

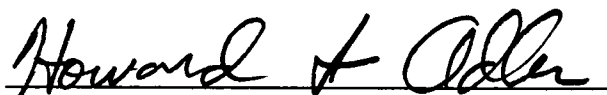
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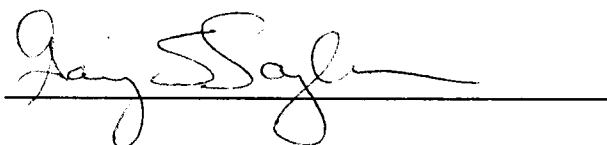
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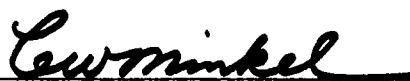
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THE EFFECTS OF INCREASED BACTERIAL METABOLIC DIVERSITY
ON THE CORROSION OF CARBON STEEL

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Richard Friedrich Jack

May 1990

To all my family and friends who have always had faith
in my abilities.

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ABSTRACT

Most studies involving microbial influenced corrosion (MIC) of metals use pure cultures of bacteria though consortia are often associated with corroding metals under in situ conditions. This study investigates the corrosion of carbon steel with increasing bacterial metabolic diversity in order to determine the additive effects of consortial members. Three physiologically distinct groups of bacteria: aerobic, fermentative and sulfate reducing were represented by Bacillus sp., Hafnia alvei and Desulfovibrio gigas, respectively. Combinations of these bacteria were used in an aerobic, freshwater system. The open cell potential (OCP) of carbon steel decreased from -250 to -700 mV/SCE for all bacterial communities while the control decreased to approximately -350 mV/SCE. The increase in corrosion rate, measured in admittance, was greatest with communities containing D. gigas. The admittance with D. gigas plus Bacillus increased from 2.06×10^{-5} to 1.35×10^{-3} mhos cm^{-2} while the coculture with D. gigas plus H. alvei increased from 3.86×10^{-5} to 2.28×10^{-3} mhos cm^{-2} while the admittance with all three bacteria increased to 1.37×10^{-3} mhos cm^{-2} . Upon completion of the experiments the carbon steel electrodes were removed for bacterial analysis. The plate counts of Bacillus and H. alvei in monoculture were similar, but when grown in diculture, the total population

size increased an order of magnitude. The population size of H. alvei also increased when cocultured with D. gigas, however the Bacillus population size decreased when cocultured with D. gigas. The D. gigas population was similar in both diculture combinations. The population size was greatest for all bacteria when grown in triculture. The bacterial communities were also analyzed based on the differences in the branching, saturation and chain length of the phospholipid fatty acids (PLFA). Cluster analysis of the PLFA confirmed the effects of D. gigas on H. alvei and the Bacillus. The second part of this investigation shows the effects of increased metabolic diversity using enrichments, from a naturally corroding pipe, of these same physiologically distinct groups. The corrosion rate was compared between an aerobic plus fermentative enrichment and an aerobic, fermentative plus sulfate reducing enrichment. The OCP decreased from -250 to -700 mV/SCE for all consortia versus -350 mV/SCE for the control. The admittance was greatest for the aerobic, fermentative plus sulfate reducing enrichment at 3.13×10^{-3} mhos cm^{-2} . The total numbers of bacteria from the electrodes were similar for both treatments based on plate counts but MPN revealed the presence of approximately 1×10^3 cells cm^{-2} sulfate reducing bacteria in the aerobic, fermentative plus sulfate reducing treatment. Clustering of the PLFA showed distinct patterns of relatedness based on the enrichments used.

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Part I

INTRODUCTION

Introduction

Microbially Influenced Corrosion (MIC) has been observed for several decades (17). The failure of structures made of metals, concrete and asphalt has an important economic impact (22). One of the first studies of MIC involved in pipeline corrosion estimated the cost at 100 million dollars per year in 1954 for the United States alone (17). Costs to the pulp and paper industry are estimated at five to nine dollars per ton of product or 20% of maintenance costs. Corrosion of concrete is most pronounced in underground sewage pipes where anaerobic water with reduced sulfides produces hydrogen sulfide gas. This gas rises to the top of the pipe where bacteria oxidize the hydrogen sulfide producing sulfuric acid (21). This phenomenon is found worldwide and cost estimates have not been accurately estimated. The phenomenon does, however, contribute to the cost of sewage transport system repairs.

Corrosion Reactions

Corrosion essentially involves dissolution of metal into solution. The anodic reaction results in an oxidized form of a metal ion and generation of one or more electrons. The fundamental corrosion reactions are shown in Table 1. Consumption of electrons generated by the anode at some other site, is known as the cathodic reaction. Oxygen,

Table 1. Abiotic and Biotic Reactions involving the Corrosion of Carbon Steel

Reactions	Examples
<u>Abiotic</u>	
I. Anodic	$\text{Fe}^0 \rightarrow \text{Fe}^{2+} + 2\text{e}^-$ $\quad \quad \quad \searrow \quad \quad \quad \text{Fe}^{3+} + \text{e}^-$
II. Cathodic	
a.) pH > 7	$\text{H}_2\text{O} + 2\text{e}^- \rightarrow \text{OH}^- + \frac{1}{2}\text{H}_2$
b.) pH < 7	$2\text{H}^+ + 2\text{e}^- \rightarrow 2[\text{H}] \rightarrow \text{H}_2$
c.) with O_2 and pH < 7	$\text{O}_2 + 4\text{H}^+ + 4\text{e}^- \rightarrow 2\text{H}_2\text{O}$
d.) with O_2 and pH > 7	$\frac{1}{2}\text{O}_2 + \text{H}_2\text{O} + 2\text{e}^- \rightarrow 2\text{OH}^-$
III. Summation	
a.) iron hydroxide	$\text{Fe}^{3+} + 3\text{OH}^- \rightarrow \text{Fe}(\text{OH})_3$
	$\text{Fe}^{2+} + 2\text{OH}^- \rightarrow \text{Fe}(\text{OH})_2$
and oxide	$3\text{Fe}(\text{OH})_3 \xrightarrow{[\text{O}_2, \text{H}^+]} \text{Fe}_2\text{O}_3 \cdot x\text{H}_2\text{O} + \text{FeOOH}$
IV. acceleration of cathodic reaction	
a.) depolarization	$\text{H}_2\text{S} + \text{e}^- \rightarrow \text{HS}^- + \frac{1}{2}\text{H}_2$
<u>Biotic</u>	
V. acceleration of anodic reaction	
a.) Fe oxidation	$\text{Fe}^{2+} \rightarrow \text{Fe}^{3+} + \text{e}^-$
b.) Fe reduction	$\text{Fe}^{3+} + \text{e}^- \rightarrow \text{Fe}^{2+}$
c.) alternate end product	$\text{Fe}^{2+} + \text{S}^{2-} \rightarrow \text{FeS}$
VI. acceleration of cathodic reaction	
a.) O_2 reduction	$\text{O}_2 + \text{C}_6\text{H}_{12}\text{O}_6 \rightarrow 6\text{CO}_2 + 6\text{H}_2\text{O}$
b.) H_2 oxidation	$6\text{H}_2 + \text{O}_2 + \text{CO}_2 \rightarrow (\text{CH}_2\text{O}) + 5\text{H}^+$
c.) depolarization	$\text{SO}_4^{2-} + 8[\text{H}] \rightarrow \text{S}^{2-} + 4\text{H}_2\text{O}$

hydrogen ions and water can all act as electron acceptors, Table 1. In alkaline environments the dissolution of water consumes electrons resulting in increased alkalinity and hydrogen formation. If the solution has sufficient oxygen present, increased alkalinity also occurs but without the subsequent production of hydrogen.

In acidic solutions with little oxygen, protons consume the excess electrons generated by the anode resulting in the production of hydrogen gas. Increased amounts of oxygen in acidic environments will result in the formation of water.

The products of the anodic and cathodic reactions, in the case of ferrous alloys, leave the metal surface and immediately react to form iron hydroxides and oxides. In oxygen saturated solutions these complexes precipitate adjacent to the metal surface forming a diffusion barrier against the bulk solution (51). This iron oxide/hydroxide layer, if stable, impedes further dissolution into the bulk electrolyte. The formation of the oxide/hydroxide layer is shown in Figure 1. Other anions, such as chloride, will also react with iron forming ferrous and ferric chlorides but do not form a metallic complex. Instead, these remain soluble in solution and accelerate metal dissolution (51). The dissolution of metal is directly proportional to the chloride concentration (51). Chloride ions readily migrate to the anode and concentrate to promote the anodic reaction.

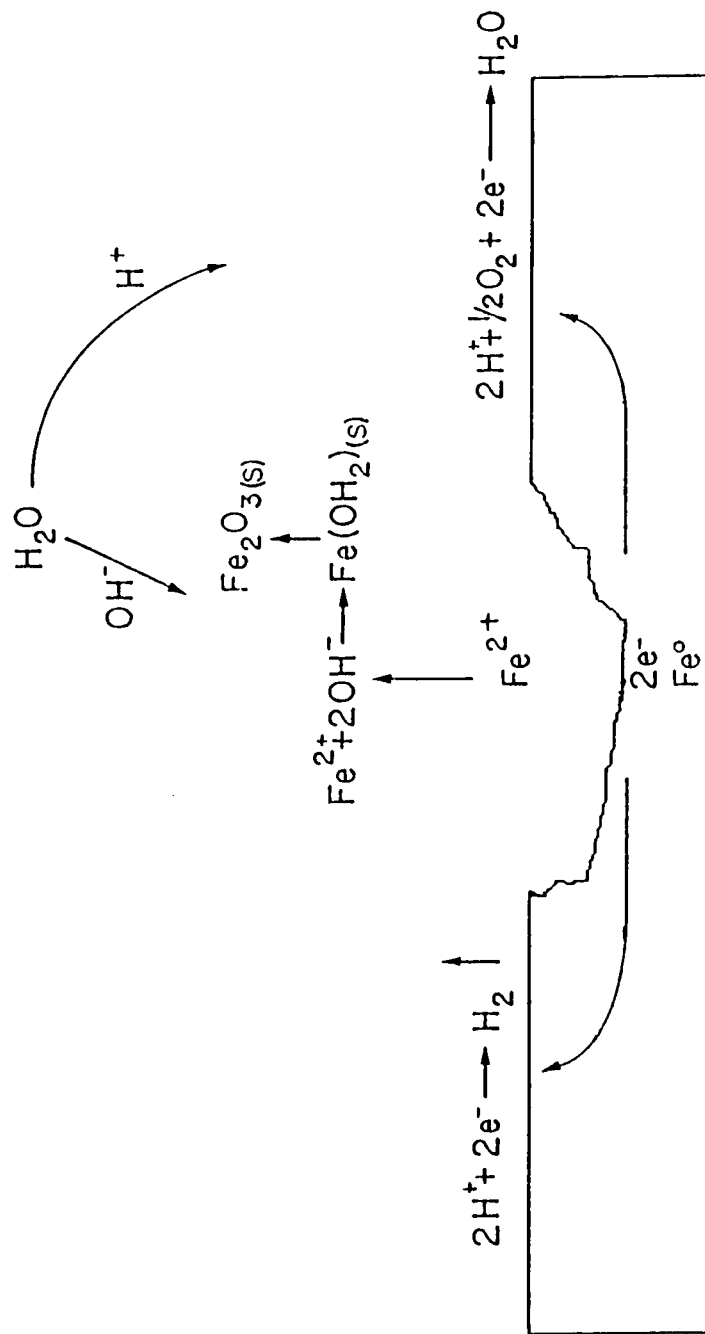


Figure 1. Anodic and cathodic reactions. As metallic iron (Fe^0) is oxidized to the ferrous form (Fe^{2+}) two electrons are released. The ferrous iron complexes with hydroxides and forms an iron oxide/hydroxide rust layer. The electrons are consumed by various reactions including protons and water and forming hydrogen and hydroxide, respectively.

Other factors that prevent formation of the iron oxide/hydroxide layer include hydrogen ions and physical particles such as sediments. Hydrogen ions accelerate the cathodic reaction and result in the formation of hydrogen. Sediments and other particles attached to a metal surface form oxygen concentration cells or differential aeration cells. The different oxygen concentrations from site to site on the metal surface leads to distinct anodic and cathodic regions.

The interference of either anodic or cathodic reactions will upset the equilibrium between metal loss and metal passivation.

Microbial Influenced Corrosion

There are many forms of microbial influenced corrosion (MIC). The mere attachment of aerobic bacteria to a metal surface results in the consumption of oxygen and causes segregation of anodic sites to areas with low oxygen tension. An anodic site will form beneath the cell and a cathodic site adjacent to it. More complex MIC associated processes involve direct metabolic interaction with a corrosion reaction, in particular hydrogen consumption (43). Other MIC forms include metal-ion interaction (26), exopolymer production (40), acid production (45), metal transformation and accelerated corrosion by sulfide production (2,34,54,56).

Bacterial Iron Oxidation

Many bacteria interact with metals in the environment, including Gallionella ferruginea, Sphaerotilus natans and members of Leptothrix sp. Some of these are directly involved in the transformation of metal ions and on occasion play a central role in metal corrosion. The autotrophic, microaerophilic G. ferruginea uses ferrous iron as an electron donor and accumulates large quantities of ferric oxide precipitates on large sheaths that it uses to attach to a surface (20). This organism requires reduced iron for it's growth needs and will not use carbon sources as electron donors. These bacteria are connected to a surface by long extracellular sheaths which can also accumulate manganese and chloride ions at the site of attachment. The bacteria S. natans and members of the Leptothrix group of bacteria also accumulate ferric oxides and hydroxides on extracellular sheaths which form long tubes around the cells. These are strictly aerobic, heterotrophic bacteria and derive no energy from the oxidation of iron. The importance of iron precipitate accumulation by these bacteria from an evolutionary or survival standpoint is not clear and perhaps is involved in co-precipitation of toxic compounds (36). The ferric ions that attach to these long polysaccharide sheaths do not form stable iron oxide/hydroxide precipitates that can impede metal dissolution.

Also, the accumulation of these large sheaths can result in excessive film formation and induces biofilm sloughing. This results in a continual loss of metal and formation of oxygen concentration cells.

Another group of bacteria that are involved in the oxidation of iron are the Thiobacilli (21). The Thiobacilli have been investigated mainly in the accelerated corrosion of concrete (50). However, the production of an inorganic acid would also affect the corrosion of carbon steel. These organisms oxidize sulfide while some species, such as Thiobacillus ferrooxidans, oxidize ferrous iron. The corrosion of concrete has been shown to involve a succession of bacterial community members as the pH drops. In sewer systems, hydrogen sulfide and carbon dioxide are produced in the anaerobic water and diffuse upward to the interior pipe surface. The surface contains a thin water film in the upper aerobic regions where Thiobacilli can use the electrons from reduced sulfur compounds producing sulfur and sulfate. A further oxidation step results in the production of sulfuric acid. Different Thiobacilli are predominant as the pH decreases. The surface pH of concrete is 12.5 but quickly lowers to 8.5 upon Thiobacilli activity. At neutral pH's the predominant bacteria have been shown to be T. thioparus, T. novellus, and T. intermedius. As the pH drops below 6, T. neopolitanus predominates and below pH 5 T. thiooxidans predominates. This organism has been found when

the pH is as low as 0.6. T. ferrooxidans oxidizes ferrous to ferric iron and has been shown to enhance corrosion of iron pipes. Because it is able to survive at low pH's, it is used in the leaching of metal from low grade ores (21).

Bacterial Iron Reduction

Ferric iron reduction to ferrous oxide accelerates corrosion by removing insoluble iron oxide/hydroxide complexes. The physiology and biochemistry of iron reduction is well established (50). All bacteria with the ability to reduce nitrate to nitrite can reduce ferric iron. The reaction is carried out by nitrate reductase which has been demonstrated by inhibition studies of nitrate reductase (52).

Ecologically, nitrate inhibits iron reduction and upon depletion of nitrate, iron reduction proceeds followed by sulfate reduction. Iron reduction inhibits sulfate reduction thermodynamically but it should be remembered that ferric hydroxides and oxides are insoluble whereas sulfate is not. Thus in a hydromorphic iron-oxide matrix in a corroding pipe, these reactions could occur simultaneously as sulfate diffuses through the matrix in a flowing system (37).

Extracellular Polysaccharides

Extracellular polymers affect the local chemistry adjacent to a metal by binding of metal ions with uronic acid, cis-hydroxyl and phosphoric acid functional groups (5). The extracellular polymer of Vibrio natriegens was shown to accelerate the corrosion of stainless steel after the cells were extracted from the metal surface (40).

Extracellular polymers help maintain a structural integrity of a biofilm and are involved in metal ion dissolution from the metal. The binding of ions by these polysaccharide matrixes is affected by pH, redox and other ions (5). Bacterial extracellular polymers may also be composed of proteins, teichoic acids and lipopolysaccharides (4), all of which may affect metal binding for different ions and in different environments.

