



Leptothrix discophora SP-6 : effects of biofilms on passive film chemistry of 316L stainless steel and modeling of growth
by Nurdan Yurt

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemical Engineering
Montana State University
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Abstract:

This thesis examined effects of manganese oxidizing bacteria, *Leptothrix discophora* SP-6 biofilms, on passive film chemistry and pitting corrosion of 316L stainless steel..

Biofilms of manganese oxidizing bacteria attach to the passive films of metals and deposit manganese oxides on the surfaces. These oxides, being in equilibrium with manganese ions in water, shift the corrosion potential of the passive metals in the noble direction, causing a phenomenon known as “ennoblement”. Ennoblement is of interest because it increases corrosion potential, which may initiate localized forms of corrosion of passive metals, pitting corrosion. The mechanism of pit initiation on the ennobled coupons still is not completely known. For localized corrosion to occur, the passive film has to be broken down, and it has been speculated that the biofilms of manganese oxidizing bacteria have a direct effect on the chemistry of the passive films.

The effect of ennoblement of 316L stainless steel by biomineralized manganese deposits on chemistry of passive films was studied using surface-sensitive analytical techniques and cyclic polarization. Depth profiles of elements in the passive films on the ennobled coupons were analyzed using x-ray photoelectron spectroscopy (XPS), and distribution of metal elements were examined by time-of-flight secondary ion mass spectroscopy (ToFSIMS). The results showed that oxide layers on the ennobled coupons were thinner than those on the control coupons.. Cyclic polarization curves showed that ennobled 316L stainless steel indicated, significant loss of passivity. It was concluded that metabolic activity of manganese oxidizing bacteria, *Leptothrix discophora* SP-6 degraded the quality of the passive film on stainless steel coupons by locally reducing its thickness and lowering the pitting potential.

To relate the kinetics of ennoblement to the rate of deposition of manganese oxides on metal surfaces, growth kinetics of *Leptothrix discophora* SP-6 were quantified in biofilms and in planktonic form. In planktonic form, double-substrate growth kinetics, using Monod growth kinetics for pyruvate and Tessier growth kinetics for oxygen, showed the best agreement with the experimental data. Monod model of microbial growth kinetics adequately represents the growth of *Leptothrix discophora* SP-6 biofilms.

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MONTANA STATE UNIVERSITY
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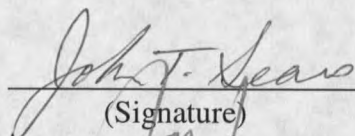
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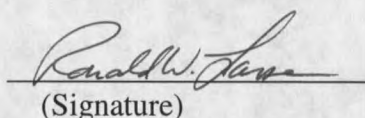
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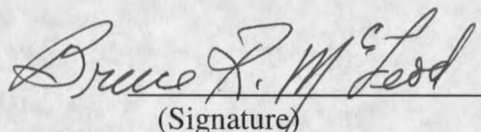
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NOMENCLATURE

B_i	Constant in Contois model for substrate i (mg substrate/mg microorganism)
D	Dilution rate (1/h)
D_{eff}	Effective diffusion coefficient of oxygen in biofilm (m^2/s)
i	Subscript refers to the growth model, M for Monod and T for Tessier
K_{sM}	Monod half rate coefficient (g/m^3)
K_{sMi}	Monod half saturation constant for substrate i
K_{sMZi}	Moser constant for substrate i
K_{sT}	Tessier coefficient (g/m^3)
K_{sTi}	Tessier saturation constant for substrate i
L_f	Biofilm thickness (m)
m	maintenance coefficient for oxygen (mg microorganism/mg oxygen)
m_i	Maintenance factor for limiting substrate i , (mg microorganism/mg substrate i)
m_M	maintenance coefficient for oxygen when growth is described according to Monod kinetics (mg microorganism/mg oxygen)
m_T	maintenance coefficient for oxygen when growth is described according to Tessier kinetics (mg microorganism/mg oxygen)
M_M	$= \frac{m_M L_f^2 X_f}{D_{eff} S_s}$

$$M_T = \frac{m_T L_f^2 X_f}{D_{eff} S_s}$$

- N Number of experiments (integer)
- Q Volumetric flow rate (L/h)
- S Oxygen concentration in biofilm (g/m^3)
- S^* Dimensionless oxygen concentration in biofilm ($=S/S_s$)
- S_{bottom} Oxygen concentration at the bottom of biofilm (g/m^3)
- S^*_{bottom} Experimentally measured dimensionless oxygen concentration at the bottom of biofilm ($=S_{\text{bottom}}/S_s$)
- $S^*_{\text{experimentally_measured}}$ Experimentally measured dimensionless oxygen concentration in the biofilm
- $S^*_{\text{predicted}}$ Calculated dimensionless oxygen concentration from the solution of the model in biofilm
- S_{ei} Substrate concentration in effluent stream (mg/L)
- S_{fi} Concentration of substrate i in influent stream (mg/L)
- S_i Concentration of substrate i (mg/L)
- SOUR Specific oxygen uptake rate (mg oxygen/mg microorganism/h)
- S_s Oxygen concentration at biofilm – air interface (g/m^3)
- SSD: Sum of squares of difference
- V Reactor volume (L)
- x Distance from the bottom of the biofilm (m)

X	Microorganism concentration in chemostat (mg/L)
x^*	Dimensionless distance from the bottom ($=x/L_f$)
X_f	Biofilm density (g/m^3)
$Y_{x/i}$	Yield coefficient for limiting substrate i (mg microorganism/mg limiting substrate)
$Y_{x/o}$	Yield coefficient for oxygen (g microorganism produced/g oxygen consumed)

Greek letters

$$\beta_M = \frac{K_{sM}}{S_s}$$

$$\beta_T = \frac{K_{sT}}{S_s}$$

Φ Thiele modulus (Φ_M or Φ_T)

$$\Phi_M = \sqrt{\frac{\mu_{\max} L_f^2 X_f}{Y_{x/o} D_{\text{eff}} S_s}}$$

$$\Phi_T = \sqrt{\frac{\mu_{\max} L_f^2 X_f}{Y_{x/o} D_{\text{eff}} S_s}}$$

λ_i Moser coefficient for substrate i

μ Specific growth rate of microorganism, (1/h)

$\mu_{\text{experimental}}$ Experimentally determined specific growth rate of microorganism from ($\mu=Q/V$), (1/h).

μ_i Specific growth rate of microorganism for limiting substrate i , (1/h)

μ_{max} Maximum specific growth rate (s^{-1})

μ_{model} Specific growth rate of microorganism predicted from the models, (1/h)

Subscripts

i Substrate

p Pyruvate

o Oxygen

n NH_4^+

ABSTRACT

This thesis examined effects of manganese oxidizing bacteria, *Leptothrix discophora* SP-6 biofilms, on passive film chemistry and pitting corrosion of 316L stainless steel.

Biofilms of manganese oxidizing bacteria attach to the passive films of metals and deposit manganese oxides on the surfaces. These oxides, being in equilibrium with manganese ions in water, shift the corrosion potential of the passive metals in the noble direction, causing a phenomenon known as "ennoblement". Ennoblement is of interest because it increases corrosion potential, which may initiate localized forms of corrosion of passive metals, pitting corrosion. The mechanism of pit initiation on the ennobled coupons still is not completely known. For localized corrosion to occur, the passive film has to be broken down, and it has been speculated that the biofilms of manganese oxidizing bacteria have a direct effect on the chemistry of the passive films.

The effect of ennoblement of 316L stainless steel by biomineralized manganese deposits on chemistry of passive films was studied using surface-sensitive analytical techniques and cyclic polarization. Depth profiles of elements in the passive films on the ennobled coupons were analyzed using x-ray photoelectron spectroscopy (XPS), and distribution of metal elements were examined by time-of-flight secondary ion mass spectroscopy (ToFSIMS). The results showed that oxide layers on the ennobled coupons were thinner than those on the control coupons. Cyclic polarization curves showed that ennobled 316L stainless steel indicated significant loss of passivity. It was concluded that metabolic activity of manganese oxidizing bacteria, *Leptothrix discophora* SP-6 degraded the quality of the passive film on stainless steel coupons by locally reducing its thickness and lowering the pitting potential.

To relate the kinetics of ennoblement to the rate of deposition of manganese oxides on metal surfaces, growth kinetics of *Leptothrix discophora* SP-6 were quantified in biofilms and in planktonic form. In planktonic form, double-substrate growth kinetics, using Monod growth kinetics for pyruvate and Tessier growth kinetics for oxygen, showed the best agreement with the experimental data. Monod model of microbial growth kinetics adequately represents the growth of *Leptothrix discophora* SP-6 biofilms.

CHAPTER 1

INTRODUCTION

This thesis examines several effects of growth and attachment of *Leptothrix discophora* SP-6 in biofilms. The effects of biofilms of these manganese oxidizing bacteria on passive film chemistry and on the pitting potentials of 316L stainless steel were investigated. Also, the growth kinetics of *Leptothrix discophora* SP-6 were determined for planktonic and biofilm growth. This introductory section briefly describes the content of the thesis, presents research questions and hypotheses to be addressed, and shows the organization of the thesis.

A research group at the Montana State University (MSU) is systematically studying the ennoblement of stainless steel by microbially deposited manganese oxides. It has been shown that biofilms of *Leptothrix discophora* SP-6 grown on 316L stainless steel (SS) increase the open circuit potential to values of 300 – 400 mV_{SCE} by depositing manganese oxides (Olesen et al., 2000a,b; Dickinson et al., 1996). This phenomenon, which increases the open circuit potential, is called ennoblement. The manganese deposition mechanism and ennoblement on stainless steel coupons have been well described by the MSU research group (Shi et al., 2002; Olesen et al., 2000a,b; Dickinson et al., 1996). The ennoblement increases risk of pitting corrosion by lowering pitting potential of stainless steel in the presence of

active ions such as chloride (Olesen et al., 2001; Amaya and Miyuki 1997; Linhardt 1996; Suleiman et al., 1994). One of the possible mechanisms that could lower pitting potential is that the passive film is degraded by ennoblement caused by manganese deposition. Continuing the efforts at MSU, the first part of this thesis investigates passive film chemistry, explores these processes, presents previous literature and states research goals (section 1.1). The research correlating passive film chemistry and pitting potential of stainless steel ennobled by biomineralized manganese was prepared as a research paper and is presented in chapter 2 (Yurt et al., 2002a). The effect of biomineralized manganese on pitting corrosion of type 304L stainless steel are examined, and the basic approach to measure pitting corrosion of stainless steel 304L used in this thesis, and tests results of pitting potentials for clean and ennobled coupons, are presented in the appendix as a published paper (Olesen, B. H., Yurt, N., Lewandowski, Z. 2001).

For ennoblement studies, the model microorganism used at MSU has been *Leptothrix discophora* SP-6, which grows in bulk liquid, attaches to stainless steel surfaces, grows as biofilms and then deposits manganese and causes ennoblement. The second part of this thesis explores quantification of the growth kinetics of *Leptothrix discophora* SP-6 in planktonic and biofilm forms. Discussion of microorganism growth in planktonic form and in biofilms along with a literature review and research goals are presented in section 1.3. The growth kinetics of *Leptothrix discophora* SP-6 in planktonic form is presented as a research paper in chapter 3 (Yurt et al., 2002b). Since there was not any adequate published method to

calculate biokinetic parameters in biofilms, I developed an algorithm to extract biokinetic parameters of microorganism growth in *Leptothrix discophora* SP-6 biofilms. The developed algorithm and biokinetic parameters for *Leptothrix discophora* SP-6 biofilms are presented as a research paper in chapter 4 (Yurt et al., 2002c). The developed algorithms to calculate biokinetic parameters for planktonic growth and biofilm growth are integrated into MATLAB programs. These MATLAB programs were added to corresponding sections as an appendix to make them available for other researchers, since they are not available in the literature.

The experimental methods and instruments used in the thesis are presented in section 1.4 for future reference.

The hypotheses addressed in this thesis are:

1. Manganese oxidizing activity of *Leptothrix discophora* SP-6 in a biofilm leads to ennoblement of stainless steel and causes changes in the passive film chemistry of the steel.
2. The presence of biomineralized manganese oxides on the surface of 316L stainless steel initiates pitting corrosion in a low-chloride aqueous environment.
3. The statistically optimum model to represent *Leptothrix discophora* SP-6 growth in the planktonic form can be determined by an algorithm, developed by the author, which optimizes parameters for the four major kinetic models.

4. The statistically optimum model to represent *Leptothrix discophora* SP-6 growth in the biofilm form can be determined by an algorithm, developed by the author, which optimizes parameters.
5. *Leptothrix discophora* SP-6 has different growth and substrate consumption kinetics in planktonic and biofilm form.

Surface sensitive techniques such as XPS, ToFSIMS were used to determine passive film chemistry and thickness. The anodic polarization technique was used to measure pitting potentials. Hypotheses 1 and 2 are addressed in chapter 2. A chemostat operated at steady state was used to determine the best growth model, along with corresponding biokinetic parameters of *Leptothrix discophora* SP-6, and hypothesis 3 is addressed in chapter 3. Dissolved oxygen microsensors were used to measure concentration profiles in *Leptothrix discophora* SP-6 biofilms. A new method was developed to extract the best representative growth model and corresponding biokinetic parameters of *Leptothrix discophora* SP-6 growth in biofilms. Hypotheses 4 and 5 are addressed in chapter 4. Chapters 2, 3, and 4 are presented in the format of a journal paper.

Manganese Biomineralization on 316L Stainless Steel

Stainless Steels

Stainless steels are iron-chromium alloys with a minimum of 10 wt % chromium. When the steel contains at least that much chromium, a thin, transparent,

protective passive film forms on the surface. The passive film forms spontaneously as a result of the reaction between the chromium in the steel and the oxygen in the air. The passive film is a barrier between the steel and the environment and prevents corrosive attack of the underlying steel as long as the passive film remains intact. This protective passive film is quite thin, on the order of nanometers, and its actual composition depends on the alloying elements in the stainless steel and the environment to which it is exposed (Figure 1.1).

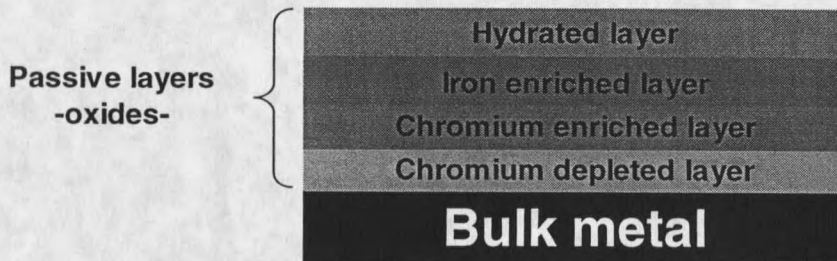


Figure 1.1. Schematic representation of passive layers in the presence of water.

Pitting Corrosion

Pitting is localized attack at a point where the passive film can no longer protect the stainless steel. Theoretically, pitting processes consist of the following stages; 1) passive film breakdown, 2) metastable pit formation, and 3) pit growth. These stages are discussed below.

Passive Film Breakdown. The breakdown of the passive film is the initiation of the pitting process. The breakdown happens rapidly on a very small scale, which

makes direct observation difficult. The passive film can be drawn schematically as a simple inert layer covering the underlying metal, blocking access of the environment to the metal (see Figure 1.2). Depending on alloy composition, environment, potential, and exposure history, this film can have a range of thickness, structure, composition, and protectiveness. Typical passive films are quite thin, and support an extremely high electric field (on the order of 10^6 - 10^7 V/cm). The passage of a finite passive current density is evidence of continual reaction of the metal, with resultant film thickening, dissolution into the environment, or some combination of the two. The view of the passive film as a *dynamic structure*, rather than static, is critical to the proposed mechanisms of passive film breakdown and pit initiation.

Theoretically, passive film breakdown and pit initiation have been categorized according to three main mechanisms; 1) passive film penetration, 2) film breaking and, 3) adsorption.

Figure 1.2 shows the penetration mechanism for pit initiation. It involves the transport of the aggressive anions through the passive film to the metal/oxide interface where accelerated dissolution of metal is promoted (Hoar 1965).

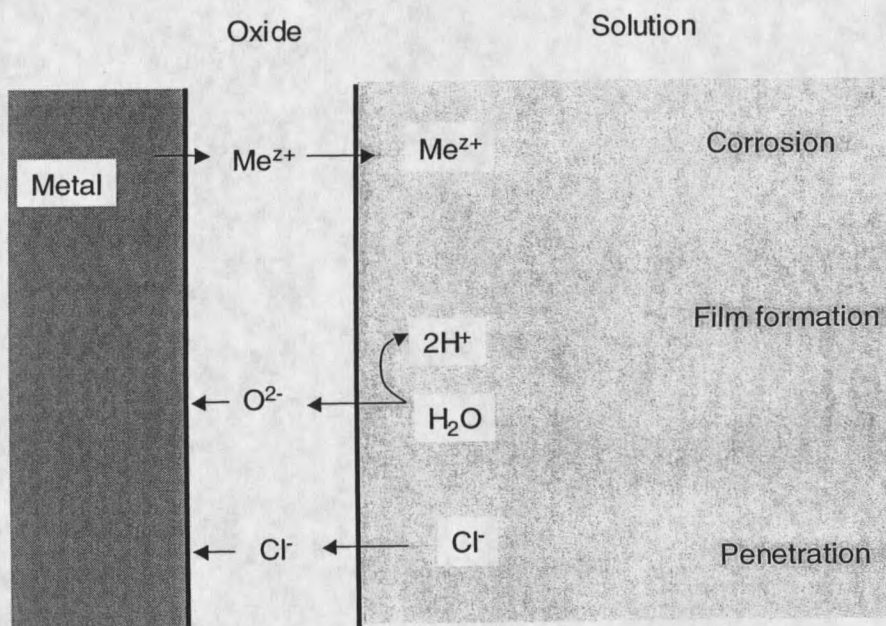


Figure 1.2 Penetration mechanism for pit initiation.

