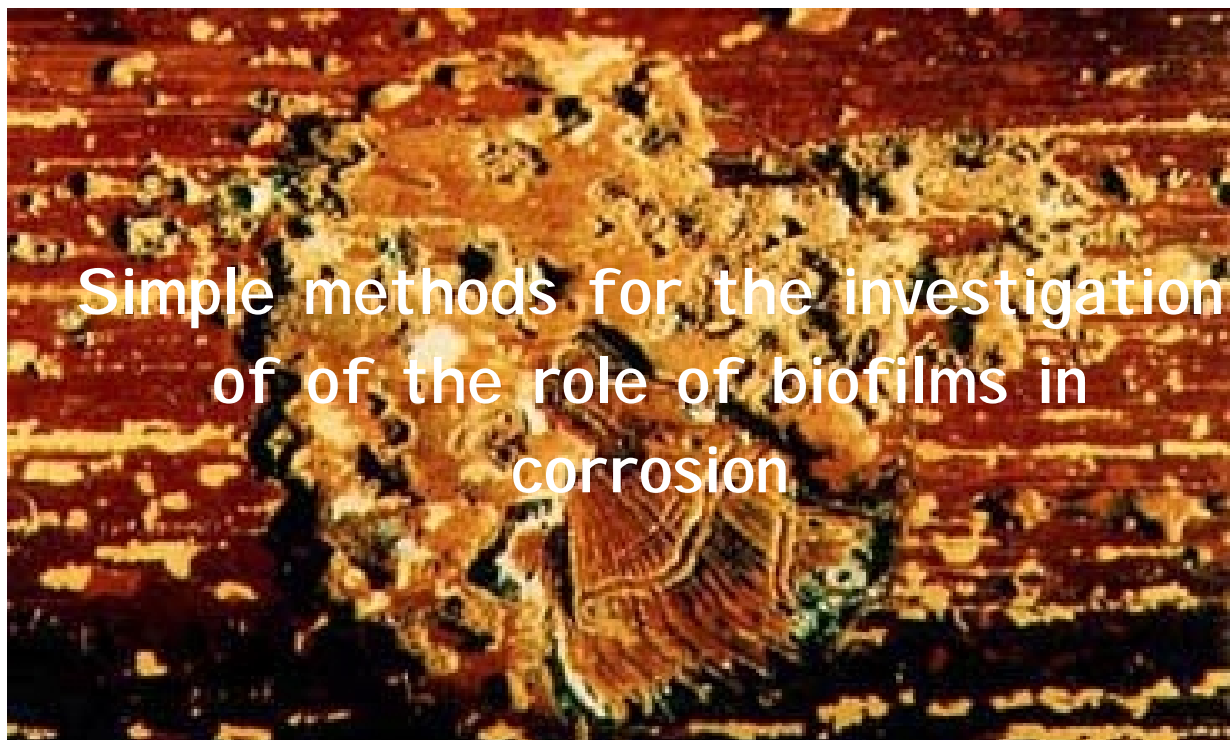


MICROBially INFLUENCED CORROSION OF INDUSTRIAL MATERIALS

- BIOCORROSION NETWORK -

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Task 1: Biofilms Publication

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SIMPLE METHODS FOR THE INVESTIGATION OF THE ROLE OF BIOFILMS IN CORROSION

**BRITE EURAM THEMATIC NETWORK ON MIC OF
INDUSTRIAL MATERIALS**

TASK GROUP 1 BIOFILM FUNDAMENTALS

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1 MICROBIOLOGICAL FUNDAMENTALS

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1.1 What is MIC?

MIC is the acronym for “Microbially Influenced Corrosion”. It refers to the possibility that microorganisms are involved in the deterioration of metallic (as well as non-metallic) materials. MIC is not a new corrosion mechanism but it integrates the role of microorganisms in corrosion processes. Thus, an inherently abiotic process can be influenced by biological effects. A suitable definition of MIC, which has been developed in task group 1, is the following:

"Microbially Influenced Corrosion (MIC) refers to the influence of microorganisms on the kinetics of corrosion processes of metals, caused by microorganisms adhering to the interfaces (usually called "biofilms"). Prerequisites for MIC is the presence of microorganisms. If the corrosion is influenced by their activity, further requirements are: (I) an energy source, (II) a carbon source, (III) an electron donator, (IV) an electron acceptor and (V) water"

A role of MIC is often ignored if an abiotic mechanism can be invoked to explain the observed corrosion. MIC, as a significant phenomenon, has thus been viewed with scepticism, particularly among engineers and chemists with little appreciation for the behaviour of microorganisms. The slow process in establishing the importance of MIC in equipment damage is also the result of the paucity of analytical techniques to identify, localize and control corrosion reactions on metal surfaces with surface-associated microbial processes. In the meantime, MIC has become increasingly acknowledged during the last decade (for reviews: see Little et al., 1990 a; Videla, 1991, 1996; Lewandowski et al., 1994; Heitz et al., 1996; Borenstein, 1998; Beech, 1999; Geesey et al., 2000). In some cases, MIC has become the “joker” in cases where there is no plausible electrochemical explanation for a given corrosion case. In order to better understand under which conditions MIC is possible, some basic questions are answered in this introduction.

The role of biofilms

In principle, corrosion is an interfacial process. The kinetics of corrosion are determined by the physico-chemical environment at the interface, e.g., by the concentration of oxygen, salts, pH value, redox potential and conductivity. All these parameters can be influenced by microorganisms growing at interfaces. This mode of growth is preferred by most microorganisms on earth (Costerton et al., 1987). The organisms can attach to surfaces, embed themselves in slime, so-called extracellular polymeric substances (EPS) and form layers which are called “biofilms”. These can be very thin (monolayers) but can reach the thickness of centimeters, as it is the case in microbial mats. Biofilms are characterized by a strong heterogeneity. It is well known that the metabolic activity of clusters of biofilm organisms can change the pH value for more than three units locally. This means that directly at the interface, where the corrosion process is actually taking place, the pH value can differ

significantly from that in the water phase. Thus, water sample values do not reflect such effects.

Thus, MIC is not a “new” corrosion process but is caused by the effect of microorganisms at the corrosion process kinetics. These can be very considerable, however, it must be made sure that microorganisms are in fact involved into the process. This is an important consideration as MIC has become a kind of “joker”, explaining all kinds of otherwise unexplained corrosion cases. Therefore, it is important to consider under which conditions the participation of microorganisms is possible.

1.2 Principles of microbial life

MIC can only occur when microorganisms are present and active. First of all, they need water. Bacteria cannot multiply if the water activity is below 0,9. Certain fungi can grow at a water activity of 0,7 but they are very much specialized and do not play a major role in MIC. However, water is not enough - growth requires always an electron donor, which is oxidized, an electron acceptor, which is reduced, an energy and a carbon source. According to these requirements, they are grouped systematically (Table 1):

Table 1: Prerequisites for the growth of microorganisms

Prerequisite	Provided by	Kind of growth
Energy source	Light Chemical substances	Phototrophic Chemotrophic
Carbon source	CO ₂ Organic substances	Autotrophic Heterotrophic
Electron donor (which is oxidized)	Inorganic substances Organic substances	Lithotrophic Organotrophic
Electron acceptor (which is reduced)	Oxygen NO ₂ ⁻ , NO ₃ ⁻ , SO ₄ ²⁻ , CO ₂	Aerobic "anoxic" anaerobic

Light is a very important energy source as it promotes photosynthesis. This process has been “invented” by microorganisms some three billion years ago and led to the existence of molecular oxygen in the atmosphere. This was a major environmental disaster for the anaerobic microorganisms which dominated life in the beginning, because oxygen was toxic to most of them. However, they managed to find ecological niches to survive, e.g., in the oxygen free areas of the subsurface, but also inside of living organisms and in the oxygen-depleted regions of biofilms. A very good place to live for an anaerobic organism is below an active colony of aerobic organisms as these consume the oxygen and create anaerobic zones which serve as habitats for the anaerobics. This is the reason why anaerobic organisms can be found in close proximity of aerobic organisms, a fact, which may be quite unexpected. Obligate anaerobes such as sulfate-reducing bacteria, which are very sensitive to oxygen, can therefore survive and multiply in aerobic habitats as they are protected by aerobic organisms.

