Abstract:
Odd indentations were found on the surface of 316L stainless steel that had been treated with biomineralized manganese and chloride solution. It was hypothesized that the sessile bacteria responsible for the mineral deposition (.Leptothrix discophor) altered the environment, which led to pitting corrosion on the surface of the metal.

Small squares were etched on the surface of polished stainless steel to enable the study of biocorrosion of a small area of the surface by various surface analysis techniques. The etched square’s region was mapped by atomic force microscopy and then mounted in a batch reactor. The reactor was inoculated with the bacteria so that manganese oxide would be deposited through microbial action. Once the manganese was deposited, the stainless steel was removed, biofilm detached and exposed to a sodium chloride solution to initiate pitting. Once pitting was observed, the stainless steel was analyzed by scanning electron microscopy and atomic force microscopy.

The pits observed when microbial colonization was present were different from pits generated by electrochemical means. Small microbially initiated pits were arranged evenly in a straight line. Indentation shapes that were the same as the microbially initiated pits were also found on the surface of the stainless steel when the coupons were not post-treated to a sodium chloride and/or manganese solution. All indentations and pits initiated by the bacteria were similar to the morphology of the bacteria in dimension and in aspect ratio (length/width). Thus it appears that the bacteria change the surface of the stainless steel to cause initiation of pitting corrosion.

It was further hypothesized that other manganese oxidizing bacteria could also initiate pitting. The same type of investigation was performed as described above with a strain of Pseudomonas putida that can oxidize manganese. It was shown that P. putida too could initiate pitting corrosion. Pits generated by the Pseudomonas putida and by Leptothrix discophora were shallow, unsheathed and had the same dimensions as the corresponding bacteria, however they differed from one another in that indentations by Leptothrix discophora are more organized.
CORROSION OF 316L STAINLESS STEEL INFLUENCED BY MANGANESE

OXIDIZING BACTERIA

by

Michael Joseph Geiser

A thesis submitted in partial fulfillment
of the requirements for the degree
of
Master of Science
in
Chemical Engineering

MONTANA STATE UNIVERSITY
Bozeman, Montana

May 2001
This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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<td>$\eta$</td>
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<tr>
<td>AFM</td>
<td>Atomic Force Microscopy</td>
</tr>
<tr>
<td>EDX</td>
<td>Energy Dispersive X-ray Analysis</td>
</tr>
<tr>
<td>MIC</td>
<td>Microbially Influenced (or Induced) Corrosion</td>
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<td>MOB</td>
<td>Manganese Oxidizing Bacteria</td>
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<tr>
<td>OCP</td>
<td>Open Circuit Potential</td>
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<td>SEM</td>
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ABSTRACT

Odd indentations were found on the surface of 316L stainless steel that had been treated with biomineralized manganese and chloride solution. It was hypothesized that the sessile bacteria responsible for the mineral deposition (*Leptothrix discophora*) altered the environment, which led to pitting corrosion on the surface of the metal.

Small squares were etched on the surface of polished stainless steel to enable the study of biocorrosion of a small area of the surface by various surface analysis techniques. The etched square’s region was mapped by atomic force microscopy and then mounted in a batch reactor. The reactor was inoculated with the bacteria so that manganese oxide would be deposited through microbial action. Once the manganese was deposited, the stainless steel was removed, biofilm detached and exposed to a sodium chloride solution to initiate pitting. Once pitting was observed, the stainless steel was analyzed by scanning electron microscopy and atomic force microscopy.

The pits observed when microbial colonization was present were different from pits generated by electrochemical means. Small microbially initiated pits were arranged evenly in a straight line. Indentation shapes that were the same as the microbially initiated pits were also found on the surface of the stainless steel when the coupons were not post-treated to a sodium chloride and/or manganese solution. All indentations and pits initiated by the bacteria were similar to the morphology of the bacteria in dimension and in aspect ratio (length/width). Thus it appears that the bacteria change the surface of the stainless steel to cause initiation of pitting corrosion.

It was further hypothesized that other manganese oxidizing bacteria could also initiate pitting. The same type of investigation was performed as described above with a strain of *Pseudomonas putida* that can oxidize manganese. It was shown that *P. putida* too could initiate pitting corrosion. Pits generated by the *Pseudomonas putida* and by *Leptothrix discophora* were shallow, unsheathed and had the same dimensions as the corresponding bacteria, however they differed from one another in that indentations by *Leptothrix discophora* are more organized.
CHAPTER 1

INTRODUCTION

Ennoblement

Microorganisms form biofilms on metal surfaces, modifying the near surface chemistries and affecting electrochemical properties of the metal. For example, stainless steels when exposed to natural waters increase their open circuit potential from about \(-100\text{mV}_{\text{SCE}}\) to approximately \(+350\text{mV}_{\text{SCE}}\) (Figure 1-1). This effect is accompanied by a 2 to 3 decade increase in cathodic current density upon mild polarization (about \(-0.2\text{V}\)).

Although many hypotheses have been formulated to explain the observed noble shift in potential (ennoblement), a general consensus was that it was caused by microbial colonization of the stainless steel surface. However, the hypotheses explaining the mechanism were not consistent. Some authors explained the noble shift in potential as a result of catalysis of oxygen reduction caused by metalo-organic complexes of iron, cobalt, and nickel in a biofilm [1, 2]. Others explained that ennoblement was caused by microbially produced hydrogen peroxide that acted as an auxiliary cathodic reactant [3, 4]. Still others explained the increase in potential as a result of algae attachment [5-7], which produces oxygen photosynthetically; thus increasing the concentration of cathodic reactant.
Linhardt [8, 9] was the first to show strong evidence for corrosion by manganese oxidizing bacteria. A severely corroded stainless steel turbine blade in a hydroelectric power plant had failed. The blade should not have failed because it was sufficiently resistant to corrosion in its environment. Upon examination, manganese dioxide and manganese oxyhydroxide were found attached to the blade. Investigation indicated that the manganese oxides deposited by bacteria contributed to the corrosion.

In our laboratory, Dickinson [10-13] showed that biomineralized manganese oxides cause ennoblement (Figure 1-2) by placing stainless steel, type 316L coupons in fresh river water for 9 days. A biofilm grew on the surface of the stainless steel and a positive shift in potential of $+350\text{mV}_{\text{SCE}}$ was observed. The coupons when analyzed by light microscopy revealed annular manganese oxide nodules deposited on the coupons.

**Figure 1-1.** Polarization curves of 316L stainless steel in river water. The continuous line is the polarization curve for untreated stainless steel. The broken line is the polarization curve for a coupon that was exposed to river water for 20 days.
Dickinson demonstrated that depositing a manganese dioxide paste on the surface of the stainless steel increased the open circuit potential of the metal to +350mV\textsubscript{SCE}. This agrees well with predictions based on the thermodynamics of the dissolution of manganese dioxide in natural waters (equation 1-1) and the exposure of stainless steel to river water.

\[ \gamma \text{MnO}_2(s) + \text{H}_2\text{O} + e^- = \text{MnOOH}(s) + \text{OH}^- \quad E'_{\text{pH=8}} = +0.360 \text{ V SCE} \] \text{(1-1)}

Only 6% of the surface needed to be covered by the paste to induce the ennoblement conditions. He also showed that a pure culture of \textit{Leptothrix discophora} could be used to ennoble stainless steel by biomineralizing manganese oxides onto its surface.

\textbf{Figure 1-2.} Ennoblement mechanism proposed by Dickinson [10-13]. Manganese dioxide is deposited by biomineralization, and then kept in equilibrium with manganese oxyhydroxide, producing an ennobled potential.

Also in our laboratory, Olesen [14, 15] furthered the understanding of the mechanism of ennoblement by biomineralized manganese oxides when he proposed a
two-step electrochemical reduction of manganese dioxide to divalent manganese ion (Figure 1-3). Manganese dioxide was plated on the surface of stainless steel, and then electrochemically reduced by maintaining a constant current between the coupon and the counter electrode. The generated potential versus time curve was repeatable so that the reduction process could be stopped at any time. Samples taken at different times in the reduction process were then analyzed by x-ray photoelectron spectroscopy (XPS). An analysis of the solution at these corresponding times indicated that manganese was progressively removed from the coupon. The XPS results showed that biomineralized manganese oxyhydroxide was formed and then reduced electrochemically to manganese divalent ion. The two reactions representing the manganese dioxide reduction can be expressed by equations 1-2 and 1-3 and their overall combined reaction expressed by equation 1-4.

\[ \text{MnO}_2(s) + H^+ + e^- = \text{MnOOH}(s) \quad E^0 = +0.81 \text{ V}_{\text{SCE}} \quad E'_{\text{pH}=7.2} = +0.383 \text{ V}_{\text{SCE}} \quad \{1-2\} \]

\[ \text{MnOOH}(s) + 3H^+ + e^- = \text{Mn}^{2+} + 2H_2O \quad E^0 = +1.26 \text{ V}_{\text{SCE}} \quad E'_{\text{pH}=7.2} = +0.336 \text{ V}_{\text{SCE}} \quad \{1-3\} \]

Overall:

\[ \text{MnO}_2(s) + 4 H^+ + 2e^- = \text{Mn}^{2+} + 2H_2O \quad E^0 = +1.28 \text{ V}_{\text{SCE}} \quad E'_{\text{pH}=7.2} = +0.360 \text{ V}_{\text{SCE}} \quad \{1-4\} \]
Recently in our laboratory, Shi [16] modified the model of ennoblement through biomineralization of manganese oxides by demonstrating that pure cultures of Leptothrix discophora, a manganese oxidizing bacteria, ennobled metals by oxidation of the divalent manganese ion to manganese oxyhydroxide; which was then further oxidized to manganese dioxide (Figure 1-4). Analysis of coupons that were partially ennobled (potential between +200mV\textsubscript{SCE} and +350mV\textsubscript{SCE}) by time of flight-secondary ion mass spectrometry (ToF-SIMS) indicated that manganese oxyhydroxide was predominant. When the coupons were fully ennobled (+350mV\textsubscript{SCE}), the oxides were mostly composed of manganese dioxide.
Figure 1-4. Possible ennoblement mechanism proposed by Shi [16]. Manganese divalent ions are first oxidized by manganese oxidizing bacteria to manganese oxyhydroxide. The oxyhydroxide is further oxidized to manganese dioxide. The manganese dioxide will then undergo reduction by electrochemical means.

Ennoblement by biomineralization of manganese onto the surface of metals has been observed in the laboratory and in the field [17] (Figure 1-5). Both cases have been observed to ennable stainless steels to approximately $+350\text{mV}_{\text{SCE}}$.

Figure 1-5. Ennoblement by biomineralization of manganese on the surface of 316L stainless steel in two different streams. Potentials reach values of $+320\text{mV}_{\text{SCE}}$ to $+350\text{mV}_{\text{SCE}}$. Graph was taken from Lafond [17].
Ennoblement can be detrimental to metals. By increasing the potential in a positive direction, the metal becomes more susceptible to corrosion.

**Hypotheses**

Galvanic corrosion requires current to pass between two electrodes. Ennoblement increases the potential energy of the metal, stored as charged ions. This increase in potential energy may exceed the binding energy of the crystalline structure of surface oxides, resulting in dissolution of the metal. This dissolution of the metal is referred to as corrosion. When corrosion occurs, it discharges the charged ions into solution in the form of current. Therefore ennoblement and corrosion cannot be present at the same time.

I desired to quantify the morphology of corrosion sites associated with ennoblement due to biomineralization of manganese oxides, and to compare these corrosion sites with the morphology of corrosion sites originating through anodic polarization of corrosion coupons. 316L stainless steel coupons were ennobled by biofilms of manganese oxidizing bacteria. Then, the biofilm was removed, and the coupons were placed in a sodium chloride solution to stimulate corrosion.

Odd shaped indentations (not round or geometrical in shape) in the passive layer of 316L stainless steel were observed by atomic force microscopy in coupons that had been ennobled by manganese oxide deposition and exposed to a chloride solution.

The following hypotheses were constructed to try to describe the origin and meaning of these indentations:
1. The indentations in the passive layer of the 316L stainless steel were formed only in the presence of the biofilm.

2. Heterogeneous manganese oxide deposition, in the biofilm and on the surface of the stainless steel, was associated with the formation of the odd shaped indentations.

3. The *Leptothrix discophora* alone were responsible for the odd shaped indentations in the passive layer of the 316L stainless steel.

4. *Pseudomonas putida*, MN-1 strain, another manganese oxidizing bacteria, can also make odd shaped indentations in the passive layer of the 316L stainless steel.

5. Observed indentations in the passive layer of the 316L stainless steel are sites for initiation of pitting corrosion.

**Outline of Thesis.**

Chapter 2 presents background information on:

- A description of the different types of corrosion, including mechanisms that pertain to iron
- Corrosion of different metals
- The effect of some microorganisms on corrosion processes
- Electrochemical methods used in this work to study corrosion
- A description of scanning electron microscopes and atomic force microscopes
Chapter 3 is a description of materials and methods used to perform experiments, and chapter 4 contains the results. Large sections found in these sections come from a paper that will be published in a special edition of the *International Biodegradation and Biodeterioration Journal*, M. Geiser, R. Avci, and Z. Lewandowski (authors). The article is attached as an appendix.

Chapter 5, the last chapter, contains the conclusions formed on the results contained in the thesis, followed by a small section pertaining to future work that could be performed to better understand remaining questions.
CHAPTER 2
BACKGROUND INFORMATION

Types of Iron Corrosion

There are many types of corrosion mechanisms; some mechanisms are purely chemical, while others require a mixture of specific conditions. In natural systems, it is possible to have multiple corrosion mechanisms.

