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FACILITATION OF CORROSION OF STAINLESS STEEL EXPOSED TO AEROBIC SEAWATER BY MICROBIAL BIOFILMS CONTAINING BOTH FACULTATIVE AND ABSOLUTE ANAEROBES

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ABSTRACT

There is increasing evidence that corrosion of metals exposed to seawater is facilitated by the presence of microbes and their products. Microbes of iffering physiological types when acting in consortia appear to be more destructive than monocultures. Methods for examining consortia based on the detection of lipid biomarkers after extraction and analysis by gas chromatography/mass spectrometry (GC/MS) that are characteristic for different classes of microbes make it possible to correlate the effects of shifts in the microbial community structure to the facilitation of corrosion. This study will present preliminary evidence that a consortia of bacteria made up of a heterotrophic facultatitively anaerobic *Vibrio natriegens* and the obligately anaerobic sulfate-reducing *Desulfobacter* form a biofilm on stainless steel surfaces exposed to aerobic seawater that facilitates corrosion.

INTRODUCTION

The actuality of microbial facilitation of corrosion (MFC) of metal surfaces has been a difficult concept for some corrosion engineers (Tatnall, 1981). Often these engineers have had little experience with microbiology and the manifold metabolic capacities that these organisms possess. Part of the problem is the difficulty in routinely identifying specific microbes that may be involved in MFC. Often these microbes are exceedingly difficult to isolate in pure culture and identify (as for example the iron-oxidizing *Gallionella* spp.).

A second and possibly more difficult problem is that these microbes often are closely associated in consortia containing bacteria of multiple physiological types. These associations greatly potentiate the enzymatic versatility and thus the metabolic potential for MFC. The best studied of the microbial associations are the consortia involved in the anaerobic fermentation of complex polysaccharides (Wolin, 1979). In these associations the hydrolysis of the complex polysaccharides by one group of organisms is coupled to the conversion of the carbohydrate monomers to short chain acids and hydrogen by another group. The metabolism of the monomer utilizing fermenters requires that the partial pressure of hydrogen be less 10^{-4} atmospheres. This is possible because of another group of bacteria that utilize the hydrogen to form reduced sulphur or hydrogen.

The problems of examining the metabolic activity of consortia of bacteria of mixed physiological types required the isolation of each component of the consortia and the study of the mixed isolates. Often the organisms proved extremely difficult to culture in isolation.

Our laboratory has been involved in the development of assays to define microbial consortia in which the bias of cultural selection of the classical plate count is eliminated. Since the total community is examined in these procedures without the necessity of removing the microbes from surfaces, any microstructure of multi-species consortia is preserved. The method involves the measurement of biochemical properties of the cells and their extracellular products. Those components generally distributed in cells are utilized as measures of biomass. Components restricted to subsets of the microbial communities can be utilized to define the community structure. The concept of 'signatures' for subsets of the community based on the limited distribution of specific components has been validated by using antibiotics and cultural conditions to manipulate the community structure. The resulting changes agreed both morphologically and biochemically with the expected results (White *et al.* 1980). Other validation experiments involved isolation and analysis of specific organisms together with their subsequent detection in mixed culture experiments, utilization of specific inhibitors with the appropriate response, and the specific responses to changes in the local environment such as the light intensity. These validation experiments are summarized in a review (White 1983).

Phospholipids are found in the membranes of all cells. Under the conditions expected in natural communities the bacteria contain a relatively constant proportion of their biomass as phospholipids (White *et al.* 1979b). Phospholipids are not found in storage lipids and have a relatively rapid turnover in some sediments so the assay of these lipids gives a measure of the 'viable' cellular biomass (White *et al.* 1979a).