Cathodic Depolarization

The increase in corrosion by promotion of the reductive half cell reaction is known as cathodic depolarization. Table 1 shows some examples of bacterial cathodic reactions. These reactions involve the accelerated removal of electrons, often through hydrogen consumption, which otherwise would stabilize the metal. Sulfate reducing bacteria (SRB) have been the most extensively studied group of bacteria involved in anaerobic corrosion processes (18). The link between hydrogenase and cathodic depolarization was

first proposed by von Wolzogen Kuhr and van der Vlugt (58). The corrosion of hydrogenase positive versus hydrogenase negative SRB has since been demonstrated (3,8,43). These reactions are depicted in Figure 2. Electrons released at the anode travel to a cathodic region where they reduce protons in solution to form hydrogen. Many SRB can then use hydrogen as an electron donor for the reduction of sulfate to sulfide (19,61).

The early studies with hydrogenase positive bacteria used Desulfovibrio sp. (3,43). Since then many hydrogenase positive bacteria in many genera have been isolated including Desulfobulbus, Desulfotomaculum, Desulfuromonas, Desulfobacter, Desulfosarcina and Desulfonema (61). This widespread utilization of hydrogen and sulfate as energy sources emphasized the large scale possibility of cathodic depolarization and resulted, in its re-investigation by many workers (43). Investigation by Cord-Ruwisch and Widdel (8) of hydrogenase positive Desulfovibrio vulgaris with hydrogenase negative D. sapovorans growing on lactate showed increased production of hydrogen sulfide when steel wool was added only with the hydrogenase positive strain. Cathodic consumption of hydrogen was then based on the stoichiometric amount of hydrogen sulfide formed based on the amount of lactate added and it's incomplete oxidation to acetate.

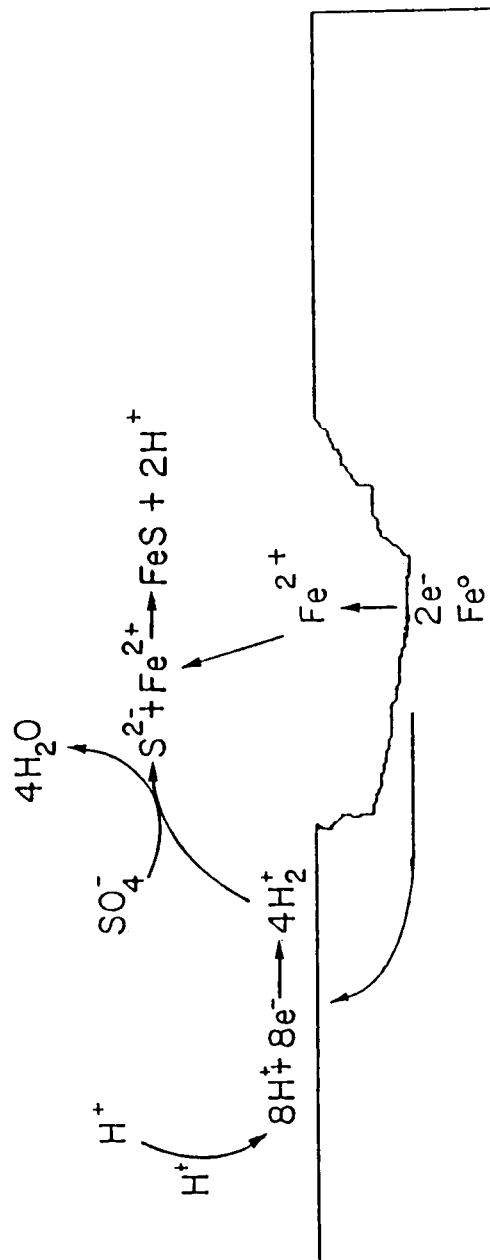


Figure 2. Cathodic depolarization by sulfate reducing bacteria. Hydrogen consumption by sulfate reducing bacteria results in the acceleration of the cathodic reaction by accelerating the removal of electrons. The reduced sulfide by-product reacts with ferrous iron preventing formation of a stable iron oxide/hydroxide layer.

A more conclusive study by Pankhania (44) using D. vulgaris showed hydrogen sulfide production while the organism was growing on acetate and sulfate only with mild steel. This organism can utilize acetate as a carbon source only if hydrogen is available as an electron donor. If the mild steel was absent, growth would not occur and concluded that the source of hydrogen must be that cathodically produced by the mild steel.

Other genera that possess a hydrogenase include the aerobic Alcaligenes, Chromatium and Nocardia. Acetogenic bacteria possessing hydrogenase include Clostridia and Methanogenic bacteria including Methanosarcina, Methanococcus and Methanobacterium. Few studies of these genera in relation to corrosion and cathodic polarization have been undertaken. Recently, Daniels (7) has shown production of methane using Methanosarcina barkeri with iron as a sole energy source while Phelps et al. (45) have implicated acetic acid production as contributing to corrosion in natural gas transmission lines.

Despite the support of the depolarization theory, several workers have proposed additional corrosion mechanisms under anaerobic conditions. One of the alternative mechanisms proposed by King, Miller and Wakerley (26) suggest that iron sulfides produced by SRB were directly associated with cathodic depolarization. This was also demonstrated by Booth et al. (2) who showed cathodic

depolarization by chemical and biologically produced iron sulfides. However, in sterile systems the iron sulfides did not act as permanent cathodes and it was proposed that SRB keep the iron sulfides cathodically active (35). Others workers such as Costello (9) and Togano et al. (56) have shown that hydrogen sulfide is also capable of cathodic depolarization. Increased corrosion was directly proportional to the amount of hydrogen sulfide produced by SRB and it was proposed the hydrogen sulfide accelerates both the anodic and cathodic reaction (56).

Consortial Corrosion Studies

Most MIC studies have involved the use of pure cultures (22). Because MIC is often associated with complex consortia under in situ conditions (27), true estimates of metallic corrosion need to be investigated using bacterial consortia.

The emphasis on pure cultures began with the work of Koch and Pasteur with investigations on anthrax toxin and wine fermentation, respectively (4). This monoculture approach is prevalent to this day despite the increasing awareness of the role of consortia in nature and industrial applications.

Though monoculture investigations are beneficial, they can not address questions of additive affects of increasing bacterial metabolic diversity and interaction associated

with MIC. Aerobic bacteria play a fundamental role in the corrosion of metals by modifying the metal surface where it is dramatically different from the bulk solution (5).

Anaerobic conditions underneath the biofilm drastically changing the local chemistry. This new environment allows establishment of a more diverse consortia with the colonization of anaerobic bacteria (19). Thus, aerobic environments can possess distinct anaerobic pockets with active anaerobic bacteria. The reduction of sulfate in anaerobic microniches has been demonstrated in aerobic marine sediments (23).

The association of bacterial consortia with MIC has been reviewed in industrial settings (47). Accelerated corrosion by Vibrio anguillarum in diculture with Desulfovibrio vulgaris was greater than either of the organisms in monoculture though the population sizes of each organism was greater in monoculture (16). This simple experiment indicates that the increased metabolic diversity resulted in greater corrosion rates.

Some Techniques used To Investigate Metallic Corrosion

When carbon steel is placed in aqueous solutions the corrosion is initially fast but slows to a steady state as an oxide/hydroxide layer forms, the speed of which is dependent upon the electrolyte. The open cell potential (OCP) of the metal is the net sum result of the potentials

of all the redox half reactions at the metal surface and may be used to predict the oxidation states of the ions at the surface (21).

An excellent method used to measure MIC is AC Impedance or electrochemical impedance spectroscopy (EIS). The use of alternating currents for corrosion measurements allows relatively non-destructive measurement of biofilms and does not alter the metal/metal oxide interface as does conventional direct current (D.C.) scans. The EIS measurement is shown schematically in Figure 3. The procedure is as follows: first the OCP is determined for a working electrode (WE) relative to a reference electrode (RE) such as a saturated calomel electrode. A potentiostat then applies this potential to the WE by impressing a current between the WE and the counter electrode (CE). Subsequently, an external 5 mV RMS sinusoidal signal is superimposed from a frequency response analyzer around the OCP. As the potential is anodically polarized, the WE becomes more positive with respect to the RE and the CE becomes more negative. As this occurs, the oxidative reactions are promoted on the WE surface resulting in production of charged ions, ie. ferrous and ferric ions. Negatively charged anions migrate to the WE in order to maintain electroneutrality. As the potential is polarized cathodically, reductive reactions occur on the WE resulting in cation migration to the surface.

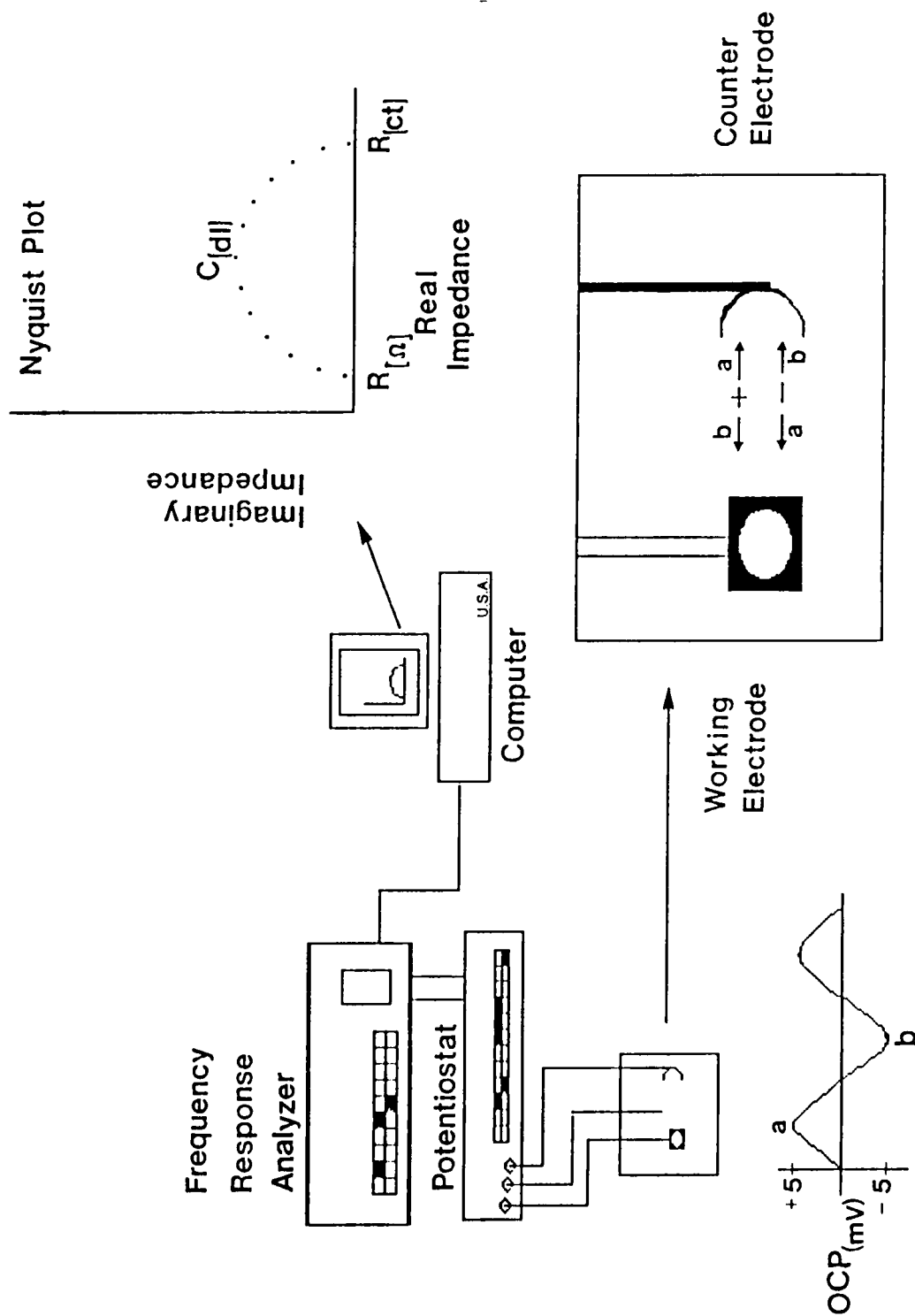


Figure 3. Diagram of A. C. Impedance test apparatus. Oscillations in the open cell potential result in anodic and cathodic reactions being localized between the working and counter electrodes.

An important aspect of these measurements is that the net current flow is zero. This current flow is impeded by the solution resistance (R_{Ω}), the double layer capacitance (C_{dl}) and the charge transfer resistance (R_{ct}).

Useful information is obtained if the oscillation rates are varied from very fast to very slow usually ranging from 10 KHz to 3 or 5 mHz. The data obtained is the current response in terms of phase shift and magnitude, in relation to the speed of oscillation, based on the Randles equation for a simple resistance-capacitance (RC) circuit given below:

$$|Z| = R_{\Omega} + \frac{R_{ct}}{1 + R_{ct}^2 C^2 \omega^2} - j \frac{R_{ct}^2 C \omega}{1 + R_{ct}^2 C^2 \omega^2}$$

R_{Ω} = the solution resistance in ohms.
 R_{ct} = the charge transfer resistance in ohms
 C = the Capacitance in Farads
 ω = frequency in radians
 Z = total impedance
 j = $\sqrt{-1}$

When the frequency (ω) is large both terms become insignificant with respect to R_{Ω} , the first term, and the current flow is essentially a function of the solution resistance. As the anodic and cathodic reactions oscillate at a slower rate the ions remain at the WE and do not migrate (are impeded) due to an induced counter charge on

the WE known as the double layer capacitance (C_{dl}). When the current is not in step with the potential ie. does not behave like a true resistor, a phase shift results. The C_{dl} is determined on the y-axis of the Nyquist plot, the y-axis is also known as the imaginary impedance (third term of the Randles eq.) while the x-axis is known as the real impedance (second term of the Randles eq.)

At very low frequencies the charge "leaks" across the C_{dl} and the corrosion approaches a resistive form. As ω becomes smaller the total impedance reduces to a summation of $R_{\Omega} + R_{ct}$. This normally occurs at 15 mHz and below. The corrosion is determined from the Stearn-Geary equation (53):

$$I_{corr} = \frac{(\beta_a \beta_c)}{2.303 (\beta_a + \beta_c) R_{ct}}$$

I_{corr} = amperes cm^{-2}

β_a = anodic Tafel parameter

β_c = cathodic Tafel parameter

2.303 = natural log conversion factor

R_{ct} = charge transfer resistance in ohms cm^2

The greater the charge transfer resistance the slower the anodic reaction and the slower the corrosion. R_{ct} is therefore inversely proportional to the corrosion.

Objectives

The objective of this Thesis was to demonstrate that as bacterial metabolic diversity is increased statistically significant increases in the corrosion will be observed.

Three bacterial isolates: Bacillus, Hafnia alvei and Desulfovibrio gigas will be used to show the additive effects of a strict aerobe, a fermenter and a sulfate reducing bacterium, respectively on the corrosion of carbon steel. The presence of the Bacillus or H. alvei are essential for the formation of an anaerobic niche and survival of the strictly anaerobic D. gigas in an aerobic environment. The freshwater sulfate concentrations will address the effect of D. gigas in nonmarine conditions. The additive effects of each isolate increases the number of corrosion reactions possible, ie. oxygen concentration cells, acid production, cathodic depolarization and FeS, H₂S production. The greater the number of corrosion reactions should result in increased corrosion.

The second part to this thesis also involves the effects of increased metabolic diversity on the corrosion of carbon steel. Bacterial enrichments from a corroding carbon steel pipe will be used to compare the corrosion between the same physiologically distinct groups.

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Part II

THE CORROSION OF CARBON STEEL BY COMBINATIONS
OF A Bacillus sp., Hafnia alvei AND Desulfovibrio gigas

Abstract

The corrosion of carbon steel was investigated using different combinations of a Bacillus sp., Hafnia alvei and Desulfovibrio gigas using a four sided electrode. The open cell potential (OCP) decreased from -200 mV/SCE to -450 mV/SCE in the sterile control over a two to three week period. The OCP for all bacterial combinations decreased to -600 to -700 mV/SCE between 40 and 70 hrs. The admittance, measured by AC impedance, was used as corrosion indicator. The admittance increased greatest when D. gigas was present in a consortia. For example, dicultures of D. gigas with Bacillus sp. and H. alvei increased the admittance to 1.4×10^{-3} and 2.3×10^{-3} mhos cm^{-2} , respectively, while the triculture increased the admittance to 1.4×10^{-3} mhos cm^{-2} compared to 5.3×10^{-4} mhos cm^{-2} for the control. The admittance in communities with D. gigas was statistically different from the control at $P < 0.01$ while H. alvei alone was statistically different from the control at $P < 0.05$. The admittance was not related to the total biomass, as determined by plate counts, MPN and phospholipid fatty acids from the electrode surface. The data indicates that D. gigas had the most significant impact on the corrosion of carbon steel in this aerobic, freshwater system.