Chemical energy can be drawn from practically all reduced chemical species. A well known example which is of importance for MIC is the oxidation of sulfide, which can be performed by sulfur oxidising bacteria. A result is a steep decrease in pH value, which is well understandable when the reaction equation [1] is considered, which demonstrates how a weak acid is turned into a strong acid:



This mechanism is important for the generation of acide drainage and for the destruction of concrete in sewerage pipelines (Sand et al., 1989).

In table 2, some important and corrosion relevant types of electron acceptors and the organisms performing the reactions are summarized (after Schlegel, 1992):

Table 2: Examples for electron acceptors

Electron acceptor	Product	Name of respiration, Organisms
Aerobic respiration		
O ₂	H ₂ O	oxygen respiration: all strictly or facultative aerobic organisms in presence of oxygen; <i>Pseudomonas aeruginosa</i> , <i>E. coli</i>
Anaerobic respiration		
NO ₃ ⁻	NO ₂ ⁻ , N ₂ O, N ₂	nitrate respiration, denitrification: <i>Paracoccus denitrificans</i> , <i>Pseudomonas stutzeri</i>
S ²⁻	SO ₄ ²⁻	sulphate respiration: obligate anaerobe bacteria; <i>Desulfovibrio desulfuricans</i> , <i>Desulfomaculum ruminis</i> , <i>Desulfonema limicola</i>
S	S ²⁻	sulphur respiration: facultative and obligate anaerobe bacteria; <i>Desulfuromonas acetoxidans</i>
CO ₂	Acetate	carbonate respiration: acetogenic bacteria; <i>Clostridium aceticum</i>
	Methane	carbonate respiration: methanogenic bacteria; <i>Methanobacterium</i> , <i>Methanosarcina barkeri</i>
Fumarate	Succinate	fumarate respiration: succinogenic bacteria; <i>E. Coli</i> , <i>Wolinella succinogenes</i>
Fe ³⁺	Fe ²⁺	iron respiration: <i>Alteromonas putrefaciens</i>
MnO ₂	Mn ²⁺	manganese respiration: <i>Shewanella putrefaciens</i> , <i>Geobacter sp.</i>

Microbial life is possible under circumstances we would consider as “extreme” from the point of view of humans. Table 3 (after Flemming, 1991) gives an impression of the spectrum of microbial life. However, it must be considered that not one single organisms is capable to live along the entire spectrum. It is always specifically adapted populations which will be possible to live at the limits as indicated.

Table 3: Spectrum of conditions under which microbial life is observed (after Flemming, 1991)

<p><i>Temperature</i> from -5 C (Salt solutions) to >120 C (hot vents at the bottom of the ocean)</p>
<p><i>pH-spectrum</i> from 0 (Thiobacillus thiooxidans) to 13 (Plectonema nostocorum, in natron lakes)</p>
<p><i>Redox potential</i> entire range of water stability from -450 mV (methanogenic bacteria) to +850 mV (iron bacteria)</p>
<p><i>Pressure</i> to 1.000 bar (barophilic bacteria at the sea floor)</p>
<p><i>Salinity</i> growth in ultrapure water (e.g., <i>Burkholderia cepacia</i>) up to almost saturated water (halophilic bacteria in the Dead Sea)</p>
<p><i>Nutrient concentration</i> from 10 µg L⁻¹ (drinking and purified water) up to life in and on carbon sources</p>
<p><i>Radiation</i> biofilms on UV lamps biofilms on irradiation units biofilms in nuclear power plants (e.g.: <i>Micrococcus radiodurans</i>)</p>
<p><i>Biocides</i> biofilms in disinfection lines</p>

As indicated earlier, MIC is a biofilm problem. The construction material of biofilms, the EPS, are mainly composed of polysaccharides and proteins with usually minor concentrations of nucleic acids and lipids (Wingender et al., 1999). This material is highly hydrated (95-97 % water) and provides a matrix in which the organisms can immobilize themselves, establishing stable synergistic communities, called “microconsortia”. This is one of the ecological advantages for microorganisms in aggregates.

1.3. Some details about microorganisms

It is obvious that microorganisms are a large diverse group of microscopic organisms such as viruses, bacteria, fungi, algae and diatoms that, with the exception of viruses, exist as single cells or cell clusters. A single microbial cell is typically able to carry out its life processes of growth, energy generation and reproduction independently of other cells, either of the same kind or of a different kind. Groups of related cells, generally derived from a single parent cell are called populations. In nature, populations of cells live in association with other population of cells in communities.

Microbial proliferation is greatly influenced by the availability of molecular oxygen (Fig. 1) and temperature.

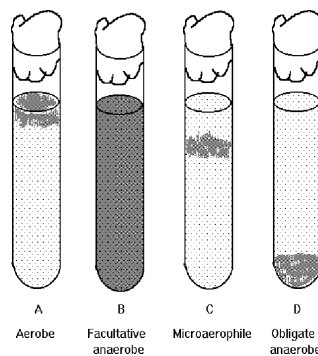


Figure 1. Relationship between the growth of various types of microorganism and the presence of oxygen. (A) Aerobes grow in the presence of oxygen on the surface; (B) facultative anaerobes grow throughout the tube; (C) microaerophiles grow in a narrow band where the oxygen tension is reduced; (D) obligate anaerobes grow at the bottom of the tube where there is no free oxygen.

Temperature influences greatly the growth and survival of microorganisms. There is a minimum temperature below which growth no longer occurs, an optimum temperature at which the growth is most rapid and a maximum temperature above which growth is not possible. Microorganisms can be divided into four groups in relation to their temperature optima: psychrophiles (e.g. *Flavobacterium* spp. with optimum growth at 13°C), mesophiles (e.g.

Escherichia coli with optimum growth at 39°C), thermophiles (e.g. *Bacillus* spp. with optimum growth at 60°C) and hyperthermophiles (e.g. *Thermococcus* spp and *Pyrodictum* spp with optimum growth at 88°C and 105 °C respectively).

1.4 Microorganisms Involved in MIC Processes

Microorganisms implicated in MIC of metals such as iron, copper and aluminium, and their alloys are physiologically diverse. Bacteria involved in metal corrosion have frequently been grouped by their metabolic demand for different respiratory substrates or electron acceptors. The capability of many bacteria to substitute oxygen with alternative oxidisable compounds as terminal electron acceptors in respiration, when oxygen becomes depleted in the environment, permits them to be active over a wide range of conditions conducive for corrosion of metals. The ability to produce a wide spectrum of corrosive metabolic by-products over a wide range of environmental conditions makes microorganisms a real threat to the stability of metals that have been engineered for corrosion resistance.

The main types of bacteria associated with corrosion failures of cast iron, mild and stainless steel structures are sulphate-reducing bacteria, sulphur-oxidising bacteria, iron-oxidising/reducing bacteria, manganese-oxidising bacteria, as well as bacteria secreting organic acids and extracellular polymeric substances (EPS) or slime. As already stated these organisms coexist in naturally occurring biofilms often forming communities able to affect electrochemical processes through co-operative metabolism which individual species have difficulty to initiate.

