial degradation of chemical compounds that act as corrosion inhibitors. Microbial effects on the cathodic reaction are mainly accomplished through the consumption or production of cathodic reactants such as oxygen (aerobic) or protons (anaerobic), that will result in slowing down the corrosion rate (polarization) or the acceleration of metal dissolution (depolarization). Microorganisms influence the corrosion process by several mechanisms operating simultaneously or consecutively. A single cause very seldom accounts for MIC (Videla, 1991). In chemical and biological corrosion mechanisms, oxygen, or its absence, plays a crucial role. There can be distinguished between aerobic and anaerobic MIC processes (Crombie *et al.*, 1980; Hamilton, 1985) and these will now be discussed.

2.1. Anaerobic corrosion caused by sulphate-reducing bacteria

According to various authors the most widely distributed and economically important organisms associated with anaerobic corrosion are the sulphate-reducing bacteria (SRB) (Boivin and Costerton, 1991; Crombie *et al.*, 1980; Ford and Mitchell, 1990; Hamilton, 1985), especially *Desulfovibrio desulfuricans*. Pitting corrosion is characteristic of the action of SRB on metals, with the pits being filled with soft black corrosion products in the form of iron sulphides. It has also been claimed that SRB are responsible for pitting corrosion in aluminium and copper containing alloys (Hamilton, 1985).

Two questions have always been central in attempts to understand the corrosion mechanisms, i.e. what is the nature of the cathodic reaction that prevents the electrolytic cell from becoming polarized due to the build-up of a hydrogen overpotential and what, if any, is the role of sulphide in the overall corrosion process (Hamilton, 1985)?

The most widely accepted theory for the mechanism of corrosion of iron and steel involves cathodic depolarization. This theory was first postulated by Von Wolzogen Kuhr and Van der Vlugt (1934) - SRB stimulate corrosion by removing the normal electrochemical film of hydrogen that forms on iron in water. The cathodic depolarization theory is summarized in the equations below:

Initial reaction of iron placed in an aqueous environment:

$2H_2O = 2H^+ + 2OH^-$		I
$Fe = Fe^{2+} + 2e^-$	(anode)	II
$2H^+ + 2e = 2H$	(cathode)	III

A protective film of hydrogen is formed on the metal surface.

Bacterial interaction:

$SO_4^{2-} + 8H = S^{2-} + 4H_2O$	(cathodic depolarization)	I

Corrosion products:

$Fe^{2+} + S^{2-} = FeS$	(anode)	I
$Fe^{2+} + 2OH^- = Fe(OH)_2$	(anode)	II

The overall result can be presented by:

$$4Fe + SO_4 + 4H_2O = FeS + 3Fe(OH)_2 + 2OH^- \quad \text{I}$$

Data in support of the cathodic depolarization hypothesis of Von Wolzogen Kuhr and Van der Vlught (1934) was obtained by various research groups (Hamilton, 1985). Results obtained by Nedwell and Banat (1981) confirmed that hydrogen was an important electron donor for SRB present in Salt Marsh Sediments. In anaerobic corrosion experiments, hydrogenase-positive *Desulfovibrio* strains, growing with limiting lactate concentrations in the presence of steel wool, formed more sulphide than observed with lactate alone. The additional sulphide originated from sulphate reduction with cathodically formed hydrogen from the steel surface. The hydrogenase negative *D. sapovorans* did not produce additional sulphide (Cord-Ruwisch and Widdel, 1986). Utilization by *D. vulgaris* of cathodic hydrogen from a mild steel electrode was demonstrated electrochemically and physiologically by Pankhania *et al.* (1986). The importance of hydrogenase activity to corrosion of steel was assessed in biofilms by using mixed populations of SRB isolated from corroded and noncorroded oil pipelines. The biofilm with active hydrogenase activity was associated with a significantly higher corrosion rate relative to the noncorrosive biofilm with SRB but without measurable hydrogenase activity (Bryant

et al., 1991). Carbon steel was exposed to 3 rganisms; *Eubacterium limosum*, *Desulfovibrio* sp. and *Desulfobacter* sp. which were provided with H_2/CO_2, buthanol, glucose and acetate as carbon and electron source. A consortium of these bacteria utilizing hydrogen gave rise to relatively high corrosion rates with respect to corrosion resulting from bacteria supplied with organic electron sources (Dowling *et al.*, 1992).

For this corrosion process to occur the bacteria must contain the enzyme hydrogenase. The rate of corrosion of steel has been related to hydrogenase activity. However, other conditions are known to be important and actively growing cultures of a number of different strains of SRB corroded mild steel at rates independent of hydrogenase activity (Crombie *et al.*, 1980). When using semicontinuous and continuous cultures of SRB in sulphate media, corrosion rates generally were low, and a thin ferrous sulphide film formed on the test specimen. After a period of some months, this film fractured, with a considerable increase in the rates of corrosion. There were minor differences in the rates of corrosion caused by hydrogenase-positive and -negative strains. The higher rates of corrosion thus seemed to be dependent on the corrosion product ferrous sulphide (Hamilton, 1985).

An alternative mechanism for cathodic depolarization has been proposed by Booth *et al.* (1968). In addition to utilization of polarizing hydrogen by the bacterial hydrogenase system, there is evidence of depolarization of the cathode by iron(II) sulphide. A more recent theory (King and Miller, 1971) proposed that the cathodic reaction, that is, hydrogen evolution, occurred on the iron(II) sulphide formed by the reaction of the iron(II) ion with the bacterially produced sulphide ion.

Research performed by Hardy (1983) indicated that SRB were utilizing cathodic hydrogen. However, the relatively low rates of sulphate respiration observed indicated that it was unlikely that cathodic hydrogen removal was the dominant mechanism whereby SRB induced corrosion. The concomitant generation of sulphide was probably of more significance in terms of corrosion. According to Deckena and Blotevogel (1990) the consumption of cathodic hydrogen as well as the sulphide production of SRB may influence the anaerobic corrosion of iron. The most important role of SRB on offshore facilities is the generation of hydrogen sulphide. The latter may have an effect on sites away from the sites of the production of hydrogen sulphide (Edyvean, 1991).

In media containing low concentrations of iron, free sulphide ions caused the formation of sulphide films, which protected the metal underneath (Crombie *et al.*, 1980). FeS may be formed as a film on the surface of the metal in the presence of actively growing cultures of SRB in media low in concentration of soluble iron, or as bulk FeS in the case were SRB were cultured in medium high in soluble iron. In the former case the film usually inhibits corrosion, but it may break down, usually with an increase in the corrosion rate. The primary film appears to be composed of mackinawite (FeS_{1-x}) and siderite ($FeCO_3$) - the latter being protective. In the case where high concentrations soluble iron was added to the culture, forming bulk iron sulphide and preventing film formation, corrosion rates were very high (Iverson, 1987; Lee and Characklis; 1991). Results obtained by Lee and Characklis (1991) indicated that iron sulphide films on metal surfaces did not offer protection from anaerobic MIC. They concluded that; (a) the anaerobic corrosion process at low hydrogen sulphide concentration at pH 8 under continuous flow conditions was initiated at defect sites in the iron sulphide film or metal matrix and propagated through film spalling and rupture, (b) the loosely accumulated iron sulphide

accelerated corrosion of mild steel due to cathodic-only depolarization, which is limited by concentration diffusion polarization and (c) accumulation of biofilm on a precoated iron sulphide film could reduce spalling of iron sulphide film but could not avoid the risk of localized corrosion.

Results obtained by Daumas *et al.* (1988) showed that, although the influence of iron sulphide deposited on the surface of metals was not negligible, the major mechanism for corrosion was the oxidation of cathodically formed hydrogen. This mechanism occurs only near the surface of the metal.

Extensive corrosion of iron was induced using spent culture media from which SRB and sulphides had been removed. The corrosive metabolite was a volatile phosphorous compound (Hamilton, 1985). The corrosive activity of SRB ascribed to the production of highly corrosive reduced phosphorous compounds, due to the lack of supporting evidence, has not been widely accepted. Weimer *et al.* (1988) reported that an increase in the phosphate content of the growth medium resulted in an increased corrosion rate of carbon steel. The involvement of phosphate in enhancing corrosion in natural or process environments containing mixed microbial populations and complex biofilms is far from clear. Many of these environments contained very low concentrations of phosphate.

Elemental sulphur has been observed around corrosion pits caused by the action of SRB. High corrosion rates of steel have been noted in the presence of elemental sulphur. Oxidation of sulphide to sulphur could occur abiotically or by the action of the reduced sulphur oxidizing bacteria (Crombie *et al.*, 1980; Hamilton, 1985).

Although anaerobic corrosion has traditionally been associated with SRB, it should be noted that many other anaerobic reactions consume hydrogen and may act to cathodically depolarize a metal. These include chemical and biological reduction of thiosulphate, sulphite, sulphur, fumerate and nitrate as well as methanogenesis and acetogenesis (Ford and Mitchell, 1990).

2.2 Aerobic processes

Information about the influence of microorganisms on mechanisms of aerobic corrosion is limited because of the assumption that abiotic oxidative processes are much more rapid (Ford and Mitchell, 1990). However, the importance of microorganisms in aerobic corrosion of metals may be significantly underestimated. The most apparent influence of an aerobic community on a metal surface is the creation of differential aeration cells. Other aerobic microbial processes that may accelerate corrosion include formation of iron concentration cells (iron and manganese deposition), bacterial polymer-metal interactions, activities of metal-transforming and acid-producing bacteria, and thermophilic reactions (Ford and Mitchell, 1990).

2.3. Concentration cell formation

A wide variety of organisms are capable of colonizing metals immersed in water. Aerobic biofilms are not uniform. These biofilms deplete the oxygen at the metal surface, thereby causing an oxygen differential and establishing corrosive, oxygen-differential cells. This may reduce the redox potential under the biofilm to levels where SRB may be established. Almost any type of microorganism which can colonize a surface may therefore be considered as a potential corrosion initiator (Iverson, 1987; Videla, 1991). How biofouling of metal surfaces can modify the

protective effect of inorganic passive films is not well understood (Videla, 1991). Information about the complex ecology of microbial communities adhering to and growing on solid surfaces is essential if we were to understand the function of microorganisms in corrosion. In natural systems the surface microbiota is controlled to a large degree by the nature of the substratum and the available nutrients in the water column. Extremely specific microbial communities can be associated with individual metals (Ford and Mitchell, 1990). Biofilm/passive layer interactions can affect the passivating action through the following mechanisms; (a) preventing the transport of chemical species necessary for the passivation of the metal surface, (b) facilitating the removal of passive layers as biofilm detachment occurs, (c) favouring differential aeration effects as a result of an uneven distribution of the biofilm, (d) altering oxygen gradients at the interface by microbial metabolism, (e) facilitating the dissolution and removal of passive layers on the metal surface and (f) creating aggressive micro-environments at the metal surface (Videla, 1991).

2.4. The role of exopolymer- metal interactions in corrosion

Extracellular polysaccharides (EPS) produced by bacteria are usually acidic and contain functional groups that readily bind metal ions. Not only are these polymers central to the structural integrity of the surface microbiota, but they may also be directly involved in metal dissolution from the corroding metal (Ford and Mitchell, 1990). Biofilm formed by *D. desulfuricans* contained large amounts of ferric sulphide, bacterial cells and high levels of EPS. Uronic acids, that can play a role in the sequestration of metal cations were present in the EPS of the biofilm formed by *D. desulfuricans*. The less corrosive *P. fluorescens* biofilm consisted of little EPS with no detectable uronic acid. The composition of biofilm EPS also differed from that of free EPS. Free EPS from either of the bacterium genera contained no

detectable uronic acid (Gaylarde and Beech, 1991). They thus concluded that the EPS in a biofilm, formed by certain organisms, that is in contact with a metal surface must have a major influence on corrosion.

2.5. Corrosive metabolic products

2.5.1. Volatile phosphorus compounds

Extensive corrosion of steel was obtained under anaerobic conditions in a filtrate of a marine culture of *Desulfovibrio* where all the iron sulphide as well as free sulphide ions were removed. The main corrosion product was found to be amorphous iron phosphide. Further results indicated that the corrosive agent was a volatile phosphorus compound produced by SRB. A similarly acting corrosive, volatile, phosphorus compound was also produced by the action of hydrogen sulphide on certain crystals of disodium phosphate and sodium hypophosphite. It was proposed that the induction of corrosion by ferrous ions was due to the prevention of iron sulphide film formation by the precipitation of sulphide ions as bulk iron sulphide in solution, thereby permitting the corrosive phosphorous compound to come into contact with the bare iron surface and initiate corrosion (Iverson, 1987).

2.5.2. Acids

Both inorganic and organic acids produced by microorganisms can be highly corrosive to metals. The main inorganic acid involved in metal corrosion is sulphuric acid produced by acidophilic sulphur-oxidizing bacteria (Iverson, 1987). Corrosion by organic acids is primarily due to the activity of fungi, although some cases of corrosion by bacterially produced organic acids have been reported. Organic

acids from a number of Gram-negative bacteria were found to cause extensive corrosion of iron (Iverson, 1987).