Introduction

Microbial Influenced Corrosion (MIC) has been observed since the early part of this century. Some of the early pioneering work with MIC was that of von Wolzogen Kuhr and van der Vlugt (26) who demonstrated increased corrosion by consumption of hydrogen at the cathode by hydrogenase positive sulfate reducing bacteria (SRB). This mechanism is known as cathodic depolarization. The increased understanding of SRB metabolism and new genera have re-emphasized the importance of this group of bacteria in MIC (7,8,11,20). Cathodic depolarization has also been demonstrated by methanogenic bacteria (4). Different corrosion mechanisms have been proposed for each bacterial group based primarily on the organism's metabolic capabilities. A Pseudomonas sp. was shown to accelerate corrosion by the addition of a carbon source (18). Other MIC mechanisms investigated have been organic acid production from fermentation by Clostridia (21) and iron reduction by an iron reducing bacterium (17). Corrosion is known to be affected by the redox potential of the solution. Because of the different redox potentials required by aerobic and anaerobic bacteria, these studies could not establish which type of organism had the most significant impact on MIC. Also, few MIC studies have demonstrated the effects of consortia composed of

physiologically different bacteria. The increase in corrosion by a diculture of Vibrio anguillarum plus Desulfovibrio vulgaris then either organism in monoculture (7). Consortia of bacteria are often associated with MIC under in situ conditions (12). The advantage of association of different types of bacteria was demonstrated by Jorgensen who showed that sulfate reduction occurred in anaerobic microniches in aerobic sediments and detritus particles (11). SRB have also been shown to be active in anaerobic niches below a biofilm of aerobically respiring bacteria on metals (9). Thus, contribution of SRB to corrosion may be significant in an aerobic environment if a biofilm has formed.

Three bacterial isolates readily found in in situ corrosion tubercles, Bacillus sp., Hafnia alvei and Desulfovibrio gigas were used to show the effects of an aerobic, fermentative and sulfate reducing bacterium, respectively, on the corrosion of carbon steel. The purpose of this study was to show that the increase in metabolic diversity results in greater corrosion rates. Also, the effect of the strictly anaerobic D. gigas can be determined in an aerobic, freshwater system.

Materials and Methods

Isolation and Characterization of Bacteria

A tubercle from a 4 in. carbon steel pipe used in transporting cooling water for a hydroelectric power plant, was the source for a strict aerobic and a facultative anaerobe. The tubercle or rust material inside the pipe was scraped with a sterile spatula and transferred to a test tube containing 1-2 mls of sterile mineral salts. The material was then finely ground and vortexed. The liquid was then streaked onto agar plates and incubated for 48 hrs at 32⁰ C. The agar media contained, in g/l,: glucose, 2; lactate, 2; NH₄CL, 0.5; KH₂PO₄, 0.1; MgSO₄ 7H₂O, 1, pH 7. The individual colonies were restreaked to ensure monoculture isolates. The isolates were then Gram stained and tested for strict aerobic and fermentative capabilities by inoculation into oxidative/fermentative tubes, (API Ind. (Plainview, N. Y.)). Several isolates tested positive for fermentation of glucose while strict aerobes were negative for this test. These isolates were further characterized by the API 20E method for identification of Enterobacteriaceae (Plainview, N. Y.) and by their fatty acids with the Microbial Identification System (MIS), (Hewlett Packard, Newark, DE.).

The strict aerobe stained Gram positive and was identified as Bacillus thuringiensis, this was a very common

isolate in over 500 bacterial isolates associated with tubercles from various cooling water systems (28). The facultative anaerobe stained Gram negative and was identified by the MIS as Hafnia alvei, also a very common isolate. Due to the difficulty in isolating pure cultures of sulfate-reducing bacteria, a strain of Desulfovibrio gigas #19365 was obtained from the American Type Culture Collection. This organism was selected because it possesses catalase and superoxide dismutase which were felt to ensure survivablity when inoculated into the aerobic media.

Preparation of Inoculum

Prior to inoculation the Bacillus sp. and H. alvei were grown in 7mls of the above medium at 25⁰C and shaken for 48 hrs. The Desulfovibrio gigas was grown in 10 mls of, g/l: lactate, 3; yeast extract, 1; MgSO₄ 7H₂O, 4; CaSO₄, 1; NH₄CL, 1; and K₂HPO₄, 0.5 at 32⁰C for 48 hrs. The medium was reduced by boiling and degassing with nitrogen prior to autoclaving. The nitrogen was passed through a glass tube filled with reduced copper fillings heated to 350⁰C. Prior to inoculation in the corrosion flask, the D. gigas cultures were then flushed with N₂ for 15 min to remove residual H₂S. All cultures were then washed by centrifugation at 10,000 rpm for 20 min., the supernatant decanted and 10 mls of reduced mineral salts was added, vigorously vortexed and recentrifuged. This procedure was

repeated 2 times. Additions of H_2S or FeS in the D. gigas inoculum drastically increased the corrosion rate.

Design of the Corrosion Test System

The corrosion test system consisted of a one liter flask which held the four sided working electrode (WE), a saturated calomel reference electrode (SCE) held in a glass tube with a Vycor Tip (E.G. & G., Princeton, N.J.) that functioned as a salt bridge and a titanium counter electrode (CE) soldered to a coaxial cable. The flask also contained a drip tube for media inlet, glass exit tube, a butyl rubber stoppered port for culture inoculation, and a stir bar. The fittings were securely fastened on a 1/2 in. polypropylene cover with 1/4 in. holes sealed with a butyl rubber ring, held firmly to a one liter flask with a metal clamp. The total solution volume was 550 mls. A figure of the test flask is given in the Appendix. The WE was gas sterilized with ethylene oxide to prevent premature pitting. Care was taken not to heat the flasks after sterilization as this would also cause premature pitting. Also, the experiments were started immediately after sterilization.

The medium for the corrosion experiments contained, in mg/l: glucose, 50; lactate, 50; KH_2PO_4 , 5; NH_4Cl , 15; $MgSO_4 \cdot 7H_2O$, 80 and was sterilized by autoclaving. The flow rate was one ml per minute with a peristaltic pump using C-flex tubing (Cole-Parmer, Chicago, IL.), spent media was

collected in a waste jug. This simple media was used to negate the effects of various anions and cations.

Electrode Preparation

Circular, one cm C1020 carbon steel coupons (Metal Samples Co., Mumford, AL.), were used. The composition was, in percentage; carbon, 0.17; manganese, 0.42; phosphorous, 0.009; sulfur, 0.006 and iron, 99.495. An insulated 26 gauge copper wire was soldered to the back of each metal coupon. Four coupons were then assembled together and embedded in epoxy to create a four sided electrode. The electrodes were then polished with 600 grit sand paper to a smooth finish, rinsed in acetone, distilled water and carefully dried with tissue. The electrode edges were then covered with Microshield lacquer (Pyramid Plastics, Hope, AK.) and dried for one hour. The wires were passed through a glass rod to prevent contact with the media then assembled into the test flask as described above.

Bacterial Biofilm Analysis

Upon completion of each experiment the four sided electrode was aseptically removed and analyzed for bacterial biomass. Two sides of the electrodes were used to compare the whole biofilm by Phospholipid Fatty Acid (PLFA) profiles and the remaining two sides of the electrode were analyzed for a comparison of the individual bacterial members by

plate counts for the Bacillus and Hafnia sp. and MPN for D. gigas. Upon completion of each experiment the electrodes were clamped to a flanged tube and sealed with a butyl rubber ring. One ml mineral salts was added and the mixture was sonicated at 20% power for two, 10 sec. intervals (Ultrasonics Inc., Model W140, Plainview, NY.). The material was then removed with a syringe and inoculated into five mls of prereduced mineral salts media with glass beads. This procedure was repeated two times. The glass beads facilitate pulverization of the rust material/biofilm. The tube was then vigorously vortexed and dilutions were used for plate counts and MPNs. The MPN media was supplemented with, 1 g/l $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ to detect FeS production, indicative of a positive reaction.

The electrodes were then lyophilized before PLFA analysis of the remaining two sides.

Lipid Extraction

Prior to any extraction, all glassware was heated at 450°C for four hours. Teflon fittings were carefully washed with chloroform:methanol, 1:2 (v/v). Solvents were high quality, capillary GC/GC-MS capable for detection in the parts per billion range from Burdick and Jackson (Muskegon, MI.).

The electrodes were carefully scraped to remove the ferrous oxide/bacterial matrix, ground and placed in a

separatory funnel. The PFLA extraction procedure has been described elsewhere (8). The following solvents were added, methanol:chloroform:0.1 M phosphate buffer at a ratio of 2:1:0.8, the total volume was 142.5 mls. The mixture was held at least four hours. After this initial extraction, 37.5 mls of Chloroform-washed distilled water and 37.5 mls chloroform were added to generate two phases, which was held an additional 24 hrs for complete separation of phases. The bottom lipid fraction was removed and dried in a rotovaporatory flask. The polarlipids, which are predominantly phospholipids in bacteria (23), were separated from neutral and glycolipids by silicic acid chromatography. Glass columns were prepared with ratios of 50:1 stationary phase:lipid dry weight and 1.7:1 cross sectional area. The silicic acid used was Unisil (100-200 mesh, Clarkson Chemical Co., Inc. Williamsport PA) activated at 120⁰C for 60 min. After the chloroform had evaporated from the rotovaporatory flask, the dried sample was removed with pasteur pipettes by three 50 μ l washes with chloroform and loaded onto the silicic acid columns. A series of mobile phases of increasing polarity was used to separate the lipid classes: neutral lipid, 10 ml chloroform; glycolipids, 10 mls acetone; phospholipid, 10 mls methanol. The phospholipid fractions were collected in glass test tubes with teflon-lined screw caps. The methanol was dried under a stream of filtered nitrogen at 37⁰C and stored at

-20⁰C before further analysis.

Derivatization of Phospholipids

Once the phospholipids were separated from the other lipid fractions, mild alkaline methanolysis was used to hydrolyze and methylate the ester-linked fatty acids. This procedure cleaves the diacyl fatty acids from the glycerol backbone to free fatty acid methylesters (FAME). The phospholipids were dissolved in one ml of methanol:toluene, 1:1 (v/v). To this, 1 ml of freshly made 0.2N KOH in methanol was added and heated at 37⁰C for 15 min. The mixture was then allowed to cool at room temperature and two mls of hexane:chloroform, 4:1 (v/v) was added and the pH adjusted to neutrality with 0.2 ml of 1M acetic acid. In order to break phase and separate the FAME, 2mls of distilled water was added which separated the FAME into the upper organic phase from the glycerol phosphate esters in the lower aqueous phase. The aqueous phase was washed twice with fresh hexane:chloroform, 4:1 (v/v) in order to completely remove all FAME from the aqueous phase. The hexane:chloroform organic phase was then dried under nitrogen at 37⁰C and stored at -20⁰C before quantification by Gas Chromatography.

Verification of FAME by Gas Chromatography

The FAME were quantified by Gas Chromatography (GC) on a Hewlett-Packard 5880 gas chromatograph. Dry FAME were diluted with 50 picomoles/microliter of C19:0 internal standard in iso-octane. Samples of one microliter were injected onto a 30 second splitless injector at 270⁰C and a flame ionization detector at 290⁰C. The initial oven temperature was 80⁰C for one min followed by a 10⁰C/min rise to 150⁰C, a 3⁰C/min to 240⁰C followed by a 5⁰C/min rise to 280⁰C, then held isothermally for 5 min. The carrier gas was H₂ at one ml/min with make up gas of N₂ at 30 ml/min. The peak heights were quantified by comparison with internal standard with the Nelson Analytical 3000 series Chromatography Data System (Paramus, N. J.).

The PLFA Profiles were calculated as mole % of the total PLFA extracted from an electrode surface. The data was then analyzed by cluster analysis using complete linkage on SPSSX (Chicago, Ill.)

Fatty Acid Nomenclature

Fatty acids are designated the total number of carbon atoms: number of double bonds with the position of the double bond closest to the end (w) of the molecule. The geometry indicated as cis (c) or trans (t). For instance, 16:1w7c is 16 total carbons with one double bond 7 carbons from the end in the cis configuration. Fatty acids that are

branched are designated as iso (i) or anteiso (a) if the methyl branch is one or two carbons from the w end (i15:0). Cyclopropyl (cy) fatty acids are designated by the position of the cyclic ring from the w if known (cy17:0).

Electrochemical Analysis

The open cell potential (OCP) was determined using a Solartron voltmeter model 7081 and a 7010 Minate scanner (Solartron-Schlumberger, Farnborough, England) interfaced with a Hewlett Packard computer. AC impedance was observed to monitor the corrosion over time. A 1286 Electrochemical Interface with a 1250 Frequency Response Analyzer (Solartron-Schlumberger, Farnborough, England) controlled by a Hewlett Packard series 300 computer was used to generate signals and analyze the magnitude and phase shift of the resulting currents. The OCP was determined for the working electrode and a 5 mV RMS sinusoidal signal applied around the OCP. The applied potentials scanned from 10 Hz to 5 mHz at 5 cycles for 5 steps/decade. The data was plotted in Complex Plane (Nyquist) plot format and the solution resistance (R_n), polarization resistance or charge transfer resistance (R_{ct}) and the double layer capacitance (C_{dl}) were obtained. Once an EIS scan was completed for one surface the four sided electrode was rotated for the next electrode to be scanned. Three electrodes were scanned per time point. The Tafel constants were not determined due to the

high current densities necessary for polarization plots which may disturb the biofilm on the electrode surface.

Therefore, the data was plotted as the inverse of R_{ct} which is the admittance, mhos cm^{-2} . The admittance is directly proportional to the corrosion rate.

Statistical Analysis

The admittance from three electrodes per bacterial community were compared to the sterile control by the student's t test. The values were taken from the final data points upon termination of the experiments and expressed as significance from the control. A preliminary experiment was performed to obtain electrochemical and bacterial parameters.

Experimental Procedure

Four or five flasks, a sterile control and flasks with different bacterial communities, were used for each experiment. Initial AC impedance and OCP measurements were determined prior to inoculation. Upon inoculation the flow was kept off for 12 hrs to prevent washout of cells. The OCP scanner was disconnected once a steady state was reached. The low flow rate and dilute media were used in order to slow bacterial growth. This was necessary due to the long analysis time required for AC impedance measurements.

Results

OCP and Bacterial Community

The OCP response to the bacterial combinations are shown in Figures 1 and 2. The control is the average of six electrodes from three experiments, all other treatments are an average of two electrodes. Figure 1 shows the change in OCP over time in the presence of H. alvei and D. gigas in pure culture, in diculture as well as the Bacillus, H. alvei and D. gigas triculture. The initial OCP was between -250 and -300 mV/SCE for all flasks and decreased over time for all bacterial treatments. The OCP in the presence of D. gigas decreased to -550 mV/SCE after 90 hrs and to -600 mV/SCE by 70 hrs in the presence of H. alvei. The D. gigas, H. alvei diculture decreased the OCP to -650 mV/SCE in only 40 hrs. The OCP in the presence of Bacillus, H. alvei plus D. gigas decreased at a constant rate from -300 mV/SCE to -610 mV/SCE in 70 hrs. The change in OCP over time in the presence of Bacillus in monoculture and in diculture with H. alvei and D. gigas compared to the sterile control as seen in Figure 2. The OCP for all combinations decreased to between -650 and -700 mV/SCE in 50 hrs, the Bacillus plus D. gigas diculture rose slightly after 90 hrs while the Bacillus monoculture and in diculture with H. alvei remained constant at -700 mV/SCE. The most rapid

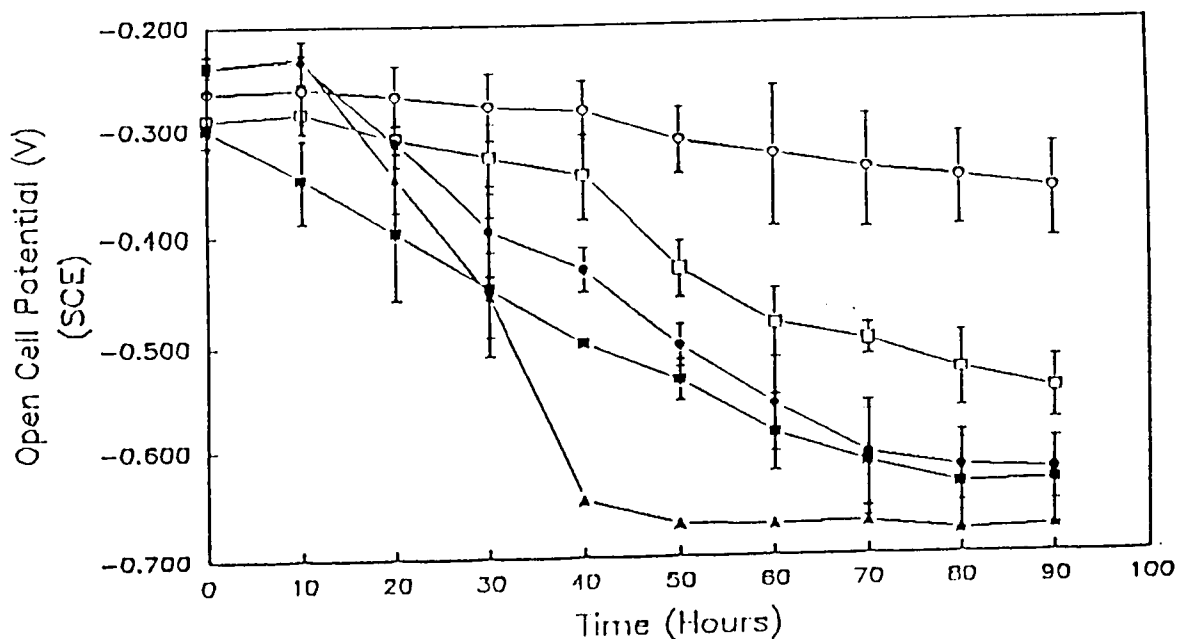


Figure 1. The open cell potential of carbon steel with the bacteria *H. alvei* (●), *D. gigas* (□), *H. alvei* plus *D. gigas* (▲) and *Bacillus*, *H. alvei* plus *D. gigas* (■) versus sterile control (O). Bars represent ranges from two electrodes.