Sulphate-Reducing Bacteria (SRB)

SRB are a group of ubiquitous, diverse anaerobes that reduce oxidised sulphur compounds, such as sulphate, sulphite and thiosulphate, as well as sulphur to H₂S. Although SRB are strictly anaerobic (obligate anaerobes), some genera tolerate oxygen and are even able to grow at low oxygen concentrations. The activities of SRB in natural and man-made systems are of great concern to many different industrial operations. In particular, oil, gas and shipping industries are seriously affected by the sulphides generated by SRB. Biogenic sulphide production leads to health and safety problems, environmental hazards and severe economic losses due to reservoir souring and corrosion of equipment. Since the beginning of the investigation into the effect of SRB on corrosion of cast iron in 1930s, the role of these bacteria in pitting corrosion of various metals and their alloys in both aquatic and terrestrial environments under anoxic as well as oxygenated conditions has been confirmed. Several models have been proposed to explain the mechanisms by which SRB can influence the corrosion of steel. These have included cathodic depolarisation by the enzyme hydrogenase, anodic depolarisation, production of corrosive iron sulphides, release of exopolymers capable of binding Fe-ions, sulphide-induced stress-corrosion cracking, and hydrogen-induced cracking or blistering. Recent reviews clearly state that one

predominant mechanism may not exist and that a number of factors are involved [5,6].

Metal-Reducing Bacteria (MRB)

Microorganisms are known to promote corrosion of iron and its alloys through reactions leading to the dissolution of corrosion-resistant oxide films on the metal surface. Thus, protective passive layers on e.g. stainless steel surfaces can be lost or replaced by less stable reduced metal films that allow further corrosion to occur. Despite of their wide spread occurrence in nature and likely importance to industrial corrosion, bacterial metal reduction has not been seriously considered in corrosion reactions until recently.

Numerous types of bacteria including those from the genera of *Pseudomonas* and *Shewanella* are able to carry out manganese and/or iron oxide reduction. It has been demonstrated that in cultures of *Shewanella putrefaciens*, iron oxide surface contact was required for bacterial cells to mediate reduction of these metals. The rate of reaction depended on the type of iron oxide film under attack.

Metal-Depositing Bacteria (MDB)

Many bacteria of different genera participate in the biotransformation of oxides of metals such as iron and manganese. Iron-depositing bacteria (e.g., *Gallionella* and *Leptothrix*) oxidize Fe^{2+} , either dissolved in the bulk medium or precipitated on a surface, to Fe^{3+} . Bacteria of these genera are also capable of oxidizing manganous ions to manganic ions with concomitant deposition of manganese dioxide. Dense accumulations of MDB on the metal surface are thought to promote corrosion reactions by the deposition of cathodically-reactive ferric and manganic oxides and the local consumption of oxygen caused by bacterial respiration in the deposit [8].

Acid-Producing Bacteria (APB) and Fungi

Bacteria and fungi can produce copious quantities of either inorganic or organic acids as metabolic by-products. Microbially-produced inorganic acids are nitric acid (HNO_3), sulphurous acid (H_2SO_3), sulphuric acid (H_2SO_4), nitrous acid (HNO_2) and carbonic acid (H_2CO_3). Sulphurous acid and sulphuric acid are mainly secreted by bacteria of the genera *Thiobacillus*. Other bacteria, such as *Thiothrix* and *Beggiatoa spp.*, as well as some fungi (e.g., *Aureobasidium pullulans*), also produce these acids. *Thiobacilli* are extremely acid tolerant and can grow at a pH value of 1. Nitric acid and nitrous acid are mainly produced by bacteria belonging to the groups of ammonia- and nitrite-oxidising bacteria. The main problem in sulphuric and nitric acid corrosion is the fact that the resulting salts are water-soluble and, hence, a formation of a protective corrosion product layer is not possible. Furthermore, due to the lowering of the pH, protective deposits formed on the surface, e.g., calcium carbonate, can dissolve. The third type of inorganic acid, the carbonic acid, is produced by many life forms. Carbonic acid, especially if present in high concentrations, can react with calcium hydroxide and forms the insoluble calcium carbonate (CaCO_3) and the water-soluble

calcium hydrogen carbonate $\text{Ca}(\text{HCO}_3)_2$. The latter is called aggressive carbonic acid.

Some bacteria e.g. *Pseudomonas aeruginosa* elaborate extracellular acidic polysaccharides such as alginic acid, during biofilm formation on metal surfaces. These acids can be highly concentrated at the metal-biofilm interface by the diffusional resistance of the biofilm exopolymers, making it impossible to accurately assess the acid concentration at the metal surface from measurements made in the overlying bulk aqueous phase. Microsensors (ultramicroelectrodes) which have been used to probe the pH gradients within microbial biofilms, revealed both horizontal and vertical variations in pH values at the biofilm / metal interface [9].

Fungi are well-known producers of organic acids, and therefore capable of contributing to MIC. Much of the published work on biocorrosion of aluminum and its alloys has been in association with contamination of jet fuels caused by the fungi *Hormoconis* (previously classified as *Cladosporium*) *resinae*, *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp. *Hormoconis resinae* utilises the hydrocarbons of diesel fuel to produce organic acids. Surfaces in contact with the aqueous phase of fuel-water mixtures and sediments are common sites of attack. The large quantities of organic acid by-products excreted by this fungus selectively dissolve or chelate the copper, zinc and iron at the grain boundaries of aircraft aluminum alloys, forming pits which persist under the anaerobic conditions established under the fungal mat [10].

In addition to fungi, bacteria that ferment organic compounds to low molecular weight organic acids such as acetic, formic and lactic acid have also been implicated in the corrosion of iron and its alloys.

2 Microbiological Investigation of MIC

The suspicion that corrosion is biologically-influenced will begin when the predisposing environmental factors are noticed. If the surroundings of the metal are moist and contain even minute amounts of any suitable nutrient source, then BIC should be considered. In the absence of light, algal growth cannot occur and suspicion will turn to bacteria and fungi. If conditions are anaerobic, then it is likely that bacteria, including the sulphate-reducing bacteria, are the causative agents.

In most cases, bacterial corrosion is accompanied by the formation of slimes or deposits, beneath which anaerobic conditions prevail. Hence it is possible that a highly aerobic environment may nevertheless encourage the growth of corrosion-causing anaerobic microorganisms.

The appearance of the cleaned metal surface can also provide a clue to the nature of the cause of corrosion. Pitting is indicative of bacterial attack, although some aerobic bacteria produce flask-shaped cavities below a pinhole penetration (Pope and Morris, 1995). A network of etch lines could indicate fungal hyphae as the cause (Videla, 1995). Uniform corrosion could

suggest that the cause may be due to acid production by microorganisms, or may not be biological at all.

In order to confirm MIC it is essential to document the presence of microorganisms. This is not possible without recourse to specialised microbiological techniques and, apart from a few exceptional tests, it is unlikely that these can be carried out on site. Thus it is necessary to obtain samples of the natural environment surrounding the metal and, if at all possible, of the slime, deposit or other layers covering the corroded metal surface. It is well documented that in order to carry out biocorrosion risk assessment surface sampling is essential as the number of cells present in the bulk phase (known as planktonic cells) does not represent the existing level of microbial population associated with a surface (termed sessile or biofilm cells). Good sampling technique and correct handling of samples once obtained are of paramount importance.

It has to be emphasised that demonstrating the presence of microorganisms at a corroding site, even those known to produce metabolic by-products aggressive toward metallic materials, is not sufficient evidence for MIC (Ghassem and Adibi, 1995). Some of the myths surrounding biocorrosion have been reviewed by Little and Wagner (1997).