The adsorption model is presented schematically in Figure 1.3. Localized adsorption of chloride ion leads to an enhanced oxide dissolution at the sites with subsequent thinning of the oxide film until, finally, a complete removal is achieved and active metal dissolution starts. Basically adsorbed chloride ions compete with oxygen, chromates, or any other passivating agent for a place on the metal surface (Uhling 1950). However, the chloride ions do not remain adsorbed because the metal – halogen reaction rate is high (Uhling 1950). The metal, therefore, is rapidly attacked at restricted areas where chlorides succeed in displacing the adsorbed passivator and passivity breaks down. This theory was originally proposed by Uhling (1950). However, based on XPS measurements later on it has been shown that exposure of Fe to chloride and other halides caused thinning of the passive film, even

under conditions where a pit had not formed (Strehblow 1995). This contradicted Uhling's adsorption theory. However, this can be explained according to Hoar's theory for chloride adsorption: when thinning occurs locally because of some local adsorbed species, the local electric field strength will increase, which may eventually lead to complete breakdown and the formation of a pit (Hoar 1965).

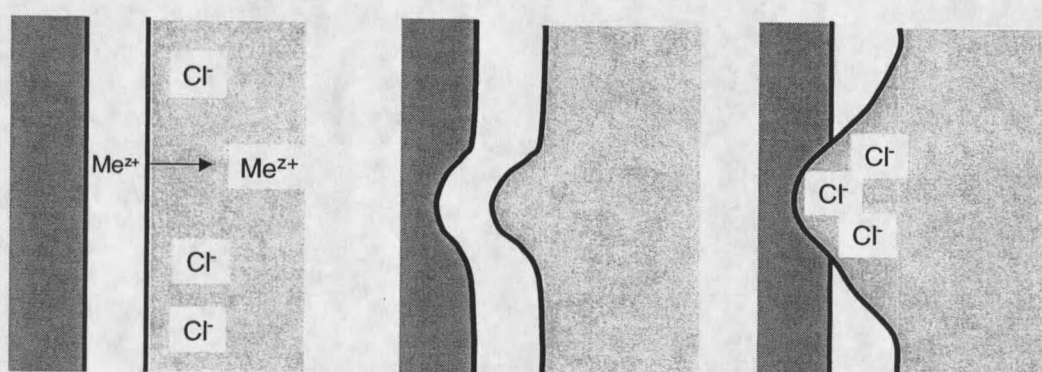


Figure 1.3 Adsorption mechanism for pit initiation.

Figure 1.4 shows pit initiation by the passive film breaking mechanism. This approach assumes that the thin passive film is in a continual state of breakdown and repair (Richardson and Wood 1970). Mechanical stresses at weak sites or flaws may cause the local breakdown events, which rapidly heal in non-aggressive environments. In fact, the background passive current density may be derived from a summation of many such breakdown and repair events (Kruger 2000). In chloride-containing solutions, however, there would be a lower likelihood for such a breakdown to heal because of the inhibition of repassivation by chloride. The passive film-breaking model really involves initiation based on pit growth stability. It

assumes that passive film breakdown will always occur, although at a rate that depends on many factors related to the passive film properties. According to this model, common and frequent breakdown will only lead to pitting under conditions where pit growth is possible (Bohni 2000). If, as stated by the passive film-breaking theory, passive film breakdown is frequent and rapid, pit initiation depends on generation of the conditions needed for a pit to be able to grow.

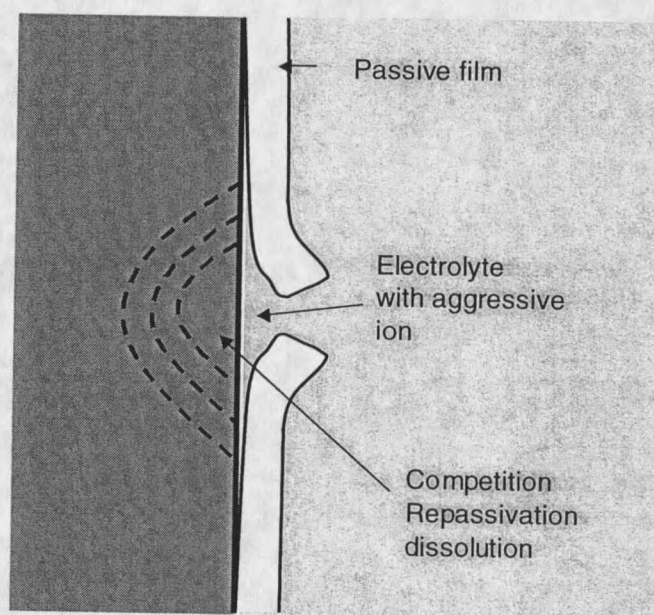


Figure 1.4. Pit initiation by passive film breaking mechanism.

Metastable Pitting. Pits which initiate and grow for a limited period before repassivating are called metastable (Bohni 2000). Large pits can stop growing for a variety of reasons, but metastable pits are typically considered to be only μm size at most with a lifetime on the order of seconds or less. Metastable pits can form at potentials far below the pitting potential (which is associated with the initiation of

stable pits). It has been shown that when stable pits are small, they behave identically to metastable pits (they open and close). Stable pits survive the metastable stage and continue to grow, whereas metastable pits repassivate and stop growing for some reason.

Pit Growth. Pit growth depends on material composition, pit electrolyte concentration and pit-bottom potential. The mass-transport characteristics of the pit influence pit growth kinetics through the pit electrolyte concentration. Pit stability depends upon the maintenance of pit electrolyte composition and pit bottom potential that are at least severe enough to prevent repassivation of the dissolving metal surface at the pit bottom. Pit growth can be controlled by the same factors that can limit any electrochemical reaction (Bohni 2000).

At the end of pit growth process, the metal corrodes and fails. Generally stainless steel considered as a metal immune to pitting. As described in the following section, recently it has been shown that stainless steel pits when microorganisms are grown at the surface and deposit minerals.

Manganese Biomineralization and Manganese Cycle

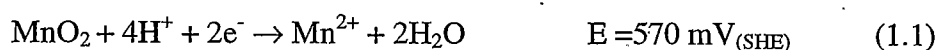
Biofilms have been reported to modify surface chemistry and electrochemistry of metals in natural waters. An example of such modification is ennoblement of passive metals. During ennoblement process, open circuit potential (OCP) of microbially colonized metal elevates from -200 mV to 350 mV SCE with an associated increase in cathodic current. This shift of OCP is called as *ennoblement*.

Mollica and Trevis (1988) were the first to report an observed increase in the open circuit potential of passive metals when immersed in natural waters. Dexter and Gao (1988) reported similar ennoblement effects from 316L exposed marine biofilm. Scotto et al., (1985) reported an increase in OCP of 22Cr3Mo0.6Ti steel up to 375 mV during 65 days of seawater exposure. Motoda et al., (1990) reported OCP values of 400 mV_{SCE} over 10 days of seawater exposure for 18 different type of stainless steels, and titanium reached 350 mV_{SCE} during the same period of time exposure. Eashwar et al., (1995) exposed titanium to seawater and changed illumination intensity from dark to strong intensity. The samples with moderate illumination only showed ennoblement. Numerous other studies have shown similar observation of passive metal ennoblement (Holthe et al., 1988 ; Little et al., 1990; Eashwar and Maruthamuthu 1995).

A variety of hypotheses were offered to explain the mechanisms of this ennoblement: 1) Depolarization of the oxygen reduction reaction caused by enzymes (Scotto and Lai 1998; Mollica et al., 1990; Scotto et al., 1985) or by iron chelators (siderophores) (Eashwar et al, 1995), 2) strong acidification of electrode surfaces due to biofilm activity (Little et al., 1991) and 3) combination of elevated H₂O₂ concentration and decreased pH (Chandrasekaran et al., 1993). Although all researchers show that ennoblement is linked to biofilm formation, none of these hypotheses has been proven either in the laboratory or in the field.

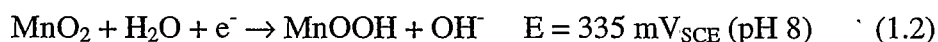
Linhardt (1996) observed a connection between microbial deposition of manganese and ennoblement of passive metals. He reported pitting corrosion of turbine runner blades (CrNi134) in a hydroelectric power plant in the Netherlands by

activity of manganese oxidizing microorganisms. SEM/EDAX and X-ray diffraction analysis of deposits on turbine blades showed significant amounts of manganese oxyhydroxide and manganese dioxide. Some of the deposits from the turbine blades were pressed into a pellet and then connected to a hydrogen reference electrode to measure the potential. A potential of 570 mV was measured. Linhardt concluded that the presence of manganese dioxide on the surface was responsible for the increase of the corrosion potential by the reaction:



This elevated potential caused pitting corrosion at a very low chloride concentration (20-170 mg/L), which normally is not enough to cause pitting itself.

In the Center for Biofilm Engineering, Dickinson et al., (1996) exposed stainless steel coupons in fresh river water and observed an increase in OCP from – 150 mV to 350 mV and 2-3 fold increase in cathodic density over 35 days. Epifluorescence and scanning electron microscopy of coupon surfaces detected Mn-rich annular deposits, associated clusters of bacterial cells, and abundant sheeted bacteria. Dickinson et al., (1996) demonstrated the same increase in potential and cathodic density by coating stainless steel surface by MnO_2 paste and concluded that the observed electrochemical changes on stainless steel were caused by microbial deposition of manganese oxides. The following reaction is proposed for ennoblement:



After Dickinson, Olesen et al., (2000a) identified types of manganese oxides deposited by pure bacteria on 316L SS coupons using X-rays photoelectron spectroscopy. To show the kinetics of reduction of manganese oxides, electroplated

manganese oxide on a steel surface was reduced electrochemically. According to XPS analysis before and after electrochemical reduction, manganese oxide reduced to divalent manganese, Mn^{2+} , obtaining 2 electrons from the anodic corrosion sites. MnOOH was determined as an intermediate product in this section. Olesen et al., (2000a) suggested the manganese cycling on stainless steel shown in Figure 1.5.

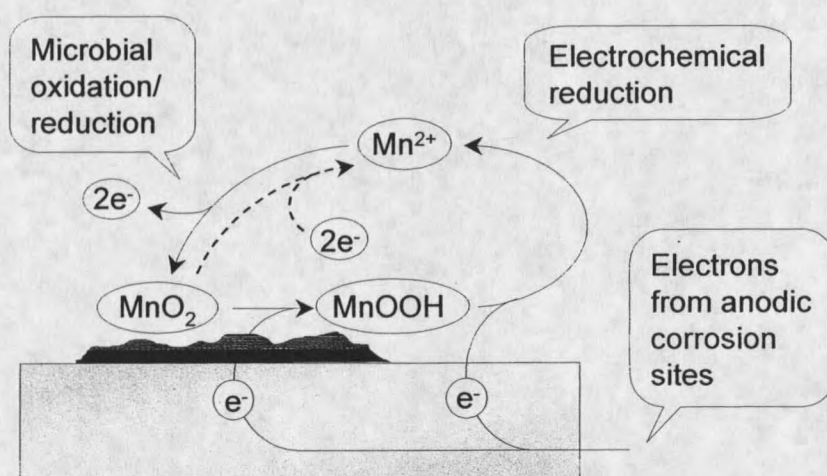


Figure 1.5. Manganese cycling at stainless steel surface hypothesized by Olesen et al., (2000).

Two years after Olesen (2000a), Shi et al., (2002), from the Center for Biofilm Engineering, improved the knowledge of the manganese cycle. They applied time of flight secondary ion mass spectroscopy to identify manganese oxides deposited by *Leptothrix discophora* SP-6. Their results indicated that the Mn^{2+} is oxidized microbiologically to MnOOH ; then MnOOH is further oxidized to MnO_2 . They schematically represented their hypothesis on the manganese cycle in Figure 1.6.

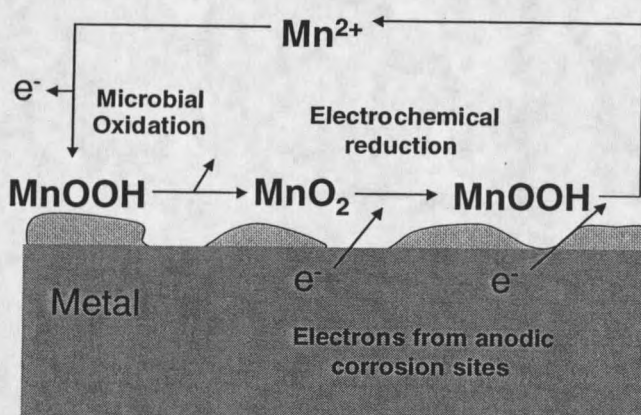


Figure 1.6. Manganese cycling proposed by Shi et al., (2002).

Role of Passive Film Layer During Manganese Biomineralization on Stainless Steel Surface

As explained above, the passive film is a protective oxide layer on the stainless steel surface. When biofilms attach on stainless steel surfaces and start to deposit minerals, they cause development of anodic and cathodic sites on stainless steel surface due to biofilm heterogeneity, as claimed by George et al., (2000). Although, George et al., (2000) found cathodic and anodic sites on surface, these sites might be not only biofilm heterogeneity, but as a result of biomineralization i.e., the manganese cycle. In another study, Maruthamuthu et al., (1996) employed electrochemical techniques to measure passive film layer chemistry with an intact biofilm. They suggested that the mechanism of ennoblement is linked to the bacterial removal of excess anions and cations from the oxide film.

Hypothesis 1. Manganese oxidizing activity of *Leptothrix discophora* SP-6 in a biofilm leads to ennoblement of stainless steel and causes changes in the passive film chemistry of the steel.

To test this hypothesis, it is required to probe chemistry of the passive films on stainless steel coupons with intact biofilms. Pendyala (1996) studied chemical effects of biofilm colonization on stainless steel. She grew a mixed culture *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* and *Klebsiella pneumoniae* and quantified passive film thickness on stainless steel sample with intact biofilms. Although her coupons were not ennobled, her study showed the possibility of measurement of passive film thickness on stainless steel coupons with an intact biofilm. Beech and Gaylarde (1999) explored how XPS can be used to quantify passive film chemistry at metal surfaces. From the same group Beech et al., (2000 a, b) later used XPS to quantify passive film chemistry of stainless steel coupons in the presence of *Pseudomonas* NCIMB2021. The method developed by Pendyala (1996) was used to measure passive film layer thicknesses to address hypothesis 1. The results of passive film thickness measurements are presented in Chapter 2.

Hypothesis 2. As stated before, biomineralization on stainless steel surface increases risk of the failure of the stainless steel. 316L stainless steel was expected to be immune to pitting in natural water, as demonstrated by Szklarsaka (1986) even at high chloride concentrations. However, there is experimental evidence that ennobled 316L stainless steel pits even at low chloride concentration (Amaya and Miyuki 1997; Suleiman et al., 1994; Felder 1994; Kobrin 1976). In another study, Neville and

Hodgkiess (2000) correlated passive film constitution with corrosion resistance; a thicker passive film makes stainless steel more corrosive resistant. If

biomineralization of manganese on stainless steel coupons compromises passive film quality, stainless steel must pit at low chloride concentration. My second research hypothesis is;

The presence of biomineralized manganese oxides on the surface of 316L stainless steel initiates pitting corrosion in a low-chloride aqueous environment.

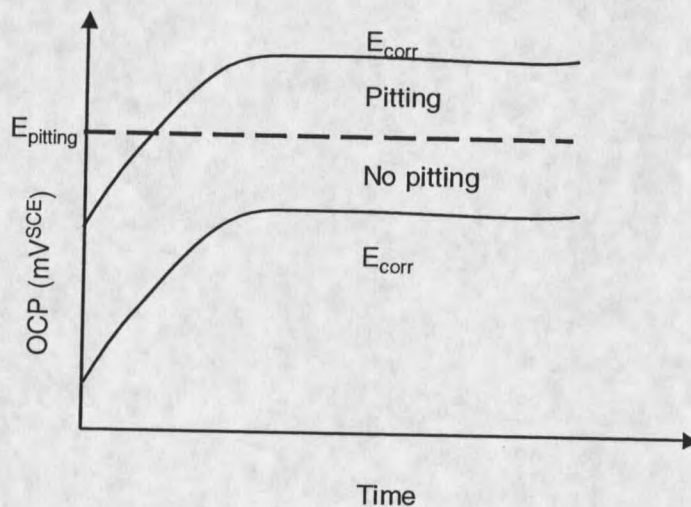


Figure 1.7. The relation between pitting (E_{pitting}) and corrosion (E_{corr}) potentials for a clean metal.

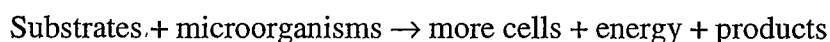
The relation between pitting (E_{pitting}) and corrosion (E_{corr}) potentials for a clean metal is presented in Figure 1.7. When corrosion potential of an ennobled metal is above the pitting potential, there is a possibility of pitting (Sedrix 1996). However, if corrosion potential of an ennobled metal is below the pitting potential, pitting does

not occur. To address my second research hypothesis given above, it is required to measure pitting potentials of clean and ennobled coupons. I developed anodic polarization techniques and tested coupons of 304L stainless steel (Olesen B. H., Yurt, N., Lewandowski, Z. 2001). It was found that ennoblement lowers pitting corrosion in a chlorinated environment for 304L stainless steel coupons. This study is presented in Appendix A. The same measurement techniques were utilized by Olesen B. H., Yurt, N., Lewandowski, Z. (2001) to measure corrosion potentials of ennobled 316L stainless steel coupons and the results are presented in Chapter 2.

Biokinetics of Microbial Growth

Microbial Growth

For microorganisms, growth is an essential response to their physiochemical environment. Microbial growth is defined as an increase in cell numbers (by replication) and changes in cell size (Shuler and Kargi, 1992). Microorganisms use substrates and in a suitable medium, temperature and pH, convert them into biological compounds. The substrates are used for microorganism growth, energy production and product formation. Simply, this process can be described by the following equation.



(1.3)

The term “substrate” is used to describe a chemical (or nutrient) which is used by the microorganisms (e.g glucose, oxygen, pyruvate, etc).