2.5.3. Sulphur and sulphur compounds

Hydrogen sulphides produced by SRB reacts with iron to form iron sulphide (see section on mechanisms of anaerobic corrosion by SRB). Under anaerobic conditions and at low pH values, where the hydrogen sulphides may be unbuffered or where the acidity may be due to organic acids, hydrogen sulphide will corrode iron with the release of hydrogen. Atomic hydrogen formed by the action of hydrogen sulphide on iron, may be responsible for embrittlement and blistering of hardened steel parts. A variety of sulphur species are able to induce intergranular stress corrosion cracking. Hydrogen sulphide may be spontaneously or biologically oxidized to elemental sulphur in the presence of oxygen, or anaerobically by biological photosynthetic oxidation by e.g. green and purple sulphur bacteria. Elemental sulphur is corrosive to mild steel under aerobic as well as anaerobic conditions. Mercaptans produced by a variety of organisms are also corrosive to certain metals (Iverson, 1987).

2.5.4. Ammonia and amines

The corrosive action of ammonia on copper and brass is well documented. Ammonia is also a very ubiquitous product of microbial metabolism. Amines have also been demonstrated to attack copper and nickel (Iverson,1987).

2.5.5. Hydrogen

Hydrogen atoms may accumulate on the surface of some ferrous alloys because of poisonous compounds (such as hydrogen sulphide). The hydrogen atoms may enter the alloys and cause hydrogen embrittlement and hydrogen cracking (Iverson, 1987). Certain bacteria are also capable of hydrogen production. Corrosion resulting from adsorption of hydrogen is especially difficult to detect and control because variations in environmental and surface conditions affect hydrogen entry (Ford and Mitchell, 1990).

2.6. Protective film disruption

Metals in natural environments form films of corrosion products, usually oxides, that may afford corrosion protection in varying degrees (Iverson, 1987). Obuekwe *et al.* (1981) reported the isolation of a ferric reducing organism that were able to remove the protective ferric film from an iron surface. The organism reduced the insoluble ferric film to a soluble ferrous iron, thus causing the protective film to be destroyed (Obuekwe *et al.*, 1981). The breakdown of iron sulphide films in cultures of SRB is usually followed by high corrosion rates (Iverson, 1987).

2.7. Tubercle formation

Tubercles are initially formed by microbiological deposition of iron and manganese oxides. Bacteria produce exopolymers that bind the mineral deposits together and limit diffusion of oxygen and nutrients in and out of the developing tubercle. Any oxygen that does penetrate is either abiotically or biotically consumed. Metabolic

activity of microorganisms reduces the pH inside the tubercle by production of CO_2 and organic acids. The outer layers of the tubercle are aerobic with a near neutral pH, while inner regions are highly reduced and acidic. Analysis of these deposits suggested the presence of sulphate- and nitrate-reducing bacteria, nitrite oxidizers and various unidentified heterotrophs (Iverson, 1987).

2.8. Combination of mechanisms

The complexity of microbial interactions in the field cannot be realistically modelled in the laboratory. It is for this reason that corrosion rates measured in the laboratory are lower than those encountered in the field. One of the primary reasons is the presence of consortia of microorganisms in surface biofilms. Consortia appear to play an important role in anaerobic MIC (Ford and Mitchell, 1990).

Under natural conditions, a number of corrosion mechanisms probably operate, simultaneously or in succession. In the case of biofilms, oxygen concentration cells may operate along with proton gradients produced as a result of the metabolic activities of the microorganisms in the biofilm. As regions under the film become depleted in oxygen, SRB may become established, which, in addition to accentuating the oxygen concentration cell effect, may further stimulate corrosion by elaboration of corrosive compounds (Iverson, 1987).

3. Bacteria other than sulphate-reducing bacteria involved in corrosion

Since early studies there has been increasing evidence that organisms, other than SRB, have been involved in the corrosion of iron as well as other metals. Examples of these organisms and their action on metals are summarized in Table 1 (Iverson,

1987). Since hydrogenase appears to be active in the reversible activation of the hydrogen molecule, hydrogenase from *Clostridium pasteurianum* was used as a test enzyme for investigating the role of cell-free hydrogenase in anaerobic biocorrosion of mild steel. Evidence was presented by Bryant and Laishley (1989) which demonstrated the activity of the hydrogenase enzyme in catalytic removal of cathodically produced hydrogen from mild steel in the presence of the appropriate electron acceptor. In many environments, methanogenic bacteria were found near SRB and could play a role in biocorrosion at these sites, as well as in environments relatively free of SRB. Methanogens can use H_2 produced by cathodic depolarization-mediated oxidation of elemental iron to produce methane (Boopathy and Daniels, 1991). According to Deckena and Blotevogel (1990) there was no evidence that anaerobic corrosion was reinforced during microbial growth, although hydrogen was consumed and the reaction balance disturbed. Cathodic depolarization does not play a dominant role in methanogenic environments.

When 2 marine isolates, a *Pseudomonas* sp and *Vibrio alginolyticus* were used to examine bacterial attachment on a 70:30 copper nickel alloy, localized corrosion underneath well defined microbial colonies was seen by using scanning electron microscopy (SEM) after short periods of exposure. Corrosion attack seems to be closely related to passive film modification by the bacterial settlement (Gomez de Saravia *et al.*, 1989).

Thin copper films were corroded by a rod shaped Gram-variable facultative anaerobic bacteria that was isolated from corroded copper coupons which were exposed to flowing municipal water. An increase in the concentration of polysaccharide material in contact with the copper thin film correlated with corrosion of the copper thin film. A different rod shaped Gram-variable facultative

anaerobic bacteria, isolated from the same source as the above mentioned bacteria, did not corrode the copper thin film, and establishment of this bacterium on the copper surface prevented corrosion of the thin film by the previously mentioned bacterium (Bremer and Geesey, 1991).

It was found that 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). Growing cells of both these bacteria in liquid batch culture of the same medium as the above experiment inhibited corrosion (Pedersen and Hermansson, 1989). Pedersen and Hermansson (1989) speculated that the difference in corrosion rate found by Pedersen *et al.* (1988) compared to their results was due to differences between free-living cells and colony forming units. The high numbers of cells in the colonies under the metal coated glass slide used by Pedersen *et al.* (1988), probably created quite different conditions compared to the ones in the experiments performed by Pedersen and Hermansson (1989). Interactions between bacteria and metal surface did not always result in enhanced corrosion rates, but could also lead to the protection of the metal. Aerobic and anaerobic biofilm populations were formed by immersing iron coupons in natural seawater under aerobic and anaerobic conditions. An anaerobic population attached to the surface increased the corrosion rate when immersed in a suspension of *Vibrio* sp. The *Vibrio* population removed oxygen and provided nutrients for the anaerobic population and thereby provided protection to the anaerobic population that in turn produced corrosive metabolites close to the metal. In contrast, coupons without a biofilm showed a decrease in corrosion when immersed in the same *Vibrio* suspension (Pedersen and Hermansson, 1991).

A variety of aerobic, anaerobic and facultatively aerobic bacteria could readily be isolated from oil, oil field water and internal pipeline encrustations. Among such bacteria were a group of facultative aerobic, oxidase positive, non spore forming, motile rods with a polar flagellum. These organisms were capable of reducing ferric to ferrous iron and reducing sulphite, thiosulphite 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 ions together with enhanced level of potentially corrosive ferrous sulphide. Changes which occurred on the surface of mild steel coupons submerged in cultures of a Fe(III)-reducing bacterium were studied microscopically. The protective surface coat of the metal was removed by the bacteria. After 2 weeks of exposure the removal of the surface coating was followed by colonization of the metal surface by the bacteria. The presence of this bacteria prevented the formation of a protective surface coat on the mild steel coupons (Obuekwe *et al.*, 1981). The corrosive pseudomonads isolated by Obuekwe *et al.* (1981) and other isolates from oil field water were identified as *Shewanella putrefaciens* (Semple and Westlake, 1987). *S. putrefaciens* has the ability to obtain energy for anaerobic growth by coupling the oxidation of hydrogen and formate to the reduction of Fe(III) or Mn(IV) (Loveley *et al.*, 1989).

4. Concluding remarks

Most of the evidence does not support the cathodic depolarization theory (Iverson, 1987). However, recent research strongly favours the original hypothesis of Von Wolzogen Kuhr and Van der Vlugt (1934) (Boivin and Costerton, 1991; Ford and Mitchell, 1990). Although there is some support for the involvement of phosphides, the mechanism is still far from clear (Ford and Mitchell, 1990).

MIC is rarely linked to a single mechanism or to a single microorganism (Videla, 1991). Hydrogenase positive bacteria can cause severe localized corrosion by cathodic depolarization. Growth of SRB is stimulated by the availability of cathodic hydrogen from corrosion cells. The production of hydrogen sulphide by sulphidogenic bacteria produces several conditions which promote corrosion processes. The reduced redox potential increases the electromotive force between cathode and anode. Dissociation of H_2S releases protons for the reaction at the cathode. Precipitation of ferrous iron produces a deposit which has an inherent ability to further depolarize cathodic reactions. Certain forms of iron sulphide will stimulate hydrogen ion reduction and this will allow the development of cathodic hydrogen for consumption by hydrogenase-positive bacteria. There is also evidence of MIC caused by other sedimentary bacteria such as *Shewanella*, *Clostridium* and methanogens, which are avid utilizers of hydrogen (Boivin and Costerton, 1991).

B. ECOLOGY AND PHYSIOLOGY OF SULPHATE-REDUCING BACTERIA

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 (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 different types of SRB-species, their main characteristics and compounds that have been reported as energy substrates are shown in Table 2 (Gibson, 1990).

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).

2. Ecological distribution of sulphate-reducing bacteria

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 2 strains of *D. desulfuricans*, *Desulfovibrio africanus*, 2 strains of *Desulfotomaculum nigrificans* and 3 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 were 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 40-50, 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 3 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).

3. Concluding remarks

SRB are an ubiquitous group of microorganisms which share an ability to couple the reduction of sulphate and other sulphur compounds 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 on CO_2, a range of organic compounds including benzoate and on fatty acids from acetate to stearate (Widdel, 1988)

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.*, 1988; Sorensen *et al.*, 1981) in freshwater and marine environments, little is known about the ecology of SRB in industrial freshwater environments, especially cooling water systems.

C. METHODS FOR THE DETECTION AND ENUMERATION OF SULPHATE-REDUCING BACTERIA

1. Isolation and growth of sulphate-reducing bacteria

1.1. Incubation conditions

SRB are strict anaerobes (Widdel and Pfennig, 1984). Handling and cultivation of SRB require techniques to effectively remove oxygen 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 ^{o}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).

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).

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, redox-poising 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 conformance 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 restricts 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 numbers of *Desulfobacter* and *Desulfobulbus*. The addition of cetyl trimethylammonium bromibe (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 calsium 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 Baars 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 X 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, based on isolation medium, 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. SEBR-medium contained 2 arbon 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* sulphate-reduction 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 any 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 being sampled 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 and 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.

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 environmental 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 and 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 plays 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 immunofluorescence (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 albumine (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).

1.3.1. Serological relationships of sulphate-reducing bacteria

a) 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 2 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 3 strains of *Desulfovibrio* MK, Hildenborough and MF, revealed that cross-reaction among strains was restricted. The 3 strains, MK, Hildenborough and NCR 49001 shared common somatic antigens and the 2 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-surface 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 count (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 of *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 2 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 has often resulted in antibodies that failed to recognize bacteria that were 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).

b) Comparative immunological study of hydrogenases of sulphate-reducing bacteria

Like many other anaerobic bacteria, some SRB have a hydrogenase 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 were 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 2 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.

c) 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 2 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* strains MK and MF. Both the antisera cross reacted with the extracts from all the strains tested. This indicated that the sulphite reductases from *Desulfovibrio* shared common antigenic determinants (Aketagawa *et al.*, 1985). The bisulphite reductase, desulfoviridin, that was present in *D. vulgaris* occured in most of the classical lactate- or hydrogen-utilizing *Desulfovibrio* species. However, desulfoviridin was 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 was not present in all the described SRB genus and species (Widdel, 1988).

d) Comparative imunological study of APS-reductase of sulphate-reducing bacteria

All sulphate-reducing bacteria investigated to date contained a soluble adenosine 5'-phosphosulphate (APS) reductase which catalyzed 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 extract from 14 species of SRB comprising the genera *Desulfovibrio*, *Desulfotomaculum*, *Desulfobulbus* and *Desulfosarcina* and from 3 species of sulphide-oxidizing 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 cross-reaction. Extracts from colorless 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).

1.4. Alternative detection methods

1.4.1. Detection of marker genes using nucleic acid probes

Nucleic acid probes that were 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 (Woese *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 (Olsen, *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 2 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 7 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 labeled 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 comprised 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 that encoded hydrogenase from *D. vulgaris* could 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 could be labeled and used to probe filters on which denatured chromosomal DNA from relevant standards have been spotted. The latter technique was referred to as sample genome probing, since it was the reverse of the usual practice of deriving probes from reference bacteria for analyzing a DNA sample. Reverse sample genome probing allowed 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).

1.4.2. The use of biochemical methods for the detection of sulphate-reducing bacteria

Biochemical methods fall into 2 basic groups: those designed to detect molecules that are a part of the microbial cells themselves and those designed to dectect metabolic products. The former can again be subdivided into 2 groups: those techniques which give an estimate of the total number 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 were partly successful during a study on the distribution of different genera of SRB in sediment and other natural environments (Taylor and Parkes, 1983).