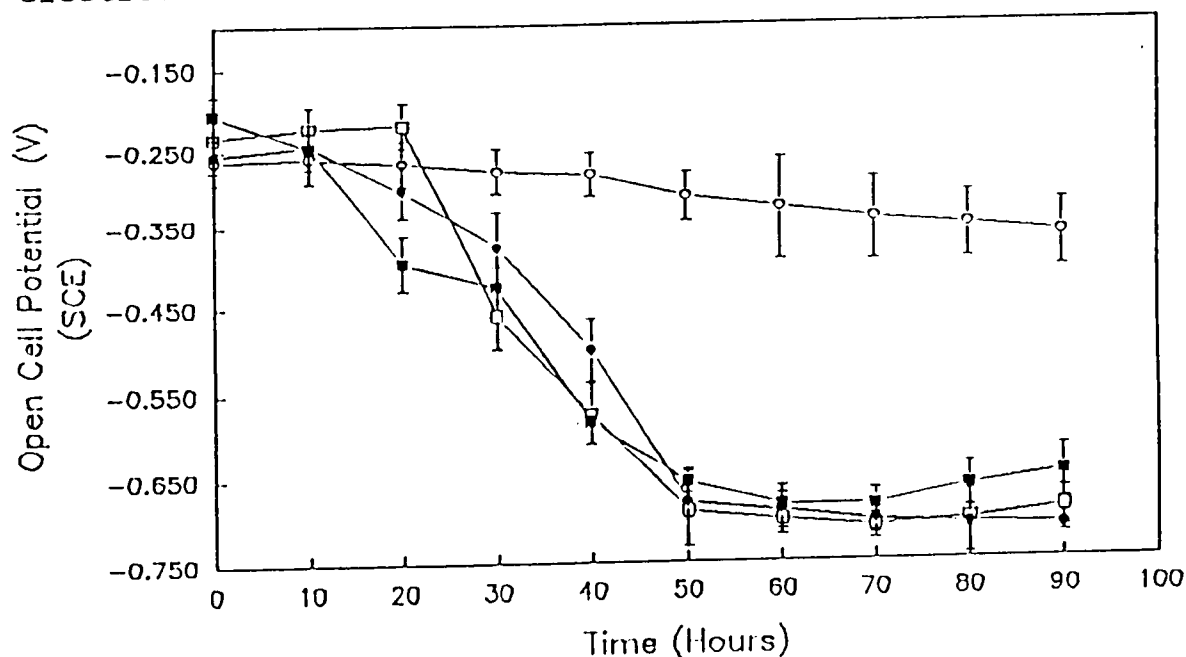


Figure 2. The effect of *Bacillus* (●), *Bacillus* plus *H. alvei* (□) and *Bacillus* plus *D. gigas* (■) on the open cell potential of carbon steel compared to sterile control (O). Bars represent ranges from two electrodes.

decrease in the OCP resulted with the diculture D. gigas plus H. alvei while the slowest occurred with D. gigas in monoculture. Addition of the Bacillus in monoculture as well as in di- and triculture appeared to unify the rate of OCP decrease.

Admittance and Bacterial Community

The corrosion, as measured in admittance mhos cm^{-2} over time, is shown in the Figures 3 and 4. All values are average admittance values from three electrodes over time. Figure 3 shows the admittance over time of carbon steel in the presence of H. alvei and D. gigas in monoculture, diculture and the H. alvei, D. gigas, plus Bacillus triculture. The admittance increased in the first four days to between 3.5×10^{-3} and 1.2×10^{-3} mhos cm^{-2} for the H. alvei and D. gigas mono- and diculture treatments while the triculture increased to 0.78×10^{-3} mhos cm^{-2} and the control to 0.19×10^{-3} mhos cm^{-2} . The admittance in the presence of both H. alvei and D. gigas monocultures decreased in 12 days to 0.9×10^{-3} mhos cm^{-2} while the admittance for the diculture remained at 2.28×10^{-3} mhos cm^{-2} after 16 days. By contrast, the admittance with the Bacillus, H. alvei plus D. gigas triculture treatment rose at a steady, constant rate to a maximum of 1.34×10^{-3} mhos cm^{-2} in 12 days and remained constant till the end of the experiment.

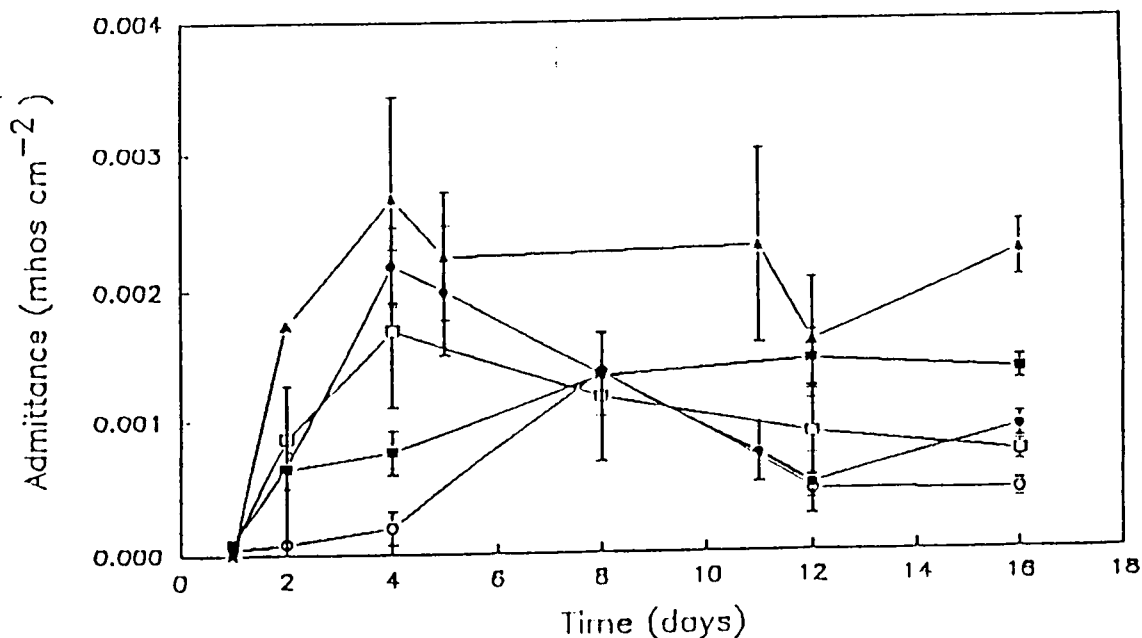


Figure 3. Admittance versus time with the bacterial combinations *D. gigas* (□), *H. alvei* (●), *H. alvei* plus *D. gigas* (▲), *Bacillus*, *H. alvei* plus *D. gigas* (■) and control (○). Error bars are +/- one std., n=3.

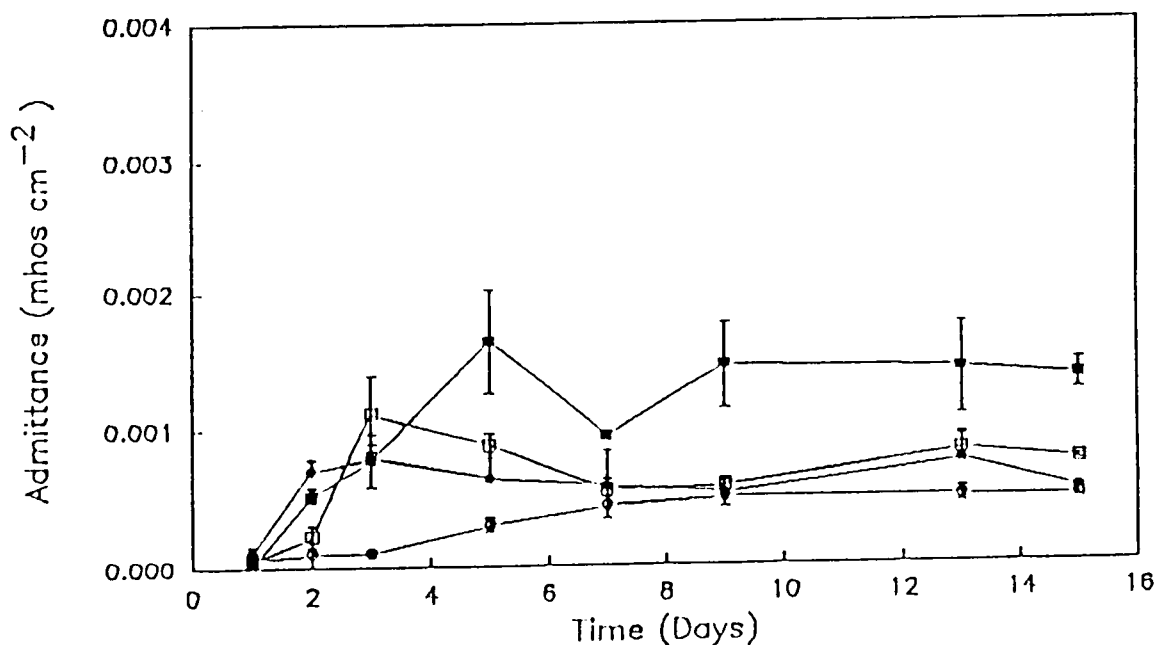


Figure 4. The effects of bacterial combinations on the admittance of carbon steel over time. *Bacillus* (●), *Bacillus* plus *H. alvei* (□), *Bacillus* plus *D. gigas* (■) and control (○). Error bars are +/- one std., n=3.

The admittance for the Bacillus in monoculture and in diculture with H. alvei and D. gigas compared to a sterile control is shown in Figure 4. The admittance increased for all treatments in the first three days. After five days the admittance increased to 1.64×10^{-3} mhos cm^{-2} for the Bacillus plus D. gigas diculture while the Bacillus and the Bacillus plus H. alvei treatments decreased to 0.636×10^{-3} and 0.87×10^{-3} mhos cm^{-2} , respectively. After nine days the control, Bacillus and Bacillus plus H. alvei treatments were similar at 0.5×10^{-3} mhos cm^{-2} while the Bacillus plus D. gigas diculture was greater at 1.44×10^{-3} mhos cm^{-2} . These values remained more or less stable for the remainder of the experiment.

Upon completion of the experiments, the largest increase in corrosion was with the H. alvei plus D. gigas diculture, followed by the Bacillus plus D. gigas and the Bacillus, H. alvei plus D. gigas triculture which had similar admittance values of 1.35×10^{-3} and 1.37×10^{-3} mhos cm^{-2} , respectively. The admittance and standard deviations of carbon steel for all the bacterial communities and sterile control upon termination of both experiments are given in Table 1. The admittance from the bacterial communities was tested for statistical significance from that of the control. All communities with D. gigas were significant at $P < 0.01$, except for the D. gigas in monoculture.

Table 1. The average admittance of carbon steel associated with Bacillus sp., Hafnia alvei and Desulfovibrio gigas in monoculture and in consortia after at least 15 days.

Bacterial Community	admittance ^a 10 ⁻³ mhos cm ⁻²	t test significance from control
Control	0.53 ± 0.12	
<u>Bacillus</u>	0.53 ± 0.01	
<u>H. alvei</u>	0.86 ± 0.23	P < 0.25
<u>Bacillus</u> <u>H. alvei</u>	0.74 ± 0.02	P < 0.20
<u>D. gigas</u>	0.72 ± 0.08	P < 0.20
<u>Bacillus</u> <u>D. gigas</u>	1.40 ± 0.1	P < 0.01
<u>H. alvei</u> <u>D. gigas</u>	2.30 ± 0.25	P < 0.01
<u>Bacillus</u> <u>H. alvei</u> <u>D. gigas</u>	1.40 ± 0.11	P < 0.01

a. average admittance ± one standard deviation (n=3).

The admittance of carbon steel associated with all other bacterial combinations was not statistically significant below $P < 0.20$.

Population Size versus Community Structure

The population sizes from the electrode surfaces after 16 days based on plate counts and MPN are shown in Table 2. The population sizes varied between 2.75×10^6 to 3.2×10^9 cells cm^{-2} for the Bacillus plus D. gigas diculture and the Bacillus, H. alvei plus D. gigas triculture, respectively. The Bacillus and H. alvei had similar population sizes when grown in monoculture, however, when cocultured the Bacillus appeared to dominate the community. D. gigas could not be isolated from the electrode surface from the monoculture treatment. The Bacillus had an average lower population size, 2.75×10^6 cells cm^{-2} , when grown in diculture with D. gigas than in monoculture, 1×10^7 cells cm^{-2} . Conversely, H. alvei had a greater population size when grown in diculture with D. gigas, 3.85×10^8 cells cm^{-2} , than in monoculture, 4.25×10^7 cells cm^{-2} . The triculture community had the largest total population size as well as the largest population sizes of each isolate than the mono- and diculture communities.

Table 2. Population Sizes^a from Bacterial Communities on electrode surfaces.

Bacterial Community	Electrode 1	Population Size Electrode 2	Total
<u>Bacillus</u>	1.0×10^7	1.0×10^7	1×10^7
<u>Bacillus</u>	$9.6 \pm 3 \times 10^7$	$11.6 \pm 3 \times 10^7$	1.19×10^8
<u>H. alvei</u>	$6.0 \pm 3 \times 10^6$	$2.0 \pm 4 \times 10^7$	
<u>H. alvei</u>	$3.5 \pm 2 \times 10^7$	$5 \pm 1.5 \times 10^7$	4.25×10^7
<u>D. gigas</u>	n.d.	n.d.	
<u>Bacillus</u>	$3.5 \pm 1 \times 10^6$	$2.0 \pm 1 \times 10^6$	2.8×10^6
<u>D. gigas</u>	6.5×10^4	3.56×10^4	
<u>H. alvei</u>	$2.7 \pm 5 \times 10^8$	$5.0 \pm 3 \times 10^8$	3.8×10^8
<u>D. gigas</u>	5.0×10^4	0.9×10^4	
<u>Bacillus</u>	$2.0 \pm 3 \times 10^8$	$2.4 \pm 4 \times 10^8$	3.2×10^9
<u>H. alvei</u>	$4.3 \pm 2 \times 10^9$	$1.7 \pm 5 \times 10^9$	
<u>D. gigas</u>	1.27×10^7	1.13×10^6	

a. Bacillus and H. alvei were determined by aerobic plate counts. D. gigas was determined by MPN, both are expressed as cells cm^{-2} . The totals are averages of two electrodes.

PLFA versus Bacterial Community

The PLFA of Bacillus and H. alvei inoculum, mono- and diculture from the electrode surface is shown in Table 3. The Bacillus contained predominantly branched, saturated fatty acids, 27% i15:0 and 19% i17:0, as well as i14:0, a15:0, i16:0 and a17:0. This organism also contains the branched, monoenoic fatty acids, i16:1 and i and a17:1. The Bacillus also contained straight chain, saturated 16:0 and straight chain, monoenoic 16:1w7c. The mole% of i15:0 increased from 27% to an average of 36.75% when removed from the electrode surface after 15 days. There was also a slight increase in a17:1 and slight decreases in i16:0, i17:1, i17:0 and 16:1w7c. Interestingly, 16:1w7t was detected in the bacterium from the electrode surface, approximately six%, but was not detected in the inoculum.