The microbial growth is characterized by the *specific growth rate* (μ), which is defined as

$$\mu = \frac{1}{X} \frac{dX}{dt} \quad (1.4)$$

Here X is the dry weight microorganism concentration, usually (g/L), t is time, (h), and μ is the specific growth rate, (h^{-1}). As engineers, when we use the term “rate” we expect to see variation of a quantity by unit time. However, the specific growth rate is described as the normalized growth rate by dividing the microbial growth rate (dX/dt) by the microorganism concentration, as given in Equation 1.4. A typical growth curve showing the correlation between microorganism concentration and time for a batch growth is presented in Figure 1.8.

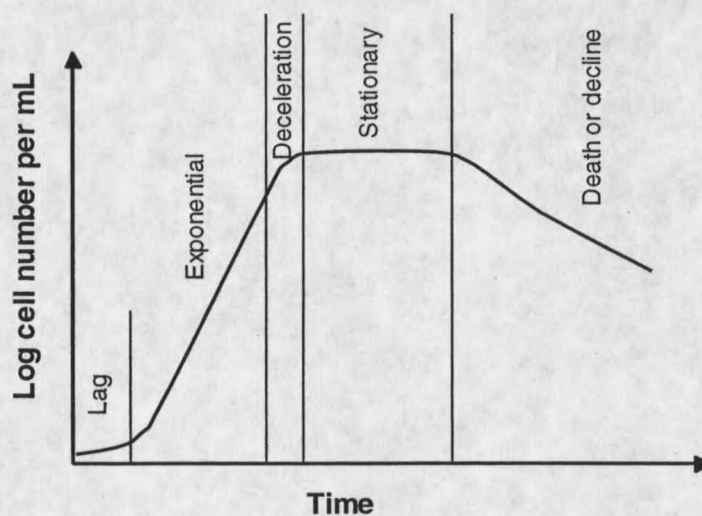


Figure 1.8. Typical growth curve for a microbial growth.

When a liquid medium is inoculated with a seed microorganism (inocula), the microorganism uses substrate in the medium and increases cell numbers as shown in Figure 1.8. The growth consists of five different phases; 1) the *lag phase* occurs immediately after inoculation and it is the time required for the adaptation of the cells to the new environment, 2) the *exponential growth phase*, also known as the logarithmic growth phase, occurs when, the microorganism concentration, or numbers, increase exponentially and the specific growth rate is described by equation 1.4, 3) the *deceleration phase* follows the exponential phase and the growth decelerates due to depletion of one or more essential substrates or accumulation of toxic microbial products, 4) the *stationary phase* starts at the end of deceleration phase, when the net growth rate is equal to zero, 5) the *death phase* (or decline phase) follows the stationary phase and in this phase total microorganism concentration decreases.

Microbial Growth Kinetics

Microbial growth kinetics is the relationship between specific growth rate (μ) of a microorganism and the substrate concentration (S). It is a basic tool used to describe microbial growth and was established in the 1940s and 1950s with the key publications by Hinshelwood (1946), Monod (1949 and 1950), van Niel (1949), Novick and Szilard (1950), Herbert et al., (1956), Málek (1958), Pirt (1965). In these early papers, Monod (1949 and 1950) presented a model describing his experimental data and correlated specific growth rate with growth limiting substrate

concentration. A sample figure correlating growth limiting substrate concentration to specific growth rate is presented in Figure 1.9.

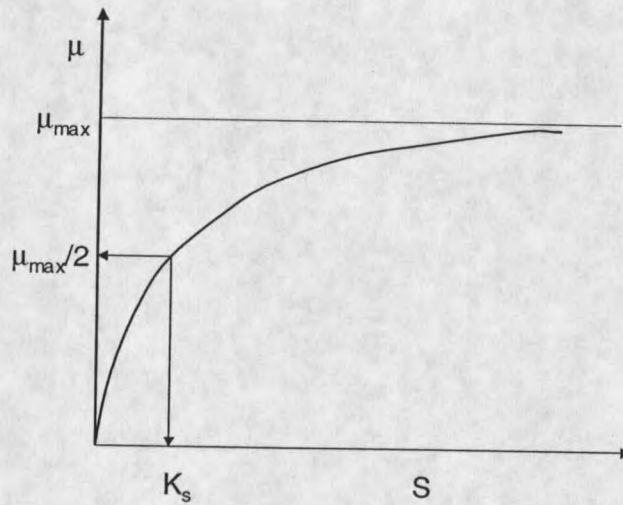


Figure 1.9. Effects of substrate concentration on specific growth rate.

As an example growth model, the Monod model is selected and discussed in the following sections (Monod 1949 and 1950). According to Monod and as seen from Figure 1.9, the specific growth rate is described as a function of substrate concentration given by the following equation.

$$\mu = \mu_{\max} \frac{S}{K_{SM} + S} \quad (1.5)$$

Two constants are used to describe the growth rate; (1) $\mu_{\max}$ is the maximum growth rate when the substrate concentration is no longer limiting, [time^{-1}]. K_s is the Monod half saturation constant, [mass (or moles) per unit volume]. K_s is equal to substrate concentration when $\mu = \mu_{\max}/2$.

Note, that the Monod growth kinetic model given above is valid only when microbial growth is limited by a single substrate. For many years the Monod equation was recognized as “fundamentally true” and some microbiologist considered it one of the major laws, such as Newton’s mechanics, Coulomb’s laws of electricity, etc. In the 1950s, there were a few studies devoted to testing the Monod equation (Moser 1958; Contois 1959). These authors found that not all experimental data could be reasonably well fitted by Monod’s equation. A better fit could be obtained by using the following, entirely empirical, equations.

$$\text{Moser} \quad \mu = \mu_{\max} (1 + K_{sMZi} S^{-\lambda_i})^{-1} \quad (\text{Moser, 1958}) \quad (1.6)$$

$$\text{Contois} \quad \mu = \mu_{\max} \frac{S_i}{B_i X + S_i} \quad (\text{Contois, 1959}) \quad (1.7)$$

Another model used in the literature was developed by Tessier (1942) by hypothesizing that the dependence of specific growth rate on the substrate concentration was proportional to the difference between μ and $\mu_{\max}$:

$$\frac{d\mu}{dS_i} = \frac{1}{K_{sT}} (\mu_{\max} - \mu) \quad (1.8)$$

Equation 1.8, when integrated, gives the well-known form of the Tessier Equation (Equation 1.9).

$$\text{Tessier} \quad \mu = \mu_{\max} (1 - e^{-S_i/K_{sT}}) \quad (\text{Tessier, 1942}) \quad (1.9)$$

Equations 1.5, 1.6, 1.7, and 1.9 are general growth kinetics equations to describe single substrate limited growth. Since for the present study I am interested

only in saturation type of kinetics, I did not include any inhibition type of growth kinetics.

Multiple Substrate Limited Growth

When microbial growth is limited by more than one substrate, three forms of multiple substrate growth kinetics can be considered (Bailey and Ollis 1986; Shuler and Kargi 1992):

(1) An interactive or multiplicative form,

$$\mu/\mu_{\max} = [\mu(S_1)] [\mu(S_2)] \dots \dots [\mu(S_i)] \quad (1.10)$$

(2) An additive form,

$$\mu/\mu_{\max} = [\mu(S_1) + \mu(S_2) + \dots \dots + \mu(S_i)]/i \quad (1.11)$$

(3) A non interactive form,

$$\mu/\mu_{\max} = \min [\mu(S_1) \text{ or } \mu(S_2) \text{ or } \dots \dots \text{ or } \mu(S_i)] \quad (1.12)$$

Substrate Utilization Rate

Substrate utilization rate in a batch chemostat is described as the variation of substrate concentration by time and it is given by Equation 1.13.

$$-\frac{dS}{dt} = \frac{1}{Y_{X/S}} \frac{dX}{dt} \quad (1.13)$$

Equation 1.13 was originally recommended by Monod (1949 and 1950); later Powell (1967) and Pirt (1965) added a maintenance term (Equation 1.14) to define substrate consumption rate because Equation 1.13 could not represent their experimental data:

$$-\frac{dS}{dt} = \frac{1}{Y_{x/s}} \frac{dX}{dt} + mX \quad (1.14)$$

Determination of Microbial Growth Kinetics

Microbial growth kinetic parameters are usually determined using a chemostat (Shuler and Kargi 1992) operated at batch or at steady state. Batch systems generally are not preferred because of difficulty in determining correct substrate concentration and specific growth rates. By operating a chemostat at steady state and at different dilution rates, one can calculate biokinetic parameters as described in Chapter 3. This is the correct and best way of determining growth kinetics; however, it requires considerable time and effort (Panikov 1995). In a chemostat operated at steady state, the specific growth rate is considered as an independent variable and it is equal to the dilution rate, D , as given by Equation 1.15.

$$\mu = D = Q/V \quad (1.15)$$

The term “growth kinetics” is used to refer any of the growth models described by Equations 1.5, 1.6, 1.7 and 1.9, and the term “biokinetic parameters” is used to refer the constants in these equations. I also used the term “biokinetic parameters” to describe a group of constants used to describe microbial growth and substrate utilization as presented in chapter 4.

Leptothrix discophora SP-6

Leptothrix discophora SP-6 is a member of the *Sphaerotilus Leptothrix* group, which are manganese and iron oxidizing sheathed bacteria that thrive in iron- and manganese-rich environments such as iron seeps, swamps, and springs (Emerson and Ghiorse, 1992). Although *Leptothrix discophora* is not a pathogen, it is considered a nuisance in water distribution systems because it oxidizes iron and manganese, causes color problems, and in extreme cases clog water distribution conduits. In the Center for Biofilm Engineering, the metabolic activity of biofilms of manganese oxidizing bacteria, *Leptothrix discophora* SP-6 and their effect on microbially influenced corrosion (MIC), specifically on pitting corrosion of stainless steels, have been extensively studied (Dickinson and Lewandowski, 1996; Dickinson et al., 1996; Olesen et al., 2000a,b). For that reason, I used *Leptothrix discophora* SP-6 as a model microorganism in my thesis. Figure 1.10 shows an SEM picture of *Leptothrix discophora* SP-6 colony on a stainless steel surface. Similarly, Figure 1.11 illustrates a single *Leptothrix discophora* SP-6 on a stainless steel surface.

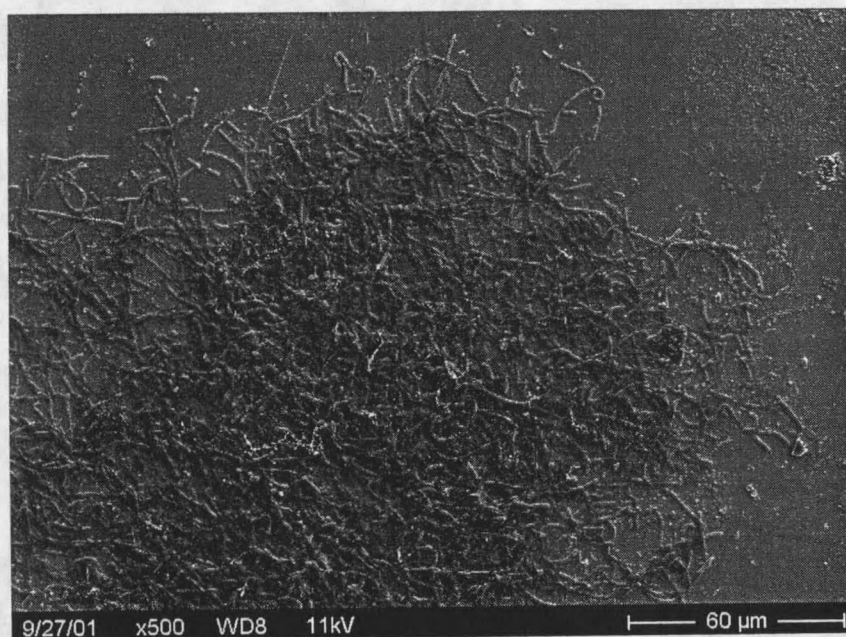


Figure 1.10. An SEM picture of *Leptothrix discophora* SP-6 colony on stainless steel surface.

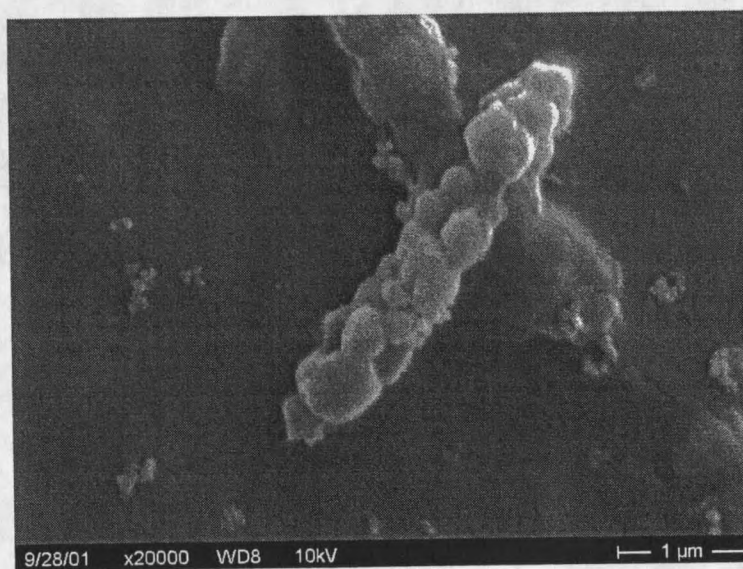


Figure 1.11. An SEM picture illustrating a single *Leptothrix discophora* SP-6.

Growth Kinetics of *Leptothrix discophora* SP-6

To construct mathematical models of microbially induced corrosion caused by manganese oxidizing bacteria it is important to evaluate two factors: (1) growth kinetics of the microorganisms involved and (2) rate of manganese oxide deposition in biofilms deposited on metal surfaces. However, studying all these factors was difficult. For that reason, I focused on the first factor and studied the growth kinetics of *Leptothrix discophora* SP-6 with the following hypothesis.

Hypothesis 3. *The statistically optimum model to represent Leptothrix discophora SP-6 growth in the planktonic form can be determined by an algorithm, developed by the author, which optimizes parameters for the four major kinetic models.*

To address hypothesis 3, my specific goals were 1) operate a chemostat at steady state and measure substrate and microorganism concentrations, 2) test equations 1.5, 1.6, 1.7 and 1.9 in representing experimental data, 3) find the best representative equation, 4) calculate biokinetic parameters, along with standard deviations, using Monte Carlo simulations.

Biofilm Growth

Microbial growth in biofilms is similar to microbial growth for planktonic microorganisms. However, generally attached cell number per unit surface area, on

attached biomass or surface coverage of biomass, are used to describe biofilm growth.

The progression of biofilm accumulation is schematically presented in Figure 1.12.

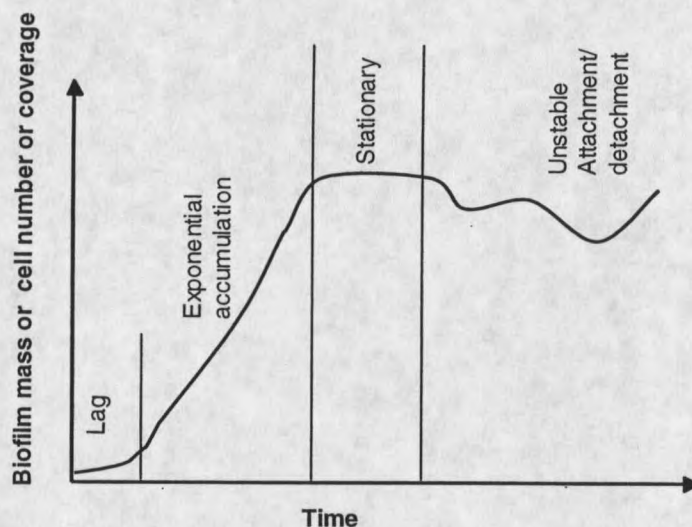


Figure 1.12. The progression of biofilm accumulation.

When a biofilm reactor such as a flat plate flow cell (Lewandowski et al., 1999) is inoculated with a seed microorganism (inocula), microorganisms attach to the reactor surfaces. The *lag phase* occurs immediately after attachment. During this time microorganisms adapt to the surface. The *exponential accumulation phase* is also known as logarithmic growth phase. In this phase, the microorganisms on the surface grow exponentially and increase cell number or total biomass or surface coverage. The *stationary phase* starts at the end of exponential accumulation phase. In this phase the biofilm thickness or total biomass or total number of cells reaches to a constant value. The *unstable phase* follows the stationary phase and in this phase,

there are detachments from the biofilm, attachments to the biofilm and biofilm growth.

The biofilm growth up to stationary phase was experimentally examined by Lewandowski et al., (1999), and later the reproducibility of biofilm growth up to the stationary phase was experimentally presented by Jackson et al., (2001).

Microbial Growth in Biofilms

The microbial growth in biofilms is described using exactly the same growth models used for planktonic microorganisms. Biokinetic parameters for different microorganisms and substrate are well studied and presented in the literature for planktonic growth (see previous section). Most authors have used biokinetic parameters obtained from planktonic culture experiments to describe microbial growth in biofilms (Kreft and Wimpenny 2001; Eberl et al., 2000; Noguera et al., 1999; Beyenal et al., 1997; Bakke et al., 1989). Because the physiology and species composition of biofilm systems are expected to be different than suspended growth systems, it is not proven that biokinetic parameters from suspended growth systems are accurate predictors of attached growth systems (Grady et al., 1996; Van Loosdrecht et al., 1990).

There are some studies which used biofilm grown cultures to calculate biokinetic parameters, but they measured the biokinetics after disruption of the biofilm structure and then treated the system as a pseudo suspended growth culture (Cao and Alaerts, 1995; Jih and Huang, 1994). However, it is unclear that biokinetic parameters are not affected by disruption of this biofilm structure. I believe

destruction of EPS will affect the biokinetic parameters because there is not a biofilm after destruction.

Several methods have been developed to measure biokinetic parameters from cultures within biofilms. These are presented below.

Bakke et al., (1984) calculated Monod growth kinetic constants using bulk substrate concentrations for steady state biofilms. They concluded that *P. aeruginosa* does not behave differently in a suitable medium, temperature and pH in biofilms than in planktonic cultures.