*Galvanic corrosion* results from a current generated from a potential difference caused by an oxidation and reduction reaction occurring in a corrosive media. The current produced is due to chemical reactions occurring in the corrosive media. The oxidation of one of the metals leads to dissolution, with possible structural failure. Galvanic corrosion is important because it often times participates with other types of corrosion.

*Localized corrosion* (pitting and crevice) results from local attack, as opposed to uniform attack, on metal surfaces. *Pitting corrosion* is a local assault on the surface of metals to aggressive ions such as chloride and bromide. Pitting corrosion got its name from small holes or pits in the metal as a result of the corrosion. Another type of local corrosion due to aggressive ions is *crevice corrosion*. Crevice corrosion takes place at narrow separations of materials and not on smooth surfaces. Narrow gaps between the materials are places where accumulation of aggressive ions due to mass transport limitations arises.
*Microbially influenced corrosion* (MIC) is the term applied when microbial activity alters the local chemistry of a metal surface, which causes the corrosion. MIC is typically a mixture of many corrosion mechanisms, but always results in localized corrosion. Many cases that were not understood 40 years ago are now being tied to microbial corrosion mechanisms.

The three corrosion types that pertain to this study are discussed in greater depth. Galvanic corrosion is explained by mixed potentials. Material selection affects corrosion types; stainless steel corrodes differently than mild steel. A principle failure mechanism for stainless steel is localized corrosion, the second corrosion type discussed. Indentations in the passive layer of 316L stainless steel that were formed by colonization of the surface by bacteria resulted in pitting corrosion when treated with a chloride solution and ennobled by biomineralized manganese oxides. The third corrosion type, MIC, has been known to contribute to corrosion in various systems.

Researchers studying corrosion use electrochemical and surface analysis techniques to better understand different mechanisms of corrosion. Different electrochemical techniques are presented to explain how corrosion is better understood by their use. AFM and SEM are also explained because they were the principle surface analyses that were used in this study to examine the metal surfaces.
Galvanic Corrosion

Rust as an Example

An example of galvanic corrosion is mild steel corrosion, which can occur by exposure to water in the presence of oxygen (rust) [18]. The anodic reaction proceeds by the following reactions:

\[ Fe \rightarrow Fe^{2+} + 2e^- \quad \text{Eo} = +0.20\text{V}_{\text{SCE}} \quad \{2-1\} \]
\[ Fe^{2+} \rightarrow Fe^{3+} + e^- \quad \text{Eo} = -1.01\text{V}_{\text{SCE}} \quad \{2-2\} \]

The anodic reactions supply the electrons needed for the cathodic:

\[ O_{2(g)} + 4e^- + 4H^+ \rightarrow 2H_2O \quad \text{Eo} = +0.99\text{V}_{\text{SCE}} \quad \{2-3\} \]

The ferric ions that were products of the cathodic reaction combine with oxygen to form ferric oxide, which is then hydrated to form rust (equation 2-4).

\[ 4Fe^{2+} + O_{2(g)} + 2xH_2O \rightarrow 2Fe_2O_3 \cdot xH_2O \cdot rust'' + 8H^+ \quad \{2-4\} \]

Thermodynamics

Galvanic corrosion stems from redox reactions, where the anodic reaction is the dissolution of metal. An anode is the electrode where oxidation occurs (anodic reaction). Corrosion results from metal at its zero oxidation state being oxidized to its positively charged ion, commonly soluble in water. The electrode where reduction is taking place is called the cathode (cathodic reaction).
Redox reactions occur as a result of potential differences. The potential difference between the anode and cathode drives the reactions. This potential difference can be calculated by equation 2-6, which relates the potential to the Gibbs energy of the system.

\[ \Delta G = -nF \cdot E \]  

{2-6}

From the thermodynamic equation 2-12, the Nernst equation (equation 2-12) can be derived.

\[ \Delta G = \Delta G^\circ + R \cdot T \cdot \ln Q \]  

{2-7}

By definition,

\[ \Delta G^\circ = -R \cdot T \cdot \ln (K). \]  

{2-8}

Equation 2-8 then becomes,

\[ \Delta G = R \cdot T \cdot \ln \left( \frac{Q}{K} \right). \]  

{2-9}

Applying equation 2-6,

\[ E = \frac{R \cdot T}{n \cdot F} \cdot \ln \left( \frac{Q}{K} \right). \]  

{2-10}

By definition \( E^\circ \) is the potential at standard conditions. It is described by the following equation:

\[ E^\circ = \frac{R \cdot T}{n \cdot F} \cdot \ln (K). \]  

{2-11}

By using equation 2-11 in equation 2-12, the Nernst equation is formed:

\[ E = E^\circ - \left( \frac{R \cdot T}{n \cdot F} \right) \cdot \ln Q. \]  

{2-12}

The Nernst equation takes into account non-equilibrium conditions to calculate the potential.
Kinetics

Current can be measured or related to the corrosion rate. The Butler-Volmer equation (equation 2-13) [19] explains the behavior of the current density of an electrode in solution.

\[
i = i_o \cdot \left\{ e^{\frac{-\eta F}{RT}} - e^{\frac{-\beta F}{RT}} \right\}
\]  

(2-13)

The value \(i_o\) is derived from the intercept of the high field approximation portion of a Tafel plot with the current axis. A Tafel plot contains three portions that are explained by approximation methods. The low field approximation section pertains to overpotentials with a magnitude less than 0.1V. The limiting current density section of the Tafel plot is controlled by mass transport limitations. This is because the ions produced at the electrode surface can only travel as quick as mass transport allows.

The high field approximation (Tafel approximation) is valid for overpotentials with a magnitude greater than 0.1V. The Tafel approximation applies to both anodic and cathodic overpotentials. In the case where the overpotential is negative (cathodic) the left exponential term in equation 2-13 become negligible:

\[
i = i_o \cdot \left\{ -e^{\frac{-\beta F}{RT}} \right\}
\]  

(2-14)

which, can be rearranged to:

\[
\ln(i) = \ln(i_o) - \frac{\beta \eta F}{R T}.
\]  

(2-15)

Equation 2-15 can be solved for the overpotential, producing equation 2-16, which is linear on the Tafel plot (Figure 2-1).

\[
\eta = -\frac{R T}{\beta F} \cdot \ln(i) + \frac{R T}{\beta F} \cdot \ln(i_o)
\]  

(2-16)
For positive overpotentials (anodic) the right exponential term in equation 2-13 becomes negligible:

\[ i = i_o \cdot e^{\frac{(1-\beta)\eta F}{RT}} \]  \hspace{1cm} (2-17)

which, can be rearranged to:

\[ \ln(i) = \ln(i_o) + \frac{(1-\beta)\eta F}{RT} \]  \hspace{1cm} (2-18)

Equation 2-18 can be solved for the overpotential, producing equation 2-19, which is also linear on the Tafel plot (Figure 2-1).

\[ \eta = \frac{RT}{(1-\beta)F} \ln(i) - \frac{RT}{(1-\beta)F} \ln(i_o) \]  \hspace{1cm} (2-19)

The current can then be related to the number of electrons used by equation 2-22. This equation is derived by the following definitions:

\[ current = \frac{q}{t} \]  \hspace{1cm} (2-20)
\[ n = \frac{q}{\zeta} \]  \hspace{1cm} (2-21)

Combined they form:

\[ n = \frac{current \cdot t}{\zeta} \]  \hspace{1cm} (2-22)

The number of electrons is then stoichiometrically related to the number of atoms of metal undergoing dissolution by its half-cell oxidation reaction. The number of metal atoms can then be converted to mass or moles, and this value divided by time yields the rate of dissolution.
Figure 2-1. Example of Tafel plot when an overpotential is applied to a corrosion system. The variable $i_0$ can be derived from the graph to be used in the Butler-Volmer equation.

**Mixed Potentials**

Anodic and cathodic reactions in a system do not exhibit their individual potentials at a zero current, rather they express a potential that is measured between the two individual potentials. This effect is primarily due to the thermodynamics of the system. Both reactions prefer to have their energies the lowest, and this minimum energy is found in between the two individual potentials. The two individual reactions are not at equilibrium so a current results, driving the two individual potentials to some common potential. A polarization curve of the two reactions best illustrates this principle (Figure 2-2. Cathodic reactions are straight with a negative slope. Anodic reactions of pure
substances are also straight with a positive slope. These reactions are straight lines because the reactions at the surfaces of the electrodes do not change as a function of potential.

Corrosion potential ($E_{\text{corr}}$) (also known as the open circuit potential) and current ($i_{\text{corr}}$) are established at the intersection of the anodic reaction and cathodic reaction on a plot of the current versus potential applied. If one of the reactions changes, a shift in the corrosion current and potential will result. For example, as seen in Figure 2-2, a positive shift in the anodic reaction will increase the corrosion potential while decreasing the corrosion current.

![Figure 2-2. Plot of typical anodic and cathodic reactions. The corrosion potential ($E_{\text{corr}}$) is determined by the intersection of the two lines. A shift in one of the reactions results in a shift in corrosion potential and current.](image-url)
Material Selection Effect on Corrosion

Different metals have different standard potentials that affect their behavior in corrosive solutions. When both anodic and cathodic reactions are active, the corrosion potential measured will come from redox reactions that are the furthest separated in formal potential. If it is desired to inhibit corrosion, the untreated metal chosen should not be the lowest potential among all the electrodes in solution. Many times it is necessary to protect a metal that has the lowest potential among all the electrodes in solution. One technique of protecting the metal is to use a sacrificial anode, an inexpensive metal with a very low potential. One example is zinc, commonly added to mild steel, protecting the steel because zinc has a lower potential.

Stainless steel falls into the group of metals classified as passive. Passive metals are those that form an oxide layer on their surface that make them quite corrosion resistant to most environments [20, 21]. These layers are sometimes referred to as films because they are only a few nanometers thick. Other metals that are passive are aluminum and titanium.

Metals that are not passive are considered active. Active metals will corrode in a neutral environment without aggressive ions. Examples of active metals are cast iron, copper and mild steel. Metal underneath a passive film is also considered to be active. This is because once the passive layer fails, the metal underneath cannot respond quickly enough to reoxidize and be protected before active corrosion begins. The environment in which metal is placed determines the properties the metal will exhibit.
Stainless Steel Composition

Stainless steels contain a large quantity of chromium and perhaps nickel, which protect the bulk metal from attack by aggressive ions. Chromium or nickel form oxide layers that inhibit mass transport of aggressive ions into the active portion of the metal. Stainless steel, type 316L contains in its bulk approximately: 16-17% chromium, 10-11% nickel and trace elements of phosphorus, sulfur, silicon, manganese, molybdenum and copper, with the remainder of the balance iron [15, 22].

Passive film composition depends on the environment in which the metal is placed. Water is a large factor in passive layer composition and thickness [23-25]. If water is present, the passive layer may become hydrated and spongy, making the total thickness greater. It is believed that the water forms hydrogen bonds with the metal oxides, providing more resistance to chemical attack [25]. The environment in which the oxide is formed may lead to inclusions of impurities in the passive layer, making it weaker. Many times pickling is used to remove surface anomalies, making the passive layer stronger against attack [26]. Temperature and pH have also been shown to change the oxide composition [27, 28].

A typical passive layer for 316L stainless steel consists of many thin layers. The passive layer while in the presence of water is depicted in Figure 2-3. At the passive layer/ bulk metal interface, on the bulk metal side there is a layer that is depleted in chromium and enriched in iron [29]. On the passive layer side of the same interface, there is a layer enriched in chromium oxide [24, 27]. Most of the passive layer is a mixture of iron oxides. This layer’s thickness is a function of the pH of the water and the
The overall potential of the metal [24]. At the interface of the oxide film and water, on the oxide side, there exists an oxide layer that is saturated with water [25]. Although nickel is present in the bulk of stainless steel, it is only found in very small quantities within the passive layer.

![Figure 2-3. Schematic of general composition of stainless steel passive layer in the presence of water. Chromium enriched layer is believed to be responsible for protection of the bulk material.](image)

The formation of the passive layer results in different surface oxidation reactions when a potential is applied, as compared to a surface without a passive layer. This difference results in a curved anodic polarization curve, instead of straight lines, when the metal is in water (Figure 2-4). The curved plot is obtained by experimentation. In the case of stainless steel, a change from cathodic reaction to an anodic reaction is marked by a sharp decrease in current. The sharp decrease in current is the open circuit potential. A positive increase in overpotential results in a region of constant or sharp decrease in current density, which is due to passivation of the metal surface that impedes the dissolution of the bulk metal. A lack of free ions being released into the solution is the
principle reason for a decrease in the measured current density. However, a small current ($i_{\text{pass}}$) is required for constant protection of the surface. When the overpotential ($E_{\text{pit}}$) is large enough, the passive layer no longer protects the bulk and begins to corrode, and the current sharply increases.

![Diagram](image)

**Figure 2-4.** Potentials of the anodic reaction of passive metals in water are given as a function of current. Corrosion does not occur until $E_{\text{pit}}$ is reached due to passivation of the metal surface. Figure taken from Corrosion Doctors [30].

The anodic polarization curve for stainless steel can be plotted with the cathodic reaction of the reduction of oxygen to find the corrosion potential of the system (Figure 2-5). As in the case for mild steel, a shift in either reaction can lead to a shift in the corrosion potential.
Fresh waters lead to corrosion potentials much lower than $E_{\text{pit}}$ for most stainless steels because they contain little chloride in solution. However, in the presence of chloride ions, the anodic passive metal reaction will be shifted down, causing the corrosion potential to occur at higher values than in fresh water systems. When $E_{\text{corr}}$ is equal to or greater than $E_{\text{pit}}$, active corrosion will occur.

**Pitting Corrosion**

Pitting corrosion is a form of localized corrosion frequently seen in passive metals. This is because the passive layer protects the surface in a general fashion.
However, if the surface is compromised in a local area, corrosion will begin at that area, forming a pit.