The ester-linked fatty acids recovered from the phospholipids (PLFA) are presently both the most sensitive and the most useful chemical measures

of microbial biomass and community structure thus far developed (Bobbie and White 1980; Guckert *et al.* 1985). The specification of fatty acids that are ester-linked in the phospholipid fraction of the total lipid extract greatly increases the selectivity of this assay as most of the anthropogenic contaminants as well as the endogenous storage lipids are found in the neutral or glycolipids fractions of the lipids. By isolating the phospholipid fraction for fatty acid analysis it proved possible to show bacteria in the sludge of crude oil tanks. The specificity and sensitivity of this assay has been greatly increased by the determination of the configuration and position of double bonds in monoenoic fatty acids (Nichols *et al.* 1985; Edlund *et al.* 1985) and by the formation of electron capturing derivatives which after separation by capillary GLC can be detected after chemical ionization mass spectrometry as negative ions at femtomolar sensitivities (Odham *et al.* 1985). This makes possible the detection of specific bacteria in the range of hundreds of cells. Since microbial consortia from many environments such as marine sediments often yield 70 ester-linked fatty acids derived from the phospholipids, a single assay provides a large amount of information. Combining a second derivatization of the fatty acid methyl esters to provide information on the configuration and localization of the double bonds in mono-unsaturated components provides even deeper insight (Nichols *et al.* 1986). By utilizing fatty acid patterns of bacterial monocultures, Myron Sasser of the University of Delaware in collaboration with Hewlett Packard has been able to distinguish between over 8000 strains of bacteria (Sasser 1985). Thus analysis of the fatty acids can provide insight into the community structure of microbial consortia as well as an estimate of the biomass.

Despite the fact that the measurement of PLFA cannot provide an exact description of each species or physiologic type of microbes in a given environment, the method provides a quantitative description of the predominating microbiota in the particular environment sampled. With the techniques of statistical pattern recognition analysis it is possible to provide a quantitative estimate of the differences between samples with PLFA analysis. The sulfate-reducing bacteria are of particular importance in MFC (Pope *et al.* 1984). These organisms contain lipids which can be utilized to identify at least a portion of this class. Some contain a unique profile of branched saturated and mono-unsaturated PLFA (Edlund *et al.* 1985; Parkes and Taylor 1983; Taylor and Parkes 1983, Dowling *et al.* 1986) that allows differentiation between those utilizing lactate and those using acetate and higher fatty acids. Detailed analysis of the PLFA recovered from sulphate-reducing bacteria in this laboratory strongly suggests that the majority of sulphate-reducing bacteria found in marine sediments and in waters used in the secondary recovery of oil are the acetate-utilizing strains.

The effects of these 'obligate anaerobic' bacteria in essentially aerobic environments are frequently both spectacular and extensive (Puckorius, 1983).

How are these obligate anaerobes capable of operation in apparently aerobic environments? Hamilton (1985), among other workers, envisages a biofilm effect where sulphate-reducing bacteria only actually function when protected from the predominantly aerobic environment by voracious oxygen consumers and facultative anaerobic consortia. How then do the bacteria withstand the rigors of oxygen exposure in transit to the anaerobic site? Hardy and Hamilton (1981) demonstrated that five strains of the sulphate-reducing bacterium *Desulfovibrio vulgaris* were able to remain viable for over 72 hours of oxygen stress in aerated water due, at least in part, to the presence of the enzymes superoxide dismutase and catalase. Subsequently Cypionka *et al* (1985) showed that other genera of sulphate-reducers, including the acetate-oxidizing *Desulfobacter*, were also resistant to oxygen stress.

If indeed sulphate-reducing bacteria do exhibit oxygen tolerance in the environment, as well as in monocultures, then a constant injection of cells into the aerobic mainstream in order to colonize new substrata would provide the inoculum. However, in order to grow and by their metabolic activity facilitate corrosion, they must exist in an anaerobic microniche. Not only do the sulphate-reducers rely upon other bacteria to remove oxygen but also to supply carbon and energy sources.

In this study evidence is presented from preliminary experiments for increased corrosion rates associated with the presence of sulphate-reducing bacteria in coculture with facultatively aerobic fermenting bacteria in aerobic seawater medium. Data is also presented which demonstrates that the characteristic PLFA of *Desulfovibrio* type sulphate-reducing bacteria are found in thin biofilms formed on stainless steel surfaces exposed to rapidly flowing highly-aerated seawater. This shows the rapid recruitment of these organisms to various metal surfaces exposed to aerobic seawater.