A relative easy method is based on the presence of the pigment, desulfoviridin, in *Desulfovibrio*. *Desulfovibrio* cells appeared red after the addition of NaOH (Sharma and Hobson, 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).

1.4.3. Electrical methods

Electrical methods (conductance, impedance and capacitance) are the established methods of monitoring microbial growth and estimating bacterial numbers. Instruments have recently been developed which produce bacteriological results much sooner than conventional methods. One such system is based on automatically monotoring the electrical conductance of cultures (Richards *et al.*, 1978). 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, It was found that the time taken for a noticeable change in conductance is reversely proportional to the logarithm of the number of viable organisms inoculated into the material assayed so that the instrument could be used for determining counts. The change detected is thought to be due to the metabolism of the constituents of the culture medium by the organisms, in particular an increase in the number of ion carriers because of the catabolism of high molecular weight compounds, e.g. ammonia, amines etc. Thus only actively growing cells contribute to the metabolic activity of the culture and hence to the conductance change. The time lapsed between inoculation and a noticeable but small change in conductance is termed the detection time. The higher the count, the shorter is the detection time (Gibson, 1985).

Electrical conductance have successfully been used to detect the presence of *Salmonella* spp. in foods and feaces (Smith *et al.*, 1989; Ogden and Cann, 1987). The bacteriological quality of chill-stored packaged fish was determined rapidly from automated conductance assays (Gibson, 1985). The method offers savings in

media and operating costs over conventional standard culture methods and produces results within 48 h (Smith *et al.*, 1989)

1.5. Commercial available test kits for sulphate-reducing bacteria

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 contain 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 2 carbon and energy sources.

5. Concluding remarks

SRB are an ubiquitous group of microorganisms which share an ability to couple the reduction of sulphate, other oxidized sulphur compounds or elemental sulphur, to the oxidation of a variety of electron donors (Postgate, 1984). Despite this common metabolic feature, these organisms are exceedingly diverse from both morphological

and biochemical perspectives. A wide range of different carbon sources can be utilized by SRB (Widdel, 1988).

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; Sorensen *et al.*, 1981) available in freshwater and marine environments, little is known about the ecology of SRB in industrial environments especially industrial cooling water systems.

The use of culture methods for detection of microorganisms offers excellent sensitivity, being theoretically able to detect a single cell. Various media and modifications of these media are available for the detection and isolation of SRB (Ferodak *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 (Herbert and Gilbert, 1984). However, not all SRB are able to grow on lactate. Some strains of SRB will not grow on widely used media. These strains are usually not detected in environmental samples. 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.

The use of either polyclonal or monoclonal antibodies offers a potentially sensitive and specific means of identifying environmental important bacteria (Pickup, 1991). Serological work on SRB has yielded conflicting results (Abdollahi and Nedwell, 1980; Aketagawa *et al.*, 1985; Baker *et al.*, 1962; Postgate and Campbell, 1963; Singleton, 1985). However, 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. Antisera prepared against various SRB-type strains were used as a polyvalent antiserum mixture for the detection of SRB in natural samples (Bobowski and Nedwell, 1987; Gaylarde and Cook, 1987).

Antiserum prepared against the APS reductase enzyme has potential use as detection method for *Desulfovibrio* species (Odom *et al.*, 1991). Oligonucleotide probes complementary to regions of 16S rRNA molecule of SRB were prepared for the detection of SRB (Amann *et al.*, 1990; Amann *et al.*, 1992). Various other techniques that include the use of biochemical methods such as fatty acid fingerprinting (Taylor and Parkes, 1983) and radiorespirometric assays (Maxwell and Hamilton, 1986) can be used for the detection of SRB. Commercial kits for the detection of SRB are also available.

Antisera prepared against certain ecologically important strains can thus be used in an ecological study to determine the dominant SRB species present in industrial cooling water systems.

Table 1. Organisms involved in the corrosion of iron as well as other metals.

Group of organsims	Examples of organisms	Corrosion mechanisms
Sulphur oxidizing	*Thiobacillus thiooxidans* *T. concretivorus* *T. ferrooxidans*	Production of sulphuric acid
Iron bacteria	*Gallionella* *Sphaerotilus* *Crenothrix* *Leptothrix* *Clonothrix* *Lieskeela*	Oxidation of ferrous to ferric ions which results in the deposition of ferric hydroxide (tubercle formation)
Hydrogen consuming	Sulphate-reducers Methanogens	Cathodic depolarization
Hydrogen producing	Sulphate-reducers *Clostridium*	Hydrogen embrittelment
Thermophilic	*Desulfovibrio thermophilus* *Thermodesulfobacterium* *Pyrodictium* *Thermoproteus* Methanogens *Sulfolobus*	Cathodic depolarization H_2S-production Cathodic depolarization Acid production
Miscellaneous	*Pseudomonas* *Lactobacillus delbrueckii* Cellulose degrading bacteria *Clostridium*	Reduction of ferric to soluble ferrous iron, Slime and acid production, formation of oxygen concentration cells Organic acid production Acetic and butyric acid production Organic acid production
Fungi	*Cladosporium resinae*, *Aspergillus niger*, *A. amstelodami*, *Penicillium cyclopium* and *P. brevicompactum* *Scopulariopsis brevicaulis*	Carboxylic acid production Acid production, concentration cell formation Mechanisms unclear
Algae Blue green algae	*Nostoc parmelioides* and *Anabaena sphaerica*	Production of oxygen, organic acids and nutrients for other organisms
Red algae	*Graciollasia*	Slime formation, concentration cells formation

(Iverson, 1987)

Table 2. Classification of sulphate-reducing bacteria

Bacterium	Morphology	Major electron donors
Desulfovibrio		
desulfuricans	Vibrio	Lactate, pyruvate, ethanol
vulgaris	Vibrio	Lactate, pyruvate, ethanol, H_2
gigas	Spiral	Lactate, pyruvate
africanus	Vibrio	Lactate, pyruvate, malate
salexigens	Vibrio	Lactate, pyruvate, malate
baculatis	Rod	Lactate, pyruvate, malate
sapovorans	Vibrio	Lactate, pyruvate
baarsii	Vibrio	Acetate, formate
thermophilus	Rod	Lactate, pyruvate
sulfodismutans	Vibrio	Lactate, ethanol
carbinolicus	Vibrio	H_2, alcohols, lactate
simplex	Vibrio	Lactate, pyruvate, glucose
furfuralis	Vibrio	Furfural, lactate, ethanol
Desulfotomaculum		
orientis	Vibrio	Lactate, pyruvate
ruminis	Rod	Lactate pyruvate
nigrificans	Rod	Lactate, pyruvate
antarticum	Rod	Lactate, glucose
acetoxidans	Rod	Acetate, butyrate
sapomandens	Rod	Ethanol, higher fatty acids
kuznetsovii	Rod	Acetate, lactate, ethanol
guttoideum	Rod	Lactate, H_2
Desulfomonas		
pigra	Rod	Ethanol, pyruvate
Desulfotobacter		
postgatei	Cocco-bacillus	Acetate
curvatus	Vibrio	Acetate, ethanol
latus	Oval	Acetate
Desulfobulbus		
propionicus	Lemon	Propionate, lactate, pyruvate
elongatus	Curved rod	Propionate, lactate, H_2
Desulfococcus		
multivorans	Round	Acetate, lactate
niacini	Oval/round	Nicotinic acid, acetate pyruvate
Desulfosarcina		
variabilis	Packets	Acetate, ethanol, H_2, pyruvate

Desulfonema		
magnum	Filaments	Acetate, malate, benzoate
limicola	Filaments	Acetate, Benzoate, pyruvate
Desulfobacterium		
phenolicum	Vibrio	Phenol, acetate
indolicum	Oval/rod	Indole, acetate, formate
autotrophicum	Oval	Ethanol, formate, H_2, betaine
macestii	Oval/rod	Lactate, ethanol, H_2
vacuolatum	Oval/rod	Lactate, H_2, formate
catecholicum	Cocco-bacillus	Cathecol, formate
Desulfomicrobium		
apsheronum	Rod	Lactate, pyruvate, ethanol
Thermodesulfobact		
commune	Rod	Lactate, pyruvate, H_2
Archaeglobus		
fulgidis	Round	Lactate, pyruvate

Gibson (1990)

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Chapter III.

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

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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 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.

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 (49). The importance of these bacteria in microbial induced corrosion (MIC) has been widely recognized for many years (11, 35, 36, 47).

Although research has been performed regarding the incidence and species diversity of SRB (3, 4, 25, 31, 46) and utilizable carbon sources (30, 44) 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 (19). 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 (20). 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 (10, 21, 45). Successful application of fluorescent antibodies can be affected by a range of factors, including specificity and cross reactivity (33).

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 (5, 38, 42); 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* (1). 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 (43). In natural samples, higher counts of SRB were obtained with a similar polyvalent cocktail compared to the MPN technique (19, 6). 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.

MATERIALS AND METHODS

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 refinery in Sasolburg, South Africa. A total of five different samples were used, of which four were biofilm deposits and one, a water sample. Iron sulphite (IS)-medium (27) or synthetic medium (32) 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 (22) 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 ^oC. Titanium (III) citrate (50) 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 ^oC 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.

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 (27). 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} (28). 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 (18). Blood samples were left to clot overnight at 4 ^{o}C and the serum was then collected by centrifugation at 3 000 X g for 20 min and stored at -12 ^{o}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).

Immunodiffusion. The prepared antisera were examined for immunological similarities as described by Garvey (18) 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 (27) 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 (18). The immunodiffusion plates were incubated at 4 ^{o}C and inspected every 24 h for precipitation bands and stained with Coomassie blue (18).

Preparation of fluorescent antibodies. The immunoglobulins were precipitated from the prepared antisera by using polyethyleneglycol 6000 (12% w/v) (9). The final precipitates were dissolved in PBS and the protein concentration determined using spectrophotometry (48).

The purified immunoglobulin fraction of the prepared antisera was conjugated with fluorescein isothiocyanate isomer I (FITC) (9). After conjugation the unbound FITC was removed from the conjugate by gelfiltration through Sephadex G-25 (9).

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 (34). 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 (27), (b) Homologous SRB-cells cultivated in synthetic medium (32) with lactate as carbon source, (c) Heterogeneous SRB-cells cultivated in IS-medium (27), (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.

Sodium dodecyl sulphate-polyacrylamide- gel electrophoresis (SDS-PAGE). Cells of *D. gigas* and *D. nigrificans* were cultured in IS-medium (27) 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 (32) 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.* (14). 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 (26), modified according to Kiredjan *et al.* (24). 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 (23) and destained according to Anderson and Anderson (2). 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.

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. 1).

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. 4). 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.

The membrane protein profiles of the different SRB-species cultivated in IS and synthetic medium are shown in Fig. 2. 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 dendrogram (Fig. 2). 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. 3. 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.

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. 4). 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 (43) 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 (43). 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 (19, 6). The antisera prepared by Bobowski and Nedwell (6) were prepared against cell extracts and not whole cells. These authors all used Postgate's media (37) 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 (13). 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 (8). The use of FA-stains has nevertheless been successfully applied for the detection and enumeration of many microorganisms in their natural habitat (11, 39). Various studies indicated that FA-staining reactions were highly specific (16, 40, 41).

Several techniques have been developed to reduce autofluorescence and nonspecific staining (17, 45, 21). A major concern in the application of any marker technique in ecological studies is the stability of the marker under different conditions and environments (8). Only a few antigens have been tested for their stability in the environment and have appeared to be relatively stable (17). For example, rhizobia have been extensively tested on different media, in different soils and as bacteroides in nodules (7). 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. 2 and 3). 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 (29) 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 (15). The results from our study are similar to those of Davies *et al.* (12) 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 (12).

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.