There are three stages in the pitting process [21, 32, 33]. (1) *Initiation* is the most debated and least understood stage [34]. What is known is that certain conditions must be met to initiate a pit. There must be a harsh environment, typically including chloride ions [21, 34]. Many hypotheses have been presented on how chloride ions weaken the passive layer [34]; a local breakdown of the passive layer must occur if pitting is to begin [32, 36]. Also a certain potential must be exceeded if pitting is to initiate [33, 35, 36]. Temperature, fluid velocity, and pH are variables that also affect pitting initiation [32, 33, 36].

(2) *Metastable pitting* follows initiation [21]. Metastable pits exist for a short time. This stage is an intermediate phase between initiation and active pitting. Metastable pitting is the process by which a group of initiated pits open and close. They do so until circumstances are such that the pits repassivate and stop corroding, or they become active pits. Conditions necessary for active pitting are sufficiently large (critical) aggressive ion concentration in the pit, and a large enough pit radius and depth [21, 37]. Statistical models best describe this stage because the process is stochastic in nature [21, 38].

(3) *Active pitting* is the best-understood step of pitting corrosion. Active pitting is considered autocatalytic [21, 32], meaning that the products accelerate and maintain the pitting corrosion process. There are many processes occurring at the pit site, which further the pit growth (Figure 2-6).
Figure 2-6. Model of active pitting. Many different processes act simultaneously to contribute to the autocatalytic nature of active pitting corrosion.

One important process that propagates corrosion is the migration of chloride ions into the pit. Chloride ions will initially migrate into the pit due to concentration gradients and potential attraction forces on the negatively charged chloride ion. As time progresses, the attraction forces will continue to attract ions, while the concentration in the pit increases, decreasing the concentration gradient until it eventually ceases to be a transport driving force. Eventually the chloride concentration in the pit will be larger than the bulk chloride concentration, causing the two driving forces to cancel each other and equilibrium is reached. Meanwhile, as the chloride concentration increases in the pit, chloride begins to complex bulk material, oxidizing the metal. The electrons liberated from the metal are used at a cathodic reaction elsewhere. The free metal ions will form
metal chloride salts at the bottom of the pit [32, 39], thus maintaining a constant high concentration of chloride ions in the pit. The metal chloride will act as a buffer for the chloride concentration. If the concentration of chloride ions in the pit is too large, the metal ions will precipitate and form more metal chloride. However, if the concentration of chloride begins to drop, the chloride ions in the salt will dissolve and maintain the chloride concentration in the pit. Metal ions that do not react with the chloride will migrate out of the pit due to concentration gradients and the repulsive forces of the positive potential. Frequently the surface of the pit does not dissolve completely, forming a thin protective sheath [21]. This sheath also inhibits convection, thus slowing down the mass transfer of ions in and out of the pit.

Migration of metal ions out of the pit and entering chloride ions need positively charged ions to maintain electroneutrality of the solution within the pit. This is typically accomplished by migration of $\text{H}^+$ ions into the pit that preexist in solution. The accumulation of $\text{H}^+$ ions decreases the pH and accelerates the oxidation of the metal.

It has been shown that 316L stainless steel should not pit in natural waters, even at high chloride concentrations [34]. Figure 2-7 shows that $E_{\text{pit}}$ of 316 stainless steel in near-seawater chloride concentrations is approximately +200mV$_{\text{SCE}}$. 316L stainless steel has an open circuit potential of approximately -150mV$_{\text{SCE}}$ in seawater. It is therefore difficult for the stainless steel to corrode in these settings. Fresh water contains much less chloride, which increases $E_{\text{pit}}$. Also natural waters do not provide high enough chloride concentrations to maintain the reactions of pitting, once initiated. However, 316L stainless steel has been observed to pit in fresh waters in the presence of microorganisms.
Microbially Influenced Corrosion

Microbially influenced corrosion (MIC) is a process by which microorganisms affect corrosion by changing the chemistry near the surface of metals. It has been demonstrated previously that microorganisms can accelerate corrosion [22, 40-44]. In the case of *differential aeration cells*, the heterogeneous nature of the growth of the microorganisms inhibits oxygen mass transfer in localized areas, but not in others (Figure 2-8) [45]. The areas, where the oxygen can penetrate uninhibited by biofilm to the surface of the metal will form a cathodic site. On the surface of the metal oxygen can be reduced. The areas where the biofilm inhibit rapid mass transfer of oxygen to the surface of the metal will become anodic. At the surface oxygen cannot be reduced, instead the
metal will be oxidized to provide the electrons used at the cathode. The development of the cathode and anode is better understood by analyzing a modified version of the Nernst equation where ‘ox’ represents the molar concentration of the oxidized species and ‘red’ represents the molar concentration of the reduced species:

\[
E = E^\circ + \left(\frac{R T}{n F}\right) \ln\left(\frac{\text{red}}{\text{ox}}\right).
\]  \(\text{(2-23)}\)

The concentration of the oxygen (the reducible species) at the substratum of the biofilm will be lower due to resistance to mass transportation, causing the potential to be lower \((E_{anodic})\). Therefore, the region in which there is oxygen freely transported to the surface will have a potential higher than at the biofilm \((E_{cathodic})\). Because \(E_{cathodic}\) is higher than \(E_{anodic}\) the site is said to be “fixed”, and corrosion becomes more probable under the biofilm.

Figure 2-8. Differential aeration cells form as a result of differences in mass transfer of oxygen to the surface of the metal. Anodic areas are formed by a slower mass transfer of oxygen to the surface of metal due to the biofilm. \(E_{anodic}\) is less than \(E_{cathodic}\) because less oxygen is present.
The presence of sulfate reducing bacteria (SRB) can also lead to corrosion. SRB have been studied for nearly a century, and therefore the corrosion mechanism is fairly well understood [42, 46-49]. SRB are found in salt water and fresh water [50]. The SRB are anaerobic, meaning oxygen inhibits their growth. For this reason they are found at the bottom of biofilms. Oxygen is consumed in respiration of aerobic microorganisms at the top of the biofilm and diffusion limitations through the biofilm restrict oxygen penetration to the bottom of the biofilm. They reduce sulfur-containing compounds in the environment to form hydrogen sulfide [42]. The hydrogen sulfide will adsorb onto the surface of the metal (equation 2-24) [51] and there the adsorbed species will be oxidized (equation 2-25) and then be incorporated into the corrosion products (equation 2-26)[52]. The most common SRB is *Desulfovibrio desulfuricans* [43, 49].

\[
\begin{align*}
Fe + H_2S & \rightarrow FeSH^-_{ads} + H^+ \quad \{2-24\} \\
FeSH^-_{ads} & \rightarrow FeSH^+_{ads} + 2e^- \quad \{2-25\} \\
FeSH^+_{ads} & \rightarrow FeS_{1-x} \text{"mackinawite"} + xHS^- + (1-x)H^+ \quad \{2-26\}
\end{align*}
\]

The mechanism for corrosion of metals by manganese oxidizing bacteria (MOB) is still not well understood. MOB have been observed to corrode metals in fresh waters as well as in salt waters. Knowledge of their influence on corrosion came in the last decade [53]. Microorganisms that oxidize manganese form half of a biological manganese cycle that recycles manganese in nature [54-56]. Their part in this cycle is to oxidize free manganese ions to form metal oxides. It is still not understood why these
organisms oxidize manganese [54, 57]. Our lab showed, as shown in chapter 1, that manganese oxidation occurs as shown in equations 1-2 through 1-4. Some MOB are *Leptothrix* [57, 58], *Pseudomonas putida (MN-1 strain)*, *Siderocapsa, Gallionella* and *Sphaerotilus* [50].

\[
\begin{align*}
\text{MnO}_2(s) + H^+ + e^- &= \text{MnOOH}(s) \quad E^\circ = +0.81 \text{ V}_{SCE} \quad E'_{pH=7.2} = +0.383 \text{ V}_{SCE} \quad \{1-2\} \\
\text{MnOOH}(s) + 3H^+ + e^- &= \text{Mn}^{2+} + 2H_2O \quad E^\circ = +1.26 \text{ V}_{SCE} \quad E'_{pH=7.2} = +0.336 \text{ V}_{SCE} \quad \{1-3\} \\
\text{Overall:} & \quad \text{MnO}_2(s) + 4H^+ + 2e^- = \text{Mn}^{2+} + 2H_2O \quad E^\circ = +1.28 \text{ V}_{SCE} \quad E'_{pH=7.2} = +0.360 \text{ V}_{SCE} \quad \{1-4\}
\end{align*}
\]

The hypothesis for corrosion by manganese oxidizing bacteria is closely related to the hypothesis proposed by Shi [16] for ennoblement. MOB deposit the oxidized manganese on the surface of the metal so that it is in electrical contact with the metal. The manganese oxides are in equilibrium with the divalent manganese ions in solution. This equilibrium has a potential, which is a strong function of pH and divalent manganese ions in solution. The manganese oxide dissociation reaction ennobles metals to a potential of approximately +350mV in fresh water environments. Because the oxide is in electrical contact with the surface of the metal, the increased potential due to the dissociation of the oxide increases the metal potential. The increased potential also increases the attractive force on the chloride ions, increasing the chance for corrosion to occur [22, 41, 42, 59, 60].
Electrochemical Methods Used in this Thesis

A variety of electrochemical methods can be used to understand the corrosion characteristics of different metals in different corrosive media. The methods that were used in this study were anodic polarization and open circuit potential monitoring.

Anodic polarization is one of the most common electrochemical methods used to understand corrosion [26, 60-64]. The utilization of this method requires a three-electrode system: one working electrode (the metal of interest), one reference electrode, and one counter electrode (typically platinum or graphite). The three electrodes are then submerged in the solution of interest and the electrodes are connected to a computer via a potentiostat. The computer records the potential, which is controlled by the potentiostat, and the measured current. The potentiostat increases the potential at a constant rate, starting at a potential that is cathodic to the metal. It is increased in the positive direction until reduction of dissolved oxygen begins (greater than 1000mV$_{SCE}$ in neutral pH waters). The potential is then reversed and returned to the starting point at the same rate. For cyclic voltametry this potential loop is repeated many times; for anodic polarization the test is terminated at the end of one cycle.

Important information about the corrosion properties of metals can be gained using the data acquired from anodic polarization [63, 64]. For example, experiments with stainless steel produce information about the potential at which the passive layer forms and when it fails. Figure 2-9 illustrates anodic polarization of a passive metal with lettered points. A is the starting point. As the potential is increased the current decreases because it is nearing the open circuit potential (OCP) of the metal. The OCP is the
potential of the metal in electrical equilibrium with its applied potential. The OCP can be found on the chart at point B. As the overpotential is further increased, the metal begins to corrode like an active metal, until the potential reaches a critical value where the current becomes constant. This change in current is due to the passive film formation, which inhibits corrosion by forming an oxide layer. The oxide layer inhibits diffusion of ions in the passive metal to migrate out of the passive layer. As the potential is further increased from the open circuit potential, no change in the current occurs until the potential is so large that the repulsive forces on the metal ions in the passive layer break through the passive layer. Active pitting begins as these particles burst through the passive layer, compromising it in local areas. Because the film has been compromised, the moving particles are responsible for a sudden increase in current. Point C is called the pitting potential, occurring at a sudden increase in current. The pitting potential is thought of as the minimum potential at which active pitting will begin. D is the end of the forward scan. As the overpotential is decreased, the plot of the polarization curve will not follow the same path. If the passive layer was not compromised, the reverse scan will pass on the left side of the forward scan, indicating increased resistance to corrosion by increased thickness in the passive film. If the passive layer is compromised, the reverse scan will fall on the right side of the forward scan, as seen in Figure 2-9. As the potential continues decreasing, the metal will again repassivate (reform the oxide), lowering the current. Point E is the repassivation potential and is thought to be indicative of the potential, at which initiation of pitting may begin. Point F is the end of the cycle.
Although pitting potentials can be compared for all types of metals, the methods used to generate them are not standard. Polarization curves generated from anodic polarization can change if the scan rate, temperature, or mixing conditions are changed [63, 64]. Fast scan rates give smooth ideal plots, but tend to give pitting potentials higher than expected. Slow scan rates give jagged, sometimes hard to interpret, plots. Ideally, as the scan rate approaches zero, the measured pitting potential will be the pitting potential found in natural corrosion circumstances. Higher temperatures result in a lower pitting potential. Rapid mixing conditions can cause difficulty in interpreting the graph. Variances in the surface conditions of the metal also result in different pitting potentials.
Another electrochemical method used to monitor corrosion is simply to record the open circuit potential as a function of time (Figure 2-10) [26, 35]. In the case of ennoblement in stainless steels, an increase of potential due to a decrease in the anodic reaction is monitored. The passive layer will not pit until the OCP reaches the pitting potential. When this occurs, active corrosion will begin. A sudden drop in potential would be expected as a result of the discharging of the potential stored in the metal.

![Figure 2-10](image)

**Figure 2-10.** Ennoblement was monitored upon addition of hypochlorite to 304 stainless steel. $E_p$ marks the point at which active pitting began, $E_B$ marks the point at which metastable pitting began. Figure taken from Daufin [35].

**Surface Analysis Methods Used in this Thesis**

AFM and SEM surface analysis techniques are used quite frequently throughout the project. The acquired information enables the determination of the morphologies of the indentations in the passive layer of 316L stainless steel generated by manganese oxidizing bacteria and the morphology of the bacteria used in the experiments. SEM was used to produce an overhead view of the pits so they could be analyzed with clarity.
SEM displays a difference in signal quantity and displays the result in a gray-scale image. Both height and chemical composition cause a change in signal quantity, making it difficult to determine if dark or light colored objects on the surface of a metal are created by chemical difference or height difference. Therefore AFM was then used to verify whether objects (typically black) identified by SEM were pits or manganese oxide deposits.