EXPERIMENTAL

Experiments were set up to determine if enhanced corrosion of 304 stainless steel coupons could be detected in cocultures of *Vibrio natriegens* and the sulphate-reducing bacterium *Desulfobacter postgatei* strain 2ac9 when compared to cultures of *Vibrio natriegens* alone. In the first experiment the bacteria were cultured in half strength 2216 Difco marine broth in Erlenmeyer flasks. (It had previously been determined that

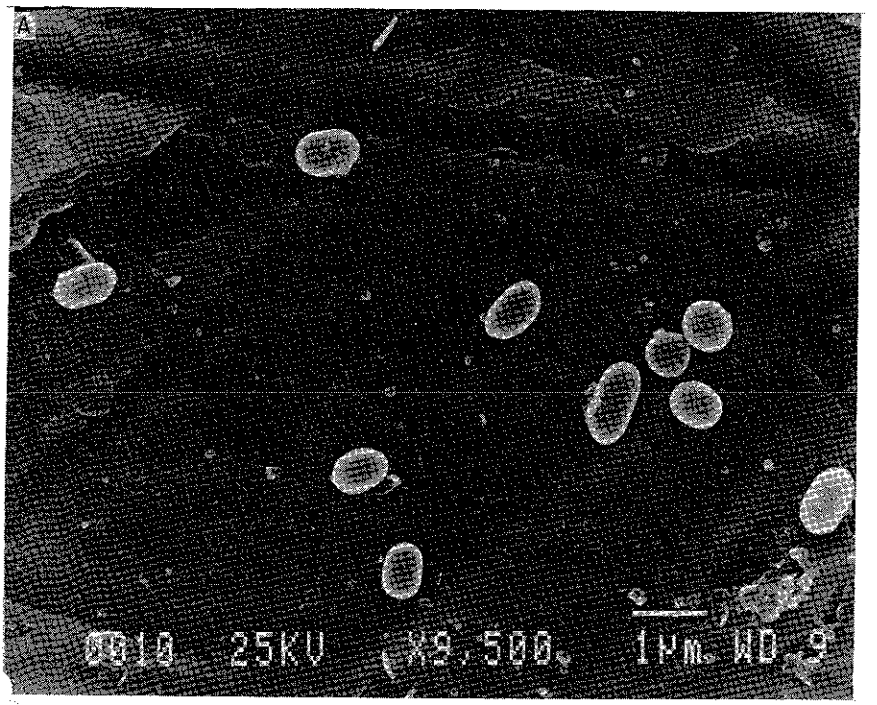


FIG 1(a) Scanning electron micrographs of (a) bacterial cells attached to a steel coupon from batch cultured *V. natriegens*, (b) cells and glycocalyx attached from chemostat cultured *V. natriegens*, and (c) cells and glycocalyx from chemostat cocultured *V. natriegens* and *Desulfobacter postgatei*.

Desulfobacter sp. could grow satisfactorily in 2216 MB when the medium was reduced with sodium sulphide and provided with acetate as carbon and electron source). Duplicate flasks were set up for each treatment, each flask containing five coupons (four for corrosion analysis, and one for scanning electron microscopy, SEM). The different treatments were:

1. Vibrio inoculated alone with the flask sealed with cotton wool and incubated with rotory shaking (aerobic).
2. Vibrio and Desulfobacter inoculated with the flask sealed with cotton wool and incubated with rotory shaking (aerobic).
3. Vibrio and Desulfobacter inoculated with the flask sealed with a bung (limited oxygen).