Rittmann et al., (1986) measured reactor influent and effluent substrate concentrations, then by normalizing these data they defined a series of design curves. Due to the inherent error in the sample analysis and visual comparison, it appears difficult to achieve accurate matches between the design curves and the experimental data (see Rittmann et al., 1986 and Riefner et al., 1998). Thus, the accuracy of the retrieved biokinetic parameters is uncertain.

Nguyen and Shieh, (1995) developed a batch method for determining biokinetic parameters in fluidized bed reactors. In this approach, mass transfer limitations are lumped into the model along with the biokinetic parameters and are not accounted for explicitly. Since biokinetic parameters are not calculated independently, the reliability of the calculated biokinetic parameters were questioned. These values depended on correct estimation of mass transfer parameters, such as diffusivity and external mass transfer coefficient (Riefner et al., 1998).

Zhang and Huck (1996) developed a technique to determine biokinetic and substrate diffusion coefficients in water treatment biofilm systems. Basically they

operated their reactor at different steady states and used steady-state biofilm equations developed by Rittmann and McCarty (1980). They determined the diffusion coefficient for substrate in water, the minimum substrate concentration to maintain a steady-state biofilm, and the half-saturation coefficient. Because of the assumptions used in the model, the sensitivity of measurements limited final accuracy of their approach.

Samb et al., (1998), Reifler et al., (1998) and Cheton et al., (2001) used a respirometric technique, integrated with a unsteady state diffusion + reaction model, and predicted Monod growth constants. However, these authors did not verify the assumptions used in their models.

In summary, the techniques presented above are limited. First, because some of these studies rely on measuring concentrations at steady-state conditions, long time periods are needed to bring the systems back to steady state between tests. These studies defined steady state in terms of substrate concentration, however, none of them considered biofilm structure dynamics. It is expected that the biofilm structure will not reach steady state (Figure 1.12) although substrate concentrations may reach pseudo steady state. Second, the techniques using previously developed biofilm models suffer from the accuracy of parameters (not directly measured) in the models such as effective diffusivity, biofilm density, external mass transfer coefficient, etc. Most of the biofilms grown under liquid flow have heterogeneous structure and these parameters show local variability (Yang and Lewandowski 1995). None of the above studies considered such biofilm structure in their model. A third problem is that none of these studies verified their results (not tested under different operation conditions).

The developed models predict biokinetic coefficients using a fitting technique. The iterations continue until experimentally measured bulk concentration and/or flux mathematically are calculated. All these models somehow predict substrate concentration profiles in biofilms, but predicted and experimental concentration profiles were never compared!

To overcome these problems, first we need to examine what happens to substrates in the biofilm. Substrates form concentration profiles in biofilms and substrate concentration decreases toward the bottom, because of mass transfer limitations and microbial consumption. For that reason bulk concentrations cannot represent true substrate concentrations in biofilms and it is important to use biokinetic parameters and concentrations directly determined in biofilms. This can be accomplished only by using concentration profiles from microelectrode measurements in biofilms.

Lewandowski et al., (1991) noticed the problems explained above and developed a method to calculate biokinetics parameters for biofilms. They exemplified the method by calculating biokinetic parameters from dissolved oxygen concentration profiles measured across a biofilm. The technique, however, was applicable to only the Monod kinetics and the computational procedures employed were performing well only at low dissolved oxygen concentrations, near the half saturation concentration for oxygen.

In other reports, addressing similar problems, Berg et al., (1998) developed a method of predicting substrate concentration profiles from reaction rates in different zones in the sediments. By increasing the number of reaction zones, they successfully

predicted the concentration profiles of NO_3^- , O_2 , using Nelder and Mead 's (1965) down hill simplex method. Even though the application of this method to biofilms is difficult, the authors showed that the simplex method could be used to predict substrate concentration profiles, and their approach could, in principle, be used in biofilms. Similarly, in Robinson (1985)'s review paper about determining microbial kinetic parameters using nonlinear regression analysis, he mentioned that simplex algorithm is superior to Gaussian methods. Many of the techniques used in nonlinear regression, such as Levenberg-Marquart method, are modified versions of the Gaussian method (Robinson 1985).

Visser et al., (2002), also at the Center for Biofilm Engineering, used the Levenberg-Marquart algorithm along with least squares estimates to find biokinetic parameters by minimizing the least squared differences between experimental data and the model prediction. The authors used MATLAB's *leastsq routine* (in version 6.1 it is called *lsqnonlin*), which requires good guesses of the initial values, and for these reason Visser et al., (2002) had to define a method to select initial values to reduce the number of iterations. Moreover, their method did not allow predicting concentrations at *exactly* the same location as obtained by microsensor measurement location. To overcome this problem they used an interpolation technique (the cubic spline interpolation option in MATLAB), which added unknown errors to the solution. Although this method has the problems stated above, it might be used for the purpose of quantifying biokinetic parameters in biofilms. However, this method and computer program is not generally available.

The quantification of biokinetic parameters from microelectrode data is possible from the solution of the diffusion reaction model of biofilm. The steady state diffusion-reaction equation, including a maintenance factor through, a biofilm in one dimension is given by the following nonlinear differential equation:

$$D_{\text{eff}} \frac{d^2S}{dx^2} = \frac{\mu X_f}{Y_{x/o}} + m X_f \quad (1.16)$$

The following assumptions were made with respect to equation 1.16.

1. The growth limiting substrate is oxygen.
2. The biofilm is uniform.
3. The substrates in the biofilm are transported only via diffusion and described according to Fick's law .
4. The biofilm density and diffusivity can be represented by their average values.
5. Mass transfer of oxygen is one-dimensional only.
6. The biofilm (in terms of oxygen consumption rate) is at pseudo steady state.

It is also assumed, the specific growth rate, μ , can be described by one of growth models (Equations 1.5, 1.6, 1.7, or 1.9). None of the previous studies were tested to calculate biokinetics parameters for growth models other than Monod, and did not include maintenance factor for substrate consumption. The biokinetic parameters for growth models other than Monod, including a maintenance factor for substrate consumption, can be calculated using the following hypothesis:

Hypothesis 4. The statistically optimum model to represent *Leptothrix discophora* SP-6 growth in the biofilm form can be determined by an algorithm, developed by the author, which optimizes parameters.

To address this hypothesis, I had the following specific research goals: 1) Produce experimental data from which biokinetic parameters could be calculated. 2) Verify the assumptions stated above. 3) Develop a superior method to calculate biokinetic parameters. 4) Test the different biokinetic models and find the best representative model. For these purposes, I constructed dissolved oxygen microelectrodes and measured oxygen concentration profiles in repeated experiments. I used a specifically designed biofilm system which satisfied the assumptions used in the model.

Hypothesis 5. The physiology and species composition of biofilm systems are expected to be different than suspended growth systems. It has not been proven experimentally that biokinetic parameters from suspended growth systems are accurate predictors of attached growth systems (Grady et al., 1996; Van Loosdrecht et al., 1990). To test this expectation experimentally, I hypothesized that;

Leptothrix discophora SP-6 has different growth and substrate consumption kinetics in planktonic and biofilm form.

To address hypothesis 5, I compared microbial growth models and corresponding parameters for planktonic and biofilm systems as determined from hypotheses 4 and 5. The hypothesis is addressed in chapter 4.

Experimental Methods

Passive Film Examination

Stainless Steel Coupon Preparation. Type 316S stainless steel coupons (1.6 cm outer diameter) were punched from 1 mm sheet metal (composition given in Table 1.1). Coupons were epoxy embedded in polycarbonate holders using a slow hardening epoxy (Buehler Epoxide). Figure 1.13 illustrates a mounted steel coupon. The holders were constructed from a hollow polycarbonate tube approximately 10 cm long and 0.9 cm inner, and 1.9 cm outer diameter, respectively. Each application of epoxy/glue was allowed 24 hours to harden. All the coupons were polished using 240, 360, 400 and 600 grit size sand papers, followed by fine polishing with 5.0-, 0.3-, and 0.05-micron Al_2O_3 particles placed on micro cloths. Following the mounting on the polycarbonate coupon holders, the electrical connections were made by attaching a copper wire (spring) to the back of each coupon. The contact resistances of the coupons were measured to make sure that the values were less than 1 ohm. The coupons were sterilized in ethyl alcohol solution under the biohood for three hours before placing in sterilized growth medium.

Table 1.1. The composition of 316L stainless steel used in this study (% w/w).

Fe	Cr	Ni	Mo	Mn	Si	P	N	C	S
69.3	16.4	10.1	2.10	1.71	0.39	0.03	0.03	0.01	0.00
4	9	9				4		7	1

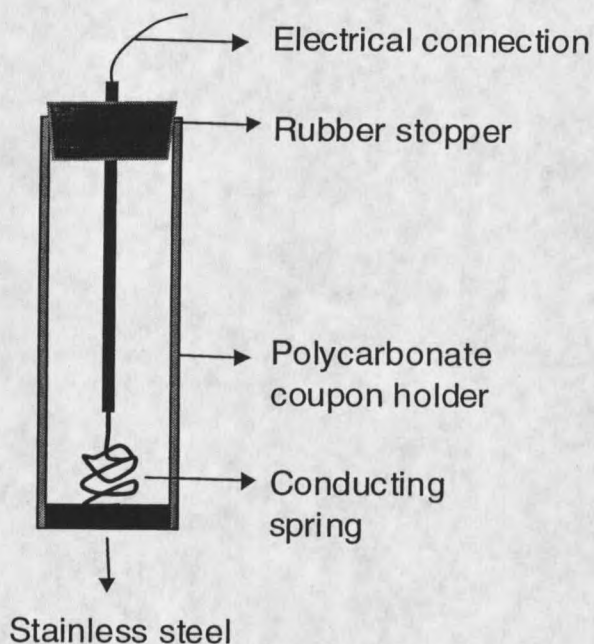


Figure 1.13. Polycarbonate coupon holder and accessories.

Ennoblement Experiments. A stock culture of manganese oxidizing bacteria *Leptothrix discophora* SP-6 was obtained from American Type Culture Collection (ATCC no. 51168). The organisms were grown in a mineral-salt-pyruvate-vitamin (MSPV) medium (ATCC no. 1917). The composition of the MSVP medium is given in Table 1.2. The organisms were centrifuged and re-suspended in MSVP medium containing 20% glycerol and preserved in a freezer at -70°C . These stock cultures were used to inoculate the reactor.

Table 1.2. ATCC Culture Medium 1917 MSVP for *Leptothrix discophora* SP-6.

(NH ₄) ₂ SO ₄	0.24 g
MgSO ₄ ·7H ₂ O	0.06 g
CaCl ₂ ·2H ₂ O	0.06 g
KH ₂ PO ₄	0.02 g
Na ₂ HPO ₄ ·7H ₂ O	0.06 g
HEPES	2.383 g
FeSO ₄ 10 mM	1.0 mL
Distilled water	984 mL

Adjust to pH 7.2 with NaOH or H₂SO₄. Autoclave at 121°C for 15 minutes. Cool to approximately 50°C and aseptically add 1.0 mL of the following filter-sterilized solutions:

20% Sodium pyruvate

Vitamin solution (see below)

Biotin	20.0 mg
Folic acid	20.0 mg
Thiamine HCl	50.0 mg
D-(+) -calcium pantothenate	50.0 mg
Vitamin B12	1.0 mg
Riboflavin	50.0 mg
Nicotinic acid	50.0 mg
Pyridoxine HCl	100.0 mg
p-Aminobenzoic acid	50.0 mg
Distilled water to	1.0 L

The coupons, prepared as described above (up to 8 coupons) were then placed into the autoclaved (1 atm, 30 minutes) polycarbonate reactor with 500 mL- solution containing MSVP medium, but excluding FeSO₄ (FeSO₄ is only used in stock culture preparations). Manganese was added as MnSO₄ (0.2 mM) using a sterile syringe filter. The reactor (Figure 1.14) was 11 cm inner diameter and 10 cm high. The reactor was continuously aerated via a glass tube. The inlet air and outlet air were filter sterilized. A glass tube (1 mm diameter) with a porous glass tip was placed into

the reactor before autoclaving. We added 0.1 g/L autoclaved agar containing 0.1 M Na_2SO_4 into the glass tube which provided a salt bridge for reference electrode. The reference electrode was placed at the end of agar layer immersed into the saturated KCl solution. This method of reference electrode positioning was recommended by Olesen (2000a,b) because direct insertion of a reference electrode into the medium caused Cl^- ion leakage into the solution. We used Saturated Calomel Electrode (SCE) as the reference electrode.

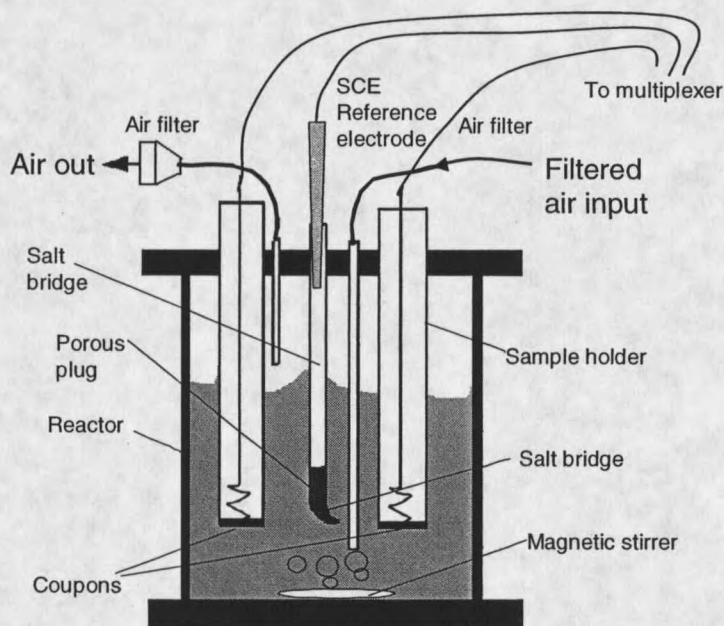


Figure 1.14. The reactor used to ennoblement experiments.

Potentials between SCE and coupons were measured hourly by a data acquisition system (Hewlett Packard 3497A multiplexer connected to a computer) against an SCE. A photograph of reactor and data acquisition system is shown in

Figure 1.15. After at least 6-7 days, the OCP reaches a stable reading, $\sim +350 \text{ mV}_{\text{SCE}}$. At this time the coupons are considered ennobled and are removed from the reactor and taken for analysis immediately.

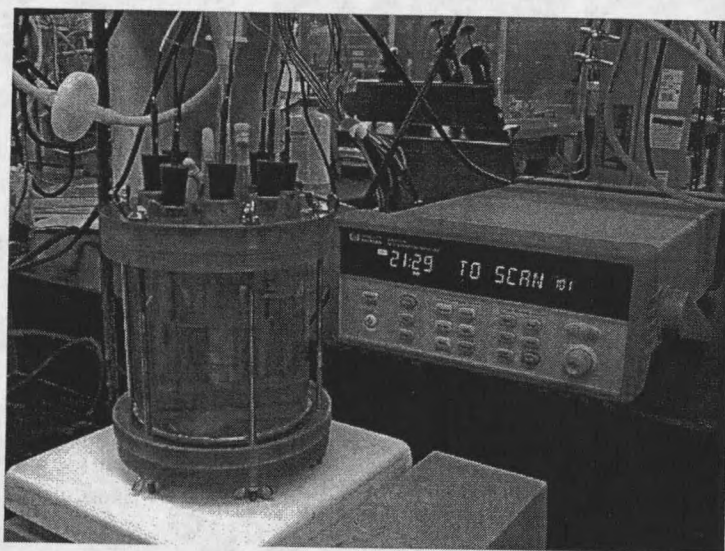


Figure 1.15. A photograph of the reactor and data acquisition system.

Coupon Classifications for Different Experiments. All the coupons were first subjected to the polishing and cleaning procedure described above. The XPS, ToFSIMS and anodic polarization experiments were conducted on five groups of coupons, four of which served as controls. The first group of coupons was subjected to a series of *Leptothrix discophora* SP-6 induced ennoblement processes as described above. These coupons are called as “*ennobled*”. One of the four groups of control coupons was composed of *as-received* coupons, subjected only to the polishing and cleaning procedure described above. The second group of control coupons was immersed into pure sterile water for the same period of time as the other

control coupons. The XPS and ToFSIMS experiments conducted on the two groups of coupons described above serve as the background measurements with respect to which other results were compared. The third group of control coupons was treated with sterile MSVP and a 0.2 mM Mn^{2+} solution without any bacteria (herein termed *growth medium treated coupon*) in order to determine the effect, if any, of the growth medium on the stainless steel passive film. The fourth group of control coupons was exposed to MSVP and the bacteria solution without any Mn^{2+} (called *biofilm treated coupons*) in order to determine the effect of bacteria on the passive film without the Mn^{2+} ions in the medium. Prior to XPS and ToFSIMS analysis, coupons were wiped or rinsed, as explained below in detail, and then the coupons were removed from the polycarbonate holders mechanically, making sure that no epoxy was left on back of the coupons before inserting them in the UHV chamber.

Anodic polarization experiments were performed using as-received, biofilm treated and ennobled coupons.

XPS Analysis. XPS experiments were performed using a PHI Model 5600ci MultiTechnique system located in ICAL laboratory, Physics, MSU. A monochromatized Al K alpha (1486.6 eV) x-ray source was used. An 800-micron diameter equivalent area of the sample surface was analyzed while a $3 \times 3 \text{ mm}^2$ area was sputtered using Ar^+ ions for each cycle of depth profiling. In the sputtering process, Ar^+ ions, accelerated to $\sim 2 \text{ keV}$ at $0.25 \text{ }\mu\text{A}$ current, were used to bombard the sample surface for selected time intervals ($\sim 0.5 \text{ min}$) until Fe and Cr oxides were removed and metallic Fe and Cr (bulk SS) were shown to be present. Following each

cycle of sputtering, multiplex XPS spectra of selected core level emissions were taken to determine the depth profiles of Mn, Fe, C, O and Ni for each of the control and the ennobled coupons. Ni depth profile was omitted in the figures because of low Ni concentration in the passive film and because no variation with any treatment was observed in the Ni profile.