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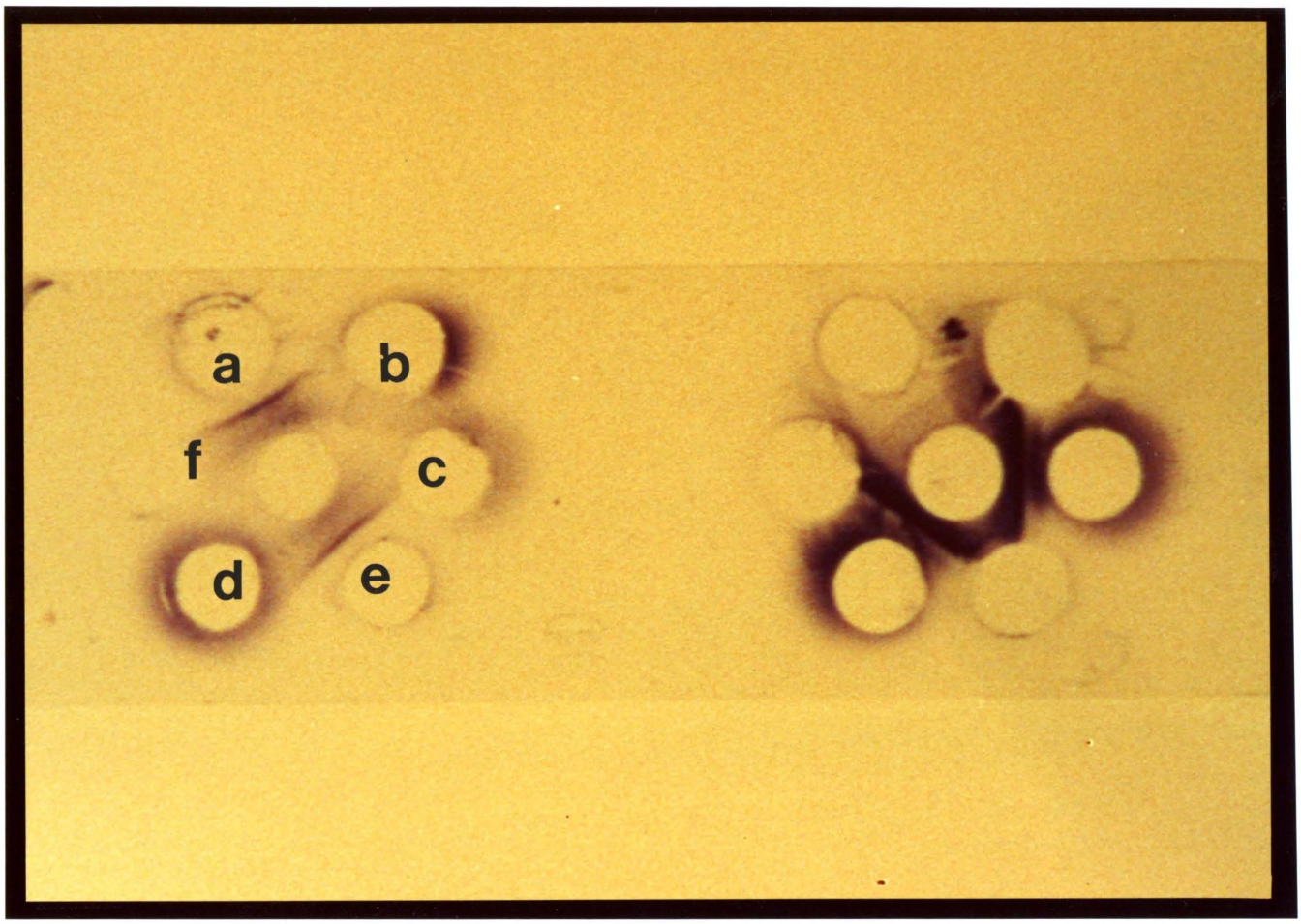


FIG. 1. Double immunodiffusion agar plate with antisera prepared against *D. desulfuricans* in the center well and the following antigens cultured in IS-medium (16) placed in the outer wells; a, e, *D. desulfuricans*; b, *D. gigas*; c, *D. orientis*; d, *D. guttoideum* and f, *D. nigrificans*.

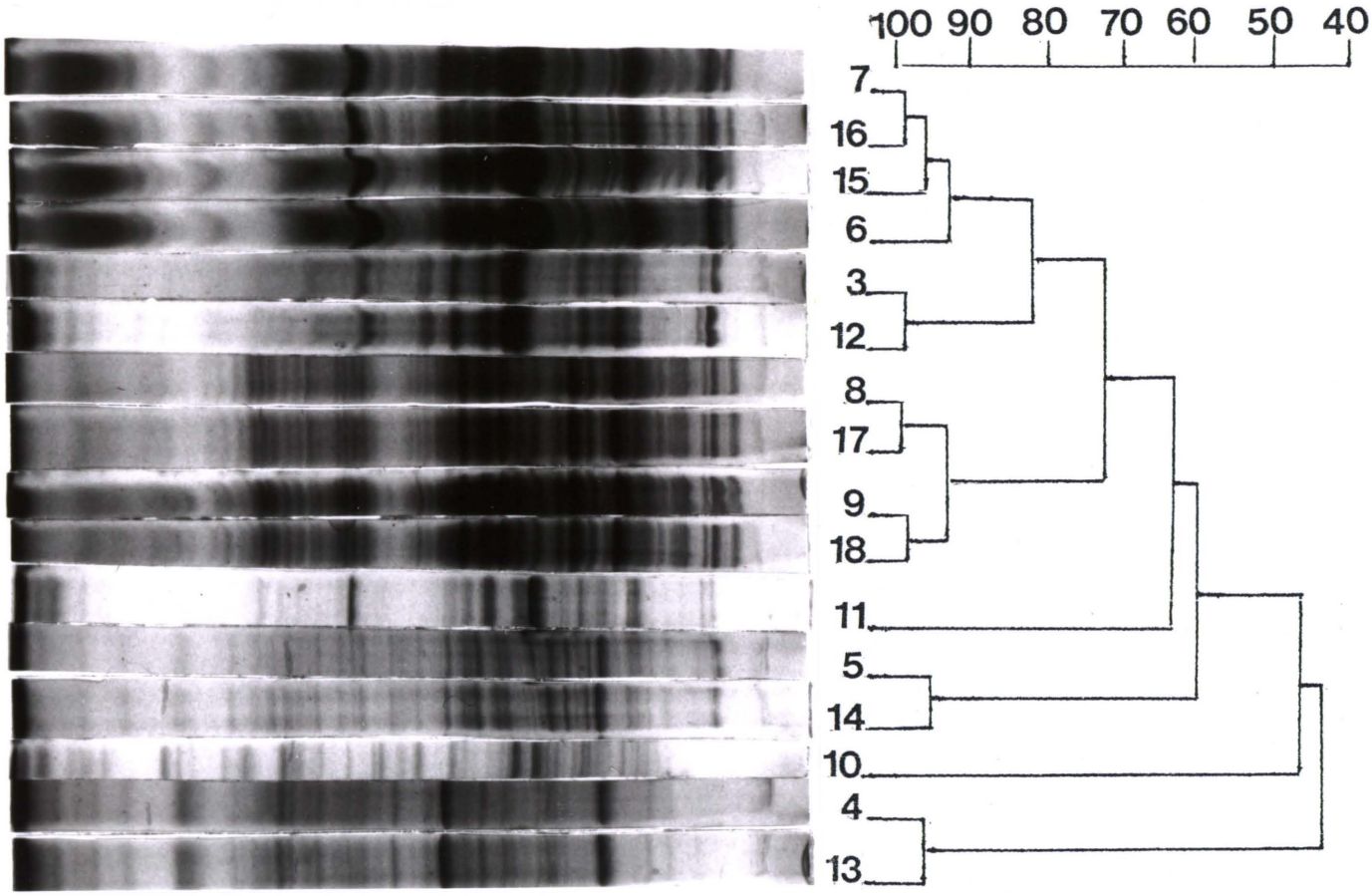


FIG. 2. Dendrogram of the relationships of different SRB-strains cultivated in IS-medium (16) and synthetic medium (17) based on r values, as calculated by the unweighted average pair group method using SDS-PAGE of total membrane proteins. Lanes 3, 12 represent the protein profile of *D. gigas*; 4, 13, *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).

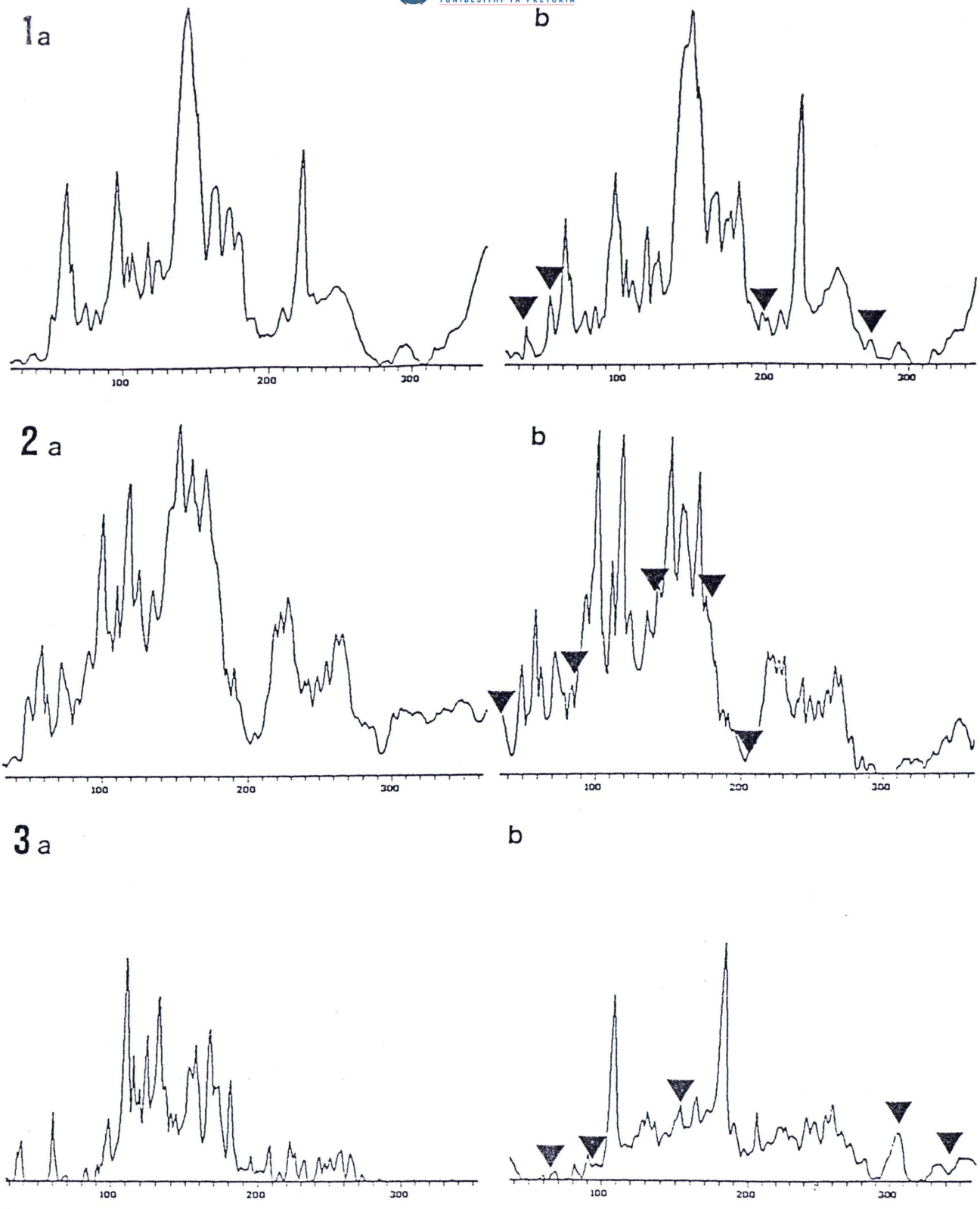
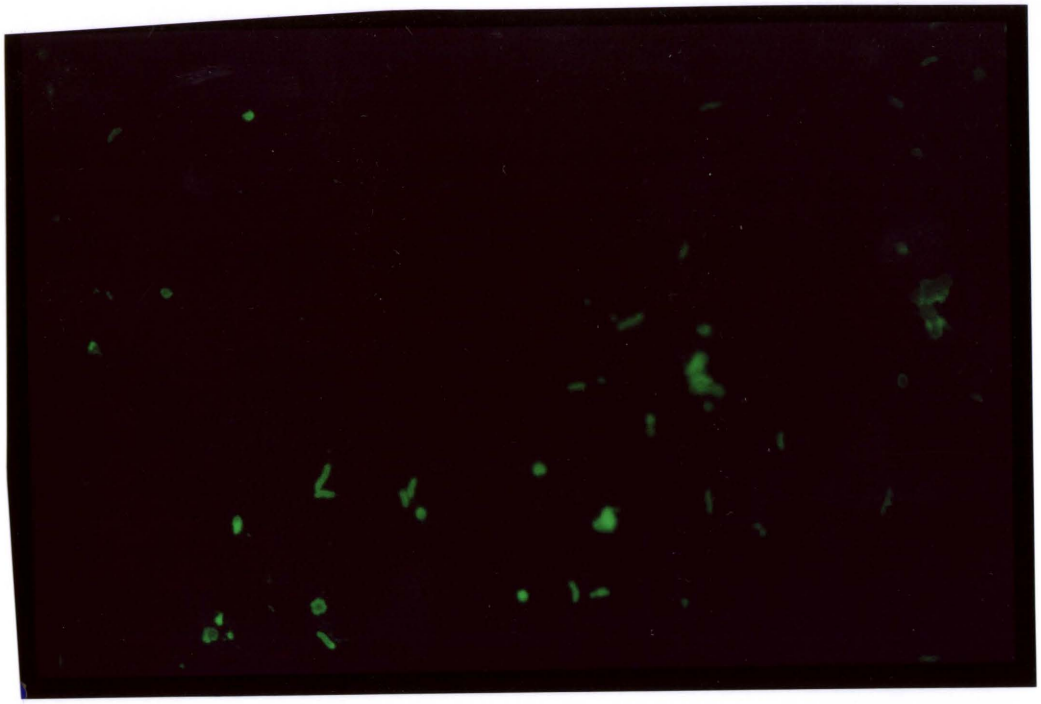


FIG. 3. Scans of; 1, the membrane protein profile of *D. desulfuricans* cultured in; a, IS-medium (16) and b, synthetic medium (17), 2, *D. africanus* cultured in; a, IS-medium and b, synthetic medium and 3, *D. orientis* cultured in; a, in IS-medium and b, in synthetic medium. Arrows indicate prominent differences in the profiles of the SRB-species cultured in synthetic medium from the species cultured in IS-medium.