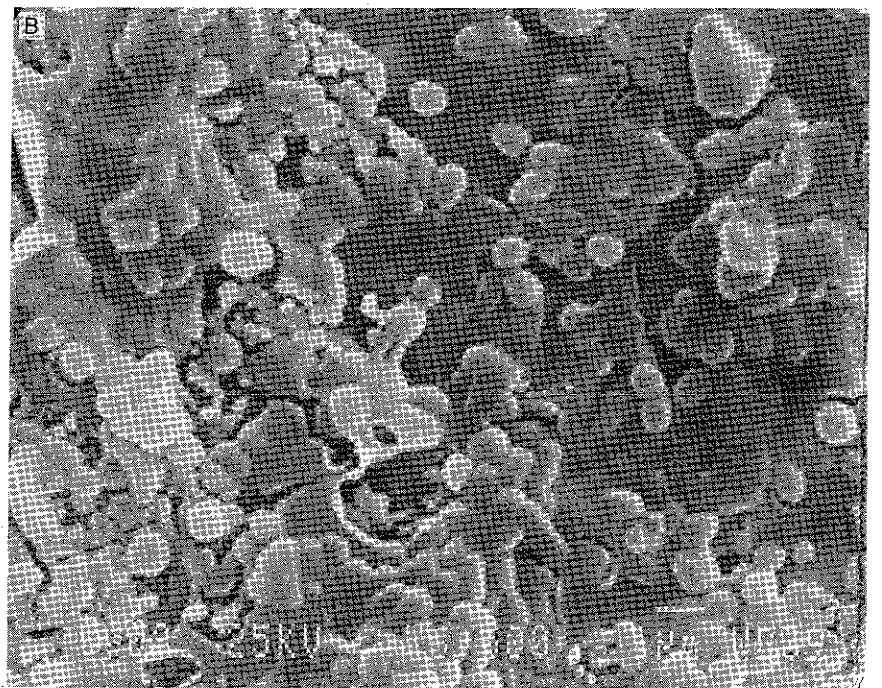
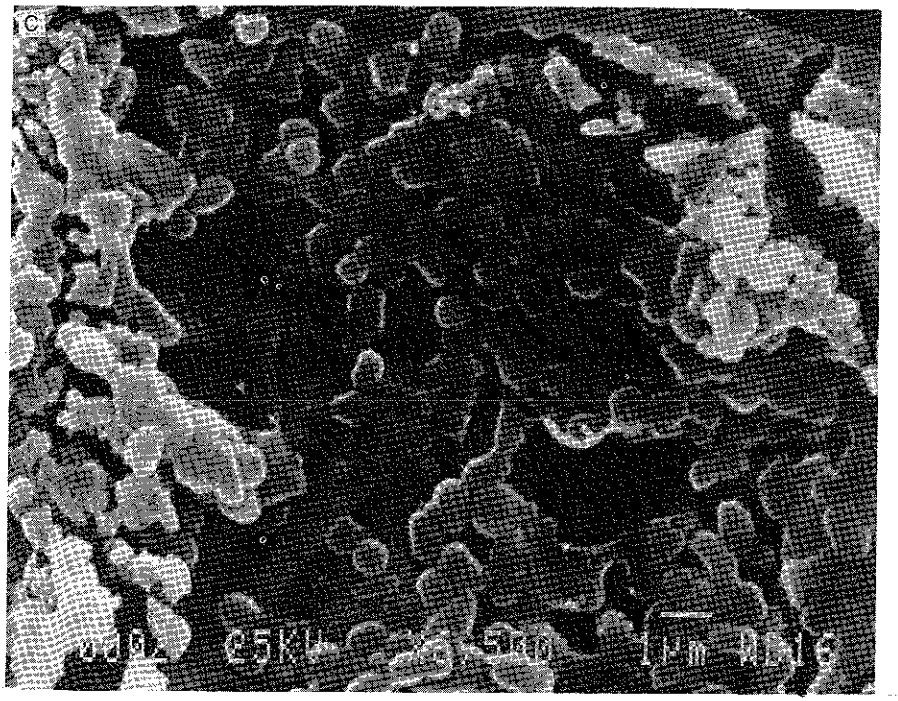


FIG 1(b).

The flasks were incubated for four weeks at room temperature on an orbital shaker. At the end of this period four coupons from each flask were analysed by an EG & G PARC model 350 A corrosion measurement console for Tafel constants and polarization resistance from which Icorr. values were calculated as described (Nivens *et al.* 1986). The remaining coupons were examined by SEM after critical point drying and shadow contrasting.

The treatments had the following average Icorr. values: 1) 6.5 nA/cm^2, 2) 10.4 nA/cm^2, 3) 23.0 nA/cm^2 (control with no bacteria was 4 nA/cm^2). The SEM micrographs showed that very few cells remained attached to the coupons after the latter were removed from the media (Figure 1). The Icorr. values appear to indicate an extremely small enhanced corrosion rate with the presence of a coculture with *Desulfobacter*.

The second experiment was designed to observe any differences in corrosion rates in organisms selected to form a biofilm on the stainless steel coupons. The organisms were selected in a continuous culture apparatus



(Figure 2). The nutrient content of the media feed was decreased 10-fold and was supplied at a rate of 1.5 ml/min./ reaction vessel. The working volume was 800 ml with a dilution rate of 0.1/ hr. The medium used was 1/10th strength 2216 Difco marine broth diluted in 'Forty fathoms' marine salts. The continuous culture system was run for one month after which the coupons were recovered and analysed as in the batch culture experiment.