ToFSIMS Experiments. A Phi-Evan's Trift-I system, located in the ICAL laboratory, Physics, MSU, was used for the ToFSIMS experiments. The system consists of a liquid metal (Ga^+) ion gun for obtaining high mass and high spatial resolutions for chemical imaging. In these experiments 25 keV Ga^+ primary ions were used to generate secondary ions. These ions are separated and detected by a triple-pass 90° spherical sector electrostatic mass analyzer. The output is a high-resolution mass spectrum plotted as a function of intensity vs. mass-to-charge ratio. For chemical maps a 256×256 pixel area (typically corresponding to a 120×120 square micron size) with each pixel containing the full mass spectrum was stored in the computer media as raw files. Chemical maps of Fe, Cr and Mn (Ni was omitted for the same reason as explained above) were extracted retrospectively following the raw data acquisition.

Measurement of Pitting Corrosion. Anodic polarization experiments were conducted using a potentiostat/galvanostat (EG&G Princeton Applied Research, model 273A) with two graphite electrodes and an SCE as reference placed into an electrochemical cell (Figure 1.16). The 352 SoftCorr III corrosion measurement

software (EG&G, Princeton Applied Research) was used to set up anodic polarization processes. OCPs were measured before each experiment, versus the SCE using a handheld multi meter (Wavetek DM23XT, internal resistance 10m Ω).

Pitting potentials of the 316L stainless steel coupons were obtained through potentiodynamic polarization. Scans were initiated 10 mV below the OCP and scanned, using a scan rate of 5mV/s, to 0.8 V_{SCE} or 1mA, whichever was reached first. Experiments were conducted at room temperature in 0.1M Na₂SO₄ solutions with NaCl at different concentrations.

Anodic polarization is mostly used to understand corrosion behavior of metals (Silverman 1998). The anodic polarization technique requires a three electrode system: 1) a working electrode: the metal to be tested, 2) a counter electrode: platinum or graphite are generally used ones, and 3) a reference electrode. These three electrodes are connected to a potentiostat. The potentiostat is used 1) to apply potential between working electrode and reference electrode, 2) to measure current between counter electrode and working electrode, 3) to set a range of potential to be tested, and 4) to set the scan rate (or the rate potential variation by time). The electrochemical cell used in anodic polarization experiment is given in Figure 1.16. A computer is used to control the potentiostat.

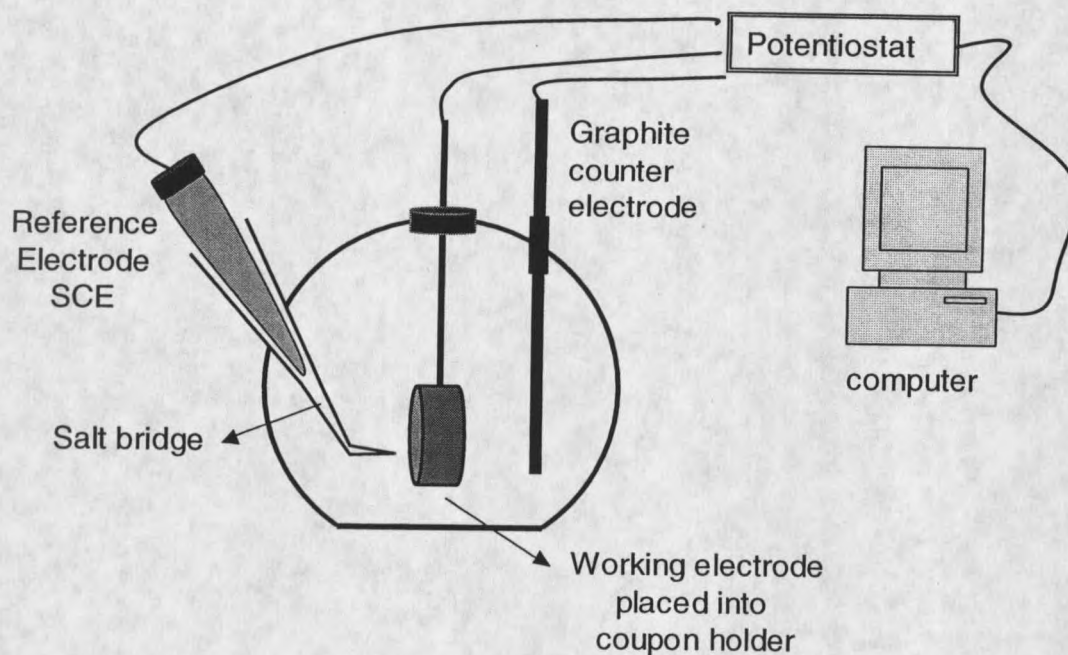


Figure 1.16. The electrochemical cell used for anodic polarization consisted of three electrodes, a potentiometer, and a computer.

The test coupons were placed into a specifically designed coupon holder. The electrochemical cell is filled with the electrolyte solution depending on the measurement.

For a stainless steel coupon used in this study a typical anodic polarization curve is presented in Figure 1.17.

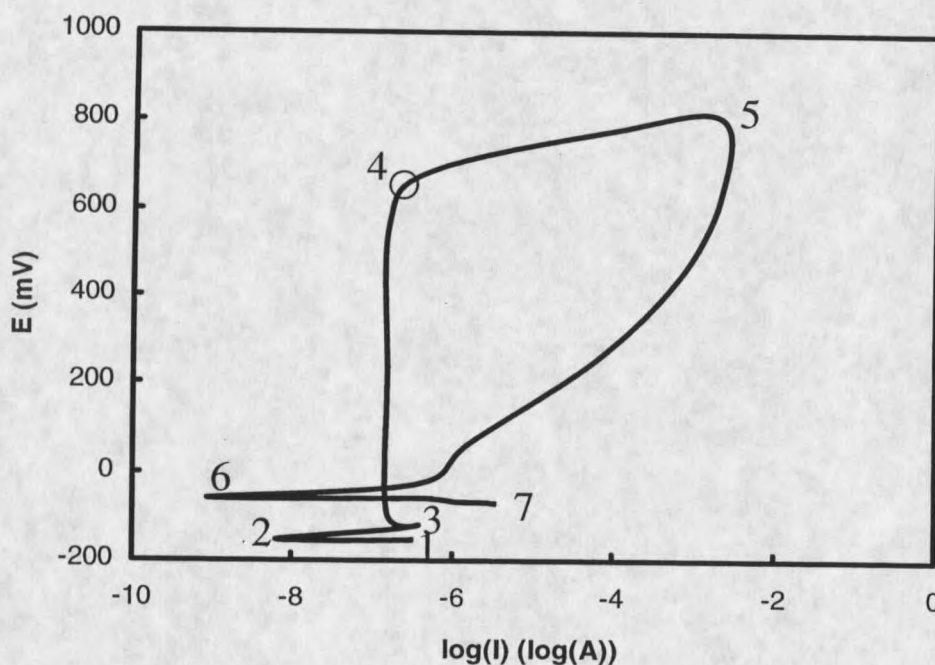


Figure 1.17. A typical anodic polarization curve.

The y axis is logarithm of the measured current between working electrode and counter electrode with a unit of ampere. The point 1 is corrosion potential or OCP of the stainless steel sample. Potential is applied from the point 1. From points 1 to 2, the current decreases with slightly increased potential because of the proximity of the metal's OCP. The point 2 is OCP of the stainless steel, since OCP measured in the medium the potentials at points 1 and 2 are almost the same ($\pm 10\text{mV}$). Between points 2 and 3, the current increases with increased potential, and metal begins to corrode like an active metal until the potential reaches a critical value where the current becomes almost constant (point 3). At this stage, the current does not change with increased potential (which shows passive film formation) until point 4. The

oxide layer prevents diffusion of chloride ions through passive layer. With increased potential the passive film breaks down at the point 4 and suddenly current starts to increase significantly. The potential at point 4 is called the pitting potential, as the passive film breaks down and ions attack metals. The pitting potential is the minimum potential at which active pitting will began. Any potential above the pitting potential will open active pits on metal's surface. The point 5 is end of the forward scan, and is set by the user. After point 5 the backward scan is started. If the passive film was not compromised, the backward scan will pass the left side of forward scan, indicating increased resistance to corrosion by increased passive film thickness. If the passive film has been compromised, the backward scan will fall on the right side of the forward scan, as seen in Figure 1.17. As the potential continues decreasing, the metal will repassivate by forming oxide layer and will lower the current. The point 6 represents the repassivation potential. The repassivation potential shows the potential at which initiation of pit may begin. The point 7 is the end of the cycle.

The pitting potential measured from anodic polarization curves are functions of temperature, scan rate, pH, mixing rate, and chloride concentration. For these reasons, the same conditions for anodic polarization experiments were maintained throughout. All anodic polarization experiments were performed at room temperature (25 °C) and repeated at least four times to produce a representative data (Burststein and Ilevbare 1996).

Planktonic Growth Kinetic Measurements

Chemostat. The microorganisms were grown in a New Brunswick (BioFlo 2000) chemostat with a working volume of 2 L, equipped with pH, agitation, and temperature controllers as shown Figure 1.18. The sensitivities of the control units for pH, temperature and agitation rate were respectively: 0.1 unit, 0.1 °C, and ± 1 rpm. Prior to use, the chemostat, including growth medium, filters, and tubings, were autoclaved for 30 minutes at 121°C. The pH was controlled by pumping solutions of 0.2 N NaOH and 0.2 N H₂SO₄. The solution in the chemostat was stirred by a blade type double impeller at 350 rpm. The dissolved oxygen electrode is calibrated by saturating with air and sparging the reactor with nitrogen (zero oxygen). The dissolved oxygen concentration was controlled by sparging filtered air or filtered mixtures of air + pure oxygen or air + nitrogen in various proportions, depending on the needs, at the flow rates between 1 L/h and 5 L/h.

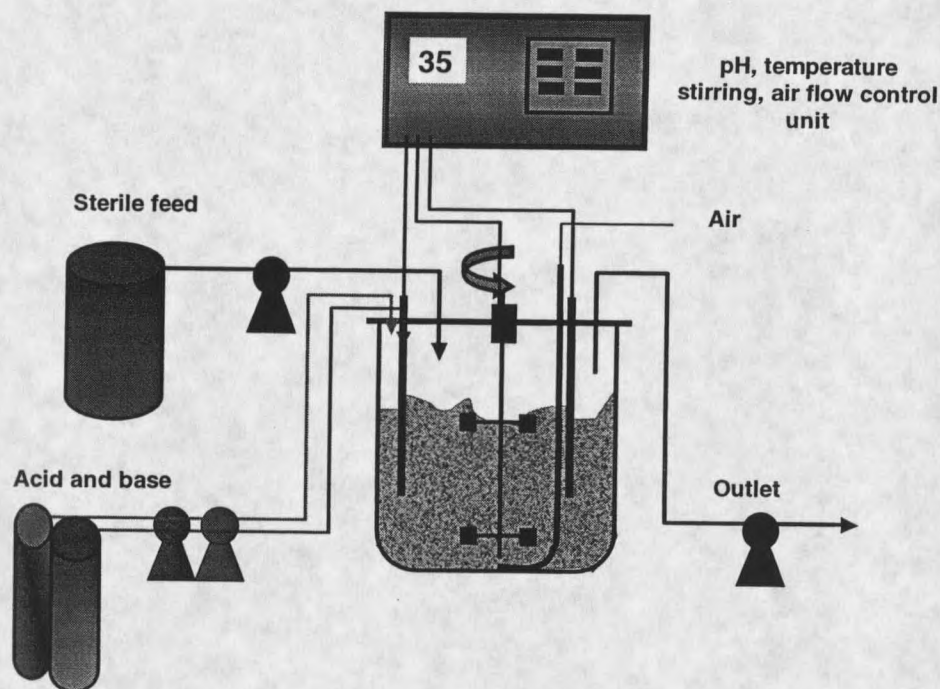


Figure 1.18. Schematic diagram of chemostat used in this study.

Biofilm Growth Kinetic Measurements

Colony Biofilms. The colony biofilms of *Leptothrix discophora* SP-6 were prepared according to the procedure described by Anderl et al., (2000). Forty –eight hour old cultures of *Leptothrix discophora* were diluted to an optical density of 0.02 at 600 nm (using 1-cm path length) in growth medium. Ten μL of diluted culture was used to inoculate one sterile, polycarbonate membrane filter (25 mm ID, 0.2 micron pore size) lying on agar plate. The membrane filters were sterilized by UV light for 15 min for each side prior to inoculation. The Petri plates were kept at room

temperature and the membrane filters were transferred to fresh agar plates every 12 h. The growth substrates were supplied by the agar, and transferred through the membrane to the colony biofilm. The oxygen required for growth was transported from air to the colony, and all substrates were transported via diffusion. A schematic diagram of colony biofilm is presented in Figure 1.19.

To grow the biofilms, we used ATCC Culture 1917 MSVP for *Leptothrix discophora* SP-6 (American Type Culture Collection Catalogue, 1992). It consisted of 0.24 g $(\text{NH}_4)_2\text{SO}_4$, 0.06 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.06 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.02 g KH_2PO_4 , 0.06 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, and 2.383 g HEPES in 950 mL distilled water. The pH of this medium was adjusted to 7.2 with NaOH or H_2SO_4 . We added 15 g Agar Noble (Difco 214230) and autoclaved this solution at 121 °C for 15 minutes (for 1 L volume of the solution); after cooling it to approximately 50 °C, we added 1 mL vitamin solution (see below), 1 mL FeSO_4 (10 mM) and 48 mL of pyruvate solution (at different concentrations) aseptically by filter-sterilizing (0.2 micron sterile syringe filter, Corning, 431219). One L of vitamin solution consisted of 20 mg Biotin, 20 mg Folic acid, 50 mg Thiamine HCl, 50 mg D-(+) -calcium pantothenate, 1 mg Vitamin B12, 50 mg Riboflavin, 50 mg Nicotinic acid, 50 mg Pyridoxine HCl, 50 mg p-Aminobenzoic acid. After adding vitamin solution, we poured this solution into sterilized petri dishes and kept these dishes in a clean and cold place.

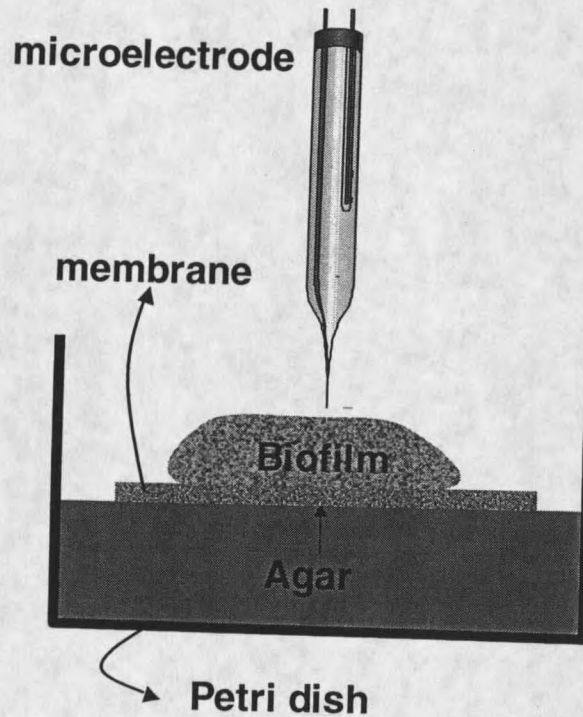


Figure 1.19. Diagram of a colony biofilm grown on top of a membrane filter. The growth substrate is supplied by the agar and transferred through the membrane to the biofilm. The microorganisms receive oxygen from the air.

Dissolved Oxygen Microelectrode Measurements. Dissolved oxygen concentration profiles were measured using Clark type microelectrodes with a guard cathode, as described by Revsbech, (1986). Details of the construction process is presented in the Center for Biofilm Engineering microsensors workshop materials (Microsensors – Workshop, Center for Biofilm Engineering, August 2001). The microelectrodes had tip diameters less than $10\ \mu\text{m}$ to prevent damaging the biofilm structure during measurements. The oxygen concentrations were measured at $5\ \mu\text{m}$ intervals. The response time of the microelectrodes was less than 0.3 s. The

microelectrodes were calibrated using air and pure nitrogen gases in tap water. The calibrations before and after the measurements were within the range of acceptable experimental error. Figure 1.20 shows schematic diagram of a dissolved oxygen sensor used in this study.

To measure dissolved oxygen concentration, a -0.8 V is applied between the cathode (Pt) and anode (Ag/AgCl wire) and the current is measured between them. A HP 4140B pA meter / DC voltage source was used to measure the current. The measured current is directly proportional to the oxygen concentration in the vicinity of the microelectrode tip. The data is collected and the microelectrode can be moved simultaneously using a custom written software package known as Vscan.

Microelectrodes were mounted on a micromanipulator (Model M3301L, World Precision Instruments, New Haven, CT) equipped with a stepper motor (Model 18503, Oriel, Stratford, CT) controlled by the Oriel Model 20010 interface. The microelectrode was introduced to the biofilm from the top perpendicular to the biofilm. The stepper motor was interfaced with a computer, and the microelectrode movement was handled by a controller (CTC-283-3, Micro Kinetics) with a positioning precision of 0.1 μm . Custom software was used to control and to coordinate microelectrode movement and the data acquisition. The position of the microelectrode was monitored using a stereo microscope (Leica Stereo Zoom 7) at 40 – 70 times magnification.