a



b

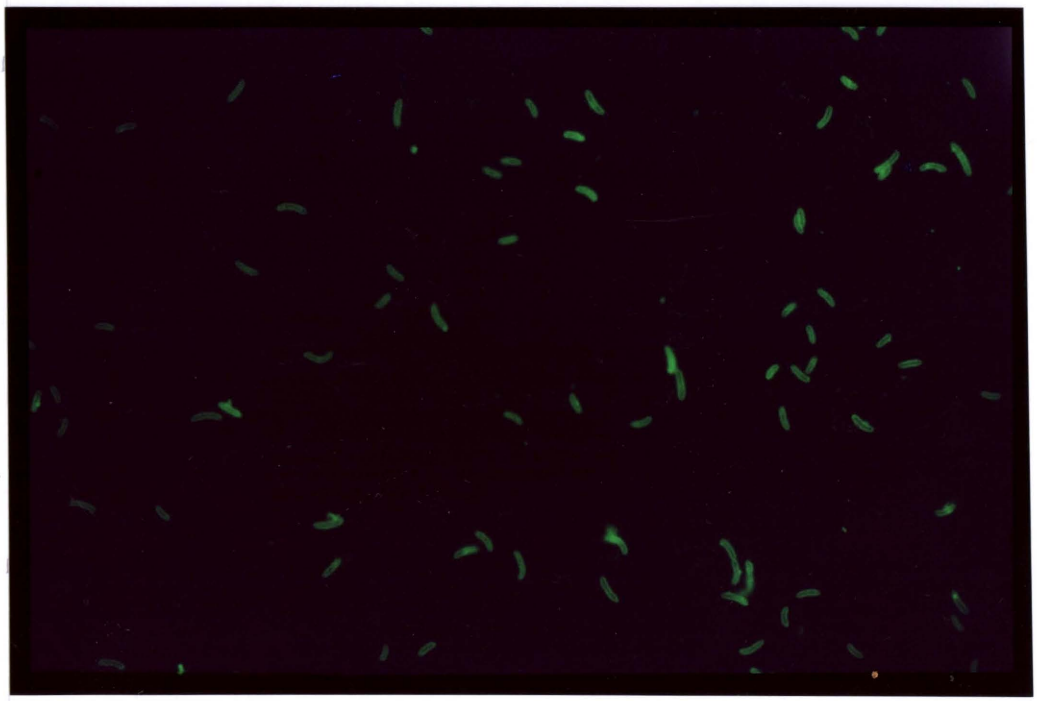
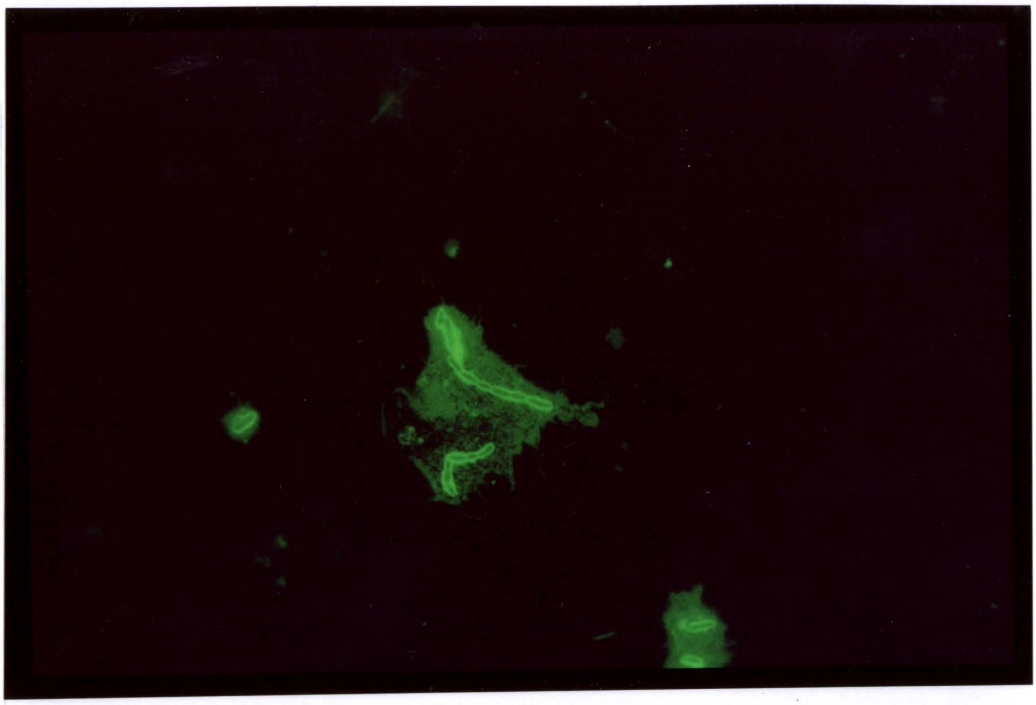


FIG. 4a. Cross-reactions between the fluorescent-antibody conjugates and the homologous cells of a; *Desulfovibrio desulfuricans* and b; *Desulfotomaculum orientis* cultured in the same medium as used for the preparation of antisera.

a



b

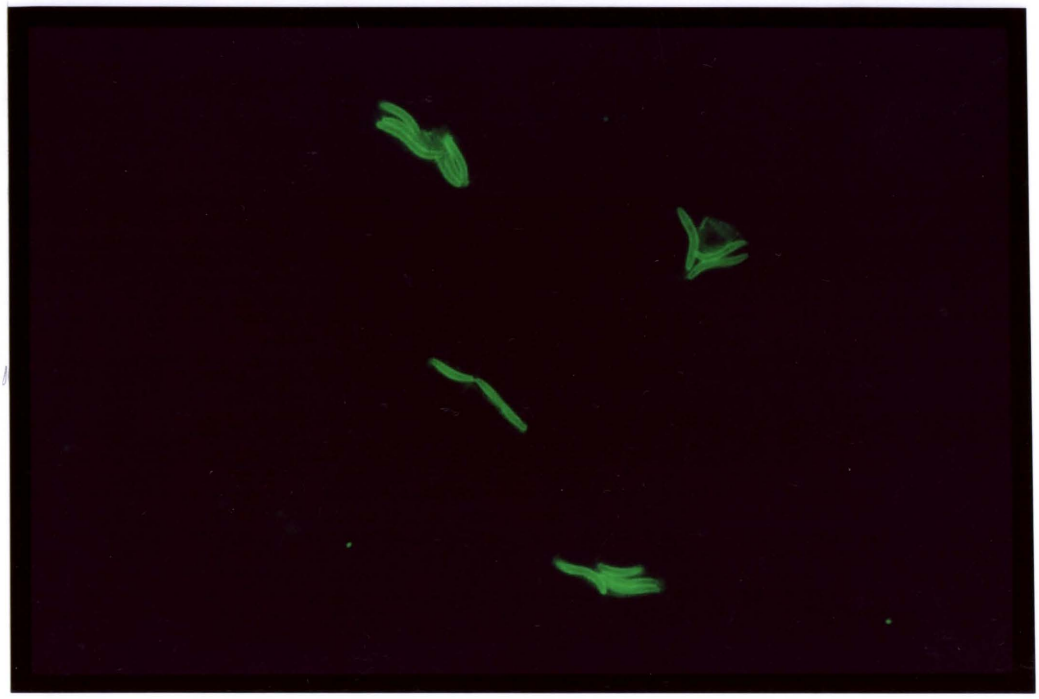


FIG. 4b. Cross-reactions between the fluorescent-antibody conjugates and the homologous cells of a; *Desulfotomaculum guttoideum* and b; *Desulfovibrio gigas* cultured in the same medium as used for the preparation of antisera.

Chapter IV.

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

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(Language and style used in this Chapter are in accordance with the requirements of the Journal of Microbiological Methods*)

Summary

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.

Key Words: Industrial water systems, Isolation media, Microbiological induced corrosion, Sulphate-reducing bacteria

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 [5]. Although many organisms can play a part in the corrosion processes, the chief culprits are the SRB [1].

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 [3]. Despite this common metabolic feature, these organisms are exceedingly diversified from both morphological and biochemical perspectives. Growth is possible on CO_2, a range of organic compounds including benzoate and on fatty acids from acetate to stearate [6].

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 [2, 3, 4, 5, 7, 8].

Hamilton [1] 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 [4]. The isolation of a large number of new types of SRB which use carbon sources other than lactate [9] 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.

Materials and methods

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 [10], the modified medium of Postgate C with a lower calcium content [11], The modified iron sulphite (IS)-medium [12], 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. [13] 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. [13]. 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), 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) [14] was used as reducing agent. The agar roll tube technique [15] 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 ^{o}C. After the inoculation and preparation of agar roll tubes, the tubes were incubated at 30 ^{o}C. All the black colonies that developed after 14 d were counted as being SRB.

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.

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 [12], SABS-medium and the modified synthetic medium [13] with lactate as carbon source were used to count SRB in industrial water systems with the agar roll tube technique.

Modified synthetic medium [13] 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 [13] 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 [6].

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 ^{o}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 [16], oxidase and catalyze activity and fermentation on Hugh and Leifson medium [17] 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 [12], Postgate mediums [3] and API-medium. Different modifications of IS-medium (Table 1) 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 ^{o}C. Black discoloring of the media was noted as positive for sulphide production.

Results

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 Figure 1. The highest numbers of D. desulfuricans cfu ml^{-1} of pure culture were obtained when Oxoid-medium for SRB and IS-medium [12] were used. IS-medium [12] 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.

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 2. 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.

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 Figure 2. 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 [13] with lactic acid, acetate, formic acid or palmitic acid as different carbon sources (Figure 3). The organisms that were isolated from industrial water samples using the modified medium [13] 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.

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 1) iron sulphide production by these organisms occurred only when cultured on modifications 1, 4 and 5 of IS-medium (Table 1). 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.

Discussion

When comparing API-medium, SABS-medium, IS-medium [12], Oxoid-medium and the modified synthetic medium [13], 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 (Figure 1).

Sulphate-reducing bacteria are a diverse group of organisms that are able to utilize various carbon sources [6]. 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 [13] 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 (Figure 3). 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 (Figure 2). Aerobic, facultative aerobic and anaerobic bacteria were isolated from corrosion lesions on metal surfaces [18]. 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 [18]. According to Atlas and Bartha [19], 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 [20] provided evidence for both a dissimilatory and a assimilatory pathway for sulphate reduction in Clostridium

pasteuranium 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 [21] and Hardy [22] 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 [23-25]. 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 [18].

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.

Acknowledgment

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Table 1. Medium composition of the various modifications of iron sulphite medium.

Medium composition	Amounts in g 1000 ml^{-1} of the various modifications						
	1	2	3	4	5	6	7
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

Iron sulphite medium (Mara and Wilians, 1970)

Table 2a. 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 2b. Analysis of Variance by using the Duncan's multiple range test for variables when comparing different 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