SEM micrographs of the chemostat coupons showed that bacteria were more adherent under these conditions than those of the batch experiment, possibly correlating with the large quantities of glycocalyx produced (Figure 1). Corrosion values were also elevated: Coupons incubated with *V. natriegens* had a mean Icorr. value of 25.5 nA/cm^2. Coupons incubated with both *Vibrio* sp. and *Desulfobacter* sp. had a mean Icorr. value of 93.3 nA/cm^2. It still remains, however, to be shown that these differences in corrosion rates were solely due to the metabolic activity of sulphate-reducing bacteria. Unfortunately the SEM micrographs showed no

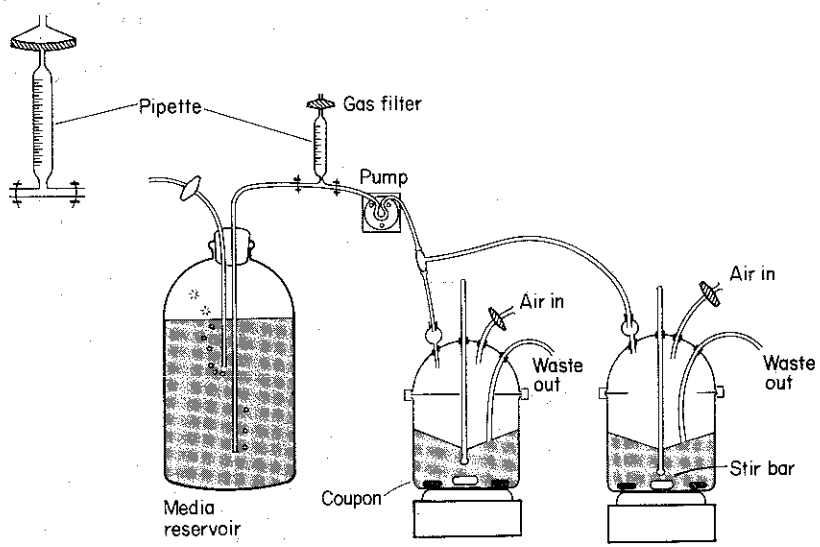
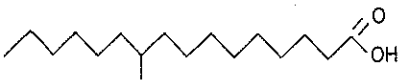


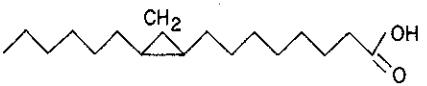
FIG 2 Continuous culture apparatus used to culture *Vibrio natriegens* and coculture *V. natriegens* and *Desulfobacter postgatei*. Both culture vessels contained 304 stainless steel coupons which were analysed after one month of the apparatus' operation to determine if the presence of the sulphate-reducing bacterium enhanced the corrosion rate.

difference in cell morphology in the biofilm. It may be unreasonable, however, to expect to observe a difference when the largest concentration of metabolically active sulphate-reducing bacterial cells are expected to be under the Vibrios in anaerobic microniches. Further experiments are in progress to study the mechanism of the enhancement of corrosion.

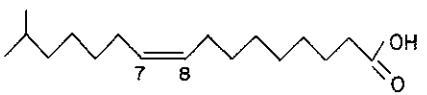
From the above two experiments it appears reasonable to expect recruitment of sulphate-reducing bacteria to metal surfaces in the aerobic, oligotrophic marine environment. At the IFREMER laboratories in Brest, France, several types of metal tubing were exposed to seawater over periods of 2, 5, 10, 15 and 30 days (Guezennec, 1985). Seawater was passed through the tubes at varying rates, from 0.1 to 1.5 m./sec. After exposure to seawater the lipids were extracted from the biofilm with a one phase chloroform/methanol/water mix (Bligh and Dyer, 1959), fractionated by silicic acid column (Gehron and White, 1983) and the phospholipids recovered. Mild akaline methanolysis released the PLFA as methyl esters. The PLFA methyl esters were characterized



10Me16:0
(Δ^{10}-methyl hexadecanoic acid)



Cyclopropyl 17:0
(Δ^{9},Δ^{10}-methylene hexadecanoic acid)



iso17:1ω7cis
(Δ^{15}-methyl-Δ^{9},Δ^{10}-hexadecanoic acid)

FIG 3 Fatty acid biomarkers used to detect members of the genera *Desulfobacter* and *Desulfovibrio*.

and quantified by gas chromatography and gas chromatography-mass spectrometry.