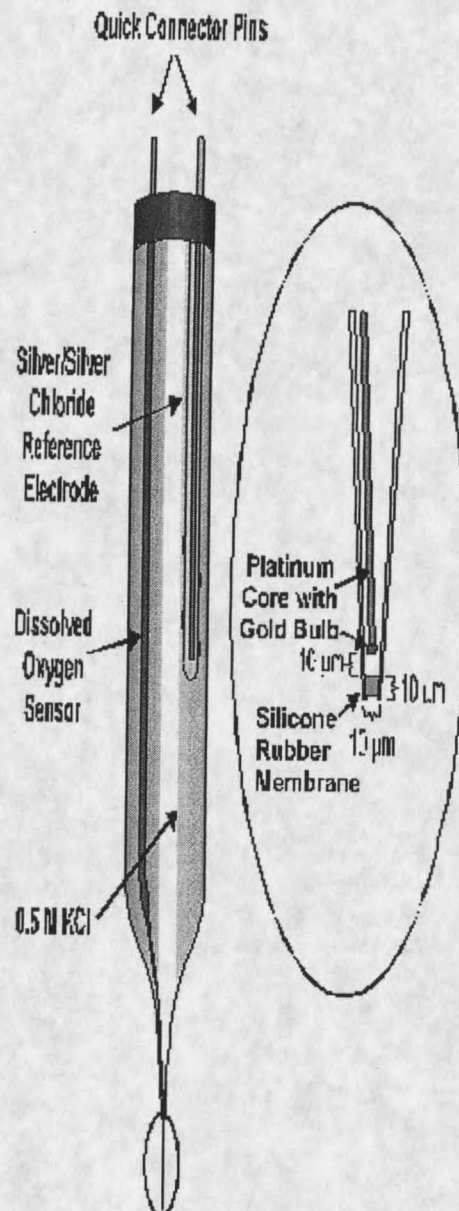


Figure 1.20. Schematic diagram of a Clark type dissolved oxygen sensor. The insert shows the position of the cathode, the reference electrode, and the silicone rubber membrane.

CHAPTER 2

BIOMINERALIZED MANGANESE DEGRADES PASSIVE FILMS AND
LOWERS PITTING POTENTIAL OF STAINLESS STEEL 316L

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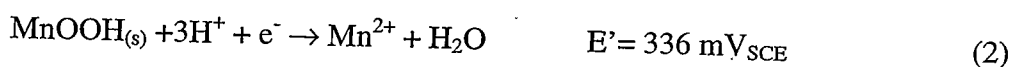
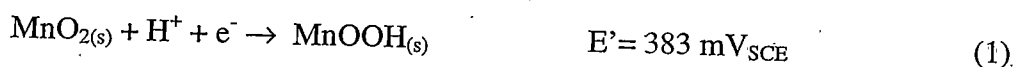
Abstract

The effect of ennoblement of 316L stainless steel by biomineralized manganese deposits on chemistry of passive films was studied using surface-sensitive analytical techniques and cyclic polarization. Under well-defined laboratory conditions, stainless steel coupons were ennobled to $\sim +350\text{mV}_{\text{SCE}}$ by biofilms of manganese-oxidizing bacterium *Leptothrix discophora* SP-6. Passive films on the ennobled coupons were analyzed by x-ray photoelectron spectroscopy (XPS) and time-of-flight secondary ion mass spectroscopy (ToFSIMS). From the XPS depth profiles of Fe, Cr, O, Ni, C and Mn, we evaluated thickness of the passive layers before and after ennoblement, while from the ToFSIMS depth profiles, spatial distribution of Mn, Cr, Fe, and Ni on the metal surface were evaluated. Because the ennobled coupons were covered with biomineralized deposits, the deposits were removed by sputtering in ultrahigh vacuum (UHV) before probing the chemistry of the underlying passive layers. The results show that oxide layers on the ennobled coupons are thinner than those on the pre-ennobled coupons. Pitting potentials in 0.2 M NaCl for as received, polished clean coupons and biofilm covered coupons (non-ennobled) were $573 \pm 67 \text{ mV}_{\text{SCE}}$ and $497 \pm 64 \text{ mV}_{\text{SCE}}$; in contrast the ennobled coupons pitted spontaneously in 0.2 M NaCl solution. We conclude that the biomineralized deposits degraded the quality of the passive film on stainless steel coupons by reducing its thickness and lowering the pitting potential.

Introduction

Numerous studies have reported that the open circuit potentials (OCP) of stainless steels immersed in natural waters shift spontaneously in the noble direction from $-200 \text{ mV}_{\text{SCE}}$ to $+350 \text{ mV}_{\text{SCE}}$. This process is known as *ennoblement* and may facilitate pitting corrosion in the presence of aggressive ions such as Cl^- even at low concentrations that do not cause pitting of non-ennobled coupons (Amaya and Miyuki 1997; Linhardt 1996; Suleiman et al., 1994; Felder 1990).

Several mechanisms have been proposed to explain ennoblement: They include enzymatic depolarization of the oxygen reduction reaction (Mollica et al., 1990 ; Scotto et al., 1985), strong acidification of the surface as a result of microbial activity in biofilms (Chandrasekaran and Dexter 1993; Little et al., 1990), combined effects of increased H_2O_2 concentrations and decreased pH, both biologically originated (Feron et al., (1997), and reduction of MnO_2 deposited by manganese-oxidizing microorganisms. This latter mechanism of ennoblement is the only one that has been verified experimentally in the laboratory as well as in the field (Braughton et al., 2001; Olesen et al., 2000; Linhart 1996; Dickinson and Lewandowski 1996). According to this mechanism, the microbiologically deposited MnO_2 is electrochemically reduced to MnOOH , and then further to Mn^{2+} . Reactions (1) and (2) illustrate this mechanism. Note that these reactions have the same electrode potential (E') as that of the 316L stainless steel coupons ennobled in the laboratory and in the field.



The net reaction is:



Where $\text{pH}=7.2$, $[\text{Mn}^{2+}] = 1 \times 10^{-6} \text{ M}$.

Unexpected pitting of microbially ennobled stainless steel has been reported. For example, Olesen et al., (2001) showed that 304L stainless steel coupons ennobled by the biomineralized deposits pitted at 0.35 % NaCl concentration, which is generally not sufficient to cause pitting. In another study, Neville and Hodgkiess (2000) correlated passive film composition with corrosion resistance and demonstrated that a thicker passive film makes stainless steel more corrosive resistant. Combining these two factors, we hypothesize that microbial ennoblement of stainless steel degrades the passive film and, therefore, can cause pitting of stainless steels immersed in low chloride solutions, e.g., fresh waters.

To test this hypothesis we have quantified the effect of ennoblement on the chemistry of passive layers on 316L stainless steel. Using coupons that are 1) polished as received, 2) ennobled, and 3) biofilm covered (but not ennobled), we measured pitting potentials and passive film thickness to relate the chemistry of passive layers to the susceptibility to corrosion.

To quantify the chemistry and thickness of passive films, we used XPS and ToFSIMS to determine the atomic concentration profiles of such elements as Fe, Cr, Ni, O and Mn with micron-scale resolution. Pitting potentials were measured in 0.2 M NaCl solution, as 316L stainless steel normally does not pit in 0.2 M NaCl. To enoble 316L stainless steel coupons, we used biofilms of manganese-oxidizing bacterium *Leptothrix discophora* SP-6. Measurements were repeated to determine average chemical composition of the passive films and average pitting potentials.

Materials and Methods

Ennoblement Experiments

A stock culture of MOB *L. discophora* SP-6 was obtained from the American Type Culture Collection (ATCC no. 51168). The organisms were grown in a mineral-salt-pyruvate-vitamin (MSPV) medium (ATCC no. 1917), Table 2.1. The organisms were harvested by centrifuging, re-suspended in the MSVP medium containing 20% glycerol, and stored in a freezer at -70°C . These stock cultures were used to inoculate the reactor, which contained 316L stainless steel coupons.

Table 2.1. ATCC Culture Medium 1917 MSVP for *Leptothrix discophora* SP-6.

(NH ₄) ₂ SO ₄	0.24 g
MgSO ₄ ·7H ₂ O	0.06 g
CaCl ₂ ·2H ₂ O	0.06 g
KH ₂ PO ₄	0.02 g
Na ₂ HPO ₄ ·7H ₂ O	0.06 g
HEPES	2.383 g
FeSO ₄ 10 mM	1.0 mL
Distilled water	984 mL

Adjust to pH 7.2 with NaOH or H₂SO₄. Autoclave at 121°C for 15 minutes. Cool to approximately 50°C and aseptically add 1.0 mL of the following filter-sterilized solutions:

20% Sodium pyruvate

Vitamin solution (see below)

Biotin	20.0 mg
Folic acid	20.0 mg
Thiamine HCl	50.0 mg
D-(+)-calcium pantothenate	50.0 mg
Vitamin B12	1.0 mg
Riboflavin	50.0 mg
Nicotinic acid	50.0 mg
Pyridoxine HCl	100.0 mg
p-Aminobenzoic acid	50.0 mg
Distilled water to	1.0 L

Type 316L stainless steel coupons (composition given in Table 2.2) were polished, sonicated in acetone, and mounted in holders made of polycarbonate tubes using a slow-hardening epoxy resin. The coupons, 1.6 cm in diameter, mounted in the polycarbonate coupon holders, were polished using 240, 360, 400 and 600 grit size sandpapers, followed by fine polishing with 5.0-, 0.3-, and 0.05-micron Al₂O₃ particles placed on microcloths. Electrical connections between the coupons and the external circuit were made by attaching a copper wire to the back of each coupon, within the holder, and extending the wire beyond the coupon holder. Contact resistances of the coupons were measured, and the coupons were accepted only if these values were less than 1 ohm. The coupons in their holders were sterilized in 95% ethyl alcohol under the biohood for three hours, and placed into the autoclaved polycarbonate 500 mL-reactor containing MSVP medium without FeSO₄ (FeSO₄ was

used only to preserve the stock culture). Manganese was added to the reactor as MnSO_4 (0.2 mM) using a sterile syringe filter. Potentials were measured hourly against the saturated calomel electrode (SCE). After 6-7 days, when the OCP stabilized at $\sim +350 \text{ mV}_{\text{SCE}}$, holders with the coupons were removed from the reactor and the coupons were subject to surface analysis. The coupons were divided into five groups depending on the treatment;

- Group I : Working coupons (ennobled)
- Group II : As received coupons
- Group III : Sterile DI water treated coupons
- Group IV : Growth medium treated coupons (MSVP and Mn^{2+})
- Group V : Biofilm treated coupons (MSVP and bacteria)

The working coupons (group I) were ennobled by the MOB, as described above. As received coupons (group II) were polished and cleaned by the procedure described above. Group III coupons were immersed in the sterile DI water for the same period of time as the other control coupons (6-7 days). Growth medium treated coupons (group IV) were exposed to sterile MSVP containing 0.2 mM Mn^{2+} to determine the effect of the growth medium on the composition of the passive film. Biofilm treated coupons (group V) were exposed to MSVP, growth medium inoculated with the bacteria but without Mn^{2+} , to determine the effect of the bacteria alone on the passive film composition. Prior to the XPS and ToFSIMS analyses, the

coupons were wiped or rinsed, then removed from the polycarbonate holders and immediately inserted into the UHV chamber.

Table 2.2. Composition (w/w) of 316L stainless steel.

Fe	Cr	Ni	Mo	Mn	Si	P	N	C	S
69.34	16.49	10.19	2.10	1.71	0.39	0.034	0.03	0.017	0.001

XPS analysis was conducted on samples prepared in two different ways: (1) coupon surfaces were rinsed with DI water and wiped completely with a cotton swab to remove the biomineral deposits, and (2) coupon surfaces were rinsed gently with DI water only, leaving the biomineral deposits on the surface. At least two different areas were analyzed on a given coupon. The motivation for removing the biofilms before analysis (preparation 1) is to determine whether or not substantial changes are introduced to the passive layer as a result of biofilm activity. Such a change usually manifests itself with increased passive layer thickness as compared with the controls (Olefjord et al., 1985). The motivation for leaving the biofilm intact (preparation 2) is to prevent repassivation in case the ennoblement reduces the film thickness and compromises the passive film in such a way that removal of biominerals and exposure to ambient conditions will simply cause the re-oxidation of the surface before one can determine the changes due to ennoblement.

XPS Analysis. XPS analysis was performed using a PHI Model 5600ci MultiTechnique system. A monochromatized Al K alpha (1486.6 eV) x-ray source was used. An 800-micron diameter equivalent area of the sample surface was analyzed while a $3 \times 3\text{-mm}^2$ area was sputtered using Ar^+ ions for each cycle of depth profiling. In the sputtering process, Ar^+ ions, accelerated to $\sim 2\text{ keV}$ at $0.25\text{ }\mu\text{A}$ current, were used to bombard the sample surface for selected time intervals ($\sim 0.5\text{ min}$) until Fe and Cr oxides were removed and metallic Fe and Cr (bulk SS) were reached. Following each cycle of sputtering, multiplex XPS spectra of selected core level emissions were taken to determine the depth profiles of Mn, Fe, C, O and Ni for each of the controls and the ennobled coupons. Ni depth profile was omitted in the figures because of low Ni concentration in the passive film and because no variation with any treatment was observed in the Ni profile.

ToFSIMS Analysis. A Phi-Evan's Trift-I system was used for the ToFSIMS analyses; this system consists of a liquid metal (Ga^+) ion gun for obtaining high mass and high spatial resolutions for chemical imaging. In these experiments 25 keV Ga^+ primary ions were used to generate secondary ions. These ions are separated and detected by a triple-pass 90° spherical sector electrostatic mass analyzer. The output is a high-resolution mass spectrum plotted as a function of intensity vs. mass-to-charge ratio. For chemical maps a 256×256 pixel area (typically corresponding to a 120×120 square micron size) with each pixel containing the full mass spectrum was stored in the computer media as raw files. Chemical maps of Fe, Cr and Mn were extracted retrospectively following the raw data acquisition.

Cyclic Polarizations. Cyclic polarization of 316L stainless steel was conducted using a potentiostat/galvanostat (EG&G Princeton Applied Research, model 273A) with a graphite auxiliary electrode and a saturated calomel electrode (SCE). OCPs of the coupons versus the SCE reference electrode were measured using a handheld multi meter (Wavetek DM23XT, internal resistance $10\text{M}\Omega$) before scanning. The OCP was measured to establish the initial potential for the voltage scan.

Pitting potentials were obtained through potentiodynamic polarization. Scans were initiated 10 mV below the OCP and scanned, at a scan rate of 5mV/sec, to 0.8 V_{SCE} or until the current reached to 1mA, whichever was first, as described by Olesen et al., (2001). We did not remove coupons from the reactor, because when we removed the ennobled coupon from the reactor we had reproducibility problems possibly because of sloughing of some of the biofilms or causing a random redistribution of the biofilm structure. Instead, the growth medium in the reactor was replaced with NaCl by feeding the reactor 5 L of NaCl solution (for approximately 30 minutes).

Measurements were conducted at room temperature in a mixture of 0.1M Na_2SO_4 and 0.2 M NaCl. We found that 0.2 M NaCl was the minimum concentration to cause pitting on the ennobled coupons. Each measurement was repeated at least four times using fresh coupons to determine the average and standard deviation of the pitting potential.

Results and Discussion

XPS Analysis

Stainless steel coupons with removed biomineral deposits: Each data point in the depth profile graph is generated by quantitative analysis of the individual core-level spectra acquired during each cycle of depth profiling. In Figs. 2.1-2.3 we show the XPS depth profiles corresponding to the ennobled and control coupons whose surfaces were completely cleaned by rinsing with DI water and wiping with cotton swabs. The depth profiles for the as-received and DI water treated coupons look identical to those shown in Figs. 2.1-2.3 and hence are not shown here. The passive film thicknesses of those coupons, however, are listed in Table 2.3.

Stainless Steel Coupons With Intact Biomineral Deposits. The depth profiles of O, C, Fe, and Cr of the coupons subjected to biofilms of *L. discophora* without introducing Mn^{2+} into the solution and without removal of biofilm are given in Fig. 2.4. These coupons were not ennobled, because of a lack of Mn^{2+} ions. The depth profiles of the same elements for the coupons which are ennobled because of biofilm and Mn activity without removal of biomineral deposits are given in Fig. 2.5.

The depth profiles shown in Fig. 2.1 for the control coupons (growth medium treated coupons) show two regions of interest; (1) an O- and Cr-rich region and (2) a metallic Fe- and Cr-rich (bulk SS) region. The Cr profile shows a maximum in the first region (Fig. 2.1) near the surface. As expected, the O concentration decreases

while the Fe concentration increases to its bulk value as a function of sputter depth. Other researchers (Lorang et al., 1994; Jyostna 1996) have also reported the existence of Cr-rich regions in passive films. No Ni-oxides were detected in the passive films (results were not presented), which is also in agreement with the literature (Jyostna 1996; Clayton and Lu, 1986; Olefjord 1985).

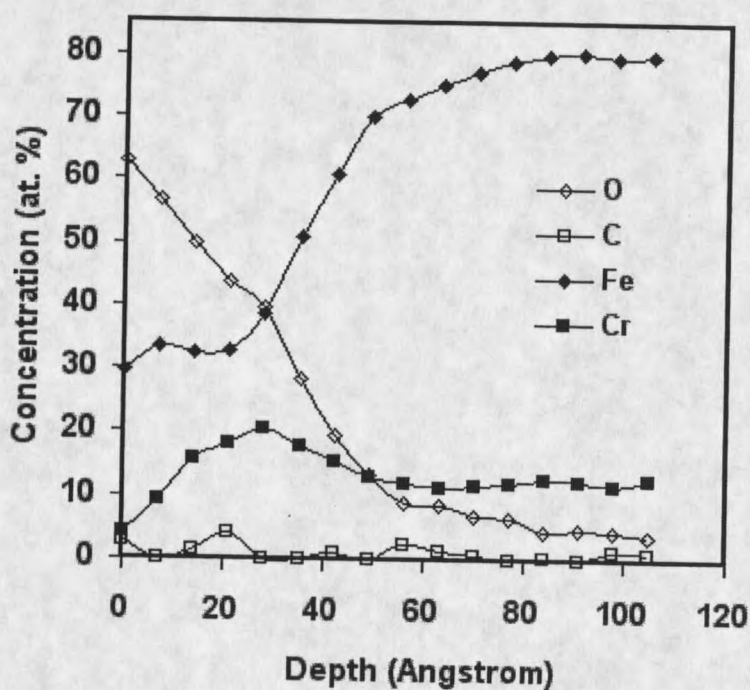


Figure 2.1. Depth profiles of O, C, Fe and Cr for the growth medium treated control coupon.