Microbiological investigation of suspected MIC will, however, involve the identification of microorganisms in samples taken from the problem site. If at all possible, these should include samples of biofilm or slimes covering the metal surface, and be processed on-site or as quickly as possible. The methods employed for identification will depend on many factors, not least of which is the speed of result required. Interpretation of the results will be aided immensely by previous experience and expert advice should always be sought.

2.1 Liquid samples

In the case of a metal immersed in or carrying water or other liquids, the sample should be representative of the bulk phase. Hence a relatively large volume (at least 100 ml) should be taken, and where samples are from lengthy or branching pipe systems, liquid should be allowed to flow from the sampling point for a minute or so before collecting the fluid for testing. The material may be placed into any suitable, clean, well-sealed container, preferably microbiologically sterile. This should be filled as nearly as possible to the rim and tightly capped, both to exclude air and to prevent contamination with microorganisms from the environment after sampling. If it is not possible to fill the vessel completely, a layer of sterile oil may be placed above the sample to help exclude oxygen. These conditions must be maintained during transport to the laboratory, during which the specimen should be kept cool. It is advisable to transport the sample to the laboratory as quickly as possible, as during transport a number of changes may occur in the container. If the liquid is non-nutritive, then microorganisms will die quite rapidly, whereas in a rich, nutritious environment cell multiplication will occur. At intermediate levels, such as in a sea water sample, the scarce nutrients in the water become concentrated on to the surfaces of the container promoting the attachment of microorganisms from the fluid phase. At first, this will artificially

lower the planktonic count, but later, as the biofilm grows and sloughs off, the concentration in the surrounding medium will increase. These processes will be slowed, but not eradicated, by maintaining the sample at a low temperature during transport.

Sampling from high temperature environments may require a different approach. Many thermophilic microorganisms do not survive at low temperatures and, if stored for any time at +4°C or less, will not be detectable by culture thereafter. In such cases, it is advisable to transport samples to the laboratory in an insulated container to try to maintain the temperature as close as possible to the original.

2.2 Solid samples

Representative samples should be taken from a previously undisturbed site on or adjacent to the metal surface. Materials suitable for sampling are soil or other environmental substances, corrosion products, deposits and microbial slimes. These may be scraped from the metal surface using any suitable tool. As with liquid samples, it is important to use clean collection vessels, to fill them as much as possible and to keep them tightly closed and cool during transport. Disturbing the materials during collection is unavoidable, but as little mixing as possible should be attempted as some anaerobes are irreversibly inhibited by exposure to oxygen. The quantity of sample need not be as large as in the case of liquids, since it may be expected that the concentration of microorganisms will be higher. However, a reasonable amount of material (up to several grams) will ensure that anaerobic conditions are maintained within at least some parts of the sample. In some instances, introducing probes such as Robbins Device (Mittelman and Geesey, 1987) would facilitate *in situ* sampling.

2.3 Pre-treatment of samples

If large concentrations of cells are expected, then dilution in a suitable liquid, such as a buffer solution, will be required. Homogeneous dispersion of the sample in this diluent is an important factor, but this can be difficult to achieve, especially in the case of solid or particulate materials. The optimal process will vary from sample to sample (Table1) and must be determined empirically.

In the case of liquid samples of low cell content, concentration may be necessary. Centrifugation or, perhaps more appropriately, filtration are available. Another advantage of these manipulations is that they allow the microbial cells to be removed from any masking or inhibitory materials in the surrounding fluid so that a true estimate of their numbers may be made.

If filtration is chosen as the method of concentration, it is important that the characteristics of the filter be considered. A pore size of 0.2 µm diameter is recommended for collecting all bacteria. However, this may rapidly become clogged, making the filtration process unacceptably lengthy. A pore size of 0.45 µm is probably sufficient for most samples. Polycarbonate filters are generally accepted as being superior to nitrocellulose, but the latter are of sufficient quality for the majority of uses. The chemical nature of the sample

must be taken into account, in addition to the manipulations which are to be performed on the sample after filtration

2.4 Detection and Identification of Microorganisms

A number of classical and modern methods are available for the detection of microorganisms which may be associated with corrosion.

2.4.1 Microscopical examination

Observation of the sample under the light microscope is often the simplest and quickest of the tests available. This method is suitable for the detection of algae, fungi and, in clean samples, bacteria. Indeed, together with the macroscopic appearance of a green colouration, it may be all that is necessary to determine the algal content of the sample.

Although direct microscopical examination of samples can indicate the presence of fungi and bacteria, it is not possible to make any identification of the genera of organisms present using this technique alone. Nevertheless, it may give a useful indication of the microbial content in qualitative terms. Different types of light microscopy are used to facilitate sample examination. In addition to the bright-field microscopy which is the most commonly used technique, methods of phase-contrast, dark-field, fluorescence and, recently, confocal laser microscopy are widely employed (Walker and Keevil, 1994; Caldwell *et al.*, 1992). If desired, cationic dyes such as methylene blue, crystal violet, safranin as well as fluorescent dyes such as acridine orange or 4'6-diamidino-2-phenylindole dichydrochloride: hydrate (DAPI), tetrazolium salts or fluorescein diacetate can be used to stain cells enabling their easier identification (Chamberlain *et al.*, 1988; Beech and Gaylarde, 1989; Wolfhaardt *et al.*, 1991; Schaule *et al.*, 1993).

Recent years have seen the development of a number of rapid methods for the specific identification and detection of environmentally important microorganisms (Sogaard *et al.*, 1986). Some of these are available in kit form (e.g. Analytical Profile Index - API or Biolog), but often they require a degree of expertise not normally encountered outside the specialist laboratory. It is necessary to determine priorities before electing to use any particular test. High sensitivity invariably goes hand-in-hand with complexity, whilst economy will often mean low sensitivity and sometimes slower results.

2.4.2 Rapid detection

The "dip stick" (dip slide) technique is a simple method which allows rapid detection of aerobic organisms without the necessity for making dilutions of the sample fluid. A piece of flexible plastic, of approximately microscope slide size, coated on one or both sides with one or two different types of solid microbial growth media, e.g. agar containing triphenyltetrazolium chloride (TTC) and potato-dextrose agar, is dipped briefly into the liquid to be sampled and then placed inside its sterile plastic container for incubation. Sampling from the surface (biofilm sampling) can also be performed. Colonies developing following incubation, can be visualised more readily by the incorporation into the medium of a dye which develops a characteristic colour

on microbial growth, and hence overall colouration, as well as number of colonies, will indicate the level of viable cells in the sample. Dip sticks cannot be applied for very dilute cell suspensions, but are a readily portable, easily used method with no special expertise needed for either application or interpretation.

Microorganisms are routinely detected and identified by their ability to grow in various liquid or on solid laboratory-prepared media (Table 2).

Table 2: Examples of media used routinely for fungal and bacterial isolation

Organisms	Medium
Yeasts	Malt extract agar*
Filamentous fungi	Malt extract agar or potato dextrose agar* (antibiotics or rose bengal may be added to the above fungal media in order to inhibit bacterial growth)
Aerobic and anaerobic bacteria	Nutrient agar*
<i>Pseudomonas</i>	<i>Pseudomonas</i> selective medium*
SRB: • Lactate utilising SRB • Non-lactate utilising SRB	Postgate's medium B (liquid) ¹ Postgate's medium E (solid) ¹ API RP-38 broth (liquid) ² Widdell's medium ¹

* These media are available in dehydrated form from manufacturers such as Oxoid or Difco.

¹ Postgate, 1984.

² API recommended practice for biological analysis of subsurface injection waters. American Petroleum Institute, Washington DC, March 1982.

In some instances, however, the microorganisms of interest may not be cultivable. Therefore methods based on immunological and nucleic acid techniques are used to determine their presence. Such techniques will be discussed in subsequent chapters.