Several workers including Boon *et al.* (1978), Taylor and Parkes (1983), Dilund *et al.* (1985), and Dowling *et al.* (1986) have proposed that the fatty acids found in the *Desulfovibrio* spp. and *Desulfobacter* spp. may be used as biomarkers in the environment. These biomarkers include iso 17:1ω7cis (15 methyl-9, 10 hexadecanoic acid) and anteiso 17:1ω7cis (14 methyl-9, 10 hexadecanoic acid) for members of the genus *Desulfovibrio*, and 10Mel6:0 (10-methyl hexadecanoic) and cyclopropyl (cy) 17:0 (9, 10-methylene hexadecanoic acid) for members of the genus *Desulfobacter*.

Examination of the biofilm fatty acid spectra obtained from aluminium, steel and titanium tubes generally showed the appearance of the fatty acid iso 17:1ω7cis within a two-day period, and the fatty acids 10Mel6:0/Cy 17:0 within a five-day period in the summer (April-May, 1985). Confirmation of the presence of sulphate-reducing bacteria was only attempted from 30-day biofilms however. From these biofilms hydrogen- and acetate-oxidizing sulphate-reducing bacteria were enriched which were presumed to be members of the genera *Desulfovibrio* and *Desulfobacter* respectively.

DISCUSSION

The preliminary experiments reported here suggest that a consortium of the facultatively anaerobic fermenter *V. natriegens* and the obligate

anaerobe *D. postgatei* in consortia when in an attached biofilm can facilitate the corrosion of stainless steel exposed to aerobic seawater. With the use of the non-destructive, Fourier transforming infrared spectrometry (FT/IR) for the analysis of biofilms produced by *V. natriegens*, Nivens *et al.* (1986) was able to show that the accumulation of the polysaccharide exopolymer containing calcium hydroxide induced a reversible acceleration of corrosion of stainless steel exposed to seawater. The fact that the consortium increased the corrosion suggests that the activity of the sulphate-reducing bacteria may be involved. Preliminary experiments have shown that the Vibrio forms butanol with traces of acetate during anaerobic catabolism of glucose. The fact that the sulphate-reducer grew (evidenced by the decrease in sulphate in the batch culture) indicates that traces of acetate were utilized by the Desulfobacter. It also suggests that the addition of another fermenter which can form acetate from butanol should potentiate the growth of the sulphate-reducer and the corrosion. Since the analysis of 'signature' PLFA involves the isolation and identification of specific fatty acid methyl esters by GC/MS, it is possible to use 13-C mass-labelled precursors to follow the incorporation into the 'signature' biomarker lipids of the various component members of the consortia as has been done for muramic acid (Findlay *et al.*, 1983).

Not only can the details of interactions between components in microbial consortia be examined in detail, but the understanding of mechanisms of potentiation of corrosion can be explored. Common substrates for the sulphate-reducing bacteria include lactate, carbon dioxide, acetate, propionate, and molecular hydrogen. Removal, and in some cases generation, of these molecules by sulphate-reducers may contribute to their corrosiveness (Pope *et al.*, 1984). The by-product hydrogen sulphide may also accelerate corrosion. Some members of the genus *Desulfovibrio* contain hydrogenases. The classic mechanism by which sulphate-reducing bacteria potentiate corrosion is by cationic depolarization with the removal of hydrogen (von Wolzogen Kuhr and Van der Vlugt (1934). Iverson (1982) has suggested that labile iron phosphide compounds are the primary factors in the anaerobic corrosion process in monocultures of *Desulfovibrio desulfuricans*. The potentiation of corrosion by the *Desulfobacter postgatei* suggested by the preliminary data in this study make clear that at least with this sulphate-reducing bacteria cationic depolarization cannot be a factor as it contains no detectable hydrogenase. MFC may prove an excellent tool with which to study the interactions of microbial consortia in biofilms. It is clear that the creation of anaerobic microniches by the metabolic activities of consortia of bacteria in biofilms in aerobic systems (White, 1986) must be considered in the attempts to control corrosion of structures in the sea with its high sulphate concentration.

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