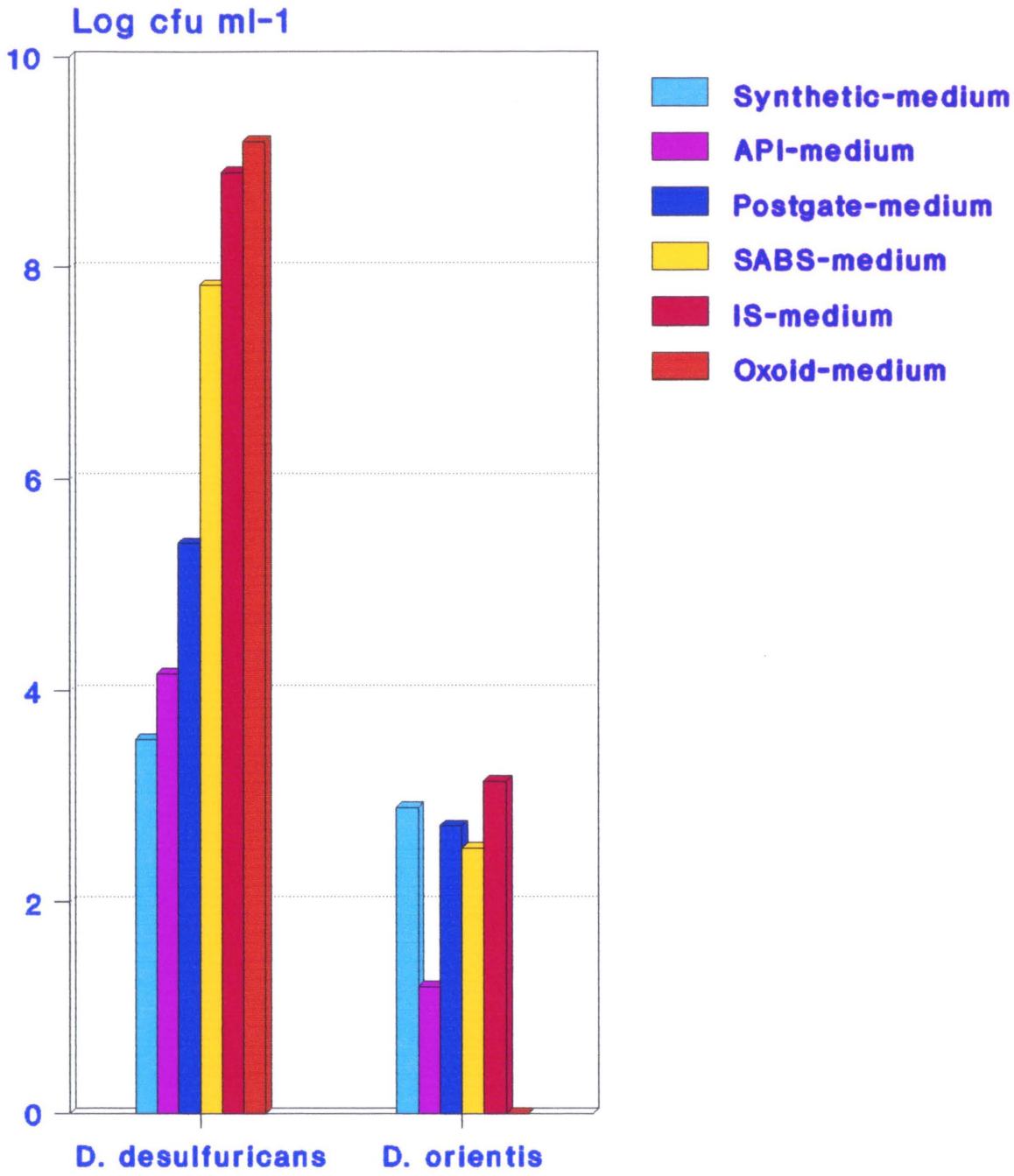
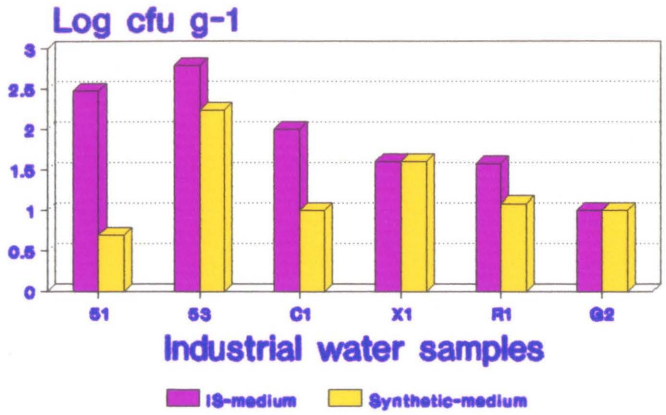
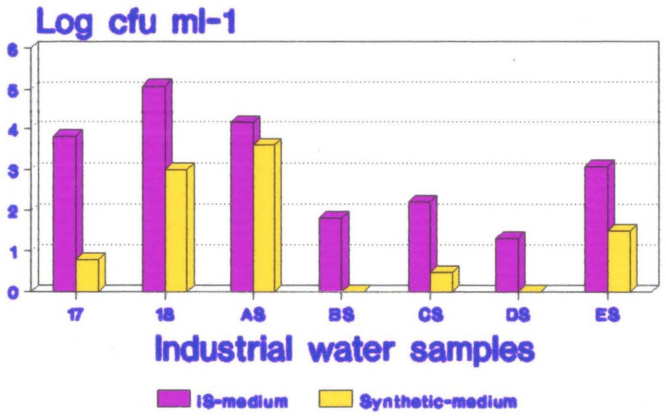


Fig. 1. Comparison of different media for the isolation of sulphate-reducing bacteria from pure cultures.





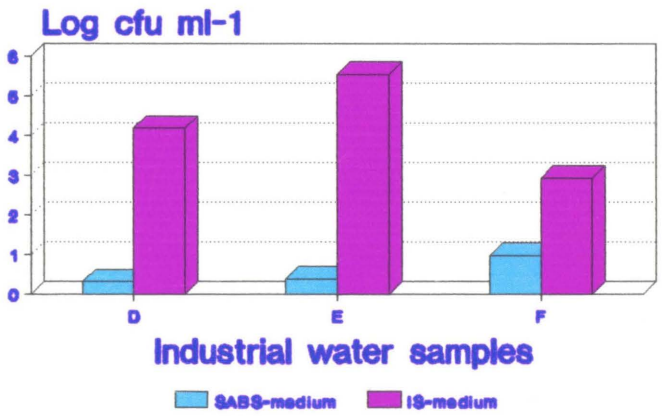
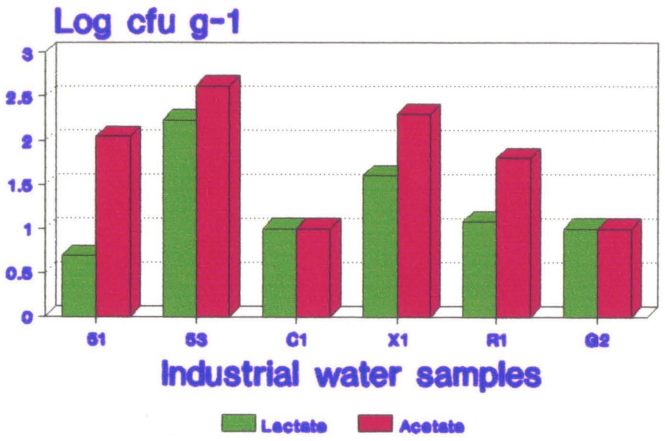


Fig. 2. The detection of sulphide-producing bacteria in industrial water samples using different isolation medium with lactate as carbon source.



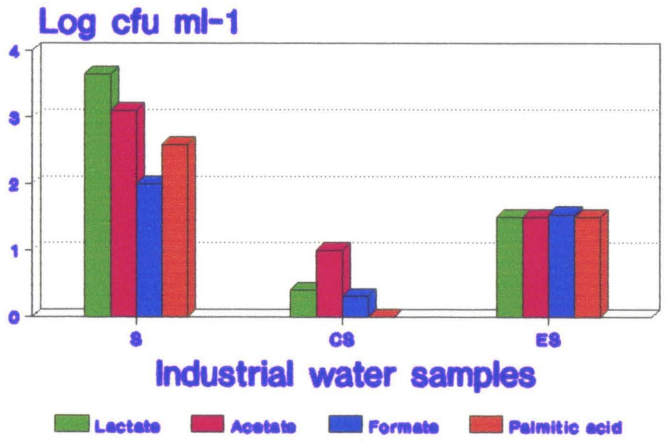


Fig. 3. The detection of sulphide-producing bacteria in industrial water samples using the modified synthetic medium [13] with different carbon sources.

Chapter V

(Submitted for publication in Journal of Applied Bacteriology*)

Shewanella putrefaciens:Facultative anaerobic H_2S-producing bacteria, isolated from industrial cooling water systems.

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Running headline: Shewanella putrefaciens

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*(Language and style used in this chapter are in accordance with the requirements of Journal of Applied Bacteriology)

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 bacteria, has been isolated from industrial cooling water systems.

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; 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.

MATERIALS AND METHODS

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 1). 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 refinery in Sasolburg, South Africa, using IS-medium (Mara and Williams, 1970). Of these isolates, 6 were used in this study for further identification.

Identification of isolates obtained from the cooling towers of a refinery in Sasolburg. i) 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 1) were cultured for 24 h at 28^{o}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 X 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 ul 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 ^{o}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.

RESULTS

Identification of H_2S-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.

Identification of H_2S-producing isolates obtained from the cooling towers of a refinery in Sasolburg using SDS-PAGE of total soluble cell proteins. The

relationships between the industrial isolates and the S. putrefaciens strains obtained from the LMG culture collection can be seen on the dendrogram (Fig. 1). According to the dendrogram 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.

DISCUSSION

The dominant bacteria isolated from 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 4 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 1). The 4 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 4 DNA-DNA homology groups of S. putrefaciens formed 4 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; 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.

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Table 1 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

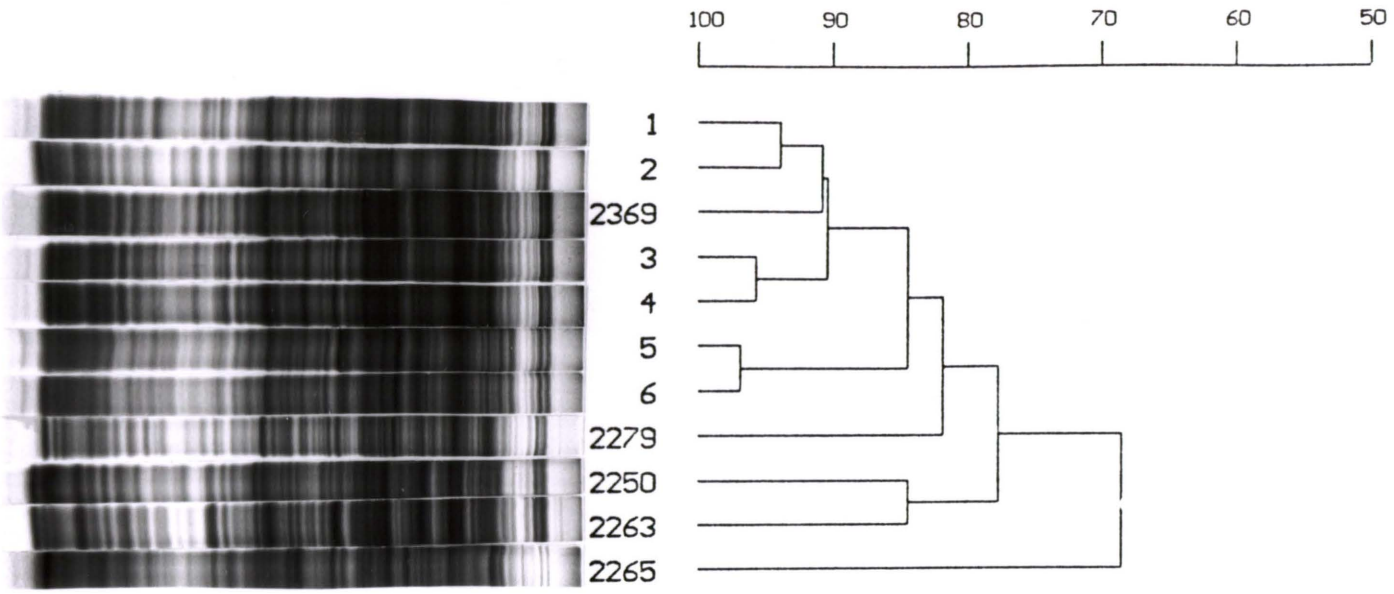

Fig. 1 Dendrogram of the relationship of different Shewanella putrefaciens strains and facultative aerobic H_2S-producing isolates obtained from industrial cooling water systems based on r values, as calculated by the average pair group method using SDS-PAGE of total soluble proteins. LMG numbers of S. putrefaciens strains and industrial isolates numbered from 1 to 6 are shown on the dendrogram.

Chapter VI.

THE ROLE OF *SHEWANELLA PUTREFACIENS* AS A CORROSION CAUSING BACTERIA

(Submitted for publication in Biofouling *)

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*(Language and style used in this chapter are in accordance with the requirements of Biofouling)

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 *Esherichia 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. Electron 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.

KEY WORDS: *Shewanella putrefaciens*, iron sulphide production, cathodic depolarization, microbiologically induced corrosion.

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 economical 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 (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. These organisms were identified as *S. putrefaciens* by using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (De Bruyn and Cloete, 1992). 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.

MATERIALS AND METHODS

Organisms: *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 (1993). 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 refinery in Sasolburg, South Africa. Of these isolates, six were further identified by using SDS-PAGE as *S. putrefaciens* (De Bruyn and Cloete, 1992). *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.

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 A 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 70 % 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 ^{o}C for up to 7 d. 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, two 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 ^{o}C for up to 7 d.

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 four weeks of incubation at 30 ^{o}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.

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 ^{o}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.

RESULTS

Screening for bacterial corrosion using silicon plates coated with a thin film of metal

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 1a and 1b. 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 in IS-medium. However, these two organisms did not corrode the copper film in the other media nor did they corrode 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 2. When *S. putrefaciens* cells were cultured on IS-medium one out of nine, one out of 10, none out of six and none out of two 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, five out of nine, six out of seven, three out of five and one out of two coupons, respectively, corroded. All the coupons corroded when *S. putrefaciens* cells were cultured on nutrient agar. *P. fluorescens* corroded four out of 11 plates when cultured on IS-medium, none out of nine when cultured on IS-Fe medium and one out of three when cultured on nutrient agar. *E. coli* corroded two 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.

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. 1a. In Fig. 1b areas of chemical attack were visible after four 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 2a and 2b). In Fig. 2b cracks in the iron sulphide film with *Desulfovibrio* cells are shown. After exposure of a 3CR12 coupons for four 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. 3a). In Fig. 3b 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. 4.

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. 5a). These areas disappeared (oxidized) after a short time and left the heavily reduced area produced by cellular (Fig 5b) 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. 5c). These results were obtained with *D. desulfuricans*, *S. putrefaciens* 2369 and a *S. putrefaciens* industrial isolate.

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 were chosen as test material mainly because of their 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. fluorescens* 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, in stead 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. 2a and 2b). 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 3a and 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.

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Table 1a. The corrosive action of various bacteria in liquid media under anaerobic conditions on copper.

Bacteria cultures	Corrosion of copper coated silicon in the following media			
	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

Table 1b. The corrosive action of various bacteria in liquid media under anaerobic conditions on nickel.