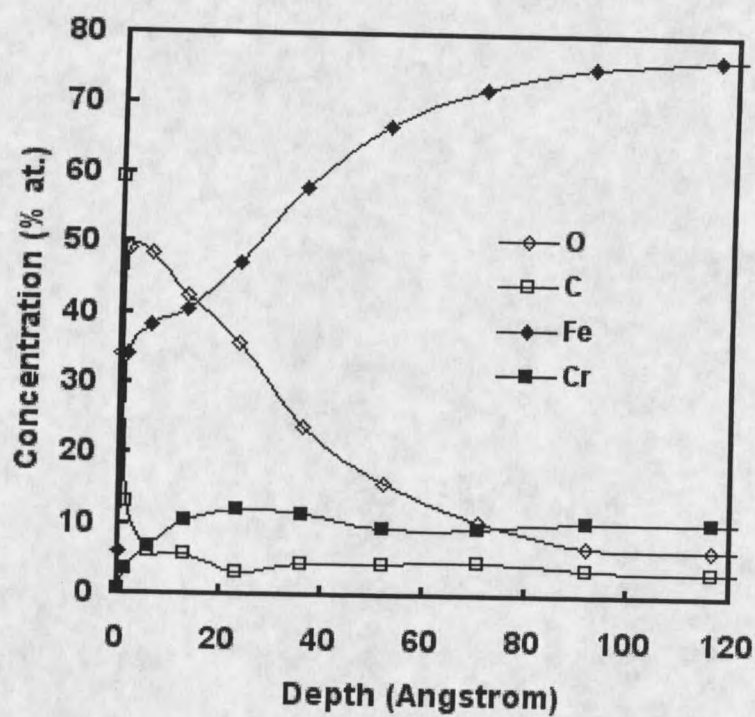


Figure 2.2. Depth profiles of O, C, Fe and Cr for the *L. discophora* biofilm treated control coupon following complete removal of biofilm.

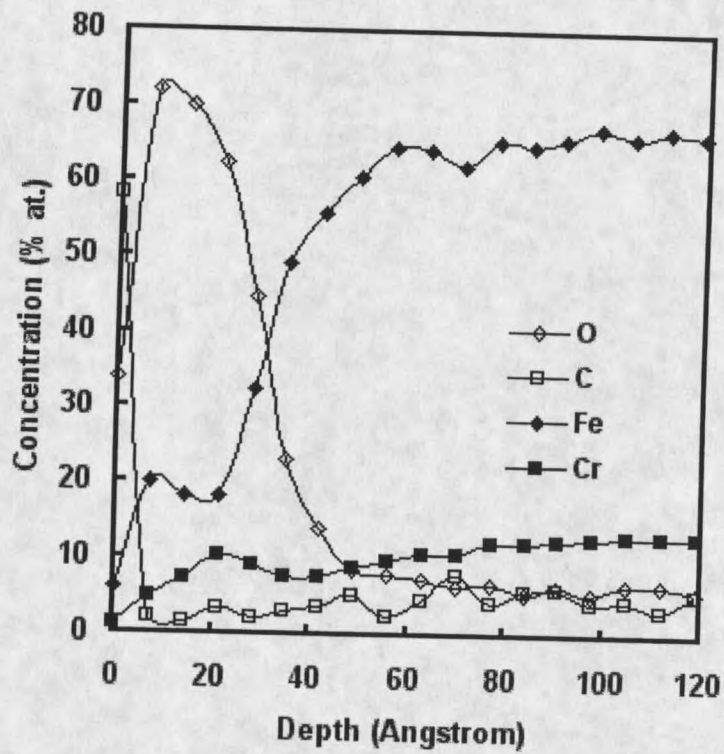


Figure 2.3. Depth profiles of O, C, Fe and Cr for the ennobled coupon after removal of biomineral deposits. Notice that the passive layer thickness appears to remain the same as that of the control coupons. This is explained in terms of re-oxidation of the surface (see text).

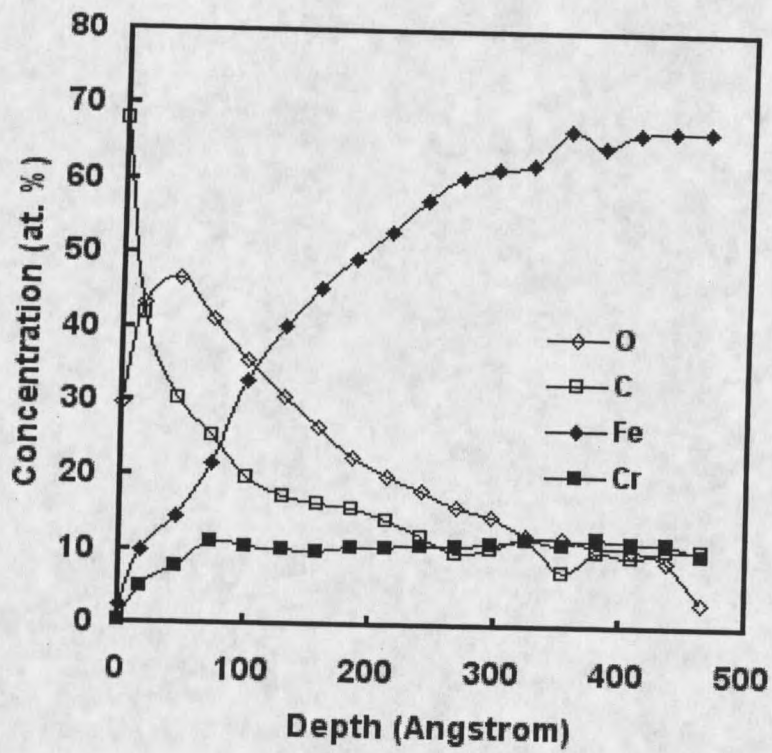


Figure 2.4. Depth profiles of O, C, Fe and Cr for the *L. discophora* biofilm treated coupon without the removal of biofilms.

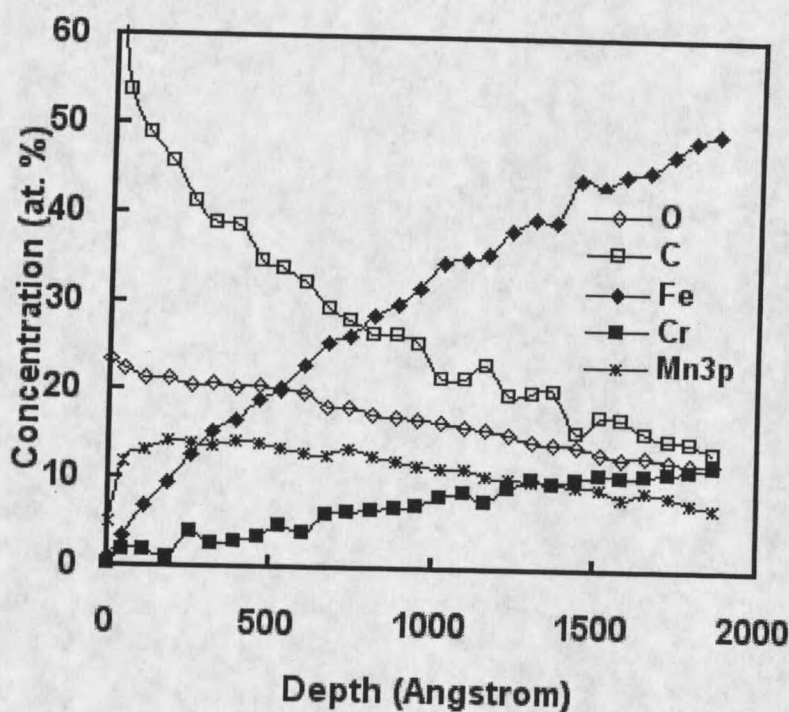


Figure 2.5. Depth profiles of O, C, Fe and Cr for the ennobled coupon without the removal of biofilms and biomineralized deposits.

In order to plot the depth profiles of the Fe-oxide and Cr-oxide layers, the Fe $2p_{3/2}$ and Cr $2p_{3/2}$ peaks after each sputtering cycle were separated into the oxide and bulk metallic components. This is because Fe $2p_{3/2}$ and Cr $2p_{3/2}$ have two contributions, one from the oxidized portion of the passive film and the other from the metallic portion (bulk SS) under the passive layer. The XPS curve fits were made to separate the oxide and bulk contributions to the Fe $2p_{3/2}$ and Cr $2p_{3/2}$ peaks, as shown in Figs. 2.6 and 2.7. Metallic Fe has a peak centered at ~ 707.6 eV, and the metallic Cr has a peak at 574.3 eV. The areas under the metallic component and the

rest of the total peak determine the relative concentrations of the two contributions. The data points shown in Figs. 2.8-2.12, corresponding to Fe and Fe-oxide and Cr and Cr-oxide depth profiles, respectively, were obtained from the areas associated with the metallic and oxide contributions. The position and the full-width-at-half-maximum (FWHM) of the metallic component are determined using the control coupons by sputtering them until the passive layer is totally removed. The resultant Fe and Cr peaks are used as standards: The position and FWHM of these peaks are used for fitting the composite peaks as in Figs. 2.6 and 2.7, by adjusting the intensity for the best fit to determine the bulk and oxide contributions. The bulk contribution was subtracted from the total area under the $2p_{3/2}$ peak to determine the oxide contributions that in general consist of multiple oxide peaks. In Figs. 2.8-2.12 we show Fe and Fe-oxides and Cr and Cr-oxides profiles for the various samples studied. The thickness determination and sputter rate were done by referencing to a SiO_2 standard, which resulted in $\sim 7 \text{ \AA}/\text{min}$ using our rate of sputtering.

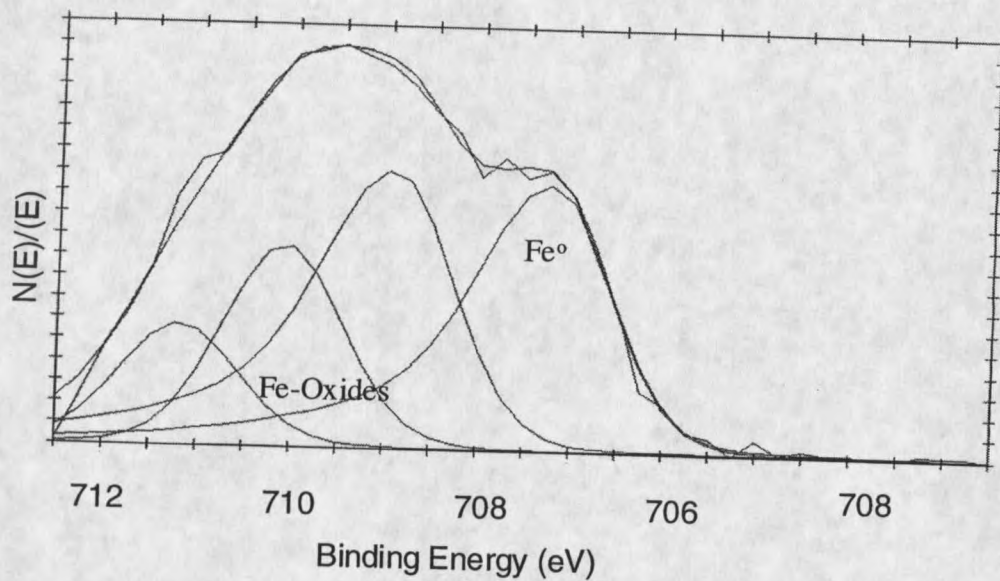


Figure 2.6. XPS curve fit for the Fe $2p_{3/2}$ profile to determine the bulk and oxide contributions.

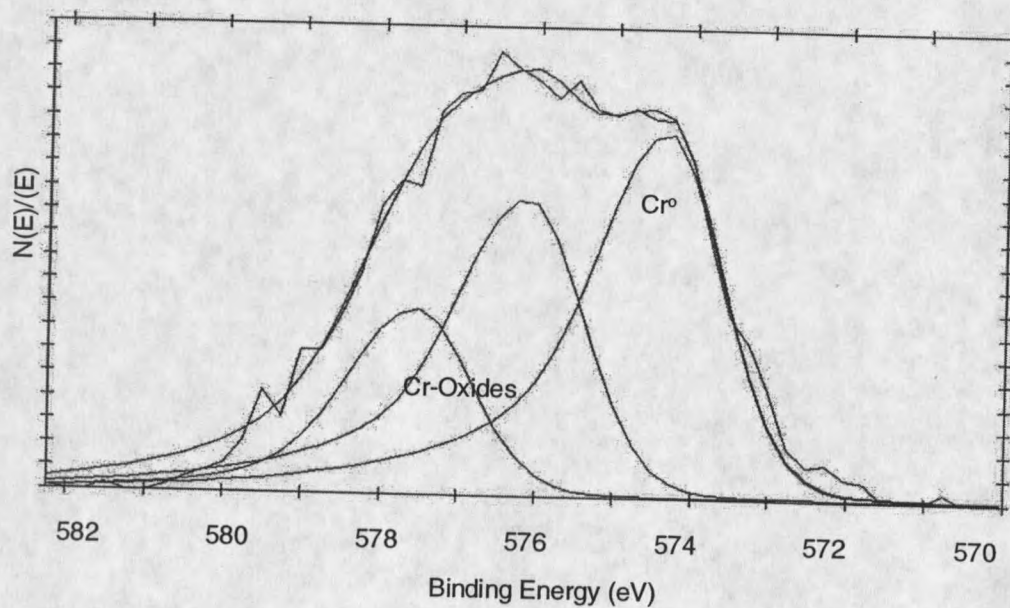
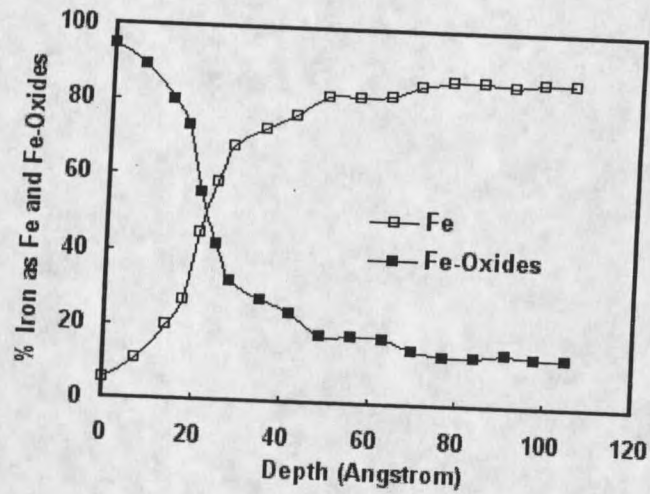
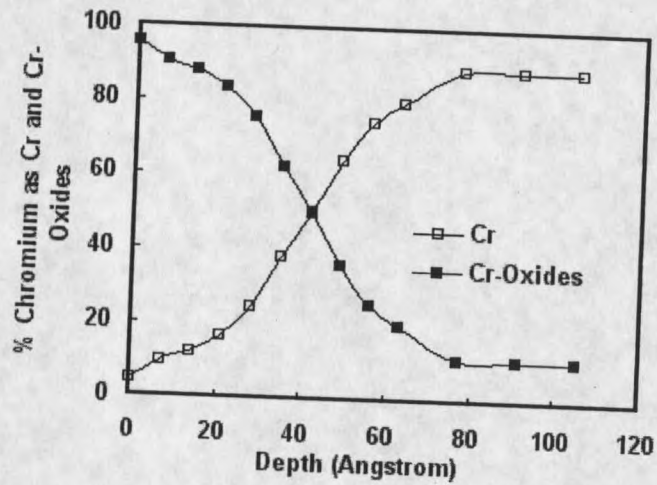


Figure 2.7. XPS curve fit for the Cr $2p_{3/2}$ profile to separate the oxide and bulk contributions.

Before the passive layer under the biofilm can be analyzed, the biofilm deposits must be sputter-cleaned in UHV. This is shown in Fig. 2.5, which gives the depth profile of an ennobled coupon containing biomineral deposits. Notice that the total Fe concentration increases linearly during sputtering, up to 2000 Angstrom. Since most of the O signal is from biominerals, which cover a large fraction of the area, it levels off at values indicating that a substantial fraction of the area is covered by biominerals. We interpret the linear increase in the Fe concentration (Fig. 2.5) in terms of exposing new areas of the passive layer as sputtering continues. This is because the biomineral deposits will form variable thicknesses across the surface, so that as the surface is sputtered, the portions having thin film deposits will be removed and the passive layer will be exposed and will contribute to both Fe and Cr concentrations. It is these areas that we are very interested in analyzing. If the thickness of the passive layer has not changed each time a fresh area is exposed, it is expected that oxidized Fe and Cr regions will be recovered. Ideally this should show an *apparent increase* in the oxide thickness, though in reality we are exposing new areas under the biofilm. In our observations we do not see the *apparent increase* in the oxide thickness (see Fig. 2.12 a,b). This we interpret as the passive layer thickness decreasing so that we can not resolve the metal oxides at each sputter cycle in the passive layer, though we can register the metallic components as we expose the passive layer which gives rise to an almost linear increase in Fe concentration with increasing sputter cycle.