H. alvei had a simpler PLFA profile of exclusively straight chain saturated and monoenoic fatty acids. H. alvei contained 28% 16:0, 24% cy17:0, and 17% of 16:1w7c and 18:1w7c, respectively. No branched PLFA were detected. There was a greater than 10% decrease in 16:1w7c and five% decrease in 16:0 from H. alvei on the electrode surface. Conversely, there was a 10% increase in cy17:0. Similar to the Bacillus, a PLFA not found in the inoculum was detected in the organism after growth on the electrode surface, a an eight% increase in cy19:0 and a 2% increase in 18:1w7t.

Table 3. Phospholipid Fatty Acids of Bacillus sp.,
H. alvei in Mono- and Diculture^a.

Fatty Acid	<u>Hafnia alvei</u>			<u>H. alvei</u> + <u>Bac.</u>		<u>Bacillus</u>		
	Inoc.	Electrode		Electrode		Electrode	Inoc.	
a13:0	0	0	0	0.6	1.1	1.9	1	1
i14:0	0	0	0	2	1.3	2.5	3	2.2
14:0	0.6	0	0	2	1.6	1.6	1.4	1.6
i15:0	0	0	0	10.6	5.7	35.5	38	27
a15:0	0	0	0	3	1.8	4.4	2	4.9
15:0	2.7	2.6	1.2	2.2	1.3	0.3	0.7	0
i16:1	0	0	0	1.4	0.8	2.7	3.1	2.7
i16:0	0	0	0	1.2	1.2	5.3	5.8	7
16:1w7c	17	5.5	5.4	6.7	6	5.4	6	9.3
16:1w7t	0.8	0	0	2.5	2.3	6.7	6.3	0
16:1w5c	0	0	0	0	0	0	0	0
16:0	28	20.9	23.6	25.4	28	3.7	4.4	7.4
i17:1	0	0	0	0.5	1	5.4	4.7	8.3
a17:1	0	0	0	1.6	3	11.4	10	6.2
i17:0	0	0	0	2.6	1.2	11	15	19
a17:0	0	0	0	0.8	1.7	1.2	1.8	2.8
17:1w8	1.2	0.9	0.6	0	0	0	0	0
cy17	24	35.4	39.2	26	16	0	0	0
17:1w6	0	0	0	0	0	0	0	0
17:0	4	5	5.3	2.1	1.3	0	0	0
18:1w7c	17	11.4	9.4	5	4.6	0	0	0
18:1w7t	0.8	2.8	2.5	1	0.7	0	0	0
18:0	1.2	4.3	3.7	1.9	2.7	1.4	2	1
cy19	0	10.3	8.7	3.6	2.3	0	0	0
Total ^b	143000	12200	21000	29900	61600	13400	15800	143000

a. Data is expressed in mole %

b. Total PLFA expressed in picomoles using C19:0 as internal standard.

The H. alvei plus Bacillus diculture from the electrode surface had mixtures of branched and straight chain fatty acids. Compared to the Bacillus from the electrode, there was a 20% decrease in i15:0 as well as decreases in i and a17:1 and i17:0. There was also a 10% decrease in cy17:0 compared to H. alvei from the electrode surface as well as slight decreases in 18:1w7c, 18:1w7t and cy19:0.

The PLFA for D. gigas inoculum, di- and tricultures with Bacillus and H. alvei are shown in Table 4. D. gigas contained a mixture of branched and straight chain fatty acids, the predominant PLFA was 16:0 at 37%, followed by branched i17:1 at 22% and 17% of 16:1w7c. The H. alvei plus D. gigas diculture contained an average of 35.8% cy17:0 and 26.35% of 16:0. Traces of i17:1, a major PLFA found in D. gigas, and traces of a17:0 were barely detectable in the diculture. No other branched fatty acids were observed. The Bacillus plus D. gigas diculture had almost half it's total PLFA as i15:0, approximately 43%, and 27% of a17:1 and i17:0. Roughly 12% of the PLFA were a mixture of 16:1w7c and 16:0. The triculture contained almost 80% straight chain PLFA, 38% of it's total PLFA was in the form of cy17:0 while the additional 37% as 16:0. An additional 15% of 16:1w7c and 18:1w7c were also found. The triculture also contained many branched PLFA but in low amounts.

The bacterial communities were clustered based on the relative relatedness of the mole% PLFA profile.

Table 4. Phospholipid Fatty Acid Profiles of *D. gigas* inoculum and Consortia^a.

		<u>H. alvei</u>	<u>Bacillus</u>	<u>Bacillus, H. alvei</u>
Fatty Acid	<u>D. gigas</u> Inoculum	<u>D. gigas</u> Electrode	<u>D. gigas</u> Electrode	<u>D. gigas</u> Electrode
a13:0	0	0	2.8	3
i14:0	0	0	1.8	1.2
14:0	1	1.8	2	0
i15:0	7	0	45.5	41
a15:0	1.9	0	2.7	2.5
15:0	0.3	4	0.2	0
i16:1	0.6	0	2.2	2.7
i16:0	0.6	0	2.6	2.7
16:1w7c	17	7.8	7.8	8
16:1w7t	0.2	1.3	1.1	0
16:1w5c	0.1	0	0	0
16:0	37	24.7	28	3.5
i17:1	22.6	0.8	0.2	4.5
a17:1	0.5	0	0	12
i17:0	4	0	0	10.4
a17:0	0.5	1.2	0.6	1
17:1w8	0.4	0	0	0
cy17	0	34	37.6	0
17:1w6	0.1	0	0	0
17:0	0.8	0.8	4.3	0
18:1w7c	2.1	8	6.7	0
18:1w7t	0	2.2	2	0
18:0	3.3	3.1	2.6	1
cy19	0.2	5.7	3.9	0
Total ^b	1630000	35800	78000	8000
				3900
				16000
				26000

a. Data are expressed in mole%.

b. Total PLFA expressed in picomoles using C19:0 as internal standard.

The results of the cluster analysis of the bacterial treatments from the electrode surface and inoculum are given in Figure 5. The first cluster division occurs between the Bacillus community, defined by predominantly branched PLFA, and H. alvei community, which is dominated by straight chain, monoenoic PLFA. Within the Bacillus cluster, the inoculum is separated from all other treatments including the electrode surface and the liquid broth of the corrosion flask from the increases in a15:0 and a17:1 and also decreases in i17:1 and i17:0. The Bacillus and D. gigas diculture is clustered separately from the monoculture because of the increase in a15:0 in the diculture as seen in Table 4.

The H. alvei dominated cluster divides into two major groups: One composed of straight chain, monoenoic PLFA and the other straight chain with some branched PLFA. These are the diculture treatments of Bacillus plus H. alvei and a H. alvei dominated group (see Table 3). The D. gigas inoculum is also within Bacillus plus H. alvei cluster due to mixtures of 16:0, 16:1w7c and i17:1, no PLFA were detected from the electrodes when D. gigas was added in monoculture. The H. alvei inoculum is separate from the monoculture, (decrease in 16:1w7c; increase in cy17:0) diculture with D. gigas due to the slight increase in 16:0, 16:1w7c and decreases in longer chained 18:1wtc and cy19:0. The triculture adds many branched PLFA resulting in further

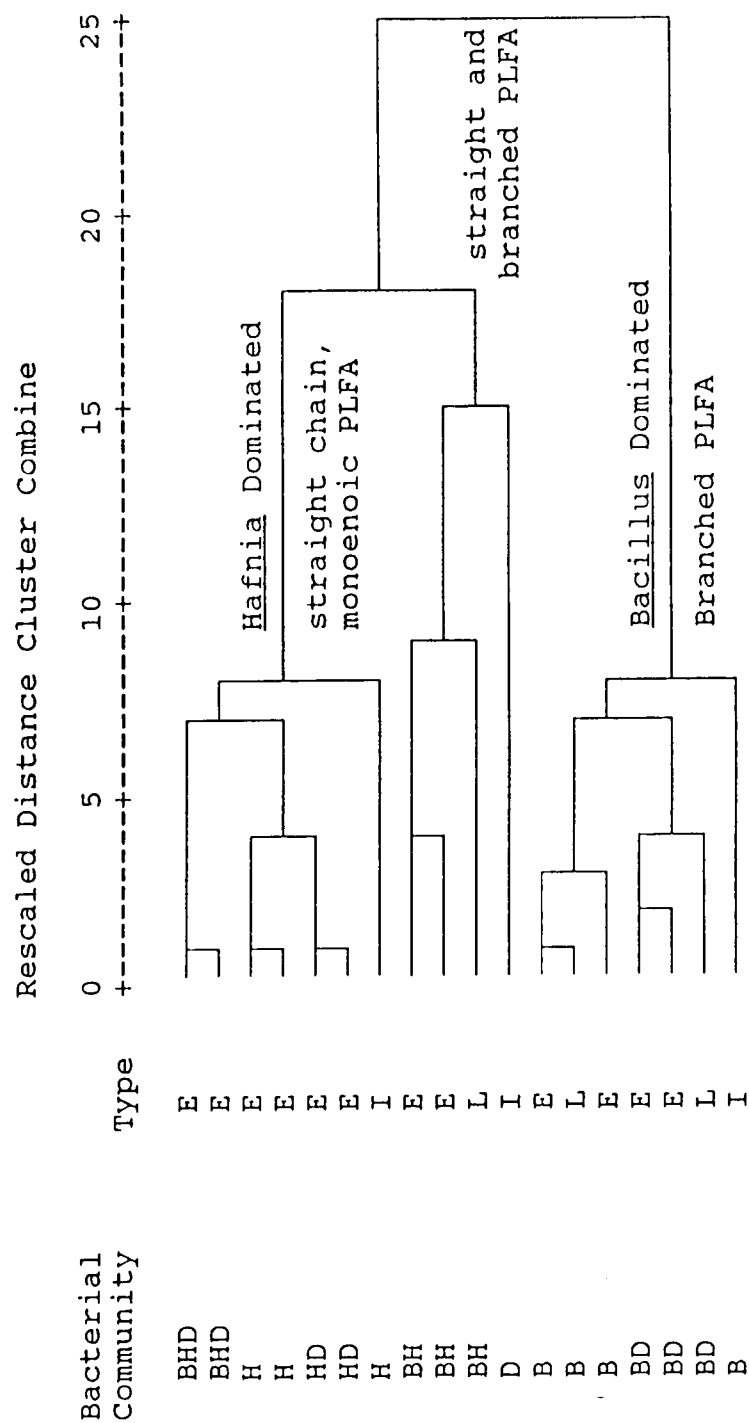


Figure 5. Cluster analysis of bacterial combinations used in corrosion experiments. The dendrogram is based on the relative relatedness of phospholipid fatty acid profiles of each respective community. The combinations are made up of *Bacillus* sp. (B), *Hafnia alvei* (H) and *Desulfovibrio gigas* (D). The Type refers to the electrode surface (E), the liquid media adjacent to the surface (L) or the inoculum in pure culture (I).

separation. It is interesting to note that the Bacillus plus H. alvei diculture and the D. gigas inoculum (composed of relatively equal amounts of branched and straight chain PLFA are in a major cluster, however, when grown together in a triculture, this community clusters with the H. alvei (predominantly straight chain PLFA) cluster. This is shown by the large amounts of 16:0 and cyl7:0 in Table 4 for the Bacillus, H. alvei plus D. gigas community. This indicates that the addition of D. gigas stimulated H. alvei or hindered the Bacillus in some way.

A comparison of the total cells cm^{-2} versus total PLFA in picomole cm^{-2} for all the bacterial communities where biomass could be measured is shown in Figure 6. The results are averages of two electrodes per method. There is a clear relationship between an increase in biomass determined by Plate counts plus MPN versus total biomass in terms of total PLFA for all but one of the bacterial treatments. This occurred with the Bacillus, H. alvei and D. gigas triculture community.

Comparison of Total Biomass versus Admittance

The relationship between the total PLFA versus admittance and the total cell number versus admittance and for the bacterial communities are shown the Figures 7 and 8, respectively. The admittance for each community is that determined at the end of the experiment.

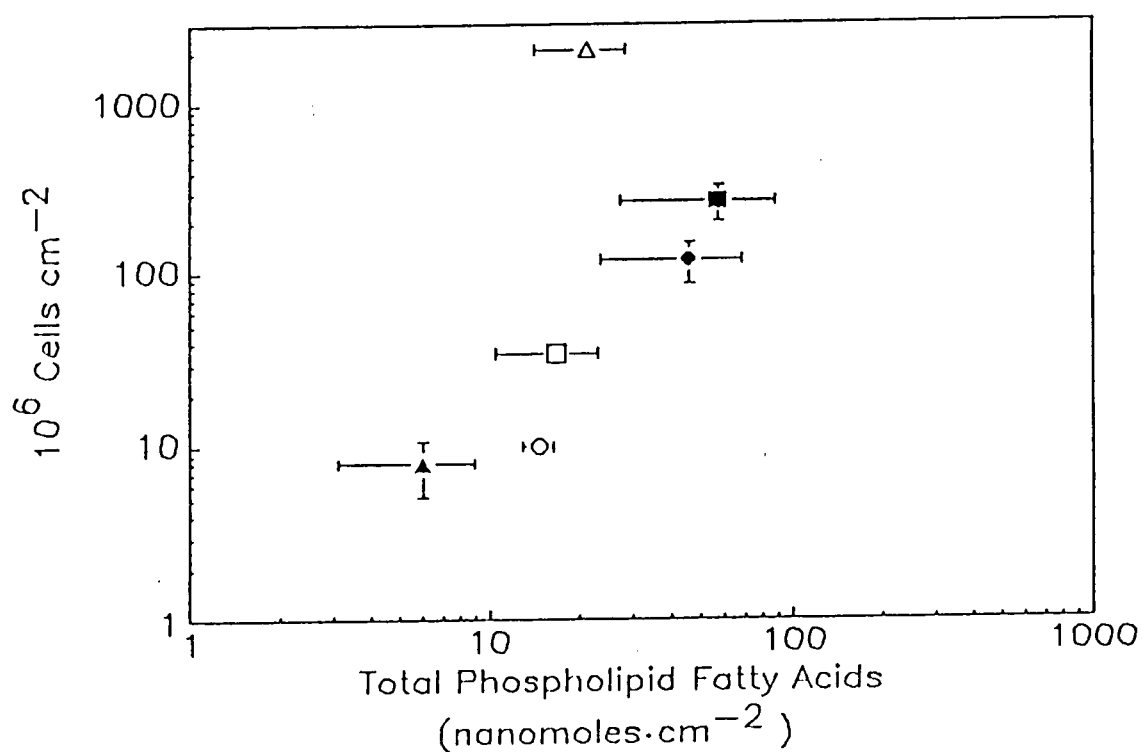


Figure 6 . Total cells versus total phospholipid fatty acids of bacterial combinations from electrode surfaces. *Bacillus* (O), *Bacillus* plus *H. alvei* (●), *Bacillus* plus *D. gigas* (▲), *H. alvei* (□), *H. alvei* plus *D. gigas* (■) and *Bacillus*, *H. alvei* plus *D. gigas* (Δ).

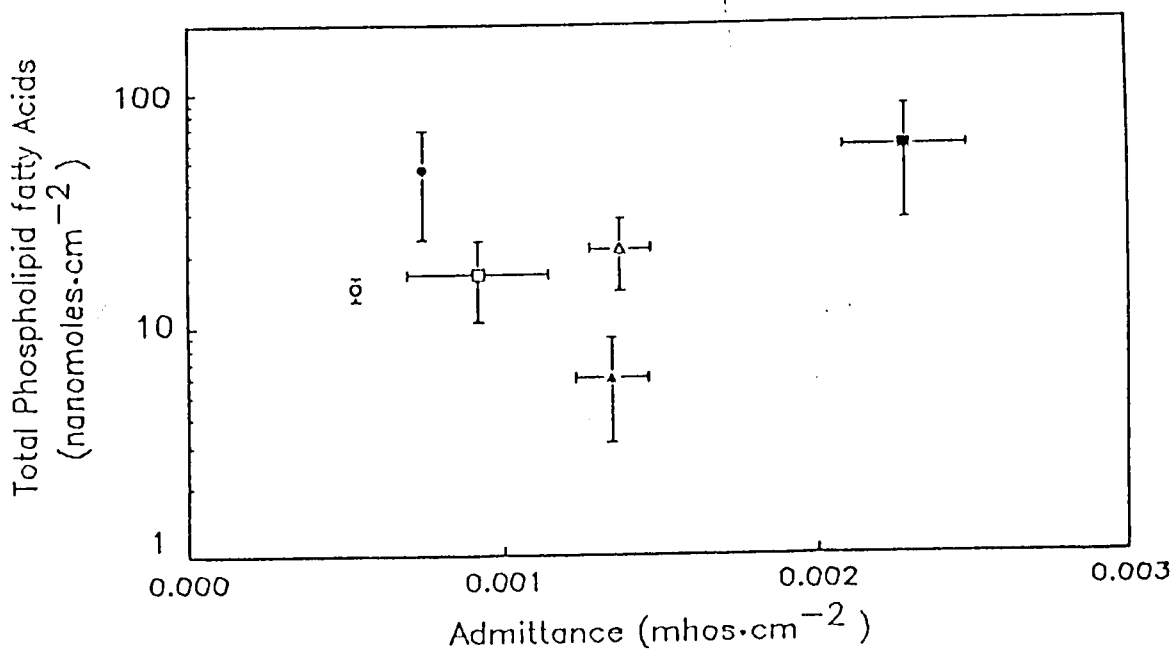


Figure 7 . The total phospholipid fatty acids versus final admittance for bacterial combinations used in corrosion experiments. Bacillus (○), Bacillus plus H. alvei (●), Bacillus plus D. gigas (▲), H. alvei (□), H. alvei plus D. gigas (■) and triculture (△).