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3 A GUIDE TO LABORATORY TECHNIQUES FOR THE ASSESSMENT OF MIC RISK DUE TO THE PRESENCE OF BIOFILMS

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3.1 Introduction

Owing their metabolic activity, microorganisms settled on metallic surfaces exposed to natural waters (referred to as sessile or biofilm communities) can considerably modify the chemistry at the surface/solution interface compared with the composition of the bulk aqueous phase. As a consequence, the presence of such a biofilm can also alter the corrosion resistance of the metallic substratum. The latter would differ from the corrosion resistance of the same material exposed in sterile water. It has already been stated in the earlier part of this communication that the deterioration of metals occurring under aerobic or anaerobic conditions as a result of the direct or indirect activity of microorganisms (including products of their metabolism) is defined as microbiologically-influenced corrosion (MIC) or biocorrosion. The modification of the corrosion behaviour due to the biofilm presence is frequently negligible (*i.e.* the biocorrosion component is insignificant) and will not create appreciable technical problems. However, in some cases, microbial colonisation of surfaces can result in severe corrosion failures (*i.e.* the MIC is a dominating process, causing the deterioration of metallic material), leading to a drastic reduction of the system's foreseen lifetime, if suitable countermeasures are not applied.

The aim of this chapter is to present an overview of relatively uncomplicated measurements which can be performed by a non-specialised personnel in order to confirm whether the system suffers from MIC and whether the involvement of an expert is required.

3.2 Methods for biofilm evaluation

In general, biofilms are slimy and mucous-like, relatively soft and highly hydrated, with organic smell and slightly coloured appearance. However, the co-precipitation of corrosion products and carbonates together with possible adsorption of corrosion inhibitors could sometimes mask these features or introduce calcareous structures and colour-evoking crustacean organisms such as diatoms.

A preliminary careful analysis of the system operating conditions should usually be sufficient to exclude the MIC risk, when these conditions are not compatible with the survival of biofilms.

Biofilm growth can be reasonably excluded under the following conditions:

- the working temperature of the system is higher than 80°C
- metallic surfaces are not accumulating any moisture (*i.e.* they are kept dry).
- organic/inorganic nutrients for the development of heterotrophic/autotrophic organisms are absent
- antifouling procedures of already proved efficiency are regularly applied

Otherwise, the possibility of a biofilm formation should be considered and the evaluation of organic matter content inside the fouling layer could be used to confirm the presence of a biofilm. This could be achieved through the measurements of the deposit weight losses after a succession of thermal treatments [1], as commonly performed in oceanography for the evaluation of organic contribution in particulate matter (seston). The procedure is not complicated and requires only a muffle furnace and a scale.

Mud or deposit samples are collected from various locations within the system. The collected material is then subjected to centrifugation at 3000 rpm (g value and not rpm value ought to be provided) to standardize the residual water content in the sample. The obtained pellet is dispersed in the small amount of distilled water, transferred into a pre-weighted melting - pot and then heated at 105°C until dryness. The dry weight of sample (D.W.) is estimated from the difference between the final and initial weights of melting-pot. The dry matter is then heated at 175-185 °C until the constant weight is recorded and the final weight loss is identified. The residual matter (R.W.) is heated at 450°-500°C until the constant weight is reached. Although the weight loss recorded after the latter treatment offers only an approximate value of the organic matter content in the sample, the method proved to provide sufficient accuracy in the majority of the investigated case-studies.

The level of organic matter in the biofilm dry weight changes with time due to the entrapment of detritus (sand, silk, inorganic corrosion products) from waters; the organic matter appears to dominate the first phases of biofilm formation, however, later on, its contribution decreases to approximately 30-40% of the biofilm dry weight.

If a high organic content is detected within the deposit, several methods can be applied to quantify the level of contribution from living organisms. The most common technique used for evaluation of the biomass is the spectrophotometric analysis of carbohydrate and protein content.

3.2.1 Spectrophotometric methods:

3.2.1.1 Carbohydrate analysis

A number of methods has been developed for the analysis of saccharides. These methods commonly involve treatment of samples with sulphuric acid to cause the hydrolysis of glycosidic linkages and to dehydrate the monosaccharides in order to allow their reaction with phenol [5], orcinol [6] or antrone [7] to yield coloured products. The brief description of the antrone method is provided below.

The biofilm samples, dispersed in 1 ml of distilled water, are added to 10 ml of an antrone solution in sulphuric acid (0.2 g of antrone is dissolved in a mixture of 8.0 ml of ethyl alcohol and 30 ml of distilled water and the solution is added to 100 ml of concentrated sulphuric acid). The reaction mixture is then heated for 10 minutes at 100°C in sealed tubes. The absorbance of the resulting blue solution is measured at 620 nm in a spectrophotometric cell against water blank prepared using the same procedure. Glucose is used as standard and calibration curve is constructed based on glucose solution series.

3.2.1.2 Protein analysis

Several methods can be used for the measurement of protein content in biofilms [8,9]. A common problem is the presence of a wide range of interfering species, which can produce false positive results. In the case of marine biofilms, the Bradford method [9] modified by Setchell [10] is often applied to samples where a change of volume ratios between sample and reaction mixture is sufficient to reduce the interference.

Unfortunately, the Setchell method, as well as the Lowry [8] approach, are highly influenced by the presence of humic acids (HA) which are always found in natural waters. Schaule *et al.* [11] have suggested a simple way to determine the contribution of HA through modification of the Lowry method.

The biofilm sample is treated with the Folin-Ciocalteus-Phenol reagent both in the presence and in the absence of Cu^{++} and, after 45 min of incubation, the absorbance of the two solutions are measured at 750 nm against their respective blanks. The reaction mixture with Cu^{++} is prepared by mixing the following reagents (A= 0,571 g NaOH and 2.8571 g Na_2CO_3 in distilled water to 100 ml, B=0.7143 g CuSO_4 in 50 ml of distilled water and C= 1.4286 g Na tartrate in 50 ml of distilled water) in the proportion A:B:C= 100:1:1.

The reaction mixture without Cu^{++} is prepared as described above replacing B solution with distilled water.

The Folin-Ciocalteu reagent is prepared by adding 6 ml of demineralized water to 5 ml of MERCK product.

The analysis is carried out mixing 500 μl of sample with 700 μl of reagent mixture (with and without CuSO_4) and adding, 10 min later, 100 μl of Folin – Ciocalteu reagent. The colour development requires about 45 min and the two absorbances measured at 750 nm are respectively indicated as ABS total (with Cu) and ABS blind (without Cu).

The absorbance due to the protein contribution alone is calculated using the following equation:

$$\text{ABS protein} = 1.25 \times (\text{ABS total} - \text{ABS blind})$$

The protein concentration is evaluated against a calibration curve drawn using bovine serum albumin (BSA) as standard.

If the above analyses confirm the presence of a biofilm, more information on its biological structure could be obtained by measuring the maximum respiratory activity of the organisms and the level of algae. The latter approach would aim to address the following issue:

3.3 Is biofilm composed of oxygen producers or oxygen consumers?

To provide an answer to this question the fouling deposit, collected from the system under investigation, is treated with anhydrous methanol and stored in dark in a refrigerator at 4°C for about 15 minutes.

The chlorophyll a (Chl a) from the algae will colour the methanol green and the absorbance measurements at 665 nm (A_{665}), once corrected for the turbidity by subtracting the absorbance at 750 nm (A_{750}), could be used in the Talling Driver (3) formula for quantitative evaluation of Chl a levels:

$$\text{Chl a } (\mu\text{g}) = 11,9 \text{ V(ml)}(A_{665}-A_{750})/ \text{C(cm)}$$

Where V is the methanol volume, expressed in ml, and C the optical length of cells, expressed in cm.