AFM provides high resolution, three-dimensional surface maps. It operates by moving a cantilever with a small tip over the surface of a sample (Figure 2-11). A computer records the position of the tip in the x-y plane. A laser is aligned to strike the back of the tip and is reflected into a sensor. As the tip moves up and down, due to the surface topography, the laser is reflected back at a different angle and the detector measures the change. This signal is sent back to the computer and converted into a z measurement. The computer records the matrix of xyz coordinates, which are used in a graphics program to generate the three-dimensional surface map.

Figure 2-11. Schematic of the operation of an AFM. Three-dimensional topography can be mapped by correlating tip position and deflection of a laser from the back of the tip.
SEM was primarily used for imaging. It operates on many of the same principles as a light microscope, only using electrons to illuminate a surface instead of visible light. Electrons are used because they have smaller wavelengths, which help to illuminate smaller objects than are possible by visible light. SEM operates by exciting a crystal to eradiate electrons (Figure 2-12). Magnets control the electron beam, focusing and positioning it to examine the entire surface [65]. The samples are grounded. Electrons that do not leave the surface build up on the sample, referred to as "charging", and form a capacitor by storing charge. Backscattered, secondary electrons or x-rays are then detected and relayed to a monitor to be viewed. The viewed image can then be sent to a camera to capture the image to be examined later.

Figure 2-12. Principles of SEM operation are shown. Samples are impacted with electrons. Reflected electrons are detected and relayed to a display.
MATERIALS AND METHODS

A series of experiments were used to test the hypotheses presented in chapter 1.

Critical Sodium Chloride Concentration

Pitting potential is a function of chloride concentration in solution. As the chloride concentration increases, pitting potential asymptotically approaches a minimum value. The point where a further increase of chloride concentration no longer results in a significant change in pitting potential is referred to as the critical sodium chloride concentration. This value was found for MSPV media with 1 ml of vitamins (Table 3-1) and trace compounds in solution, used to culture *Leptothrix discophora* in the presence of 316L stainless steel. Chloride concentration used in the following experiments must be greater than the critical sodium chloride concentration if the minimum pitting potential is to be achieved.

Coupons

316L type stainless steel was used in all experiments presented in this thesis. The metal was chosen because previous laboratory members performed their ennoblement experiments on the same metal. Stainless steel coupons 1.6 cm in diameter were cut from a 1 mm thick sheet of type 316L stainless steel purchased from Reyerson in Spokane, Washington. Coupons were mounted in polycarbonate holders with silicon-gel [12]. The
holders consisted of a hollow polycarbonate tube 10 cm long with an inner diameter of 9 mm and an outer diameter of 19 mm (Figure 3-1).

<table>
<thead>
<tr>
<th>Composition of MSPV medium</th>
<th>Composition of Vitamin Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NH₄)₂SO₄</td>
<td>Biotin</td>
</tr>
<tr>
<td>0.24g</td>
<td>20.0mg</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>Folic Acid</td>
</tr>
<tr>
<td>0.06g</td>
<td>20.0mg</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>Thiamine Hcl</td>
</tr>
<tr>
<td>0.06g</td>
<td>50.0mg</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>D-(+)-Calcium Pantothenate</td>
</tr>
<tr>
<td>0.02g</td>
<td>50.0mg</td>
</tr>
<tr>
<td>Na₂HPO₄·7H₂O</td>
<td>Vitamin B12</td>
</tr>
<tr>
<td>0.05g</td>
<td>1.0mg</td>
</tr>
<tr>
<td>HEPES</td>
<td>Riboflavin</td>
</tr>
<tr>
<td>1.15g</td>
<td>50.0mg</td>
</tr>
<tr>
<td>FeSO₄ 10mM</td>
<td>Nicotinic Acid</td>
</tr>
<tr>
<td>1.0ml</td>
<td>50.0mg</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>Pyridoxine HCl</td>
</tr>
<tr>
<td>1000ml</td>
<td>100.0mg</td>
</tr>
<tr>
<td></td>
<td>P-Aminobenzoic Acid</td>
</tr>
<tr>
<td></td>
<td>50.0mg</td>
</tr>
<tr>
<td></td>
<td>Distilled Water</td>
</tr>
<tr>
<td></td>
<td>1000ml</td>
</tr>
</tbody>
</table>

1) 316L stainless steel
2) Conducting spring
3) Polycarbonate coupon holder
4) Rubber stopper
5) Electrical connection

Figure 3-1. Coupon holder.
**Coupon Polishing**

The coupons mounted in the holder were polished to provide a surface sufficiently void of flaws for surface analysis. They were wet-sanded with tap water on Buehler-Met II metallographic grinding discs composed of silicon carbide grit of decreasing grit sizes: 120, 240, 360, 400, and 600. After the use of each grit size, the holder and coupon were rinsed with running tap water to remove any remaining grit. The coupons were then polished using Buehler aluminum oxide powder and Buehler Micropolish II powder, each suspended in water and applied with Buehler Microcloths. Polishing was initiated with suspended 5 μm aluminum oxide powder. The coupons were then rinsed with tap water. Similarly, 0.5 μm and 0.05 μm polishing powders were used to make a mirror surface on the stainless steel, with rinses applied when polishing was complete with each powder size:

**Electrochemical Cell**

The coupons were removed from the holders and mounted in a special holder (Figure 3-2). This holder provided a means of pitting the stainless steel by electrochemical means, without promoting crevice corrosion. The glass covered metal rod is in electrical contact with the steel interior, which is in contact with the metal coupon. The coupon assembly was then placed in a special glass cell, as seen in Figure 3-3. A graphite rod was used as a counter electrode. An SCE was used as a reference electrode that was placed in a salt bridge made of 0.1M Na₂SO₄ and 1.5% wt agar. At the end of the salt bridge was a glass frit.
Figure 3-2. Special sample holder used to test pitting potentials. Opening in body does not allow for crevice corrosion. Holder is designed to prevent other metals from being exposed to the solution of interest. Holder is not drawn to scale.

Figure 3-3. Setup of three-electrode electrochemical cell used to test pitting potentials in media. Salt bridge was very close to working electrode to get accurate potential measurements.
MSPV Growth Media

To prepare the growth medium, one liter of ATCC Culture Medium 1917 MSPV (Table 4-1) was autoclaved on liquid setting at 123°C and 1.2 atm for 25 minutes. After the medium was cooled to room temperature, 1 ml of syringe-filtered vitamin solution (Table 3-1) required by the MSPV medium, 4 ml of syringe-filtered 50mMol manganese sulfate solution, and 5 ml of syringe-filtered 20% sodium pyruvate solution were added. All chemicals were from Fisher Scientific.

A varying range of different sodium chloride concentrations was prepared. After the sodium chloride was allowed to dissolve, the solution was added to the glass cell with three electrodes, which were connected to an EG&G Princeton Applied Research Potentiostat/Galvanostat (model 273A) which in turn was connected to a computer. The potential was then increased from $-0.5 \text{ V}_{\text{SCE}}$ to $+0.8 \text{ V}_{\text{SCE}}$ and then returned again to $-0.5 \text{ V}_{\text{SCE}}$ at a rate of 10 V/hr. Fresh solution mixture and new polished coupons were used for each chloride mixture.

Calculation of Pitting Potentials

Polarization curves were used to calculate pitting potentials for the range of chloride concentrations. The equation of the line that passed through the passive region of the plot was obtained by linear regression (Figure 3-4). An equation for the segment of points that indicates pitting corrosion was obtained by linear regression also. The intersection of these two lines was taken as the pitting potential. This method was used
to avoid ambiguity, sometimes caused by metastable pitting. A plot of the pitting potentials versus salt concentration was created.

![Graph](image)

**Figure 3-4.** Example of pitting potential calculation using sample pps4 from critical chloride concentration experiment. Lines of regression were calculated and shown on the graph. The intersection of these lines was taken as the pitting potential, because metastable pitting may make it difficult to locate the exact pitting potential.

General Morphology of Indentations Originating from *Leptothrix discophora* with Biomineralized Manganese Oxides and Chloride Solution Treatment

It was desired to show that indentations were not an artifact, but were indeed an effect of microbial action. Two identical experiments were then performed to show that ennoblement by *Leptothrix discophora* could be reproduced, that indentations could be found, and to determine the general shapes to be expected.
Batch Reactor

The reactor was a polycarbonate batch cylinder reactor (Figure 3-5) 10.2 cm tall and 11.1 cm in diameter. The assembled reactor was the same as the one used by Olesen [66]. Tubes used for introducing air into the medium were mounted in the reactor, and Pall-Gelman bacterial air vents were attached to these tubes to prevent contamination of the reactor. Stirring was provided by a magnetic stir bar placed at the bottom of the reactor. The reactor was then sealed with the same silicon gel and autoclaved on dry setting (depressurization method) at 123°C and 1.2 atm for 30 minutes.

Figure 3-5. Reactor.

Sterile MSPV growth media, prepared as previously described, was then added to the sterile reactor. A stiff spring with a long conducting rod and stopper was connected to the coupon and coupon holder, thereby finishing the assembly (Figure 3-1).
The coupon assembly and reference electrode in a 0.1M Na$_2$SO$_4$ salt bridge were interfaced with a computer via a Hewlett Packard 34970A Data Acquisition/ Switch Unit (a multiplexer) to monitor the potential of the coupons.

Manganese-oxidizing bacteria, *Leptothrix discophora* SP-6 (ATCC 51168), were obtained from ATCC and stored at -70°C. The reactor was inoculated with the frozen stock of the bacteria. The potential was monitored until potentials indicated that ennoblement had taken place. Once ennobled, the coupons were immersed in a solution of 0.1 M NaCl over the period of three days. A Jeol JSM-6100 scanning electron microscope with the beam voltage set to 15 kV was used to take micrographs and energy dispersive x-ray analysis (EDX). The EDX analyzed chemistry of indentations and manganese oxide deposits. The features of interest were photographed using a Polaroid camera, type 665, at a working distance of approximately 11 mm.

**Manganese Plating**

It was believed that perhaps the oxides were associated with the formation of the odd shaped indentations in the surface of the metal. When manganese oxides were deposited by the bacteria *Leptothrix discophora* on the surfaces, the deposits were heterogeneous in structure (Figure 3-6) due to the heterogeneity of the location of the manganese oxidizing bacteria. These oxides could fix local cathodic sites, which would accelerate dissolution in local anodic sites. An experiment was designed to test this hypothesis by means of ion milling and electroplating. Manganese oxides can be
electrochemically deposited on metal by applying a potential, resulting in the following chemical reaction:

\[
\text{Mn}^{2+} + 2H_2O \rightarrow \text{MnO}_2 + 4H^+ + 2e^-.
\]  

\{3-1\}

**Figure 3-6.** SEM image of biomineralized manganese oxides on the surface of 316L stainless steel. Black represents manganese oxides. Deposits are distributed heterogeneously.

One 316L stainless steel coupon was polished to the same standards as previously described. The surface of this coupon was then covered with black Sharpie brand permanent marker, which is not soluble in water, but is soluble into organic solvents. Ion milling by TOF-SIMS at a setting of \(~1.5\ \mu\text{A}\) ion current at 22 KeV impact energy for two minutes on the coupon with different patterns such as circles, bars, boxes, and the letters “MIKE”.

The coupon was then placed in the electrochemical cell in the special holder. A solution of 0.1 M Na$_2$SO$_4$, with 10 mM MnSO$_4$ in nanopure water was created and then pH balanced with borax and boric acid to 7.2. Fifteen minutes prior to beginning the experiment, argon was dispersed in the solution in the reactor. Argon dispersion was maintained throughout the duration of the experiment to keep the solution free of oxygen.
A magnetic stirrer was placed on the bottom of the reactor to keep the solution well mixed. Graphite was the counter electrode. An EG&G potentiostat was used to maintain the current at 2 \( \mu \text{A/m}^2 \) for three hours. After plating the manganese on the surface of the metal, which was exposed to the solution, acetone was used to remove the ink on the surface. This left only the manganese deposited in the desired shapes etched on the surface.

The deposits were analyzed with a Digital Instruments Dimension 3100 scanning probe microscope in contact mode to verify that the manganese had been deposited on the surface. Once the deposition was verified, the coupon was placed in a 0.2 M sodium chloride solution, until the potential of the coupon decreased, indicating corrosion or discharge of manganese oxide. The surface was again studied with AFM.

Specific Morphology of Indentations Originating from *Leptothrix discophora* with Biomineralized Manganese Oxides and Chloride Solution Treatment

Stainless Steel Etching by Ion Milling. Stainless steel coupons, 316L type, were polished as previously described in their holders. The coupons were then removed from the holders and two squares (200x200 \( \mu \text{m} \)), with a small number in the corner of each for identification, were etched on their smooth surfaces (Figure 3-7). The etchings were made by ion milling, with a focused Ga+ ion beam emitted from a time-of-flight secondary ion mass spectrometer (ToF-SIMS) for seven minutes at \(~1.5\ \mu\text{A}\) ion current at 22 KeV impact energy [67]. The etching produced a trench in the stainless steel
approximately 100 nm deep (Figure 4-2). AFM was then used to map the surface of the stainless steel.

Nine polished stainless steel coupons with the etched squares were mounted to their holders again, and the holders were attached to the top of the batch reactor used in the previous experiments. The only change modification to the reactor was the addition of the reference electrode directly into the medium instead of using a salt bridge. This alteration gives a much more stable potential reading. The reactor was then sealed with silicon gel and autoclaved on dry setting (depressurization method) at 123°C and 1.2 atm for 30 minutes.

To inoculate the reactor, 150 ml of sterile MSPV growth medium was poured into a sterile 250 ml Erlenmeyer flask. Frozen *Leptothrix discophora* SP-6 (ATCC 51168)
stock was inoculated into the flask and then placed on a shaker for two days. Then the broth was aseptically added to the reactor along with 600 ml of the sterile medium.