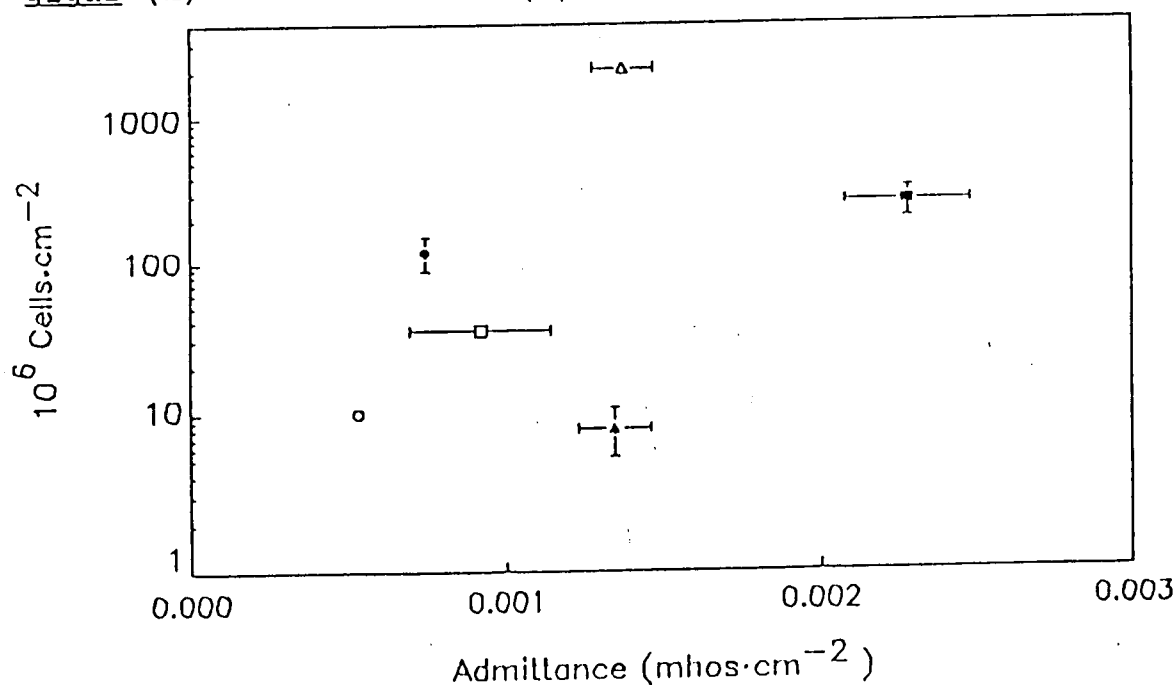


Figure 8 . Total bacterial cells from electrode surface versus final admittance. Bacillus (○), Bacillus plus D. gigas (▲), H. alvei (□), H. alvei plus D. gigas (■), Bacillus plus H. alvei (●) and triculture (△).

These Figures show no relationship between total biomass and increase in admittance. Bacillus plus D. gigas has the lowest biomass in terms of total PLFA and total cells (an average of 6000 picomoles cm^{-2} and 3.5×10^6 cells cm^{-2} , respectively) yet has the third highest admittance at 1.35×10^{-3} mhos cm^{-2} , see Tables 1,2,3 and Figure 4. The diculture H. alvei plus D. gigas had significantly greater admittance but similar PLFA and cell mass to H. alvei in monoculture. The triculture had similar admittance to the Bacillus plus D. gigas diculture but greater total biomass.

Discussion

The additive effects of metabolically distinct bacteria on the corrosion of carbon steel allow insights into the major contributors to corrosion. This study compared the additive effects of bacteria capable of producing oxygen concentration cells, organic acid production, cathodic depolarization and ferrous and/or hydrogen sulfide production by using three physiologically distinct bacteria.

The results show that D. gigas had the greatest effect on the corrosion of carbon steel in an aerobic, freshwater system when present with oxygen consuming bacteria. Whether cathodic depolarization or ferrous sulfide was the major corrosion mechanism is not known, though ferrous sulfide production has been shown to have a greater corrosion impact under in situ conditions (2,15). Because D. gigas does not consume hydrogen if lactate is available as an electron donor (19,21), it seems unlikely that cathodic depolarization was a major mechanism in these experiments.

There was no clear relationship between the change in OCP over time between the bacterial communities except for D. gigas in monoculture. This decrease was greater than that of the control but less than that of aerobically respiring communities. Because aerobically respiring bacterial communities resulted in greater decreases in the OCP, an attempt was made to relate bacterial activity (as

measured by ^{14}C -acetate into PLFA) versus OCP over time. However, incorporation of labeled acetate could not be detected until 72 to 96 hrs, which was after the drop in OCP occurred (data not shown). More work needs to be done addressing inoculum size, mixing, attachment and activity to fully address this question.

The strictly aerobic Bacillus and strictly anaerobic D. gigas (in monoculture) demonstrated the effects of oxygen concentration cells on the corrosion of carbon steel. There was little difference in the admittance of carbon steel in the presence of these bacteria. Thus, oxygen concentration cells were of minor importance in this system.

Organic acid production by H. alvei was also a minor contributor to corrosion in this system. This may be due to the continuously flowing conditions which would result in dilution of organic acids. Organic acids are believed to play a more significant role in corrosion in stagnant environments where sulfate is absent (20,21).

The increased corrosion of carbon steel with D. gigas in a biofilm clearly shows the significance of this organism in MIC at freshwater sulfate concentrations.

The greater admittance by H. alvei plus D. gigas than in the presence of Bacillus plus D. gigas indicates that a fermenter plus sulfate-reducer has a greater effect on the corrosion than a strict aerobe plus sulfate-reducer. H. alvei plus D. gigas also had a greater admittance than the

triculture. The Bacillus in the triculture may have consumed substrates that would otherwise be shunted to organic acid production and sulfate reduction. The role of sulfate-reducing bacteria in anaerobic corrosion has been extensively studied (1,2,7,9). Hamilton et al. have shown that increased corrosion in marine sediments was not indicative of SRB population size or activity (7). This agrees with current results where the D. gigas population size was greater in the triculture than in diculture with H. alvei yet had a lower admittance.

The population sizes on the electrode surface showed differing effects between mono-, di- and triculture conditions. D. gigas decreased the population size of Bacillus yet increased the population size of H. alvei compared to the total cells in monoculture. D. gigas was always present in lower amounts as shown by Plate counts versus MPN and the clustering of the mole% PLFA within the H. alvei dominated cluster or the Bacillus dominated cluster. Because D. gigas was cultured from di- or triculture communities and not in monoculture suggests that an anaerobic niche had formed when Bacillus or H. alvei were present. The use of changes in PLFA profiles to distinguish community structure between aerobic and anaerobic estuarine sediments has been demonstrated (8).

The differences in the PLFA between the Bacillus and H. alvei allowed a clear distinction between these two

organisms and how the community structures changed in mono-, di- and triculture conditions. The PLFA profiles indicate that H. alvei was the dominant organism in the triculture, which was also shown by total plate counts. However, in the H. alvei plus Bacillus diculture, the PLFA indicated that H. alvei was dominant while the plate counts indicated that Bacillus was the dominant organism. The reason for this discrepancy is unclear.

A comparison of the total biomass between total cells by plate count plus MPN versus total PLFA shows a clear relationship between the two methods, except for the triculture community. These estimates used only duplicates of each community, greater replication is needed to conclusively relate these two methods of bacterial biomass. Relationships between PLFA, acridine orange direct counts, muramic acid and lipid A compared to total gram dry weight of cells have been shown (25).

A comparison of the total biomass to the admittance shows that the type of bacterial community is more important than the total bacterial biomass. The increase in biomass for a single community versus admittance would further illustrate this point.

MIC studies using consortia are important for several reasons: they more closely mimic in situ conditions, provide a comparison between different corrosion mechanisms and give information to the major contributors of MIC.

This study has demonstrated that D. gigas is the main contributor to the corrosion of carbon steel in this aerobic, freshwater system only when present in a biofilm.

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Part III

THE EFFECT OF AEROBIC, FERMENTATIVE AND SULFATE REDUCING ENRICHMENTS ON THE CORROSION OF CARBON STEEL

Abstract

The corrosion of carbon steel using aerobic plus fermentative and aerobic, fermentative plus sulfate-reducing bacterial enrichments from a corroding pipe versus a sterile control was investigated. The corrosion, measured as admittance, was greater for the aerobic, fermentative plus sulfate-reducing community throughout the course of the experiment. The final admittance for the aerobic, fermentative plus sulfate-reducing community was 3.13×10^{-3} mhos cm^{-2} while the aerobic plus fermentative community and control had admittances of 1.38×10^{-3} mhos cm^{-2} and 0.5×10^{-3} mhos cm^{-2} , respectively. The bacterial communities were statistically significant from the control at $P < 0.01$. The open cell potential (OCP) decreased from -250 mV/SCE to -700 mV/SCE for both treatments and to -335 mV/SCE for the sterile control. There was little difference between the population sizes on the electrode surface based on total plate counts, MPN and total phospholipid fatty acid (PLFA) profiles. MPN revealed 1.05×10^3 cells cm^{-2} of sulfate-reducing bacteria. Comparison of the mole% PLFA showed differences between the communities on the electrode, inoculum and liquid adjacent to the electrode. The data indicates that low numbers of sulfate-reducing bacteria in biofilms significantly increase the corrosion of carbon steel in aerobic, freshwater systems.

Introduction

The corrosion of metals often involves the association of many organisms which make a complex consortia (14). Increasing the metabolic diversity (see Part II, this Thesis) using isolates of a strict aerobe, a facultative anaerobe and a sulfate reducing bacterium showed that the main contributor to the corrosion of carbon steel was the sulfate reducing bacterium, Desulfovibrio gigas.

Sulfate reducing bacteria have been extensively studied in microbial influenced corrosion (MIC) processes (7). Several corrosion mechanisms have been described for these bacteria including ferrous and hydrogen sulfide formation acting as cathodic depolarizers (11) and consumption of hydrogen, a process known as cathodic depolarization (1,3,13). Reduced sulfide binds with ferrous iron, accelerating the anodic reaction and hydrogen consumption accelerates the cathodic reaction by consuming electrons. Due to the formation of anaerobic niches, the sulfate reducing activity is not limited to anaerobic environments. Sulfate reducing activity has been found in anaerobic niches in aerobic sediments and associated with corroding metals below aerobic solutions (7,9). Thus bacterial consortia allow physiologically distinct bacteria to be metabolically active in otherwise inhospitable environments. Accelerated corrosion of carbon steel by a combination of

Vibrio anguillarum and Desulfovibrio vulgaris was greater than either organism in monoculture in anaerobic media (5).

The addition of physiologically distinct bacteria leads to an increase in the metabolic diversity of the total community. This increased metabolic diversity increases the number of reactions capable of affecting the complex anodic and cathodic corrosion reactions.

The purpose of this study was to show that increasing the metabolic diversity using enrichments from an actively corroding pipe would also result in an increase in the corrosion rate of carbon steel in an aerobic, freshwater environment. Due to the formation of anaerobic niches, the corrosion rate would be greatest in the presence of the sulfate reducing enrichment.

Materials and Methods

Preparation of Bacterial Enrichments

A tubercle from in a 4 in. carbon steel pipe used in transporting estuarine cooling water for a Hydroelectric power plant was used to enrich for the aerobic, fermentative and sulfate reducing communities. The tubercle or rust material inside the pipe was scraped with a sterile spatula and transferred to two test tube containing 10 mls of media. The media consisted of, in g/l,: glucose, 2; lactate, 2; NH_4CL , 0.5; KH_2PO_4 , 0.1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1. Four test tubes were inoculated for both aerobic and fermentative enrichments. The aerobic enrichment was shaken at 32°C for 48 hrs then transferred and incubated for 24 hrs before inoculation in the corrosion flask. The fermentative enrichment was tightly capped, nonshaken and incubated at 32°C for 48 hrs, transferred to fresh media 24 hrs before inoculation.

For the sulfate reducing enrichment, the tubes were inoculated into 10 mls of, g/l: lactate, 3; yeast extract, 1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 4; CaSO_4 , 1; NH_4CL , 1; K_2HPO_4 , 0.5 and $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$. The tubes were incubated at 32°C for 48 hrs. All enrichments were then transferred and incubated a second time in media that lacked $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ to prevent FeS formation. Before inoculation the sulfate reducing enrichment was flushed for 15 min. with N_2 to remove residual H_2S . All enrichments were then centrifuged

for 20 min. at 10,000 rpm, the supernatant decanted and 10 mls of reduced mineral salts was added, vigorously vortexed and recentrifuged. This procedure was repeated 2 times. Additions of H_2S or FeS in the inoculum drastically affected the corrosion rate.

Carbon Steel Preparation and Test Flask

The carbon steel elemental composition, electrode preparation and placement in the test flask and media used have been described in Part II.

Bacterial Analysis

The bacterial communities were analyzed by plate counts for aerobic bacteria and sulfate-reducing bacteria were counted using the most probable number (MPN) method. The procedure of removing the bacteria from the electrode surface and growth media used is explained in Part II. Phospholipid fatty acids (PLFA) were used to correlate community structure with inoculum and between treatments. The procedure for PLFA removal from the electrode, lipid extraction, derivatization, quantification and clustering of mole% PLFA is also described in Part II.

Electrochemical Analysis

The OCP was used to measure the potential change upon addition of the bacterial enrichments. The corrosion was

measured with AC impedance and reported as admittance mhos cm^{-2} . The apparatus and oscillation rates used are described in Part II. However, for this experiment four electrodes were scanned per time point.

Statistical Analysis

Upon completion of the experiment the admittance for the bacterial treatments and control were tested for statistical significance compared to the sterile control with the student's t test.

Experimental Procedure

One Flask was inoculated with 20 mls each of the aerobic (A) and fermentative (F) enrichments and another flask inoculated with the above plus 20 mls of the sulfate reducing (S) enrichment. The communities tested were AF and AFS which were compared to a sterile control. Previous results in Part II indicated that all communities with the sulfate-reducing bacterium D. gigas, except for the organism in pure culture, were all statistically different at $P < 0.01$ when compared to the sterile control. Therefore only the combinations AF and AFS were tested.

An initial EIS scan was performed for four electrodes per flask under constant stirring, nonflowing conditions. The OCP was then determined for a 12 to 24 hr period for 2 electrodes to check for system stability within and between

flasks. The flasks were then inoculated during OCP scanning, the flow was kept off to prevent washout of cells for an additional 12 hrs. Flow did not affect the EIS or OCP as long as the flasks were kept stirring in this system. After disconnecting the OCP scanner, EIS and OCP were determined over the course of the experiment.

The media was kept aerobic by continual stirring and flow throughout the experiment and periodically checked for dissolved oxygen with a Y.S.I. dissolved oxygen meter, model 50 (Yellow Springs, Ohio). The D.O. was kept between four and five mg/l in the broth.

When the experiment was completed the WE's were removed and the bacterial communities were analyzed as described above.

Results

OCP versus Bacterial Community

The OCP for the aerobic plus fermentative (AF) and aerobic, fermentative plus sulfate reducing (AFS) communities and sterile control are shown in Figure 1. The OCP for the AF community immediately dropped the potential at ten hrs from -245 mV/SCE to -745 mV/SCE between 60 and 70 hrs. The OCP for the AFS community did not begin to decrease until 30 hrs after inoculation, from an average of -250 to -700 mV/SCE at 60 hrs. Both treatments had similar steady state OCP at -700 mV/SCE after 80 hrs. The control stayed relatively constant and decreased to -300 mV/SCE after 100 hrs. Thus, the addition of all bacterial enrichments resulted in an increase in the cathodic reactions on the metal surface while the control became only slightly cathodic.