The Electron Transport System activity, which controls the respiratory rate of oxygen-consuming organisms, can be evaluated with the spectrophotometric method of T.T. Packard modified by Owens and King [12], through the measurement of the reduction rate of a colourless INT salt into a red coloured formazan caused by the oxidation rate of the ETS enzyme chain.

The method requires the organisms disruption with Ultra-Sound in phosphate buffer (0.1M with the 0,8% of a 25% Triton X100). The reaction mixture is formed by 1 ml of the homogenate with 3 ml of the same buffer, added with NADH(19 mg/30 ml) and NADPH₂(6 mg/30 ml) , and 1 ml of the INT salt solution (100 mg of iodofenil nitrofenil fenil tetrazolium chloride (INT) in 50 ml of water, pH 8,5). The reaction is terminated after 20 minutes with 1 ml of a solution containing 1:1 of formaline and 1M H₃PO₄ and the final adsorbance measured at 490 nm.

The ETS activity, expressed as μlO₂ consumed per hour, can be calculated using the equation:

$$\text{ETS } (\mu\text{lO}_2/\text{h}) = 1.45 \text{ H (ml) S(6ml) A}_{490}/\text{C(cm)}$$

where H is the volume of the homogenate in ml, S is the final volume of the reaction (6 ml) and C the optical length of the cell (in cm).

The ETS data reported as function of Chl a offer a tool for characterizing the ecosystem regarding oxygen utilization. Considering that a biofilm dominated by algae is characterized by a ratio ETS/Chl close to 3.8 [14], the reading from experimental data above this value [Fig. 1] would indicate that the biofilm represents mainly an oxygen consuming ecosystem and, consequently, that the risk of anaerobic niches in biofilm cannot be excluded.

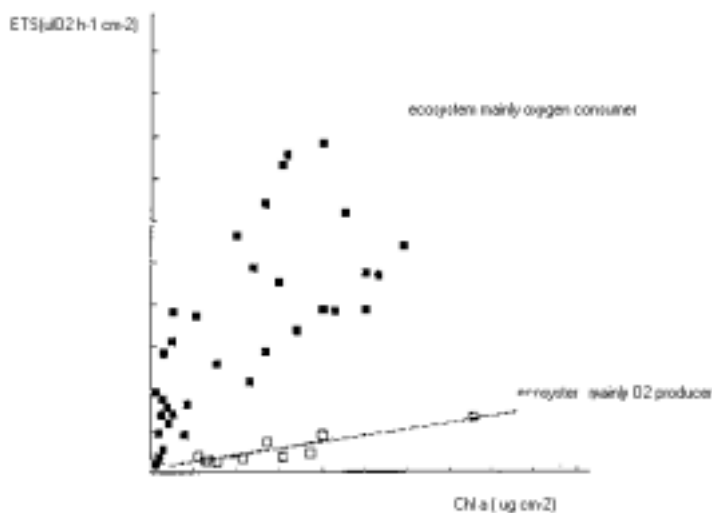


Fig.1

Regarding the bacterial presence, other observations could be performed in order to determine whether the bacterial population is mainly aerobic or anaerobic.

This could be carried out using culture techniques in selective media but such methods generally require specific skills, even if commercial kits for the detection of particular bacterial species are available. Nevertheless, some qualitative information on the nature of bacterial population can be obtained recording the redox potential of the liquid sample.

The measurement of redox-potential

The recording of redox potential of a liquid sample can be useful to signalize the ability of the industrial water system to support the growth of aerobic or anaerobic organisms. The redox values are easily monitored by immersing a platinum electrode into an aqueous phase and measuring its potential, when stable, against a reference electrode (Saturated Calomel Electrode). The data presented in Figure 2, from Memento technique de l' eau published in 1989 by Lyonnaise des Eaux and Degremont [1], show as an example, a diagram of redox potentials vs pH which can be found in waste waters.

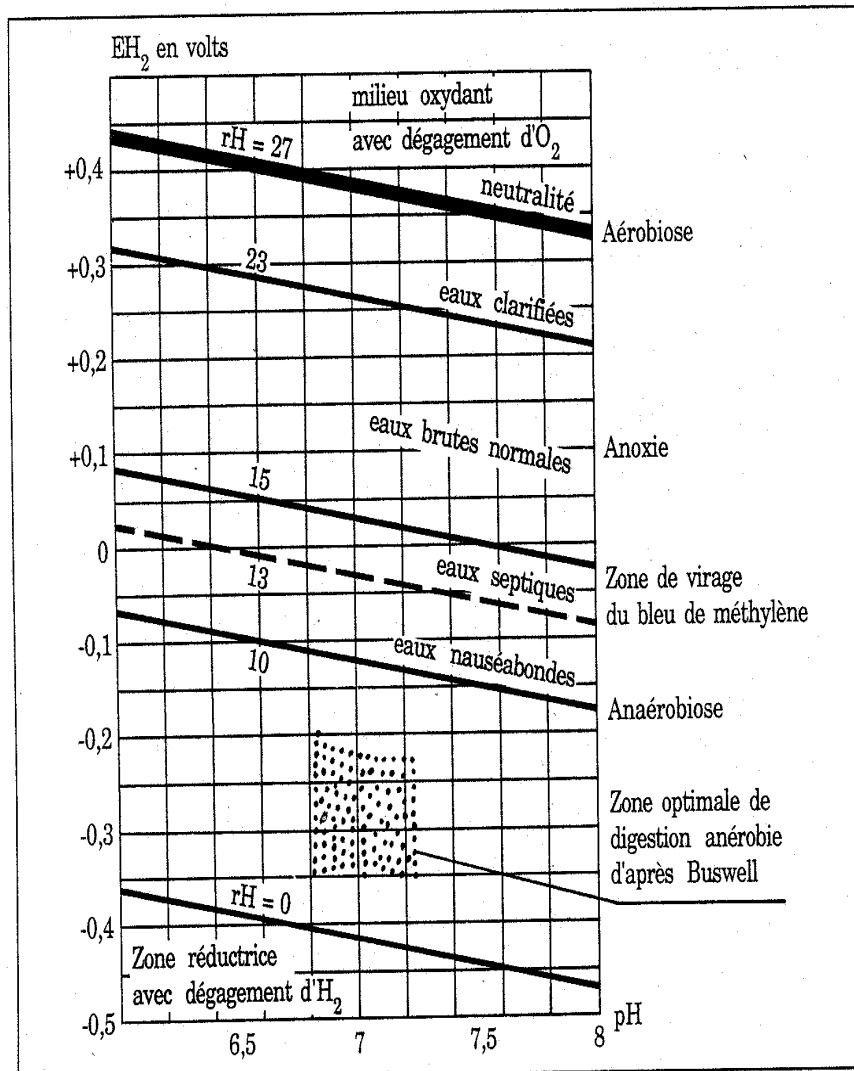


Fig.2 – Redox-potentials vs pH in waste waters

It should be emphasised that any direct measurement in liquid media is only indicative of the conditions in the bulk phase. The local conditions at the surface could vary significantly from those in the bulk due to the presence of

dissimilar microbial populations (*i.e.* aerobes and anaerobes, acid producers and pH neutral communities) existing in close proximity.

In addition, in oxygen-rich systems some anaerobic bacteria can survive as spores, thus causing the potential danger in any part of the system where there is a scope for the development of anoxic niches (*e.g.* dead legs).