Bacteria cultures	Corrosion of nickel coated silicon in the following media			
	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

Table 2. The corrosive action of various bacteria, cultured on solid media under aerobic conditions, on nickel.

Bacteria cultures	Corrosion of nickel coated silicon plates indicated as the number of plates corroded out of the total number tested when placed on the following media:		
	IS	IS-Fe	Nutrient
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
Esherichia 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

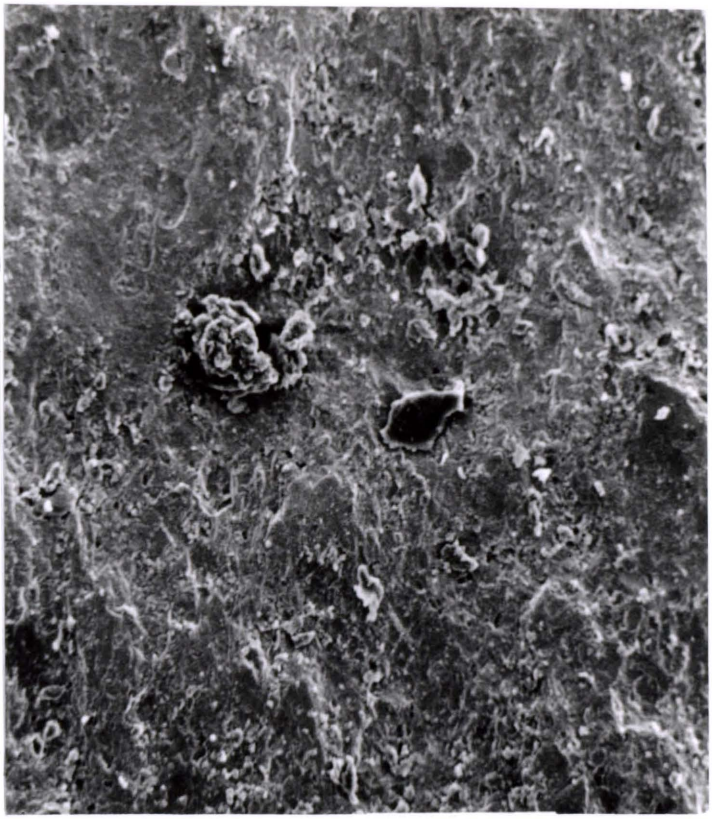

Fig. 1a SEM micrograph of the surface of a 3CR12 coupon before exposure to culture media (490 X)

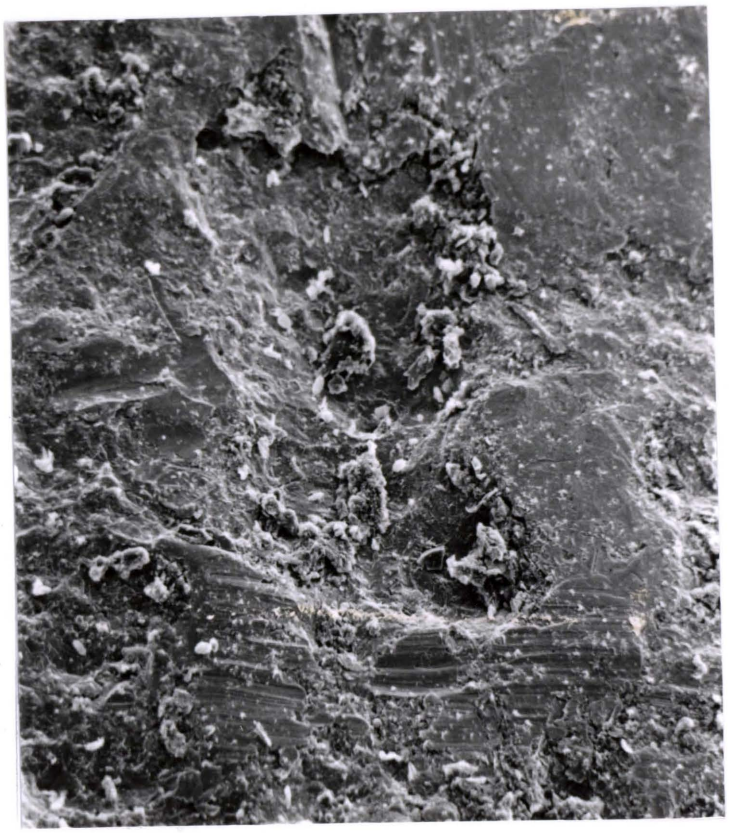

Fig. 1b SEM micrograph of 3CR12 after four weeks exposure to IS-medium under anaerobic conditions (580 X)

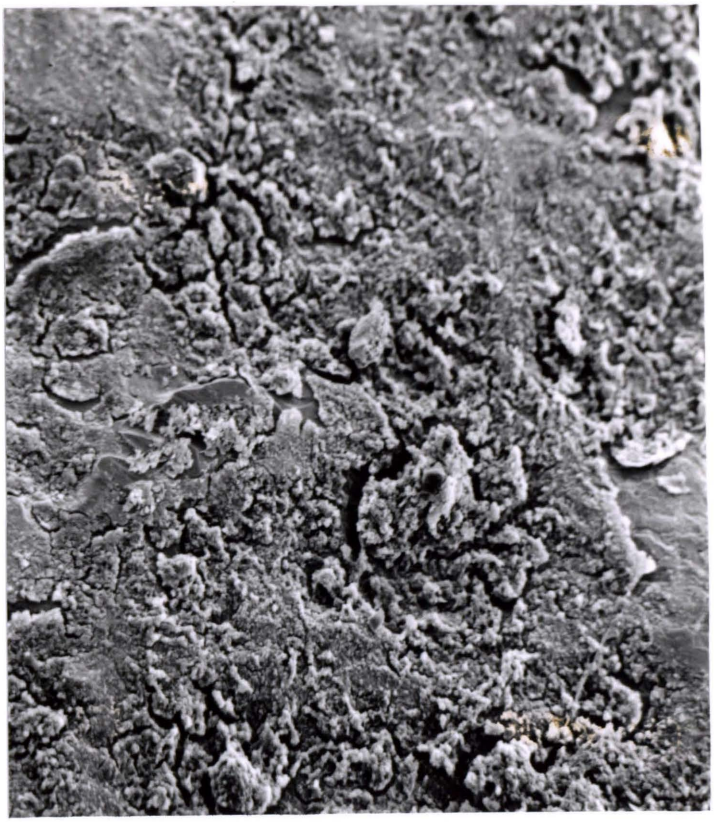

Fig. 2a SEM micrograph of 3CR12 exposed to IS-medium inoculated with *Desulfovibrio desulfuricans* (580 X)

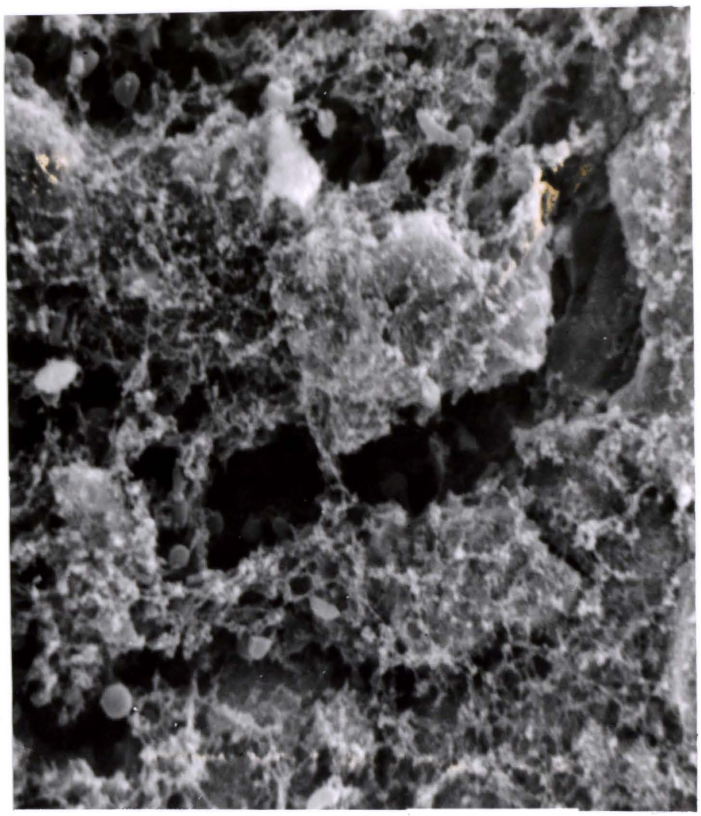

Fig. 2b SEM micrograph of 3CR12 exposed to IS-medium inoculated with *Desulfovibrio desulfuricans* (4 500 X)

Fig. 3a SEM micrograph of 3CR12 exposed to IS-medium inoculated with a *Shewanella putrefaciens* isolate (130 X)

Fig. 3b SEM micrograph of 3CR12 exposed to IS-medium inoculated with a *Shewanella putrefaciens* isolate (5 000 X)

Fig. 4 SEM micrograph of 3CR12 exposed to IS-medium inoculated with a *Shewanella putrefaciens* 2369 (1 300 X)

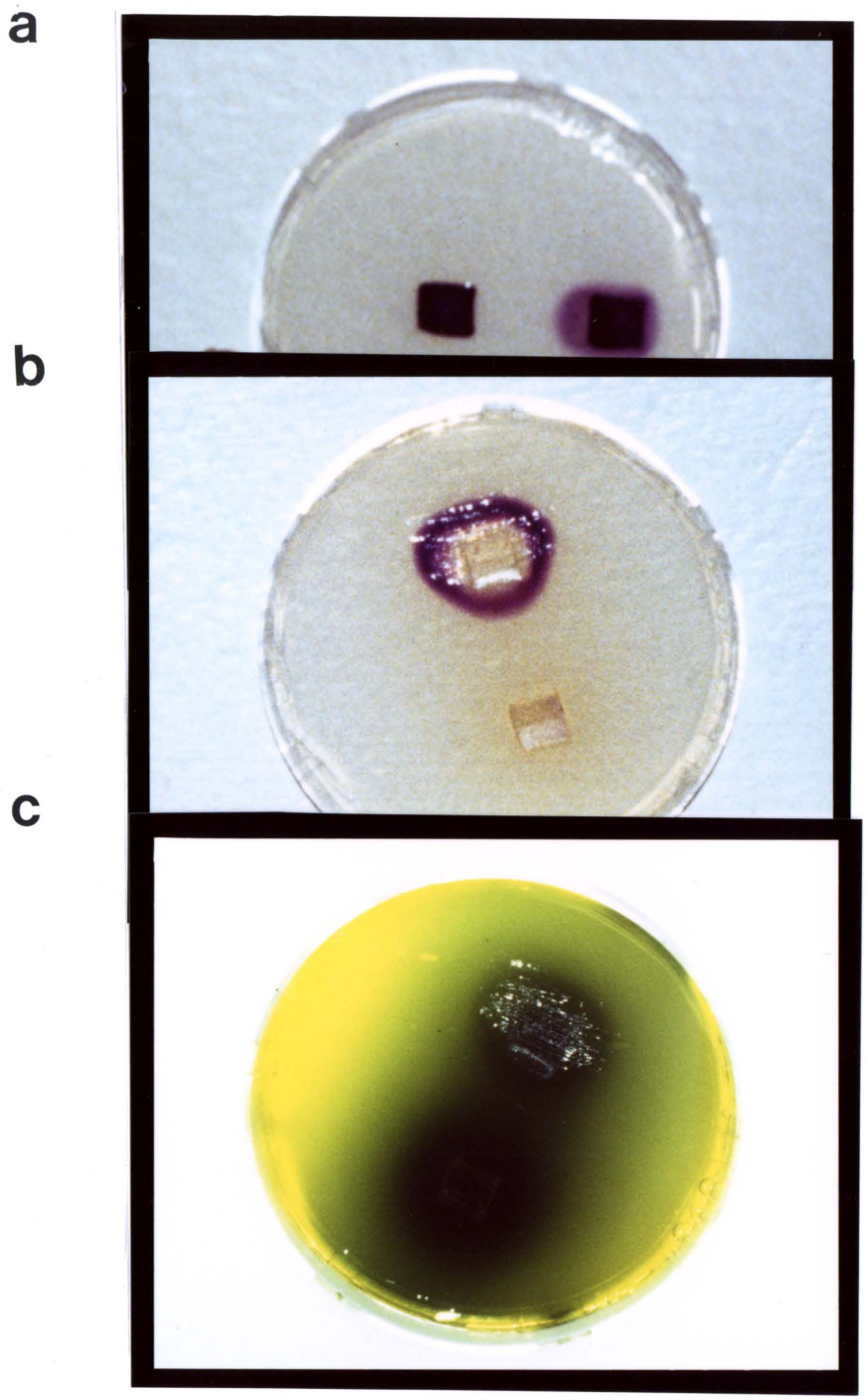

Fig. 5 Areas in agar under mild steel coupon indicating locations of reduced benzyl viologen and ferrous ions. (a) Agar surface immediately after removal of steel coupon. (b) The same plate 10 minutes later. (c) Plate after development with potassium ferricyanide showing heavy Fe^{++} concentration at the anode

Chapter VII.