Admittance versus Bacterial Community

The admittance of the carbon steel relative to the AF and AFS communities is shown in Figure 2. The initial admittance was between 1.54×10^{-4} and 2.14×10^{-4} mhos $\cdot$ cm $^{-2}$ for all flasks before inoculation. Five days after inoculation, there was a clear difference in the corrosion rates between treatments.

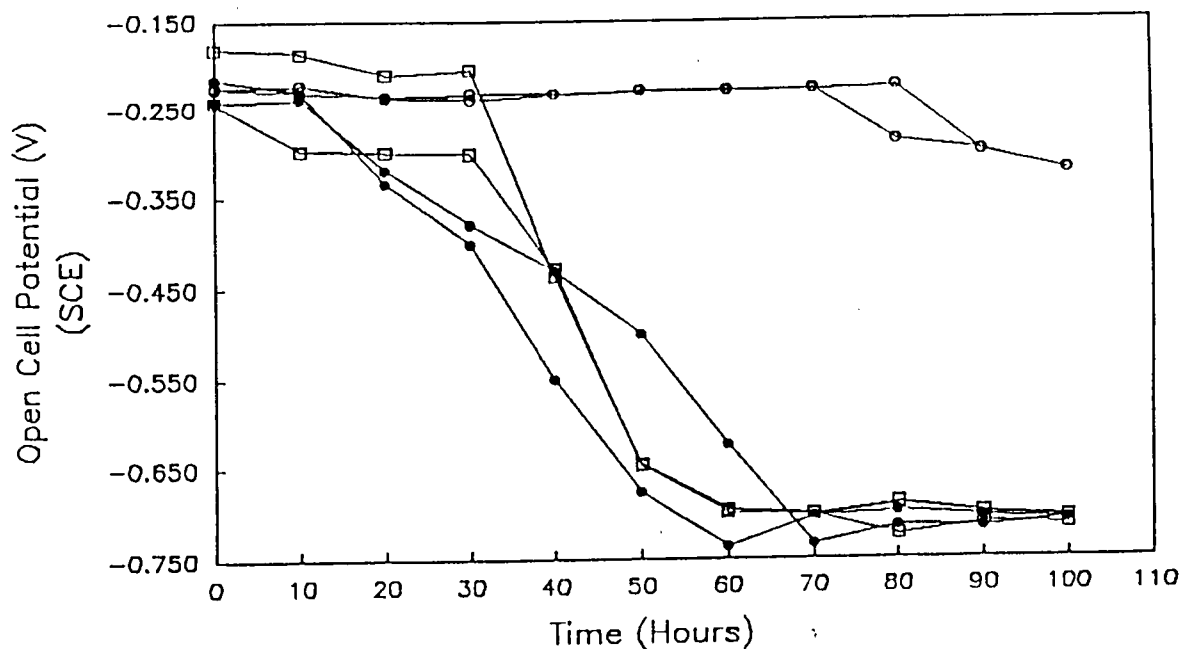


Figure 1. Open cell potential over time from two electrodes associated with different bacterial enrichments. Aerobic plus fermentative (●), aerobic, fermentative plus sulfate reducing (■) versus control (○).

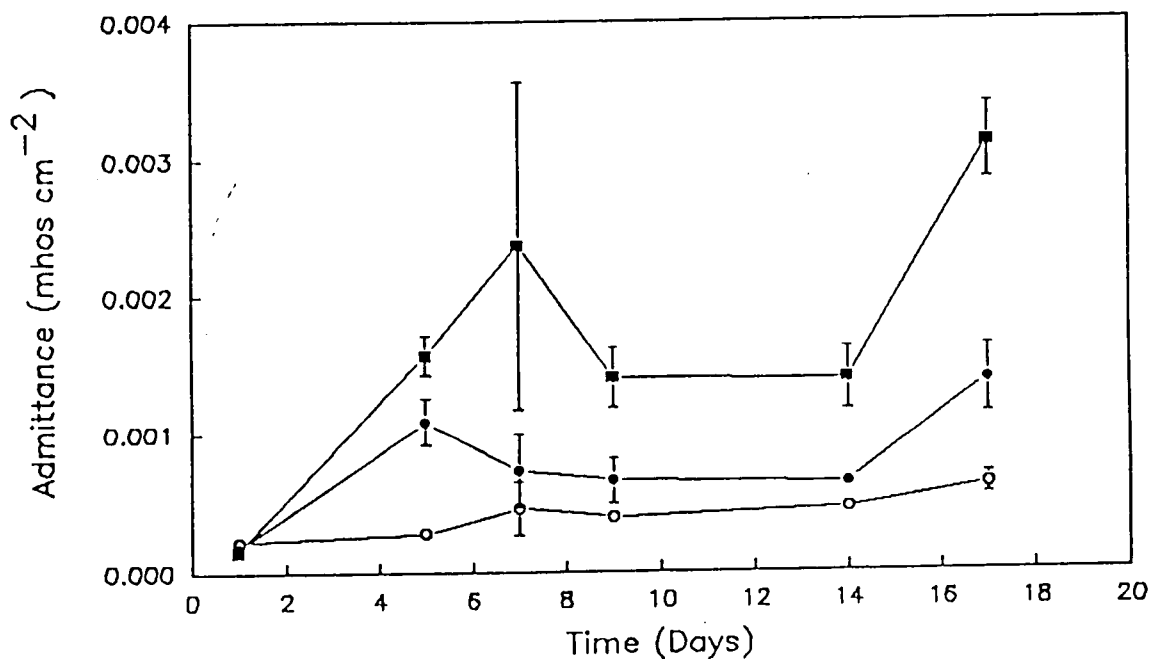


Figure 2. Admittance over time for carbon steel with different bacterial enrichments. Aerobic plus fermentative (●), aerobic, fermentative plus sulfate reducing (■) and control (○).

The admittance for the control did not increase significantly while the AF and AFS communities increased to 7.8×10^{-4} and 1.57×10^{-3} mhos cm^{-2} , respectively. The admittance for the AFS community continued to rise while the AF admittance decreased to that of the control. At nine days the admittance values reached a steady state with the AFS admittance much larger than the AF community and the control. Interestingly, all treatments showed an increase in admittance after 14 days, the largest increase was with the AFS community to 3.13×10^{-3} mhos cm^{-2} while the AF community reached an admittance of 1.38×10^{-3} mhos cm^{-2} . The admittance for the control at the end of the experiment was 6.23×10^{-4} mhos cm^{-2} . The admittances from the bacterial communities were tested for statistical significance from that of the control. This data is given in Table 1, and shows the admittance, standard deviations and significance at $P < 0.01$ using the student's t test. Both bacterial communities were statistically significant from the control.

Plate Counts and MPN versus Bacterial Community

The population sizes based on aerobic plate counts and MPN for the sulfate reducing bacteria is shown in Table 2.

Three different colony types were noticed on the aerobic plates after incubation for both the AF and AFS communities from the electrodes and broth.

Table 1. The average admittance of carbon steel from aerobic plus fermentative and aerobic, fermentative plus sulfate-reducing enrichments after 17 days.

Bacterial Community	Admittance ^a 10 ⁻³ mhos cm ⁻²	t test significance from control
Control	0.6±0.07 x 10 ⁻³	
AF	1.4±0.25 x 10 ⁻³	P < 0.01
AFS	3.1±0.28 x 10 ⁻³	P < 0.01

a. average ± one standard deviation (n=3).

Table 2. Population sizes^a of Bacterial Communities on Electrode Surfaces and Broth.

Bacterial Community	Electrode	Broth
Aerobic Ferm.	5.5±0.44 x 10 ⁸	5.85±0.45 x 10 ⁹
Aerobic Ferm.	1.16±0.7 x 10 ⁹	3.9±1.9 x 10 ⁸
SRB ^b	1.06±0.09 x 10 ³	n.d.

a. Aerobic plate counts are averages of two electrodes expressed as cfu cm⁻². The broth are expressed as cfu ml⁻¹.

b. SRB determined by MPN are expressed as cells cm⁻².

However, the total populations sizes were different between the AF and AFS communities, $5.5 \pm 0.44 \times 10^8$ and $1.16 \pm 0.7 \times 10^9$ cfu cm⁻², respectively. The aerobic bacteria in the broth contained similar colony types but had higher numbers in the AF then the AFS community. The population size from the AF broth was $5.85 \pm 0.45 \times 10^9$ and in the AFS broth was $3.9 \pm 1.9 \times 10^8$ cfu ml⁻¹. The only sulfate reducing bacteria detected were from the AFS electrode at $1.06 \pm 0.9 \times 10^3$ cells cm⁻².

PLFA versus Bacterial Community

Table 3 gives the PLFA profiles of the original tubercle and the resulting aerobic, fermentative and sulfate reducing enrichments. The tubercle is a mixture of branched, monoenoic and straight chain fatty acids such as iso- (i) and anteiso (a) 17:1; i and a 15:0. There are also large amounts of straight chain 16:1w7c, 16:0 and 18:1w7c. The aerobic and fermentative enrichments selected for bacteria with predominantly straight chain fatty acids such as 16:1w7c, 16:0, cy17:0 and 18:1w7c. The sulfate reducing enrichments select for bacteria with predominantly branched and straight chain fatty acids, note the large increase of i17:1 while the i15:0, 16:0, cy17:0 and 18:1w7c are very similar to the tubercle.

Table 3. Phospholipid Fatty Acid Profiles^a of Tubercle from corroded pipe and Aerobic, Fermentative and Sulfate Reducing enrichments.

Fatty Acid	Tubercle	Enrichment		
		Aerobic	Ferm.	Sulfate Red.
a13:0	0.04	0	0	0
i14:0	0.35	1.80	0	0
14:0	1.2	0	1.4	0.8
i15:0	5.8	0.10	0	6
a15:0	6.5	0.15	0	2.1
15:0	1	0.1	0.24	0.7
i16:1	0	0	0	0
i16:0	1.6	0	0	1.25
16:1w7c	15.8	15.3	13.26	6.7
16:1w7t	3.7	7.9	5.8	0.55
16:1w5c	0	0	0	0
16:0	16	31	32	17
i17:1	8	0	0	22
a17:1	12.8	0	0	1.6
i17:0	1.8	0	0	8.3
a17:0	4	0.13	0	3.1
17:1w8	0	0	0	0
cy17	2.3	15.8	9.6	6.2
17:1w6	0	0	0	0
17:0	1.5	0.1	0.27	1.5
18:1w7c	10	24.7	27	12.66
18:1w7t	0.4	1.5	6.6	0.7
18:0	1.6	1	1.6	5.6
cy19	0.06	0.1	0	0
Total ^b	255000	2037000	332000	85300

a. Data in mole% of total picomoles of phospholipid fatty acids.

b. Total PLFA are expressed in picomoles cm⁻².

The PLFA profiles from the AF and AFS communities on the electrode surface and broth are shown in Table 4. The fatty acids from the electrodes were similar for both the AF and AFS communities, slight increases of 16:0 and cyl7:0 (4 and 10%, respectively), a decrease of 6% for cyl9:0 is found in the AFS community and traces of branched i14:0 and a17:0 which are not found in the AF community. Little difference was seen between the broths from both AF and AFS flasks, which appear to have similar community structures. However, there are differences between the broth and biofilms within flasks. The AF broth has an increase of 19% of 16:1w7c and a 6% increase in 18:1w7c and less 16:0 and cyl7:0. The AFS broth also shows increases in 16:1w7c and 18:1w7c and decreases in 16:0 and cyl7:0. The AFS community had a larger total biomass in terms of Plate counts and total PLFA from the electrode surface than the AF community. The AF community had a larger biomass in the broth as shown by both total Plate counts and total PLFA.

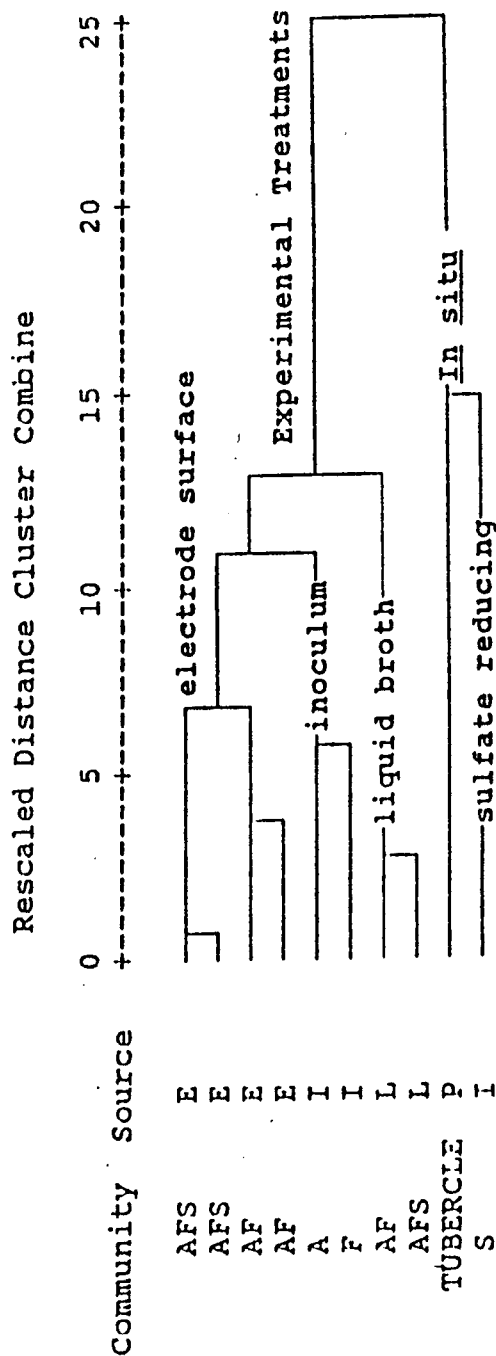
The cluster analysis of the mole% PLFA profiles from Tables 3 and 4 is given in Figure 3. The initial separation occurs between the tubercle and the resulting communities used in the corrosion studies. The sulfate reducing enrichment is the only treatment that clusters with tubercle due to the large amounts of 16:0 and i17:1 not found in the other enrichments, see Table 2. The liquid broth communities readily separate from the inoculum and the

Table 4. Phospholipid Fatty Acid profiles^a from Electrode Surfaces and Broth of Aerobic, Fermentative and Aerobic, Fermentative plus Sulfate Reducing Communities.

Fatty Acid	Community ^b					
	Electrode Surface				Broth	
	A, F	A, F, S			A, F	A, F, S
a13:0	0	0	0	0	0	0
i14:0	0	0	0.02	0.04	0	0
14:0	1.9	2.3	1.6	1.7	0.23	0.2
i15:0	0	0	0	0	0	0
a15:0	0.5	0	0.07	0.08	0.04	0
15:0	0.5	1	0.3	0.3	0.03	0.05
i16:1	0	0	0	0	0	0
i16:0	0.6	0.6	0.03	0.04	0.04	0.04
16:1w7c	10	9.3	9.8	9.8	29	25.9
16:1w7t	0.5	0.9	0.8	0.8	0.15	0.14
16:1w5c	0	0	0	0	0	0
16:0	23.84	26.98	28.30	28.60	21.3	19.4
i17:1	0	0	0	0	0	0
a17:1	0	0	0	0	0	0
i17:0	0	0	0	0	0	0
a17:0	0	0	0.04	0.06	0.05	0.03
17:1w8	0	0	0	0	0	0
cy17	16.5	16.9	26.4	26.80	13.4	14.6
17:1w6	0	0	0	0	0	0
17:0	0.6	1	0.2	0.1	0.03	0.05
18:1w7c	27	24	24	23.88	33	36.7
18:1w7t	1.3	2.4	1.2	1.2	0.07	0.07
18:0	1.8	2.8	0.7	0.7	0.7	0.7
cy19	14.26	10.8	5.8	5.7	2	1.9
Total (picomoles)	4361	1675	62841	96653	343377	226683

a. Data is in mole% of total picomoles per.cm² from electrodes and per 550 mls broth in corrosion flask.

b. A = aerobic, F = fermentative and S = sulfate reducing.



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Figure 3. Cluster analysis of bacterial communities based on phospholipid fatty acid profiles. Aerobic (A), fermentative (F) and sulfate reducing (S), enriched from a tubercle in a 4 " pipe (P). The enrichments were used as inoculum (I) which were then removed from the electrode surface (E) upon termination of the corrosion experiment.

electrode surfaces due in part to the large increase in 16:1w7c and 18:1w7c. The continuously stirring, flowing environments selected for aerobic bacteria during the course of the experiment. The A and F enrichment, which contain similar amounts of 16:1w7c, are more similar to the AF community from the electrode surface than the AF and AFS broth communities. The communities on the electrode surface allow formation of an anaerobic niche which could provide an environment for fermenting bacteria. The AF and AFS biofilm communities are more similar than the inoculum and broth. The AF and AFS biofilm communities are separated by slightly more branched PLFA such as i14:0, a17:1 and increases in 16:0 and cy17:0.