Before mounting it in the reactor, the SCE reference electrode was sterilized by soaking it in 95% ethanol for 1 hour. A stiff spring with a long conducting rod and stopper was connected to the coupon and coupon holder, thereby finishing the assembly (Figure 4-1). The coupon assembly and reference electrode were interfaced with a computer via a Hewlett Packard 34970A Data Acquisition/ Switch Unit (a multiplexer) to monitor the potential of the coupons.

The reactor was operated with the stirrer bar rotating and air bubbling through the medium until the potentials of the coupons exceeded +200mV (see Table 4-3 and Figure 4-4) which, according to the definition, indicated that the coupons were ennobled: This process usually took usually 5 days. Two of the nine coupons were removed from the reactor, sprayed with deionized water to remove the attached biofilm, and air-dried. These two coupons were then used as a control to describe the surface of the coupons that were exposed to the microorganisms but not exposed to the chloride solution. The remaining coupons were also removed from the reactor, sprayed with deionized water to remove the biofilm, and then immersed in a 0.2 M NaCl solution. Spraying removed the biofilm, but it did not remove the manganese oxides on the surface. OCP of the coupons immersed in the NaCl solution was monitored, and the coupons were removed one at a time at pre-assigned intervals over the course of two days.

Before the analysis, surfaces of all coupons were gently wiped clean with acetone and a lab tissue paper to remove the attached manganese deposits and remaining biofilm.
Absence of the manganese oxides and the biofilm was verified with a light microscope, AFM, and SEM. Any remaining attached silicon gel was gently removed or coated with colloidal graphite from Ted Pella, Inc. to minimize any charging that would occur in the SEM.

Scanning electron microscopy (SEM) and atomic force microscopy (AFM) were used to map the surface topography of the following: 1, freshly polished sterile coupons; 2, ennobled coupons after removing microbial deposits; 3, coupons after exposure to the sodium chloride solution. A Jeol JSM-6100 scanning electron microscope with the beam voltage set to 15 kV was used. The features of interest were photographed using a Polaroid camera, type 665, at a working distance of approximately 11 mm. Using a Digital Instruments Dimension 3100 scanning probe microscope in contact mode, the surface features identified by the SEM were then mapped using an AFM.

**Imaging Surfaces of Electrochemically Polarized Corrosion Coupons**

An essential part of the project was to quantify the morphology of the pits initiated on the coupons that had been ennobled and exposed to the sodium chloride solution. The working hypothesis was that the microorganisms were involved in the pit initiation. To verify this hypothesis, I compared the morphology of pits generated in the presence of manganese-oxidizing bacteria with the morphology of pits generated by anodic polarization in sterile media. The 316L stainless steel coupons were polished as previously described and placed in an electrochemical cell with the MSPV medium, vitamins, sodium pyruvate, and manganese sulfate. Sodium chloride was then added to
make a 0.1M solution. An EG&G Princeton Applied Research Potentiostat/Galvanostat, Model 273A, was used to anodically polarize the coupons. The potential was increased at a rate of 10 V/hr from $-0.5 \text{ V}_{\text{SCE}}$ to $+0.8 \text{ V}_{\text{SCE}}$ using a graphite counter electrode. Care was used to ensure that crevice corrosion did not occur near the edge of the coupon holder [68]. After the applied potential exceeded the pitting potential, the coupon was removed, rinsed with deionized water, dried, and analyzed with SEM and AFM. The corrosion pits were located on the surface, and their morphology (size and aspect ratio) was quantified.

**Imaging Surfaces of Sterile Corrosion Coupons Exposed to Sodium Chloride**

To examine the hypotheses, it was necessary to show that the observed indentations in the passive layer did not form spontaneously in the sodium chloride solution. To show this, a 316L stainless steel coupon was polished, as previously described, and then cleaned with acetone and a laboratory wipe. Two squares with the same dimensions as those used in previous experiments were etched on the surface of the coupon by the same ion milling procedure. The surface of the coupon was thoroughly examined with AFM, and then the coupon was exposed to 0.2 M NaCl solution (prepared with deionized water at room temperature) and aged for 2½ days. The coupon was then removed, rinsed with deionized water, dried, and again analyzed with AFM.
Imaging *Leptothrix discophora* Attached to the Surfaces of Corrosion Coupons

To compare the morphology of the bacteria with the morphology of the corrosion pits, I took images of the *Leptothrix discophora* attached to surfaces of corrosion coupons. Six 316L stainless steel coupons were polished to the described specification. Two 250 ml Erlenmeyer flasks each had three polished coupons placed in them. The flasks were then sealed, autoclaved for 25 minutes on the dry setting at 123°C and 1.2 atm, and cooled to room temperature. Two batches of MSPV medium were prepared in the same fashion as the previous experiments and autoclaved for 25 minutes on the dry setting at 123°C and 1.2 atm. Both batches had syringe-filtered solutions of sodium pyruvate and vitamins added, but only one had manganese sulfate added to it to make the same concentration as in the previous experiments. One sterile flask had 125 ml of the MSPV medium with manganese sulfate solution added aseptically. In the other flask, 125 ml of the MSPV media without manganese sulfate was aseptically added. The two flasks were inoculated with *Leptothrix discophora* and shaken at room temperature. A coupon from each flask was removed and dried after 6, 8, and 10 hours of bacterial growth. The coupons were gold/palladium-coated to a thickness of 15 nm and studied with the SEM.

**General Morphology of Indentations Originating from Pseudomonas putida with Biomineralized Manganese Oxides and Chloride Solution Treatment**

After the analysis of the pits and indentations initiated by *Leptothrix discophora*, it was questioned whether other bacteria could have the same effect on the surface of 316L stainless steel. I hypothesized that other manganese oxidizing bacteria could also
initiate pitting on the surface of metals. It was further hypothesized that the pit sites would have different morphologies according to the bacteria’s morphology. The lab had access to another manganese oxidizer: *Pseudomonas putida*, MN-1 strain. These hypotheses were examined by repeating the experiments discussed earlier, only changing the bacteria to *Pseudomonas putida* and media to LEP.

*Pseudomonas putida*, MN-1 strain varies greatly from *Leptothrix discophora*, SP-6 strain. *Pseudomonas* grows much quicker, and has a thicker biofilm than the *Leptothrix*. *Leptothrix* also has a sheath that protects groups of bacteria. *Pseudomonas* does not contain a sheath. One of the largest differences between the bacteria is the media required to culture them. The *Leptothrix* uses the MSPV media given in Table 3-1, containing a low concentration of salts with pyruvate as a carbon source. The *Pseudomonas* uses LEP media given in Table 3-2, containing large concentrations of organic compounds.

<table>
<thead>
<tr>
<th>Composition of LEP media</th>
<th>Composition of trace element solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast Extract</td>
<td>CuSO$_4$·5H$_2$O</td>
</tr>
<tr>
<td></td>
<td>ZnSO$_4$·7H$_2$O</td>
</tr>
<tr>
<td>Casamino Acids</td>
<td>CuCl$_2$·6H$_2$O</td>
</tr>
<tr>
<td>Glucose</td>
<td>Na$_2$MoO$_4$·2H$_2$O</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>Distilled Water</td>
</tr>
<tr>
<td>HEPES</td>
<td></td>
</tr>
<tr>
<td>Distilled Water</td>
<td></td>
</tr>
</tbody>
</table>
LEP Media Preparation

LEP media was prepared and then autoclaved for 25 minutes on the liquids or gravity setting. Once the media was cooled, 4 ml of syringe filtered manganese sulfate, and 1 ml of syringe filtered trace element solution were added. An experiment was performed with *Pseudomonas putida*, similar to the experiment for *Leptothrix discophora*, in which a general idea of corrosion morphology produced after ennoblement by manganese oxide deposition and treatment with sodium chloride. Two 316L stainless steel coupons were polished in their holders and then mounted in the polycarbonate batch reactor (Figure 4-3) as described earlier. The reactor was prepared in the same fashion as previously described. LEP media was prepared and then funneled into the reactor. Frozen stock of *Pseudomonas putida*, MN-1 strain was inoculated into the reactor. An SCE reference electrode, that was soaked in 95% ethanol for one hour prior to inoculation, was placed in the reactor. The potentials of the coupons were monitored until noble potentials of more than $+200 \text{mV}_{\text{SCE}}$ were achieved, monitored by a data acquisition unit. The coupons were then placed in a 0.2M sodium chloride solution. The potentials dropped to nearly $0 \text{mV}_{\text{SCE}}$, indicating pitting corrosion. After two days of exposure to the chloride solution, the coupons were removed from the solution and rinsed with deionized water. When dry, the coupons were analyzed by SEM.
Specific Morphology of Indentations Originating from *Pseudomonas putida* with Biomineralized Manganese Oxides and Chloride Solution Treatment

Three 316L stainless steel coupons were polished and then etched with squares as described in previously. The etched surface was mapped with AFM before treatment by bacterial colonization. The coupons were placed in the same polycarbonate reactor as in previous experiments. The reactor was then autoclaved for 25 minutes on the dry setting. LEP media was prepared as described above. The sterile reactor had 500ml of the media added to it and then inoculated with frozen *Pseudomonas putida*, MN-1 strain. A silver/silver chloride double junction electrode filled with 0.1M NaNO₃ was soaked in 95% ethanol for 1 hour before placing it in the reactor. The silver/silver chloride double junction electrode was used in this instance to mitigate slow leakage of chloride that might have been present in the SCE electrodes used previously. Open circuit potentials of the coupons were monitored, until the open circuit potentials were increased to approximately +350mV_{SCE} (converted from Ag/AgCl standard curve). The three coupons were removed and were cleaned by spraying the coupons with deionized water to remove the biofilm. Two of the coupons were immediately placed in a 0.2M sodium chloride solution. Their potentials dropped to values close to zero V_{SCE}, indicating pitting corrosion. The third coupon was allowed to dry without exposing it to the sodium chloride solution. SEM and AFM were then used to analyze the coupons.
SEM was used to image *Pseudomonas putida* in the same fashion as the *Leptothrix discophora*. Two 316L stainless steel coupons were polished and placed in a 250ml glass beaker where they were autoclaved for 15 minutes on the dry setting. LEP media was prepared, excluding the manganese addition. Only 50 ml of the media with only vitamins, not containing manganese sulfate, were then funneled into the sterile beaker containing the coupons. Frozen stock of *Pseudomonas putida*, MN-1 strain were then inoculated into the beakers. The bacteria were allowed to grow for approximately 15 hours and then the coupons were removed with the biofilm still attached. The biofilm was allowed to dry on the surface of the stainless steel under a vacuum. The dry coupons were then coated with an Ag/Pd film, 15nm thick. SEM was then used to image the dried bacteria.
CHAPTER 4
RESULTS AND DISCUSSION

Critical Sodium Chloride Concentration

At a concentration of approximately 0.1 M sodium chloride concentration, the pitting potential reaches its minimum value (Figure 4-1). The concentration of 0.1M sodium chloride was taken as the critical sodium chloride concentration, where an increase in concentration does not appreciably change the same pitting potential. The pitting potential at this salt concentration and scan rate was $+430\text{mV}_{\text{SCE}}$. The pitting potentials represented in Figure 4-1 are a little high. This is because the scan rate was too fast. A change in scan rate would shift the entire graph down.

![Figure 4-1](image-url)

**Figure 4-1.** Pitting potential as a function of chloride concentration. The critical salt concentration is taken as the point where the change of the slope is small as more salt is added (approximately 0.1M).
General Morphology of Indentations Originating from *Leptothrix discophora* with Biomineralized Manganese Oxides and Chloride Solution Treatment

After one day of exposure of the ennobled coupons to the salt solution, pitting was observed in the surface of the metal. The general shape of the indentations consisted of a variety of small pits in a line, being longer than they were wide (Figure 4-2). EDX analysis indicated that manganese was not found in the indentation, but could be found in deposits outside of the pit (Figure 4-3 and Table 4-1).

![Figure 4-2. Indentations formed in 316L stainless steel by the presence of *Leptothrix discophora* after ennoblement by manganese deposition and chloride treatment.](image-url)
Table 4-1. Percent composition of elements found in Figure 4-3: right side

<table>
<thead>
<tr>
<th>In Pit</th>
<th>In Deposit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromium</td>
<td>20%</td>
</tr>
<tr>
<td>Iron</td>
<td>71.1%</td>
</tr>
<tr>
<td>Nickel</td>
<td>8.9%</td>
</tr>
<tr>
<td>Silicon</td>
<td>1.6%</td>
</tr>
<tr>
<td>Phosphorous</td>
<td>6.1%</td>
</tr>
<tr>
<td>Chromium</td>
<td>9.8%</td>
</tr>
<tr>
<td>Manganese</td>
<td>22.7%</td>
</tr>
<tr>
<td>Iron</td>
<td>59.8%</td>
</tr>
</tbody>
</table>

Figure 4-3. EDX spectrum from the area in indentation (right) and deposit (left).

Manganese Plating

Deposition of the manganese oxide successfully created shapes on the surface of the metal as seen in Figure 4-4, representing the manganese deposited in the shape of the letters "Mike". However, when examined by AFM after exposure to the chloride solution, there were no pits or large amounts of manganese deposits observed. The potential of the deposited manganese in deionized water only achieved $+206\text{mV}_{\text{SCE}}$. 
which may not have been large enough to initiate pitting. A very small amount of the surface was covered with manganese oxide, covering enough of the surface that the surface potential had increased significantly. The plated areas would have a local potential that would be closer to an entire ennobled coupon. Because no change in the surface was evident, there is good evidence that manganese deposits are not directly responsible for the odd shaped indentations.

Figure 4-4. The first letters of “Mike” electroplated on stainless steel. Square has dimensions of 50x50 microns. White color is high; black is low.