Contamination of the system by SRB

As stated earlier, SRB can survive in adverse conditions through the production of spores. The activities of SRB can lead to localized corrosion of mild steel. The presence of these bacteria is distinguished by the accumulation of black iron sulphides inside the corrosion products and unpleasant smell due to H₂S release. The simplest way to determine whether anaerobic corrosion is linked with SRB is to treat samples of corrosion products with concentrated HCl or nitric acid to detect the characteristic smell of H₂S. If such a smell is apparent, it can be safely stated that at some stage of the system life, there was a growth of SRB marked by the production of H₂S and precipitation of sulphides.

The detection of SRB in the liquid or solid sample can be performed using method described by Chantereau [4].

A liquid Starkey medium (see below for a recipe) is sterilised by autoclaving and used with and without addition of 5% sodium sulphite (Na₂SO₃). The sulphite does not affect the SRB activity but inhibits the competition from other anaerobic bacteria.

The addition of iron sulphate into the culture media which causes precipitation of iron sulphides and the blackening of media in the vials, is used to indicate the biotic production of H₂S (*i.e.* to demonstrate bacterial activity).

The tested liquid or solid samples are placed into sterile glass tubes that, once completely filled with SRB culture medium, are closed with screw plugs. Then the vials, with and without sulphite, are incubated between 30-50°C.

Bacterial growth will be signaled by the change in medium turbidity, however, only the development of a strong smell of H₂S or precipitation of black sulphides in media enriched with iron ions, will confirm the SRB presence in the sample.

STARKEY MEDIUM

All compounds are dissolved in 1 l of either distilled water or 1% - 3% (w/v) aqueous NaCl.

Dipotassium Hydrogen Phosphate	0,5 g
Ammonium Chloride	1 g
Sodium Sulphate	1 g
Calcium chloride dihydrate	0,1 g
Magnesium sulphate heptahydrate	2 g
Sodium Lactate 70%	5 g

It should be emphasised that SRB are almost always present in coastal seawaters. However, their detection using Starkey medium supplemented with, or free from, sodium sulphite does not necessarily imply that the system is corroding as a result of SRB activity.

Based on the outcome of sample analyses and the measurements of redox potential, the following conclusions can be reached:

- If biofilm exists and the redox potential always remain within “anaerobic zone”, the presence of anaerobic bacteria, e.g. SRB, on a metal surface is thus confirmed and the associated MIC risk can be suspected (iron structures are particularly endangered).
- If biofilm exists and redox potential falls stable within “aerobic zone” it can be concluded that the upper layer of the biofilm is certainly formed by aerobic micro-organisms; nevertheless the presence of anaerobic organisms within anaerobic niches cannot be excluded. For several active-passive alloys (like, for instance, stainless steels) the presence of an aerobic biofilm acts, in itself, as a promoter of localised corrosion onset and stimulates fast propagation of active sites. The presence of SRB within the biofilm acts synergistically with the aerobic part of the biofilm as an instigator of localised corrosion attack.

3.4 The corrosion potential measurements: a tool for evaluating the MIC risk

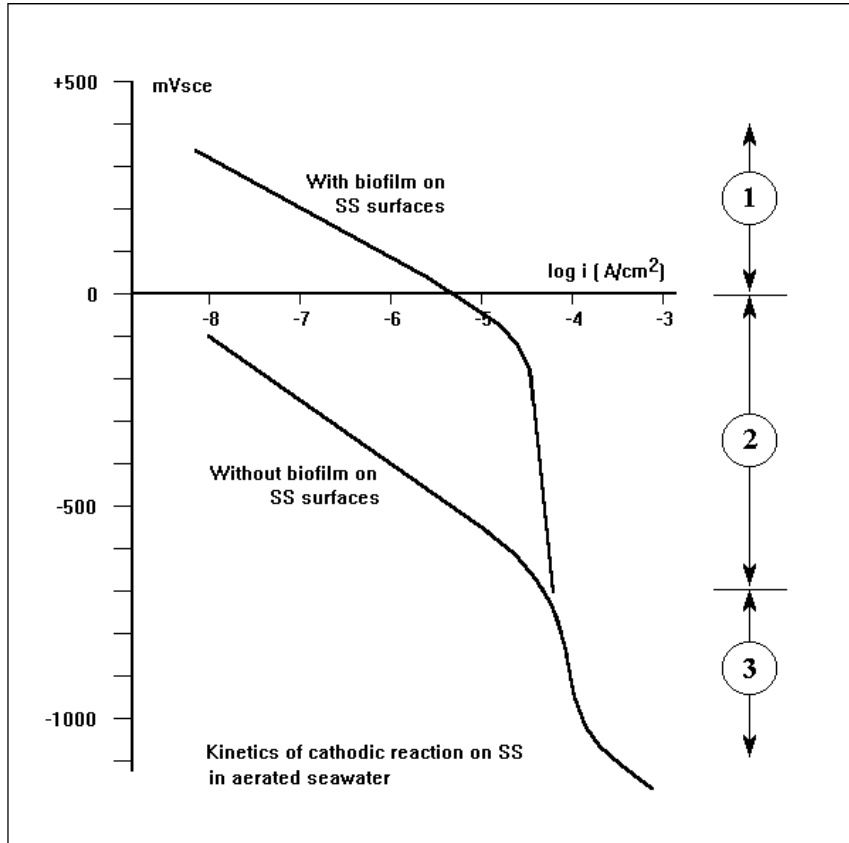
Qualitative information on the corrosive effects of a biofilm can be often obtained measuring the corrosion potentials of the metallic structure. The corrosion process is the result of two electrochemical reactions occurring concurrently on the metal surface: the metal oxidation (anodic process), which causes an accumulation of electrons, and the reduction (cathodic process) of some species present in the solution, which remove electrons from the metal. In a spontaneous corrosion process, the two reactions must run with the same rate ($I_{\text{anodic}}=I_{\text{cathodic}}$). When the kinetics of each of the two processes (anodic and cathodic) are reported in a form of E-log I plot, the crossing of the two lines will give the representative point determining the corrosion behaviour of a given metal. The co-ordinates of this point define the free corrosion potential of the system, as well as the total corrosion current ($I_{\text{corr}}=I_{\text{anodic}}=I_{\text{cathodic}}$).

The biofilm growth on metal surface can influence the kinetics of the cathodic and/or of the anodic process causing, as a consequence, a shift of the crossing point of the representative lines in the E-log |I| plot and, hence, a change of the free corrosion potential and of the corrosion current.

In several cases, the simple measurement of the free corrosion potential of a structure (and possibly the knowledge of the general trend that the potential exhibits over a period of time) can be sufficient to indicate whether there is a risk of MIC. To illustrate this point two case scenarios are presented below.

Stainless steels in aerated seawater.

It is established that the biofilm growth on surfaces of stainless steels exposed to seawater causes a change of the kinetics of the cathodic process as schematically shown in fig 3.



SS - stainless steel Fig. 3

Let us assume that we are monitoring the corrosion potentials of a structure made exclusively of stainless steel from the beginning of its exposure to natural seawater, when the steel is in a passive state and its corrosion rate is extremely low *i.e.* in the order of a few nA/cm².

The data plotted in Figure 3 indicate the following:

- In the absence of a biofilm on the steel surface the low corrosion current can be sustained by the oxygen reduction at a potential close to 0 mVsce ;
- When an aerobic biofilm is fully developed on the SS surfaces, the same corrosion current is sustained by the “biologically modified” cathodic curve at a potential close to 350 mVsce.

Such a potential increase, due to the biofilm growth, can stimulate the onset of localised corrosion which, later on, propagates at a lower potential (mean values close to 0 mVsce have often been measured).

At this potential the cathodic current of the “biologically modified” cathodic curve is high and, as a consequence, the propagation rate of localised corrosion will become very rapid.