The low numbers of sulfate reducing bacteria found by MPN are evident by the closer relationship to the aerobic and fermentative treatments than the sulfate reducing inoculum.

Discussion

The results show that an increase in the metabolic diversity of a bacterial community using enrichments from a corroding pipe increases the corrosion of carbon steel. The addition of the sulfate-reducing enrichment to the aerobic plus fermentative (AFS) had a much greater effect on the corrosion than the aerobic plus fermentative (AF) community alone. The increase in corrosion of carbon steel by a fermenter in diculture with a sulfate-reducing bacterium then either organism in pure culture has been shown in anaerobic conditions (5). These authors used batch conditions and did not monitor the bacteria attached to the metal. Few other MIC studies showing the effects of increased community structure have been reported (14).

The decrease in OCP for both communities was consistent with the results in Part II, the rate of decrease and final steady state values were not reflective of the different communities tested. However, the decrease in OCP was necessary for the increased admittance but did not reflect the extent of the admittance. Thus the OCP has an indirect effect on the corrosion rate.

The results show that relatively low numbers of sulfate-reducing bacteria cause a significant increase in the corrosion of carbon steel. Total plate counts and total PLFA indicated greater biomass on the AFS electrode than the

AF electrode. Part II has clearly shown that total biomass does not correlate with corrosion based on Plate counts, MPN dilution tubes and total PLFA. The increased corrosion was most likely due to the sulfate-reducing bacteria and not increased biomass. No SRB were detected in the AFS broth after the 17 day period. The low numbers of SRB and significant increase in the corrosion rate confirms results observed in in situ studies. Hamilton et al. has shown that the total sulfate-reducing bacteria and observed activity did not correlate with corrosion rates observed in marine sediments (8). The reduced sulfide produced by sulfate-reducing bacteria appear to play a more important role in corrosion than the rate of sulfate reduction (8,11).

Though cathodic depolarization can be caused by many genera of bacteria (possessing hydrogenase), this phenomenon was most likely a minor factor in the present experiments due to the continual supply of lactate which impedes hydrogenase activity in Desulfovibrio sp. (3,17).

Cluster analysis of the mole% PLFA showed changes in the community structure as the conditions changed. Regardless of the inocula, the AF and AFS broths were more similar to each other than to the biofilm community in the same flask. The aerobic broth selected for similar communities over the course of the experiment, however, there were no differences between colony types from the broth or biofilms. The aerobic conditions in the broth were monitored with a

dissolved oxygen meter while an anaerobic niche on the metal surface was assumed due to the presence of the sulfate reducing bacteria after 17 day incubation, sulfate reducers have not been shown to be viable in aerobic conditions after such a long incubation period (17). Thus the differences between the aerobic environment and anaerobic environment were detectable with the PLFA profiles. The use of PLFA profiles to look at changes in bacterial community structure have been reported (6). These authors showed increases in cy17:0 and cy19:0 as estuarine sediments became anaerobic. This increase in cy17:0 and cy19:0 was also shown with both AF and AFS communities on the biofilm versus the higher monosaturate PLFA's in the broth. Even the relatively low amounts of sulfate-reducing bacteria on the AFS electrode are distinguishable from the AF electrode community.

The clustering of the sulfate-reducing enrichment with the pipe tubercle indicates a large number of sulfate-reducing bacteria in the tubercle. The corrosion may not be significantly greater, however, due to the indirect relationship with sulfate-reducing bacteria to the corrosion rate (7,8).

In conclusion, the sulfate reducing community had a greater effect on the corrosion of carbon steel than the aerobic plus fermenting community. This finding is significant because it demonstrates the importance of anaerobic niches and low sulfate environments to corrosion.

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Part IV

SUMMARY

Conclusions

The use of defined cultures of bacteria allow a comparison of corrosion rate to bacterial metabolic capabilities and indicates different corrosion mechanisms. By combining physiologically distinct groups of bacteria, a comparison of the corrosion brought about by bacterial combinations to that brought about by pure cultures can be made. These comparisons indicate the major contributors to corrosion and determine whether corrosion reactions are additive or whether certain reactions predominate. Few consortial corrosion studies have been reported comparing the effects of pure vs. complex communities (2). Though in situ studies have been undertaken and bacteria isolated, comparison of the corrosion to increased physiological diversity has not been assessed (4).

Three physiologically distinct groups of bacteria were used in Part II of this Thesis to compare the effects of a strict aerobe, a fermenter and a sulfate reducer. The bacteria used were Bacillus thuringiensis, Hafnia alvei and Desulfovibrio gigas. The corrosion reactions that these bacteria promote have been previously described in the Introduction and in the literature (10). All pure isolates and different combinations showed decreases in OCP over time. There was no difference between aerobically respiring communities to the rate of OCP decrease. Consumption of

oxygen resulted in greater decreases in OCP than attachment of non-active bacteria. The role of active and non-active bacteria on the OCP needs to be investigated further in order to evaluate oxygen concentration cells and OCP.

The Bacillus in monoculture did not increase the corrosion rate significantly in this system. An aerobic, but iron reducing bacterium has been shown to increase the corrosion of carbon steel (7). A non-active bacterium, D. gigas, increased the corrosion initially but the corrosion rate decreased to that of the control by the end of the experiment. Thus, oxygen concentration cells initially accelerate corrosion but cell lysis (or removal from a metal) may allow repassivation of the metal surface and not result in lasting effects.

Fermentation has been reported to be of significance in certain environments (non-flowing) where sulfate is absent (9). In this system, corrosion with H. alvei in monoculture and in diculture with Bacillus was not significant (Part II, this Thesis). However, the admittance between the control and the aerobic plus fermentative enrichment was significant (Part III, this Thesis). Thus, the fermenting community from the tubercle enrichments had a greater effect on the corrosion than the isolate used in this experiment. These may have produced more organic acids than H. alvei. A measure of organic acid production relative to the corrosion rate is needed to fully address the role of fermentation on

the corrosion of carbon steel.

The admittance was greatest for isolate combinations and enrichments with sulfate-reducing bacteria except with D. gigas in monoculture where an anaerobic niche was not present. The diculture H. alvei plus D. gigas had a greater corrosion rate than the Bacillus, H. alvei plus D. gigas triculture and Bacillus plus D. gigas diculture. The largest D. gigas population was in the triculture. This indication that the sulfate-reducing population size does not correlate with the corrosion rate agrees with in situ studies in marine sediments. These authors found that the corrosion of stainless steel was not a function of SRB population size or activity (4). This also explains the low numbers of SRB found and significantly larger corrosion rates using bacterial enrichments (Part III, this Thesis).

The differences in community structure were measured by both Plate counts, MPN and Phospholipid Fatty acid profiles (PLFA). A comparison between the community structures using the bacterial isolates versus the enrichments shows two similar trends: 1) the more complex communities had the largest population sizes at the end of the experiments (Bacillus, H. alvei and D. gigas was larger than any other combination and aerobic, fermentative plus sulfate-reducing was larger than the aerobic plus fermentative community) 2) there were differences between the communities on the electrode surface, liquid broth and inoculum. The PLFA

using the enrichments showed that the experimental treatments were very different from the original tubercle. Thus a comparison to in situ corrosion can not be made due to the differences in community structure. The use of PLFA profiles may be used to indicate, but not substantiate, environmental (aerobic versus anaerobic) conditions (3).

The use of bacterial isolates and enrichments indicate that sulfate reducing bacteria play a major role in the corrosion of carbon steel in freshwater environments. This role will be significant even for aerobic environments due to the formation of an anaerobic niche.

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APPENDIX

Appendix

Determination of Corrosion from Raw Data

Figure 1 below gives an example of raw data collected from the four-sided working electrode. The semicircles are labeled as shown for three different treatments. When a curve did not fully intersect the x-axis at the low frequencies the approximate intersect was extrapolated and from this the charge transfer resistance (R_{ct}) was determined. The units for R_{ct} is ohms cm^2 . The impedance behaves according to the Randles equation given below:

$$|Z| = R_n + \frac{R_{ct}}{1 + R_{ct}^2 C^2 w^2} - j \frac{R_{ct}^2 C w}{1 + R_{ct}^2 C^2 w^2}$$

R_n = the solution resistance in ohms.
 R_{ct} = the charge transfer resistance in ohms
 C = the Capacitance in Farads
 w = frequency in radians
 Z = total impedance
 j = -1

At low frequencies w and $C \ll 0$ and the second x-axis intercept reduces to:

$$|Z| = R_n + R_{ct}$$

This value is a function of the solution resistance, R_n , and R_{ct} .

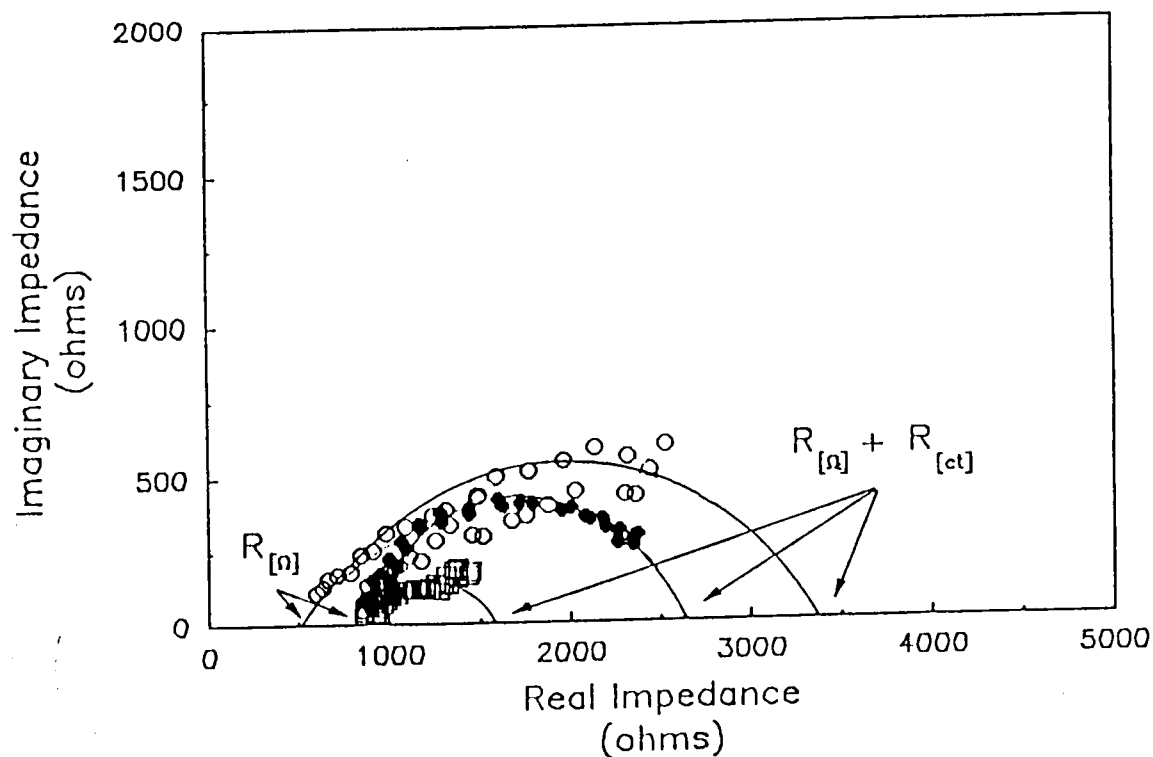


Figure 1. An example of raw impedance data of carbon steel. The figure shows the impedance values from two electrodes between 10 Hz and 5 mHz for three different treatments. Aerobic plus fermentative (●), aerobic, fermentative plus sulfate-reducing (□) versus sterile control (○).

The value for R_n is subtracted from the second x-axis intercept and R_{ct} is obtained.

In order to present the corrosion in a more conventional fashion, the inverse of R_{ct} is determined which gives the corrosion in admittance mhos cm^{-2} . Thus the admittance is directly proportional to the corrosion.

Corrosion Test Flask

The flask used for the corrosion experiments showing the four sided carbon steel, counter and reference electrodes is shown in Figure 2, below. Also shown is the drip tube for media inlet, media outlet, inoculation port, air filter and polypropylene lid. The figure is not drawn to scale.

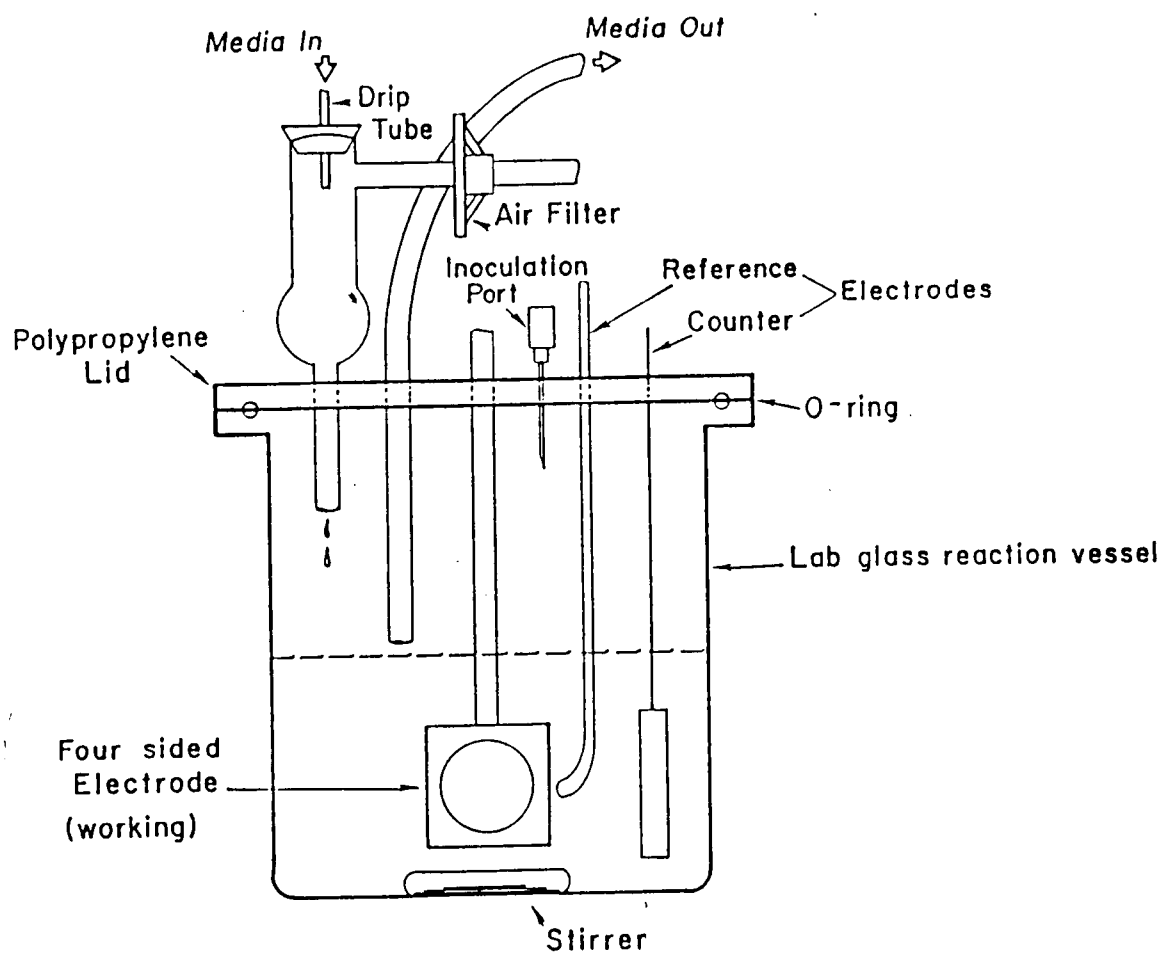


Figure 2. Diagram of corrosion test flask used in corrosion studies. Figure not drawn to scale.

VITA

Richard Fredrich Jack was born in Philadelphia, Pennsylvania on May 1, 1961. He attended St. Henry's elementary school till the fifth grade, then moved to Lynchburg, Virginia. There he completed his elementary and High School education at Holy Cross Catholic School in 1979. In the Fall of 1979 he attended Central Virginia Community College and transferred the following year to Virginia Polytechnic Institute and State University in Blacksburg, Virginia. During his undergraduate studies he was invited to travel to Antarctica and participate in a microbial ecology project for four months. In June 1984 he received his Bachelors degree in Biochemistry and minor in Chemistry. In September of 1984 he accepted a position as environmental microbiologist for Sybron Chemicals, Salem, Virginia. After approximately two years he was employed by Dr. David C. White at the Institute for Applied Microbiology/University of Tennessee. He accepted a graduate research assistanceship in the Fall of 1987 and received a Master's of Science Degree in Ecology in May 1990.

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