Specific Morphology of Indentations Originating from Leptothrix discophora with Biomineralized Manganese Oxides and Chloride Solution Treatment

The potentials of the coupons in the reactor were continuously monitored against an SCE reference electrode, Table 4-2 and Figure 4-5.
Table 4-2. Highest potentials of the coupons reached. Sample 12 leaked and Sample 34 did not enoble for an unknown reason.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leaked</td>
</tr>
<tr>
<td>12</td>
<td>224.0</td>
</tr>
<tr>
<td>61</td>
<td>301.5</td>
</tr>
<tr>
<td>23</td>
<td>282.4</td>
</tr>
<tr>
<td>24</td>
<td>299.5</td>
</tr>
<tr>
<td>45</td>
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<tr>
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<td>34</td>
<td>217.0</td>
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<tr>
<td>13</td>
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</table>

Figure 4-5. Evolution of the OCP of 316L coupons exposed to manganese-oxidizing bacteria.

When the ennobled coupons were placed in the sodium chloride solution, their potentials first dropped sharply (initiation of pitting corrosion). The potential then fluctuated, indicating formation of metastable pits (open and closing of pits). Last of all, the potential then dropped farther and remained stable, indicating that active pitting was in progress (Figure 4-6).
Figure 4-6. Drop in ennobled coupon potential upon treatment with sodium chloride solution.

AFM and SEM images (Figures 4-7 and 4-8) illustrate pits that were formed on the surfaces of microbially colonized coupons after they were placed in sodium chloride solution. These pits formed highly organized groups of small indentations oriented in long narrow rows with smooth walls and bottom. Examination of twenty-five of these images showed the depth of the pits to be $44\pm20.51$ nm, with dimensions 12 $\mu$m long by 1.4 $\mu$m wide. Fifteen of these pits could be located with both AFM and SEM.
Figure 4-7. Correlation between AFM and SEM of microbially initiated pits: SEM image, left; AFM image, right; Conditions: bacteria, present; manganese, present; chloride, 0.2M.

Figure 4-8. A single line of organized pits in a long narrow row as seen by AFM and SEM. SEM image, left; AFM image, right. Conditions: bacteria, present; manganese, present; chloride, 0.2M.

Surprisingly, the two coupons removed from the reactor prior to the addition of the sodium chloride solution had similar indentations to those in Figures 4-7 and 4-8 (Figure 4-9). They were shallower, around 6 nm deep, which is approximately equal the
thickness of the passive layer on stainless steels [29]. Using SEM, it was determined that the morphologies of pits formed in the presence of the bacteria with and without sodium chloride were similar.

**Figure 4-9.** Indentation on the edge of a square made by ion milling initiated by the microorganism without addition of chloride. SEM image, left; AFM image, right. Conditions: bacteria, present; manganese, present; chloride, absent.

**Imaging surfaces of electrochemically polarized corrosion coupons**

Figure 4-10 shows SEM images of corrosion pits on anodically polarized 316L stainless steel. The pits were round and typically larger and deeper than pits initiated by bacterial colonization. Pits initiated by anodic polarization were bowl-shaped with a thin metal sheath covering their mouths (page 24) [21]. The pits were on average 60.7 µm long and 50.2 µm wide. AFM could not be used to describe their depth morphology because their depth exceeded 6.7 µm, the maximum depth the instrument could read.
Figure 4-10. SEM images of pits initiated by anodic polarization. Left, groups of holes in the thin metal sheath covering the pit's mouth; Right, small holes surrounding a large one indicating a thin metal sheath covering the pit. Conditions: bacteria, absent; manganese, present; chloride, 0.1M.

Imaging surfaces of sterile corrosion coupons exposed to sodium chloride

The control using the abiotic sodium chloride solution and sterile corrosion coupons showed that the solution of sodium chloride alone did not initiate pits (Figure 4-11) on the surface of the 316L coupons, which was expected. Actually, the surfaces of the coupons exposed to abiotic sodium chloride became slightly smoother and had fewer scratches. The exposure time for the coupons exposed to the abiotic sodium chloride exceeded the time for which the microbially colonized 316L coupons were exposed to the solution of sodium chloride. Therefore, it can be said that the sodium chloride solution alone did not initiate pitting on the surface, but required the presence of the bacteria. The chloride propagated the pits once initiated.
**Figure 4-11.** AFM images of the control coupon with the etched square. Left – before experiment, Right – after experiment. Conditions: bacteria, absent; manganese, absent; chloride, 0.2M.

**Imaging Leptothrix discophora attached to the surfaces of corrosion coupons**

To verify the hypothesis that the pits were formed at the sites occupied by the microorganisms, SEM images were taken of the bacteria. To enhance visibility and prevent formation of manganese oxides, the bacteria were grown in the absence of manganese. *Leptothrix discophora* is a cylindrical bacterium. It connects to other *Leptothrix discophora* end-to-end in a chain. Characteristically, these bacteria form a sheath of proteins and polysaccharides around them a long, narrow protective layer [58]. The groups of bacteria in Figure 4-12 are each approximately 10 μm long by 1 μm wide. The agglomerates of bacteria in Figure 4-12 have the same shapes as the pits on the surface of the metal (Figures 4-7 and 4-8).
Surprisingly, even though the conditions of this test were designed to map the microorganisms, and chloride was not added to the solution, indentations found in the passive layer were similar in shape to those found in the presence of chloride (Figure 4-13). These indentations had the same shape as those in Figure 4-8 but were quite shallow.

To compare the dimensions of the pits with those of the bacteria, an aspect ratio, or length divided by width, was used. To compare the sizes of the indentations in the passive layer with the sizes of microbial aggregates, the dimensions of the groups of pits were quantified. I found that the 15 pits initiated by anodic polarization had an aspect ratio of $1.3 \pm 0.3$, indicating they were almost round. In contrast, 71 groups of pits found on the surface of the coupons colonized by the bacteria had an aspect ratio of $10 \pm 5$. Twenty groups of bacteria that were measured had an aspect ratio of $10 \pm 4$. Both the bacteria and the pits were ten times longer than they were wide with the same dimensions. The width was found to change little in pits found, so large changes in length are the factor that changes the aspect ratio. The bacteria found in groups will
change in length depending upon how many bacteria are found in the protein sheath. The large standard deviations on the aspect ratios of both bacteria and group of pits strongly suggest that the bacteria are responsible for pit initiation.

![Image of bacteria and pits](image)

**Figure 4-13.** Shapes with the same dimensions as those pits initiated by bacterial colonization of the surface. Left, single group of shapes; Right, groups of indentations. Conditions: bacteria, present; manganese, absent; chloride, absent.

In summary, the coupons of freshly polished 316L stainless steel did not pit in an abiotic solution of sodium chloride solution. However, the same material pitted in the same chloride solution if previously microbially colonized by *Leptothrix discophora*. The formation of pits in the presence of bacteria did not require the presence of chloride. When manganese-oxidizing bacteria were used, the pits were initiated with or without manganese present. This indicates that it is the presence of the microbes, not the microbially deposited manganese oxides, that initiates pitting. The depth of the pits depends on the concentration of the chloride solution and on the time of exposure. When no chloride was present, the pits were small, and their depth was comparable with the thickness of the passive layer. When chloride was added, the pits were deeper. The pit
aspect ratio (10±5) and size (10 μm long by 1 μm wide) were the same for the agglomerates of bacteria and the groups of pits initiated by bacterial colonization. The standard deviations of the aspect ratios are large and close to one another for both agglomerates of the bacteria and the pits, indicating that pits formed by bacteria vary the same magnitude as the bacteria.

The manganese oxides deposited on the surface elevate the potential, creating an environment where the pits initiated by microbes cannot repassivate. This is because the current due to ennoblement is 2 to 3 decades larger than the passivation current, not allowing the passive layer to form. In this light, it appears that the bacteria initiate the pits, and the microbially deposited manganese oxides stabilize the growth of the pits by maintaining a high potential. Further experiments will be conducted to determine the rate of the pit growth.

Finally, it is possible that pit locations are not random, but pertain to features of the underlying metal substratum such as grain boundaries or crystalline phases. Geesey et al. [69] showed a similar correlation between grain boundaries and microbial attachment that resulted in localized substratum changes. The hypothesis that the locations of microbially initiated pits are correlated with the grain boundaries should be verified in future experiments.
General Morphology of Indentations Originating from *Pseudomonas putida* with Biomineralized Manganese Oxides and Chloride Solution Treatment

The images from the experiment show that pit initiation was present in the stainless steel after being ennobled and exposed to a 0.2M sodium chloride solution (Figure 4-14). Pit sites were similar to those initiated by *Leptothrix discophora* in that shallow pits were generally observed as groups of small pits. The groups of pits were not nearly as organized as those initiated by *Leptothrix discophora*.

![Figure 4-14](image)

**Figure 4-14.** Pits originating from *Pseudomonas putida* are shown above. The groups of pits seem to curve around on the right and on the left they appear to be straight.

Specific Morphology of Indentations Originating from *Pseudomonas putida* with Biomineralized Manganese Oxides and Chloride Solution Treatment

The pits initiated in the principal experiment after ennoblement and exposure to a 0.2M sodium chloride solution were similar to those observed in Figure 4-14. The pits analyzed both by SEM and AFM are seen in Figure 4-15. The AFM images before colonization of the surface indicated that these pits were not present before colonization of the surface by the bacteria.
Figure 4-15. Pits are shown that were initiated by the *Pseudomonas putida* after ennoblement and exposure to 0.2M sodium chloride. Left – SEM image, black odd shapes are the pits and the gray mass in the center is a manganese oxide deposit; Right – AFM image with white being a high altitude and black being the lowest.

The coupon that was reserved for analysis after ennoblement, which was not exposed to the sodium chloride solution, showed that indentations similar to those in Figures 4-14 and 4-15 were formed. Figure 4-16 shows an indentation formed under these conditions. These indentations could be observed by light microscopy, even though they were not very deep (6 microns). For the first time, considerably deep pits were also observed, even though there was not a significant chloride ion concentration present. The depth of one such pit that was formed by microorganism action was analyzed by the AFM and found to be over 177 nm deep (Figure 4-17).
Figure 4-16. Indentations on the surface of the stainless steel coupons were found after removal of biofilm from coupons not treated in a 0.2M sodium chloride solution. Indentations are not organized like *Leptothrix discophora*. The ditch running horizontally is the edge of the box formed by etching.

Figure 4-17. A pit was found that was over 177nm deep. It was formed by the presence of *Pseudomonas putida* and ennobled potentials without treatment of sodium chloride solution. Shown above is an image of the data shown by AFM analysis of the pit.
Imaging of *Pseudomonas putida*

Images of the bacteria *Pseudomonas putida* that were coated and then analyzed by SEM indicated that the adsorbed bacteria are different from *Leptothrix discophora*.

*Pseudomonas putida* is a small bacilli bacterium that is approximately ¾ micron wide by 2 micron long (Figure 4-18). They do not have sheaths, and are generally not well organized when colonizing a surface. Sometimes they can be seen connected end to end in a straight line or bent (Figure 4-18). The disorganized nature of their colonization of the surface gives supporting evidence that the bacteria initiated the pit sites observed in Figures 4-14 through 4-16. While photographing the bacteria, it was also observed that the same pit shapes were present (Figure 4-19). Bacteria were grown in the absence of divalent manganese ions so that manganese oxides would not be biomineralized, making imaging more difficult. The fact, that manganese and subsequent ennoblement were not present when microbially initiated pit sites were formed, supports the hypothesis that the bacteria alone are responsible for the pit initiation.

**Figure 4-18.** SEM images were taken of *Pseudomonas putida* after growing on stainless steel coupon, coated by Ag/Pd. Left – cells can grow end-to-end in a straight line; Right – cells can connect while bending.
Figure 4-19. SEM images were taken of pits on the surface of the stainless steel while searching for bacteria attached to the surface. Bacteria were grown without manganese and chloride.
SUMMARY AND RECOMMENDATIONS

Conclusions

The five hypotheses presented in the introduction were tested. Four of the five were found to be correct. This section will review the results of the hypotheses with any other important information acquired during experimentation.

1. **The indentations in the passive layer of the 316L stainless steel were formed only in the presence of the biofilm.**

   This hypothesis was found to be correct. The control indicated that pits did not form with sodium chloride treatment alone. Chloride treatment actually resulted in a slightly smoother surface. Odd indentations were observed to be a result of biofilm action. These indentations were longer than they were wide.

2. **Heterogeneous manganese oxide deposition, in the biofilm and on the surface of the stainless steel, was associated with the formation of the odd shaped indentations.**

   This hypothesis was proved to be incorrect. Heterogeneous manganese deposition appears not to have any affect upon the formation of the indentions. The manganese plating experiment showed that heterogeneous manganese oxide deposition did not change the surface of the 316L stainless steel sample. Also, results showed that manganese was not required to initiate indentations. Indentations were identified after growing the bacteria on the
surface of the stainless steel in a solution containing no chloride or manganese ions.

3. The *Leptothrix discophora* alone were responsible for the odd shaped indentations in the passive layer of the 316L stainless steel.

The tests for this hypothesis proved that Leptothrix discophora was responsible for the odd indentations in the surface of the 316L stainless steel. This was accomplished by comparing the pits formed by anodic polarization with those originating from colonization of the surface. Obvious differences existed that indicated that the pits were initiated only due to the presence of the bacteria (Figure 5-1). The indentations originating from the microorganisms were shallow, narrow and long, and highly ordered. The pits generated from anodic polarization were round, deep, and covered by a sheath.

![Figure 5-1. The difference between pits generated by microorganisms (left) and anodic polarization (right) are shown above. Pits initiated by bacteria are long, shallow and highly organized, while those formed by anodic polarization are circular, deep, and sheath covered.](image-url)
4. *Pseudomonas putida*, MN-1 strain, another manganese oxidizing bacteria, can also make odd shaped indentations in the passive layer of the 316L stainless steel.