Perforation of SS pipe walls of about 2 mm in thickness were often observed in natural seawater after only a few months of exposure.

- The monitoring of the trend of the free corrosion potential can provide a wealth of information on the MIC evolution as demonstrated in Figure 4.
- After a time t_0 the aerobic biofilm starts to develop on the steel surface as demonstrated by the increase in the free corrosion potential.
 - After a time t_1 some forms of localised corrosion is initiated.
 - Later on, the localised corrosion, sustained by the “biologically modified” cathodic curve, rapidly propagates.

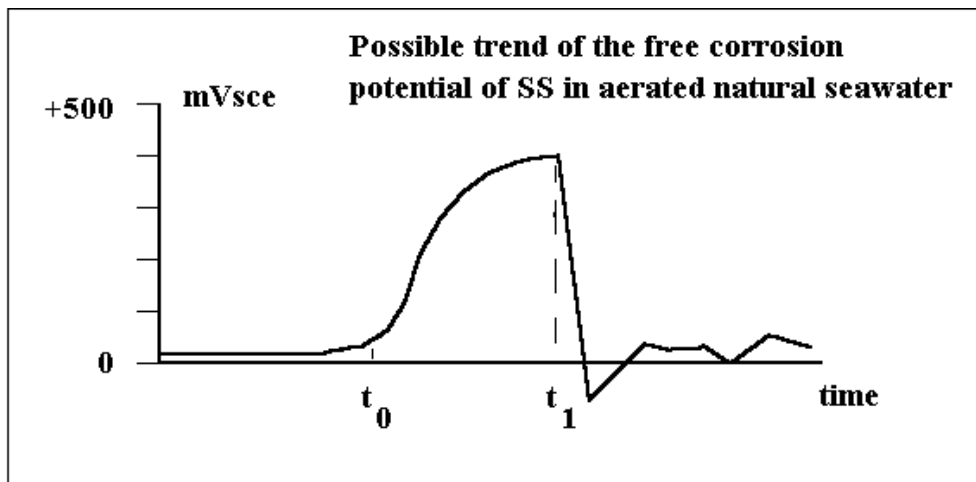


Fig. 4

As a general rule, if the free corrosion potential of a stainless steel structure clearly increases above about 0 mVsce (range 1 in Fig. 3) the risk of MIC has to be considered.

One can also propose a different scenario in which a stainless steel structure is galvanically coupled to a less noble alloy and exposed to aerated seawater and, as a consequence of the coupling, the mixed corrosion potential of the system falls below, -700 mVsce (range 3 in Fig. 3). In this case, the less noble alloy will experience corrosion and the cathodic reaction on steel (which sustains the galvanic current) will occur at the same rate both, in the presence or without the biofilm.

However, if the free corrosion potential of the structure falls between 0 and -700 mV sce (range 2 in Fig. 3) risk of stimulated galvanic corrosion induced by biofilm growth should be considered.

What is stated for stainless steel in aerated seawater is also applicable to other active-passive alloys such as Ni-Cr, Ni-Cu, and titanium alloys.

Mild steel in anaerobic seawater.

Figure 5 shows, qualitatively, two cathodic curves measured on mild steel in anaerobic seawater with and without presence of SRB. In the latter case the reduction of biologically produced sulphide is added to the hydrogen reduction reaction observed in sterile anaerobic seawater.

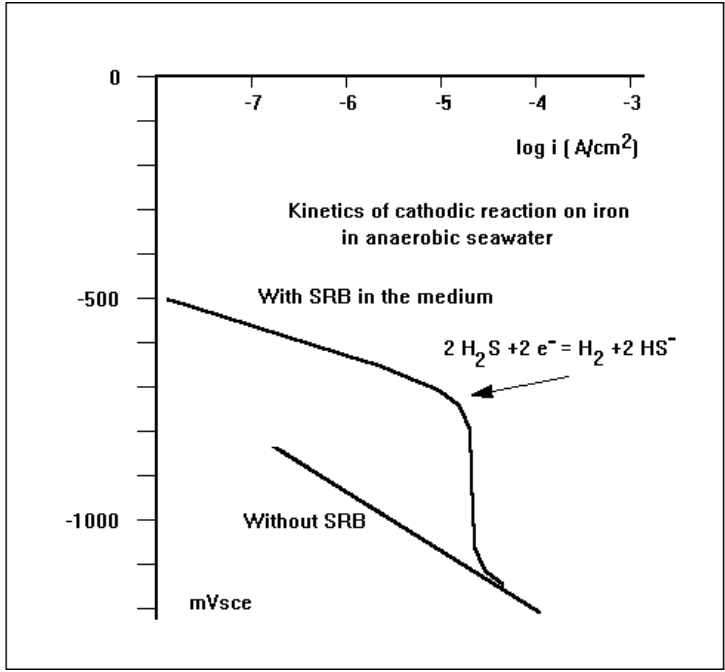


Fig 5

Based on the above described analysis it can be concluded that if the free corrosion potential measured on iron in anaerobic seawater increases above -800 mV sce, the risk of MIC due to the presence of SRB should be considered.

If a clean surface of iron alloy is exposed in anaerobic seawater containing SRB, a trend of the free corrosion potential is similar to that reported in Figure 6.

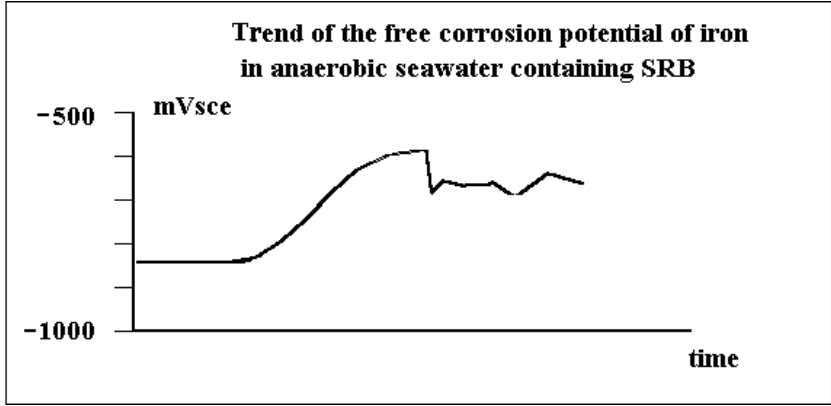


Fig. 6

The trend shown in Figure 6 suggests the following:

- Initially, the metal corrodes at a potential close to -800 mVsce. Looking at the value of the cathodic current at this potential in anaerobic seawater containing SRB (see Fig. 5) it can be concluded that a uniform corrosion rate in the order of 0.1 mm/year is expected.

- Corrosion causes a gradual accumulation of corrosion products on the metal surface, giving rise to a form of passivation which decreases the corrosion rate and increases the potentials.
- At a later stage the passive layer can be locally disrupted, *e.g.* due to the spalling of a thick passive film, causing the onset of localised attacks. The corrosion rate in the order of several mm/year can be expected.

How to distinguish MIC from other “abiotic” attacks

When the system is biologically contaminated, one of the possible control measures against suspected biocorrosion is treatment with an antimicrobial compound such as sodium azide. If such a treatment causes reduction of corrosion rates, this could be an indication that one deals with MIC problem and that an appropriate control measure should be recommended *e.g.* the use of biocides.

It is important to emphasise that, in addition to their biocidal properties, the best antimicrobials should be able to detach the biofilm from the surfaces (*i.e.* act as biodispersants), since any organic catalysers causing corrosion could remain active, despite the death of the organisms responsible for their production.

For example, in marine environments under aerobic conditions the killing of the organisms with glutaraldehyde will not prove efficient in controlling biocorrosion, since this treatment will not impede electrochemical effects involved in MIC.

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