The Malthus system for biocide efficacy testing against *Desulfovibrio desulfuricans*

(Submitted for publication in Water SA**)

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**(Language and style used in this chapter are in accordance with the requirements of Water SA)

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.

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 by making 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 (Brzel 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 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, 1993).

Materials and methods

Test organism

Desulfovibrio desulfuricans subsp. *desulfuricans* (DSM 1924) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM).

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.

Malthus calibration experiments

D. desulfuricans was precultured in IS-medium under anaerobic conditions at 30 ^{o}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 aliquote of each of the latter dilutions were inoculated into anaerobic tubes containing molten (45^{o}C) IS-agar. After inoculation and preparation of agar roll tubes (Hungate, 1969) these were incubated at 30 ^{o}C for 14 d. All the black

colonies that developed after 14 d were counted as being *Desulfovibrio*. Subsequently, 23 Malthus tubes (the required amount for statistical analysis) were inoculated with 1 ml of the primary and secondary dilution series. Conductance readings at 30 ^{o}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. 1). Detection times were then plotted 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).

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)

Results

Malthus calibration experiment

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. 2. 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 statistical 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*.

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 1. 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.

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.

Acknowledgements

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TABLE 1

BIOCIDE EVALUATION 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

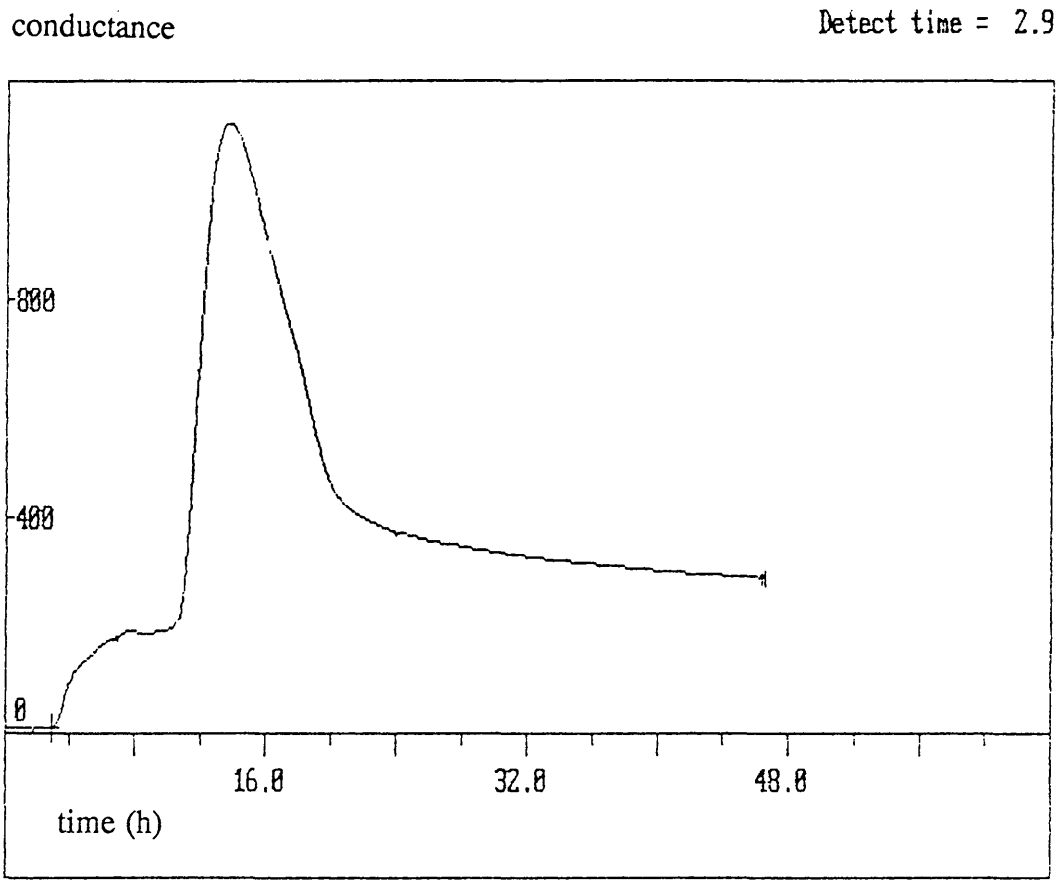

FIGURE 1

A Typical plot of the conductance change over time (h) of Desulfovibrio desulfuricans in Iron Sulphite broth

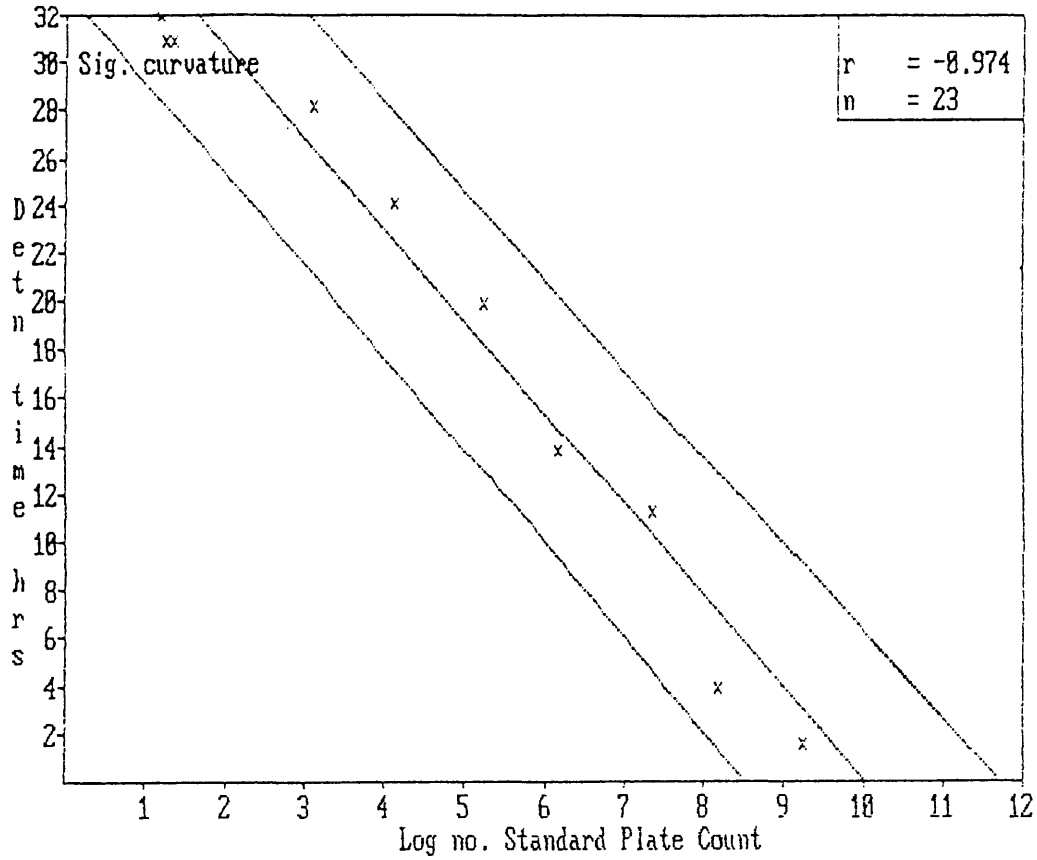

FIGURE 2

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

Chapter VIII. General discussion and concluding remarks

The importance of sulphate-reducing bacteria (SRB) in microbially 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). However, there has been increasing evidence that other organisms, in addition to SRB, have been involved in the corrosion process (Ford and Mitchell, 1990; Videla, 1991).

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; Sorensen *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 of SRB by conventional methods is very time consuming (Gaylarde and Cook, 1987) and might give rise to a biased and incomplete picture of the natural population being sampled (Hamilton, 1985). The potential of species specific fluorescent antibodies prepared using authentic SRB-strains for studying the ecology of SRB in industrial cooling water systems was investigated. Surface antigens of SRB were 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 was 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 emphasized that caution should be exercised when using fluorescent antibodies for ecological studies.

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 diverse from both morphological and biochemical perspectives (Widdel, 1988). There are many formulations of media used for enumerating SRB (Ferodak *et al.*, 1987; Gibson *et al.*, 1987; Hardy, 1981; Herbert and Gilbert, 1984; Pankhurst, 1971; Postgate, 1984). Different isolation media and carbon sources for the isolation and detection of SRB were evaluated. When comparing API-medium, SABS-medium, Iron sulphide (IS)-medium, Oxoid-medium and synthetic medium; IS-medium (Mara and Williams, 1970) yielded the highest numbers when used to enumerate SRB from pure culture and industrial water samples. The use of isolation media with 1 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. H_2S-producing bacteria that utilized lactate, acetate, formate and/or palmitic acid were isolated from industrial water samples when modified synthetic medium (Pfennig *et al.*, 1981) when these different carbon sources were used. 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 sample tested.

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. These bacteria were identified as *Shewanella putrefaciens* by using the API 20 NE identification system and SDS-PAGE of total soluble proteins. IS-medium was therefore not selective for SRB only. Medium containing sulphite can therefore not be used in the industry for selectively isolating SRB. Many organisms such as coliform bacteria, *Proteus*, *Citrobacter*, *Salmonella*, *Pseudomonas* and *Clostridia* (Atlas and Bartha, 1987; Laishley *et al.*, 1984; McMeekin and Patterson, 1975; Oltmann *et al.*, 1975) are capable of sulphide production. The presence of these organisms in an environmental sample will therefore give rise to false positive results regarding the presence of SRB. According to Costello (1974) and Hardy (1983) the generation of sulphide is of greater importance in corrosion than 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; Pope and Dziewulski, 1990; Videla, 1991). The importance of sulphide producing organisms other than SRB in industrial water systems and in MIC must be investigated in more detail and determined.

Silicon plates coated with a thin layer of copper or nickel were used for rapid screening of the corrosivety of the *S. putrefaciens* isolates, *Desulfovibrio desulfuricans*, *Pseudomonas fluorescens* and *Esherichia coli* in liquid medium under anaerobic conditions or on solid medium under aerobic conditions. The results obtained demonstrated the importance of iron sulphide produced by *D. desulfuricans* and *S. putrefaciens* in the corrosion process. 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 *D. desulfuricans* 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 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. The most widely accepted theory for mechanisms of corrosion involves cathodic depolarization of 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. When using benzyl viologen as evidence for cathodic depolarization it was demonstrated that *S. putrefaciens* has the ability to utilize cathodic hydrogen.

Microscope studies of the interaction under laboratory conditions between some bacterial isolates from crude oil and oilfield water, identified as *S. putrefaciens* by Semple and Westlake (1987), and mild steel coupons submerged in cultures of the organism were performed by Obuekwe *et al.* (1981). Under microaerobic conditions these bacteria removed the protective crystalline amorphous coat formed on the surface of the steel coupon in the absence of the bacteria. Microscope studies were also performed to determine the interaction under laboratory conditions between *S. putrefaciens* and *D. desulfuricans*, 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. 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). *S. putrefaciens* could play a important role in MIC.

The use of biocides to control biofouling and thus corrosion in industrial water systems is an accepted practice (Cloete *et al.*, 1989). However, incorrect use of biocides gives poor results and is expensive. It is therefore essential to select the correct biocide or combinations and their respective concentrations for the organisms to be killed (Cloete *et al.*, 1992). Biological techniques for determining active biocide concentrations have been developed (Hill *et al.*, 1989; Cloete *et al.*, 1990). 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 for monitoring growth and estimating bacterial numbers (Richards *et al.*, 1978). One such system (Malthus 2000 detection system, Swift Micro Laboratories) is based on automatically monitoring of electrical conductance in cultures. Conductance changes monitored using the Malthus system were evaluated as an alternative method to conventional culturing for estimating the numbers of *D. desulfuricans* for laboratory biocide evaluations. The Malthus system, using conductance measurements, proved useful in determining whether a particular biocide concentration would be effective against a SRB-strain or not. Preparations involved when using the Malthus system are less difficult and laborious than when using anaerobic culture media for enumeration of SRB because of the smaller volumes of media used than with standard methods. Samples can be inoculated directly into the system without the need for the preparation of serial dilutions. Biocide evaluations can be completed within 48 h when using the Malthus system opposed to 14 d using conventional techniques.

IS-medium would be recommended for use in the industry to detect sulphide production. A more selective method should be used for the detection of SRB as a group and the diversity of this group of organisms and the ability to utilize a wide range of carbon sources must be taken into account. No SRB enumeration method is perfect. The responsible approach is to try several methods simultaneously. Only with this approach can truly reliable SRB numbers be expected on field samples.

Concluding Remarks

Iron sulphite (IS) medium (Mara and Williams, 1970) yielded the highest numbers when used to enumerate SRB from pure culture and industrial water samples.

H_2S-producing bacteria that utilized lactate, acetate, formate and/or palmitic acid were isolated from industrial water samples when modified synthetic medium (Pfennig *et al.*, 1981) with these different carbon sources were used.

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.

These facultative aerobic Gram-negative sulphide-producing bacteria were identified as *Shewanella putrefaciens*.

S. putrefaciens could play a important role in MIC.

Antisera prepared against the surface antigens of SRB, cultured in IS-medium, were species specific and 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.

The Malthus system, using conductance measurements, could be used for enumerating SRB in pure culture.

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