The *Leptothrix discophora*, SP-6 strain and *Pseudomonas putida*, MN-1 strains, both MOB, led to indentation formation. The morphologies of the pits initiated from the two bacteria were similar in that they formed groups of small pits, they were shallow, and they did not contain a partially dissolved metal covering similar to pits formed by anodic polarization (Figure 5-2). They were different from one another in that the pits originating from the *Leptothrix discophora* are much more organized.

In the case of *Leptothrix discophora*, the indentations have the same aspect ratio and dimensions as the organisms. This test does not pertain to the *Pseudomonas putida* because there is no known way to give an aspect ratio to a cluster of bacteria that do not produce a sheath.

5. **Observed indentations in the passive layer of the 316L stainless steel are sites for initiation of pitting corrosion.**

Indentations that were formed by both types of bacteria, treated with biomineralized manganese oxides and a sodium chloride solution, were deeper than the passive layer. Beyenal showed with XPS (x-ray photoelectron spectroscopy) [70] that the passive layer thickness for 316L stainless steel in MSPV was 8 nm deep. Pit depth for the pits initiated by *Leptothrix discophora*, in the presence of ennoblement and sodium chloride, was found
to be approximately 50 nm. Pits generated in the presence of Pseudomonas putida were also 50 nm or deeper.

Figure 5-2. Comparison of pits originating from *Leptothrix discophora* (left) and *Pseudomonas putida* (right) is shown above. Pits formed from *Leptothrix discophora* are normally straight and evenly spaced. Pits formed from *Pseudomonas putida* are curved and unevenly spaced.

Indentations that were initiated by microbial colonization without the addition of chloride were generally not as deep as those with chloride. Pit initiation sites generated by bacteria were less than 10 nm deep, the approximate depth of the passive layer. The morphology of these indentations was the same as those created in the presence of chloride (Figure 5-3).
Figure 5-3. Comparison of pits initiated by bacteria in the presence of ennoblement with chloride (left) and without (right). Both pits are narrow and shallow without a sheath. Pits originating in the presence of chloride are deeper than those formed without chloride present.

Metal coupons, which had indentations that were observed to form after ennoblement, when placed in chloride solution, had their corrosion potentials decrease. As demonstrated in chapter 4, the corrosion potential was greatly reduced upon placing the coupon in the solution. The corrosion potential then oscillated between ennobled potentials and neutral potentials, until resting at a potential close to $0V_{SCE}$. This behavior is indicative of the formation of metastable pits that develop into active pits.

Marks created without manganese and chloride in solution were similar to indentations observed in Figures 5-2 and 5-3. These marks had the same morphology as those originating with chloride and ennoblement (Figure 5-4). Therefore, it is not necessary for either manganese or chloride to be present to initiate the pits created by microbial colonization. Both manganese and
chloride accelerate the corrosion process, and lead to deeper pits, as demonstrated in Figure 5-3.

Figure 5-4. Comparison of pits originated by microbial attachment in the presence of manganese (leading to ennoblement) and chloride (left) with those formed without manganese and chloride (right) is shown above. Both have the same morphology.

Recommendations

There are many questions that remain to be answered: How do the manganese oxidizing bacteria create the indentations observed? What is the difference between galvanic corrosion and pits initiated by manganese oxidizing bacteria? Are there other bacteria besides manganese oxidizing bacteria that can form pit initiation sites? Why don’t metals pit at lower potentials if pit initiation sites are created by attachment of the bacteria? Are the bacteria changing the metal surface, causing the pit initiation sites, or are the bacteria altering the imitate area of the solution that is creating a corrosive environment? Why do the bacteria form the indentations? Some of these questions can be answered by similar methods employed in this thesis.
Table 5-1. Summary of Conclusions.

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<th>Hypothesis:</th>
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<td>1.  The indentations in the passive layer of the 316L stainless steel were formed only in the presence of the biofilm.</td>
<td></td>
<td>X</td>
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<tr>
<td>2.  Heterogeneous manganese oxide deposition, in the biofilm and on the surface of the stainless steel, was always associated with the formation of the odd shaped indentations.</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>3.  The Leptothrix discophora alone were responsible for the odd shaped indentations in the passive layer of the 316L stainless steel.</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>4.  Pseudomonas putida, MN-1 strain, another manganese oxidizing bacteria, can also make odd shaped indentations in the passive layer of the 316L stainless steel.</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>5.  Observed indentations in the passive layer of the 316L stainless steel are sites for initiation of pitting corrosion.</td>
<td>X</td>
<td></td>
</tr>
</tbody>
</table>

Further experiments could be used to determine exactly how bacteria involvement is related to the indentations formed. Perhaps a confocal microscopy could be used to show this correlation. The microscope could be used to determine the location of stained bacteria with respect to the surface. This could be done in conjunction with squares etched in the surface, as used in some of the experiments in this paper. Confocal microscopy can map a three dimensional distribution of microorganisms in a biofilm after ennoblement of the 316L stainless steel. The microscope experiences difficulty with metal surfaces because the metal reflects all the light back to the microscope. This problem can be remedied by opening the pinhole of the microscope. Two different
images can be taken of the surface, one of the biofilm with microbial distribution and then one of the metal surface. The two images can be overlaid and a correlation of the microorganisms versus the etched square formed by ion milling can be made. The biofilm can then be removed and a map of the location of the pits formed versus the square can be made. Combining the different images would then form a correlation of the pits formed versus the microorganisms. This experiment would require computer graphics knowledge to combine different types of images.

Another question that remains unanswered is whether or not the pitting corrosion initiated by the bacteria behaves differently than pits initiated by anodic polarization. An experiment that could be used to quantify the corrosion behavior of the two systems is to use liquid AFM to measure corrosion as it is occurring. A volume change could be monitored in the two cases and compared. This would also give a real-time image of how corrosion proceeds. Another method that could be used to try to differentiate between the two systems is to compare their electrochemical noise.

One method that could be used to study the pits formed by microbial attachment is ToF-SIMS, to see what chemistry lies inside the pits. If there is a large enough difference in chemistry to those pits formed by electrochemical methods and those formed by bacteria, the results may suggest a mechanism that can be further studied.
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APPENDIX A

ORIGINAL TEXT APPROVED TO BE PUBLISHED IN INTERNATIONAL BIODEGRADATION AND BIODETERIORATION
Microbially Initiated Pitting on 316L Stainless Steel

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***Department of Civil Engineering and the Center for Biofilm Engineering
Abstract: Pitting corrosion of 316L stainless steel ennobled in the presence of manganese-oxidizing bacteria, *Leptothrix discophora*, was studied in a low-concentration sodium chloride solution. Corrosion coupons were first exposed to the microorganisms in a batch reactor until ennoblement occurred, then sodium chloride was added, which initiated pitting. The pits had aspect ratios (length divided by width) and shapes closely resembling the aspect ratio and the shape of the bacteria, which suggested that the microorganisms were involved in pit initiation.

**Keywords**: Pit initiation, *Leptothrix discophora*, MIC, MOB, localized corrosion, manganese oxides

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Introduction

It has been demonstrated that stainless steels and other passive metals and alloys when exposed to natural waters containing manganese-oxidizing bacteria (MOB) can increase their open circuit potential (OCP), a phenomenon termed ennoblement (5,11,13). The effect of microbial ennoblement on passive metals is analogous to that caused by polarizing of metal anodically using a potentiostat in that the open circuit potential may reach the pitting potential. In artificial seawater (30g NaCl/L) (16, 18), 316L stainless steel has a pitting potential of approximately +300mV, which means that the OCP must reach +300mV before pitting occurs. In natural waters, 316L maintains the OCP well below its pitting potential; therefore, the stainless steel should not pit. However, manganese-oxidizing bacteria depositing manganese oxide (MnO2) on the surface of the stainless steel (1) cause a positive shift in the OCP. This is because when manganese oxide is in direct electrical contact with the stainless steel, the metal exhibits the following equilibrium dissolution potential of the MnO2:

\[
\text{MnO}_2(s) + 4 \text{H}^+ + 2e^- = \text{Mn}^{2+} + 2\text{H}_2\text{O} \quad E^0 = +1.28 \text{ V}_{\text{SCE}} \quad E'_{\text{pH}=7.2} = +0.360 \text{ V}_{\text{SCE}} \quad \{3\}
\]

The standard potentials \((E^0)\) for equations 1, 2, and 3 were calculated using the energies of formation: \(\Delta G^\circ_f\) \(\text{Mn}^{2+} = -54.5\) kcal/mole, \(\Delta G^\circ_f\gamma-\text{MnOOH} = -133.3\) kcal/mole, and \(\Delta G^\circ_f\gamma-\text{MnO}_2 = -109.1\) kcal/mole (4,20).

The formal potentials \((E')\) were calculated at a pH of 7.2 and \([\text{Mn}^{2+}] = 10^{-6}\). It has been demonstrated that surface coverage of 6% by manganese oxides (4) can increase the resting potential of 316L stainless steel in a fresh water environment from ~200mV\(_{\text{SCE}}\) to +362mV\(_{\text{SCE}}\), a 500 mV increase, at a pH of 7.2 (5,12).
When studying the mechanism of ennoblement of 316L stainless steels using pure cultures of manganese-oxidizing bacteria, *Leptothrix discophora*, we have noticed oddly shaped indentations in the passive layer on the metal surface. We hypothesized that the bacteria were responsible for these indentations and that these indentations were in fact sites where corrosion pits initiated. To verify this hypothesis, we set up experiments to 1) demonstrate that *Leptothrix discophora* were responsible for the oddly shaped indentations on the surface of the ennobled stainless steel, and 2) to show that these indentations were sites where pits were initiated.

As a material to study, we used 316L stainless steel. Coupons of the metal were polished to a very smooth texture, removing as many flaws from the surface as possible. To identify sites of interest on the surface, small squares (200 x 200 μm) were etched using ion milling on the polished surface. Atomic force microscopy (AFM) was then used to examine the surface bound by the squares. The metal coupons were then exposed to *Leptothrix discophora* in a batch reactor. Electrical potential of the metal, reflecting progression of the ennoblement, was monitored versus a saturated calomel reference electrode (SCE). To initiate pitting, the ennobled coupons, once covered with microbial deposits, were removed from the reactor and exposed to a sterile solution of sodium chloride. The corrosion coupons were then removed to compare pit morphology with the shape of the bacteria *Leptothrix discophora* and their surfaces were examined with a scanning electron microscope (SEM) and atomic force microscope (AFM).

**Materials and Methods**

**Stainless Steel Coupons**

Stainless steel coupons 1.6 cm in diameter were cut from a 1 mm thick sheet of type 316L stainless steel purchased from Reyerson in Spokane, Washington. Coupons were mounted in polycarbonate holders with silicon gel (5). The holders consisted of a hollow polycarbonate tube 10 cm long with an inner diameter of 9 mm and an outer diameter of 19 mm (Figure 1).
The coupons mounted in the holder were polished to provide a surface sufficiently void of flaws for surface analysis. They were wet-sanded with tap water on Buehler-Met II metallographic grinding discs composed of silicon carbide grit of decreasing grit sizes: 120, 240, 360, 400, and 600. After the use of each grit size, the holder and coupon were rinsed with running tap water to remove any remaining grit. We then polished the coupons using Buehler aluminum oxide powder and Buehler Micropolish II powder, each suspended in water and applied with Buehler Microcloths. We initiated polishing with suspended 5 μm aluminum oxide powder. The coupons were then rinsed with tap water. Similarly, we used 0.5 μm and 0.05 μm polishing powders to make a mirror surface on the stainless steel, with rinses applied when polishing was complete with each powder size.

The coupons were then removed from the holders and two squares (200x200 μm), with a small number in the corner of each for identification, were etched on their smooth surfaces (Figure 2). The etchings were made by ion milling, with a focused Ga+ ion beam emitted from a time-of-flight secondary ion mass spectrometer (ToF-SIMS) for seven minutes at ~1.5 μA ion current at 22 KeV impact energy (17).
The etching produced a trench in the stainless steel approximately 100 nm deep (Figure 2). AFM was then used to map the surface of the stainless steel.

**Reactor**

The reactor was a polycarbonate batch cylinder reactor (Figure 3) 10.2 cm tall and 11.1 cm in diameter. Nine polished stainless steel coupons with the etched squares were mounted to their holders again, and the holders were attached to the top of the reactor. The assembled reactor is the same as the one used by Olesen (15), though slightly modified by the addition of the reference electrode directly into the medium instead of using a salt bridge. This alteration gives a much more stable potential reading. Tubes used for introducing air into the medium were mounted in the reactor, and Pall-Gelman bacterial air vents were attached to these tubes to prevent contamination of the reactor. Stirring was provided by a magnetic stir bar placed at the bottom of the reactor. The reactor was then sealed with the same silicon gel and autoclaved on dry setting (depressurization method) at 123°C and 1.2 atm for 30 minutes.

![Figure 2. Corner of the etched squares with an identification number on the surface of a polished coupon.](image-url)
To prepare the growth medium, one liter of ATCC Culture Medium 1917 MSPV (Table 1) was autoclaved on liquid setting at 123°C and 1.2 atm for 25 minutes. After the medium was cooled to room temperature, we added 1 ml of syringe-filtered vitamin solution (Table 2) required by the MSPV medium, 4 ml of syringe-filtered 50mMol manganese sulfate solution, and 5 ml of syringe-filtered 20% sodium pyruvate solution. All chemicals were from Fisher Scientific.

**Table 1. Composition of MSPV medium**

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<td>MgSO₄</td>
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<td>FeSO₄ 10mM</td>
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**Table 2. Composition of Vitamin Solution**

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<td>Thiamine HCl</td>
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<tr>
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</tr>
<tr>
<td>Riboflavin</td>
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</tr>
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<td>Nicotinic Acid</td>
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<td>Pyridoxine HCl</td>
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</tbody>
</table>
Manganese-oxidizing bacteria, *Leptothrix discophora* SP-6 (ATCC 51168), were obtained from ATCC and stored at -70°C. To inoculate the reactor, 150 ml of the MSPV medium containing vitamins, sodium pyruvate, and manganese sulfate was poured into a sterile 250 ml Erlenmeyer flask with the stock culture of the bacteria and placed on a shaker for two days. Then the broth was aseptically added to the reactor along with 600 ml of the sterile medium.

Before mounting it in the reactor, the SCE reference electrode was sterilized by soaking it in 99% ethanol for 1 hour. A stiff spring with a long conducting rod and stopper was connected to the coupon and coupon holder, thereby finishing the assembly (Figure 1). The coupon assembly and reference electrode were interfaced with a computer via a Hewlett Packard 34970A Data Acquisition/ Switch Unit (a multiplexer) to monitor the potential of the coupons.

The reactor was operated with the stirrer bar rotating and air bubbling through the medium until the potentials of the coupons exceeded +200mV (see Table 3 and Figure 4) which, according to our definition, indicated that the coupons were ennobled: This process usually took usually 5 days. Two of the nine coupons were removed from the reactor, sprayed with deionized water to remove the attached biofilm, and air-dried. These two coupons were then used to describe the surface of the coupons that were exposed to the microorganisms but not exposed to the chloride solution. The remaining coupons were also removed from the reactor, sprayed with deionized water to remove the biofilm, and then immersed in a 0.2 M NaCl solution. Spraying removed the biofilm, but it did not remove the manganese oxides on the surface. OCP of the coupons immersed in the NaCl solution was monitored, and the coupons were removed one at a time at pre-assigned intervals over the course of two days.

**Surface Analysis**

Before the analysis, surfaces of all coupons were gently wiped clean with acetone and a lab tissue paper to remove the attached manganese deposits and remaining biofilm. Absence of the manganese oxides and the biofilm was verified with a light microscope, AFM, and SEM. Any remaining attached
silicon gel was gently removed or coated with colloidal graphite from Ted Pella, Inc. to minimize any charging that would occur in the SEM.

Scanning electron microscopy (SEM) and atomic force microscopy (AFM) were used to map the surface topography of the following: 1, freshly polished sterile coupons; 2, ennobled coupons after removing microbial deposits; 3, after exposure to the sodium chloride solution. We used a Jeol JSM-6100 scanning electron microscope with the beam voltage set to 15 kV. The features of interest were photographed using a Polaroid camera, type 665, at a working distance of approximately 11 mm. Using a Digital Instruments Dimension 3100 scanning probe microscope in contact mode, the surface features identified by the SEM were then mapped using an AFM.

Other tests

**Imaging surfaces of electrochemically polarized corrosion coupons**

An essential part of the project was to quantify the morphology of the pits initiated on the coupons that had been ennobled and exposed to the sodium chloride solution. The working hypothesis was that the microorganisms were involved in the pit initiation. To verify this hypothesis, we compared the morphology of pits generated in the presence of manganese-oxidizing bacteria with the morphology of pits generated by anodic polarization in sterile media. The 316L stainless steel coupons were polished as previously described and placed in an electrochemical cell with the MSPV medium, vitamins, sodium pyruvate, and manganese sulfate. Sodium chloride was then added to make a 0.1M solution. An EG&G Princeton Applied Research Potentiostat/Galvanostat, Model 273A, was used to anodically polarize the coupons. The potential was increased at a rate of 10 V/hr from $-0.5 \text{ V}_{\text{SCC}}$ to $+0.8 \text{ V}_{\text{SCC}}$ using a graphite counter electrode. Care was used to ensure that crevice corrosion did not occur near the edge of the coupon holder (9). After the applied potential exceeded the pitting potential, the coupon was removed, rinsed with deionized water, dried, and analyzed with SEM and AFM. The corrosion pits were located on the surface, and their morphology (size and aspect ratio) was quantified.
**Imaging surfaces of sterile corrosion coupons exposed to sodium chloride**

To justify our conclusions, it was necessary to show that the observed indentations in the passive layer did not form spontaneously in the sodium chloride solution. To show this, a 316L stainless steel coupon was polished, as previously described, and then cleaned with acetone and a laboratory wipe. Two squares with the same dimensions as those used in previous experiments were etched on the surface of the coupon by the same ion milling procedure. The surface of the coupon was thoroughly examined with AFM, and then the coupon was exposed to 0.2 M NaCl solution (prepared with deionized water at room temperature) and aged for 2½ days. The coupon was then removed, rinsed with deionized water, dried, and again analyzed with AFM.

**Imaging Leptothrix discophora attached to the surfaces of corrosion coupons**

To compare the morphology of the bacteria with the morphology of the corrosion pits, we took images of the *Leptothrix discophora* attached to surfaces of corrosion coupons. Six 316L stainless steel coupons were polished to the described specification. Two 250 ml Erlenmeyer flasks each had three polished coupons placed in them. The flasks were then sealed, autoclaved for 25 minutes on the dry setting at 123°C and 1.2 atm, and cooled to room temperature. Two batches of MSPV medium were prepared in the same fashion as the previous experiments and autoclaved for 25 minutes on the dry setting at 123°C and 1.2 atm. Both batches had syringe-filtered solutions of sodium pyruvate and vitamins added, but only one had manganese sulfate added to it to make the same concentration as in the previous experiments. One sterile flask had 125 ml of the MSPV medium with manganese sulfate solution added aseptically. In the other flask, 125 ml of the MSPV media without manganese sulfate was aseptically added. The two flasks were inoculated with *Leptothrix discophora* and shaken at room temperature. A coupon from each flask was removed and dried after 6, 8, and 10 hours of bacterial growth. The coupons were gold/palladium-coated to a thickness of 15 nm and studied with the SEM.
Results and Discussion

The potentials of the coupons in the reactor were continuously monitored against an SCE reference electrode, Table 3 and Figure 4.

Table 3. Highest potentials of the coupons reached. Sample 12 leaked and Sample 34 did not ennable for an unknown reason.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>12</th>
<th>61</th>
<th>23</th>
<th>24</th>
<th>45</th>
<th>56</th>
<th>35</th>
<th>34</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potential (mV)</td>
<td>Leaked</td>
<td>224.0</td>
<td>301.5</td>
<td>282.4</td>
<td>299.5</td>
<td>251.5</td>
<td>267.5</td>
<td>165.0</td>
<td>217.0</td>
</tr>
</tbody>
</table>

Figure 4. Evolution of the OCP of 316L coupons exposed to manganese-oxidizing bacteria

When the ennobled coupons were placed in the sodium chloride solution, their potentials first fluctuated, indicating formation of metastable pits, then dropped, indicating that active pitting was in progress (Figure 5).
Figure 5. Potentials measured versus SCE as a function of time.

The control run using the abiotic sodium chloride solution and sterile corrosion coupons showed that the solution of sodium chloride alone did not initiate pits on the surface of the 316L coupons as was expected (Figure 6). Actually, the surfaces of the coupons exposed to abiotic sodium chloride became slightly smoother and had fewer scratches. The time for which the coupons were exposed to the abiotic sodium chloride exceeded the time for which the microbially colonized 316L coupons were exposed to the solution of sodium chloride. Therefore, it can be said that the sodium chloride solution alone did not initiate pitting on the surface.
Figure 6. AFM images of the control coupon with the etched square. Left – before experiment, Right – after experiment. Conditions: bacteria, absent; manganese, absent; chloride, 0.2M.

AFM and SEM images (Figures 7 and 8) illustrate pits that were formed on the surfaces of microbially colonized coupons after they were placed in sodium chloride solution. These pits formed highly organized groups of small indentations oriented in long narrow rows with smooth walls and bottom. Twenty-five of these images showed the depth of the pits to be 44±20.51 nm, with dimensions of 12 µm long by 1.4 µm wide. Fifteen of these pits could be located with both AFM and SEM.

Figure 7. Correlation between AFM and SEM of microbially initiated pits: SEM image, left; AFM image, right; Conditions: bacteria, present; manganese, present; chloride, 0.2M.
Figure 8. A single line of organized pits in a long narrow row as seen by AFM and SEM. SEM image, left; AFM image, right. Conditions: bacteria, present; manganese, present; chloride, 0.2M.

Figure 9 shows SEM images of corrosion pits on anodically polarized 316L stainless steel. The pits were round and typically larger and deeper than pits initiated by bacterial colonization. Pits initiated by anodic polarization were bowl-shaped with a thin metal sheath covering their mouths as previously described (8). Our pits were on average 60.7 μm long and 50.2 μm wide. We could not use AFM to describe their shape because their depth exceeded 6.7 μm, the maximum depth the instrument could read.

Figure 9. SEM images of pits initiated by anodic polarization. Left, groups of holes in the thin metal sheath covering the pit's mouth; Right, small holes surrounding a large one indicating a thin metal sheath covering the pit. Conditions: bacteria, absent; manganese, present; chloride, 0.1M.
Surprisingly, the two coupons removed from the reactor prior to the addition of the sodium chloride solution had similar indentations as those in Figures 7 and 8 (Figure 10). They were shallower, around 6 nm deep, which is approximately equal the thickness of the passive layer on stainless steels (2). Using SEM we perceived that the morphologies of pits formed in the presence of the bacteria with and without sodium chloride were similar.

Figure 10. Indentation on the edge of a square made by ion milling initiated by the microorganism without addition of chloride. SEM image, left; AFM image, right. Conditions: bacteria, present; manganese, present; chloride, absent.

To verify our hypothesis that the pits were formed at the sites occupied by the microorganisms, we took SEM images of the bacteria. To enhance visibility and prevent formation of manganese oxides, we grew the bacteria in the absence of manganese. To provide some background concerning the bacteria that were used, *Leptothrix discophora* is a cylindrical bacterium. It connects to other *Leptothrix discophora* end-to-end in a chain. Characteristically, these bacteria form a sheath of proteins and polysaccharides around them a long, narrow protective layer (6). The groups of bacteria in Figure 11 are approximately 10 μm long by 1 μm wide. The agglomerates of bacteria in Figure 11 have the same shapes as the pits on the surface of the metal (Figures 7 and 8).
Surprisingly, even though the conditions of this test were designed to map the microorganisms, and we did not add chloride to the solution, we found indentations in the passive layer to be of similar shape to those we found in the presence of chloride (Figure 12). These indentations had the same shape as those in Figure 7 but were quite shallow. It appears, therefore, that the pits are initiated when the bacteria are present, and the presence of chloride only accelerates their progression.

To compare the dimensions of the pits with those of the bacteria, we used an aspect ratio, the length divided by the width. To compare the sizes of the indentations in the passive layer with the sizes of microbial aggregates, we quantified the dimensions of the groups of pits (see Figure 8 as an example). We found that the 15 pits initiated by anodic polarization had an aspect ratio of $1.28 \pm 0.27$, meaning they were almost round. In contrast, 71 groups of pits found on the surface of the coupons colonized by the bacteria had an aspect ratio of $9.97 \pm 5.50$. Twenty groups of bacteria we measured had an aspect ratio of $10.0 \pm 3.7$. Both the bacteria and the pits were ten times longer than they were wide.
In summary, the coupons of freshly polished 316L stainless steel did not pit in an abiotic solution of sodium chloride solution. However, the same material pitted in the same chloride solution if previously microbially colonized by *Leptothrix discophora*. The formation of pits in the presence of bacteria did not require the presence of chloride. When we used the manganese-oxidizing bacteria, the pits were initiated with or without manganese present. This indicates that it is the presence of the microbes, not the microbially deposited manganese oxides, that initiates pitting. The depth of the pits depends on the concentration of the chloride solution and on the time of exposure. When no chloride was present, the pits were small, and their depth was comparable with the thickness of the passive layer. When chloride was added, the pits were deeper. The pit aspect ratio (10±5) and size (10 μm long by 1 μm wide) were the same for the agglomerates of bacteria and the groups of pits initiated by bacterial colonization. The standard deviations of the sizes and the aspect ratios are large for both agglomerates of the bacteria and the pits.

The manganese oxides deposited on the surface elevate the potential, creating an environment where the pits initiated by microbes cannot repassivate. In this light, it appears that the bacteria initiate the
pits, and the microbially deposited manganese oxides stabilize the growth of the pits by maintaining a high potential. Further experiments will be conducted to determine the rate of the pit growth.

Finally, it is possible that pit locations are not random but pertain to features of the underlying metal substratum such as grain boundaries or crystalline phases. Geesey et al. (8) showed a similar correlation between grain boundaries and microbial attachment that resulted in localized substratum changes. The hypothesis that the locations of microbially initiated pits are correlated with the grain boundaries will be verified in future experiments.

Conclusions

- Pits in 316L stainless steel were initiated in the presence of bacteria *Leptothrix discophora*.
- The presence of chloride ions made the pits deeper but was not required for the initiation of the pits. The presence of the bacteria was sufficient.
- We did not see any evidence of pitting when a sterile 316L stainless steel coupon was immersed in a sterile solution of sodium chloride.
- Pits formed in the presence of bacteria had the same sizes and aspect ratios as the agglomerates of the bacteria.
- Pits formed in the presence of bacteria had morphologies different from those initiated by anodic polarization of the material in the same solution.
- The evidences presented here indicate that the bacteria were involved in pit initiation on 316L stainless steel. However, this conclusion is based on indirect evidences -- corrosion pits and microbial aggregates had the same morphology.

Further tests are needed to verify these conclusions and to determine the mechanism by which the microbes influence the integrity of passive layers.
Acknowledgments

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References