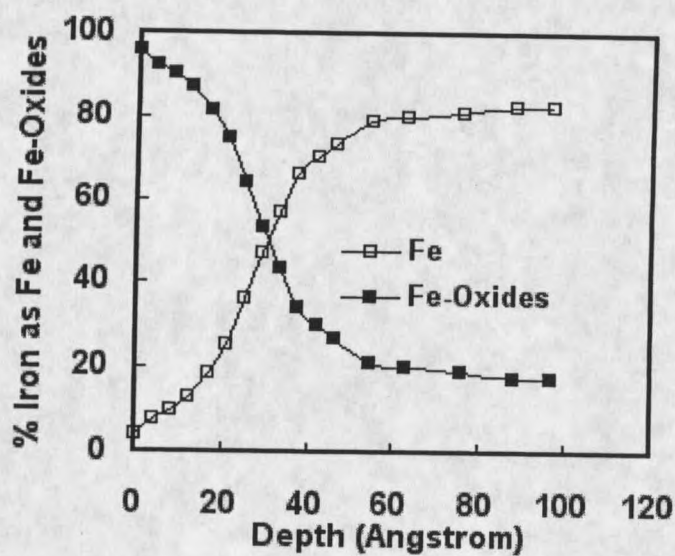


(a)

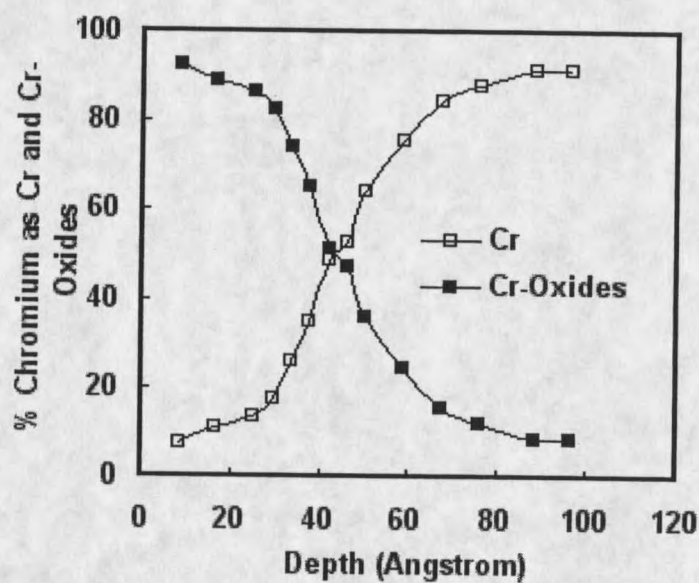


(b)

Figure 2.8 a) Fe and Fe-oxides b) Cr and Cr-oxides depth profiles for growth medium treated coupon.

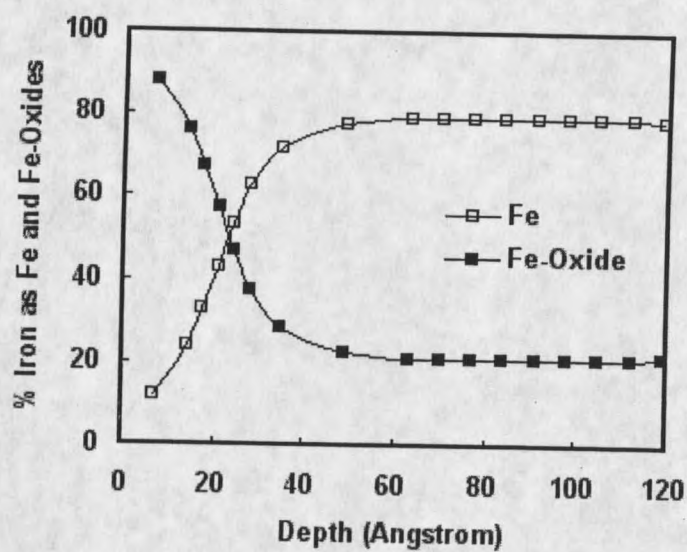


(a)

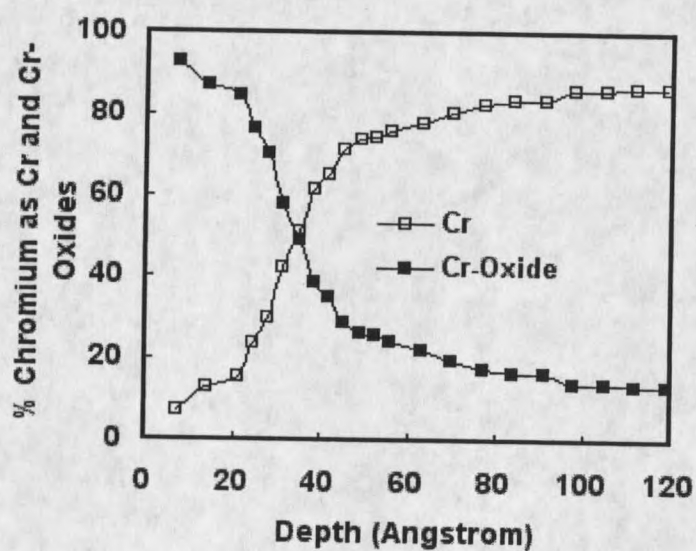


(b)

Figure 2.9 a) Fe and Fe-oxides b) Cr and Cr-oxides depth profiles for *L. discophora* biofilm treated coupon after removal of biofilms.

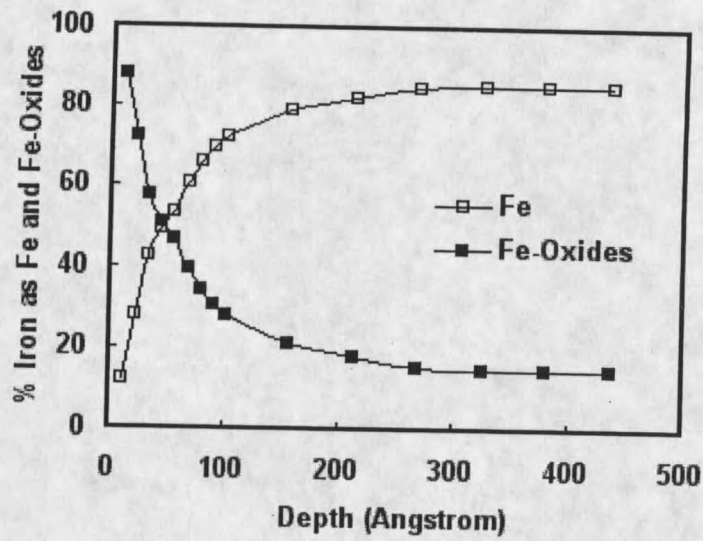


(a)

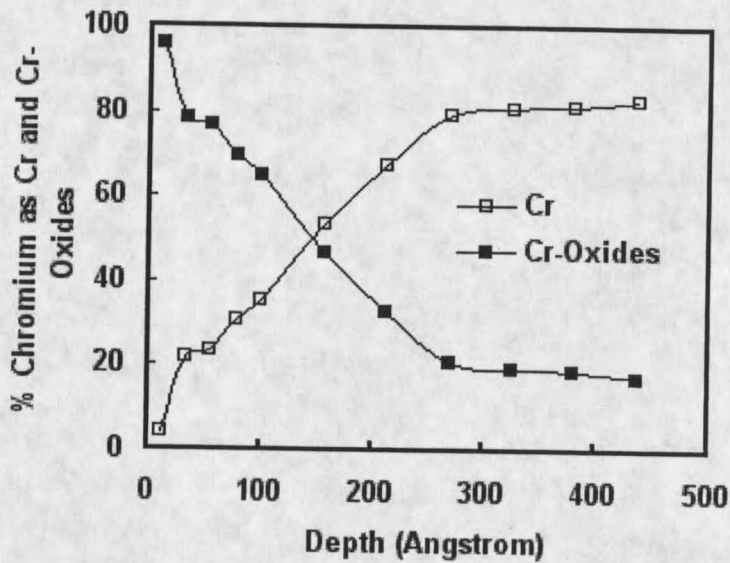


(b)

Figure 2.10 a) Fe and Fe-oxides b) Cr and Cr-oxides depth profiles for ennobled coupon after removal of biofilms and biomineralized deposits.

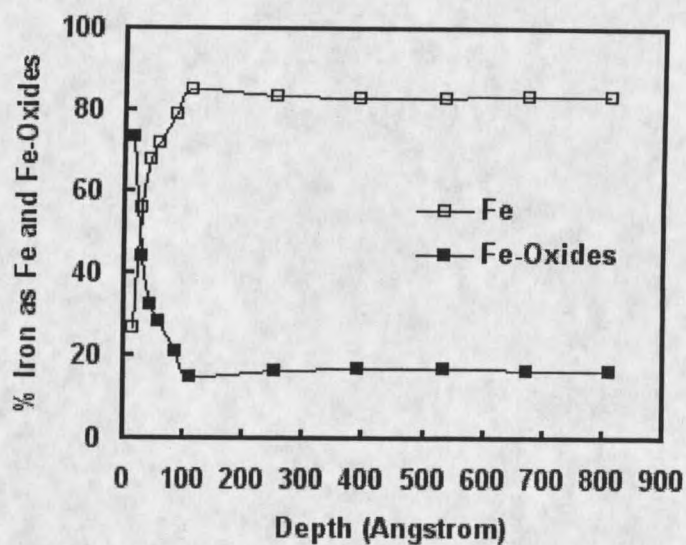


(a)

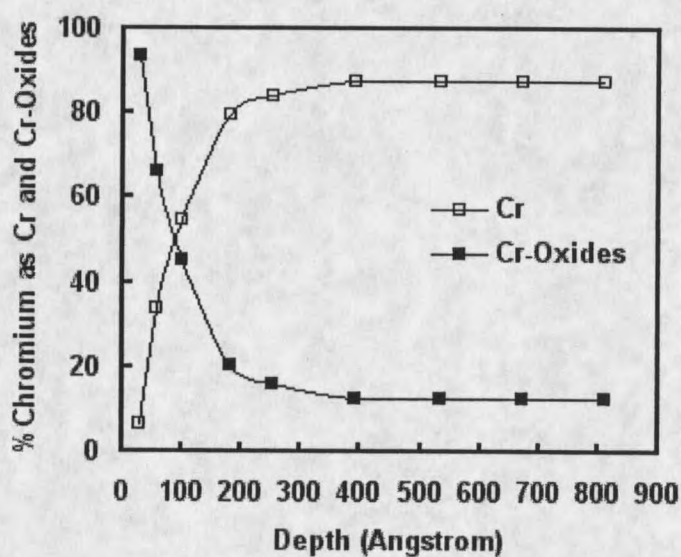


(b)

Figure 2.11 a) Fe and Fe-oxides b) Cr and Cr-oxides depth profiles for *L. discophora* biofilm treated coupons (without Mn^{2+} ; no ennoblement) without removal of biofilm.



(a)



(b)

Figure 2.12 a) Fe and Fe-oxides b) Cr and Cr-oxides depth profiles for ennobled coupon without removal of the biomineral deposits.

The thicknesses of the Fe-oxide and Cr-oxide layers on coupons after the removal of Mn-rich biominerals can be determined by making use of Figs. 2.8-2.12. The depth of the appropriate oxide was determined from the value at which the metallic contribution becomes constant. The data suggest that the Fe-oxide layer was sputtered away before the Cr-oxide layer. This shows that the passive film is composed of a Fe-rich outer layer and a Cr-rich inner layer. This duplex characteristic of stainless steel passive film has been confirmed by other researchers (Da Cunha Belo et al., 1998; Hakiki et al., 1995; Lorang et al., 1988; Lorang et al., 1987; Brooks et al., 1986).

The thicknesses of the Fe-oxide layer and the Cr-oxide layer on the coupons after removal of biomineral deposits do not change significantly (Table 2.3). As discussed earlier, the possibility of repassivation makes it impossible to differentiate between the actual film thicknesses on the ennobled coupons (the film thickens before the biofilm was removed). There is no easy way to remove biomineral deposits from the surface without causing repassivation except for sputtering them off in the UHV environment during XPS analysis. The results of such analyses of ennobled coupons were rather surprising, as seen in Fig. 2.12 (a,b) corresponding to the Fe and Fe-oxide and Cr and Cr-oxide profiles. Even though the total Fe atomic concentration increases linearly (Fig. 2.5) with sputtering, (which was interpreted as the sputtering process removing the thin portions of the bio-deposits and opening up new areas under the passive films), we do not observe the expected *apparent increase* in the passive layer thickness. This increase in the apparent thickness is expected because, considering that the rate of sputtering is about $\sim 3\text{-}5 \text{ \AA}$ per cycle, it would be

impossible to miss the passive layer by oversputtering unless the passive layer thickness under the biofilm of the ennobled coupon were reduced, perhaps down to 5-10 Å! This is reinforced by the observation that if the atomic Fe and Fe-oxide and atomic Cr and Cr-oxide profiles shown in Fig. 2.12 (a,b) (ennobled coupons) are compared with the control coupon profiles shown in Fig. 2.11 (a,b) (biofilm treated coupon) the *apparent increase* in the passive layer thickness is observed, as anticipated, provided that the passive film under the biofilm is not changed. We conclude, therefore, that the extent of ennoblement causes reduction in thickness of the passive films.

Table 2.3. Passive film thicknesses in Angstroms for the coupons after removal of biofilm/-Mn-deposits.

	Passive film thicknesses in Angstrom for the coupons				
	As-received Polished (group II)	DI water treated (group III)	Growth medium Treated (group IV)	Biofilm treated (group V)	Ennobled (group I)
Fe-Oxide	30	40	50	60	40
Cr-Oxide	35	70	70	80	80

ToFSIMS Results

To elucidate the nature of the chemical processes that cause the reduction of the passive layer, we investigated whether or not there is material dissolution in the passive layer. Taking advantage of the imaging capabilities and high sensitivities of ToFSIMS we carried out depth profile investigations on ennobled coupon surfaces. Figures 2.13 and 2.14 show the ToFSIMS images of Mn^+ , Cr^+ and FeH^+ ions for the ennobled coupons at 420 and 2600-Angstrom depths, respectively (referenced to the depth measurements conducted on Si-wafers). The dimension of each image is $\sim 60 \times 60$ microns. The white spots show high concentrations of the related element.

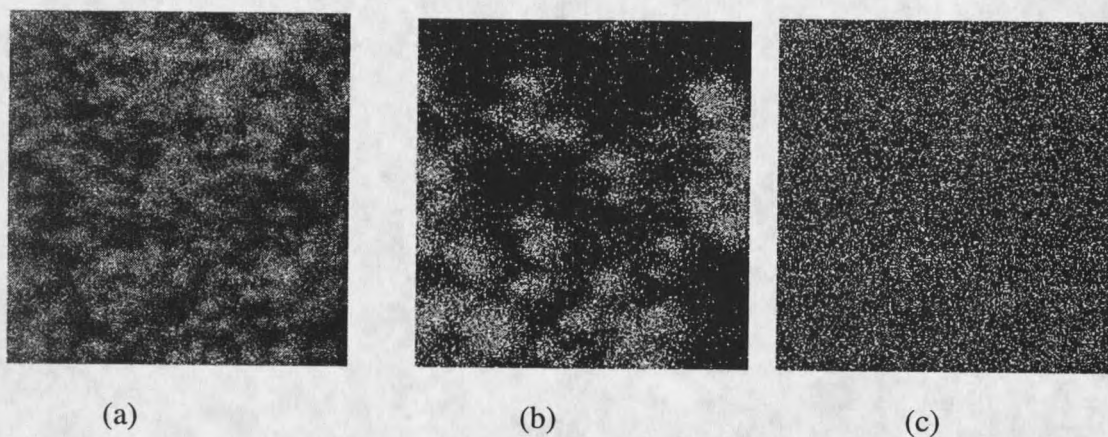


Figure 2.13. ToFSIMS images of (a) Mn^+ , (b) Cr^+ and (c) FeH^+ for an ennobled coupon at 420-Angstrom depth.

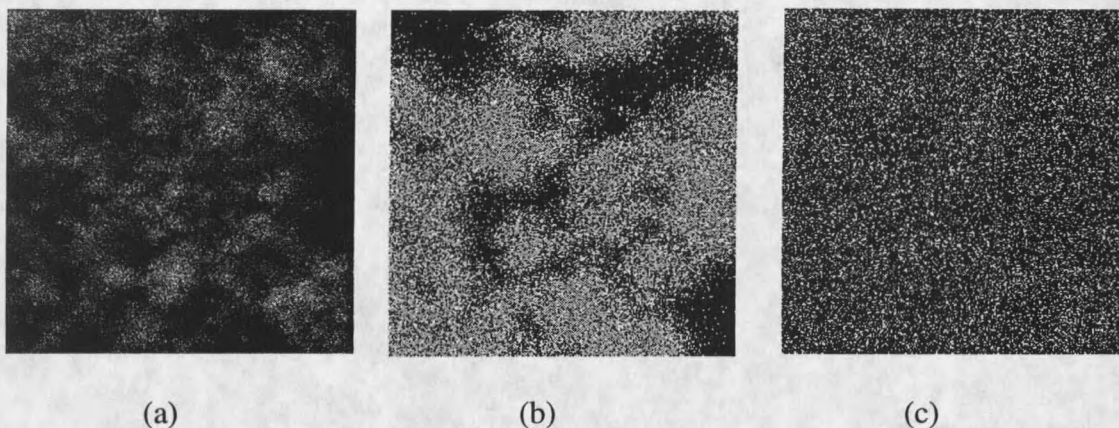


Figure 2.14. ToFSIMS images of (a) Mn^+ , (b) Cr^+ and (c) FeH^+ for an ennobled coupon at 2600-Angstrom depth.

The decrease in Mn and increase in Cr images at higher depths show that the sputtering process removes some of the manganese deposits and opens up fresh metallic surface. When Mn and Cr images from the two depths are compared, it is clear that the two elemental images are complementary in that Cr is present where minimal or no Mn is present. This means Cr originates from the passive layers or bulk of the stainless steel surface. However, the Fe images (as represented by FeH^+ rather than Fe^+ to prevent confusion with MnH^+) indicate that Fe is present both mixed with the Mn deposits inside the biofilm and on bare SS surface. Since the concentration of total Fe in the treatment solution was below 0.005 ppm before treatment and 0.009 ppm after treatment, the observation of Fe in the biofilm may be interpreted as Fe dissolving from the passive film and mixing into the biomineral deposits. A region-of-interest ToFSIMS analysis of Cr-rich and Mn-rich areas shows no Cr in the black

areas (Mn-rich areas) but shows proper concentrations of Cr, Ni and Fe in bright areas (bulk SS) from the clean stainless steel surface, as expected.

Pitting Potentials

After the growth medium in the reactor was replaced with 0.2 M NaCl solution, OCP of ennobled coupons first became unstable, and then dropped sharply (Figure 2.15). The reason for such behavior is not clear, as ennoblement potential was $364 \text{ mV}_{\text{SCE}}$, while the pitting potential of clean 316L stainless steel coupon was $573 \pm 67 \text{ mV}_{\text{SCE}}$. Even though the potential of the ennobled coupons did not exceed the pitting potential, Figure 2.15 indicates formation of metastable pits. This indicates that the ennobled coupons may have had lower pitting potentials than "as received" coupons.

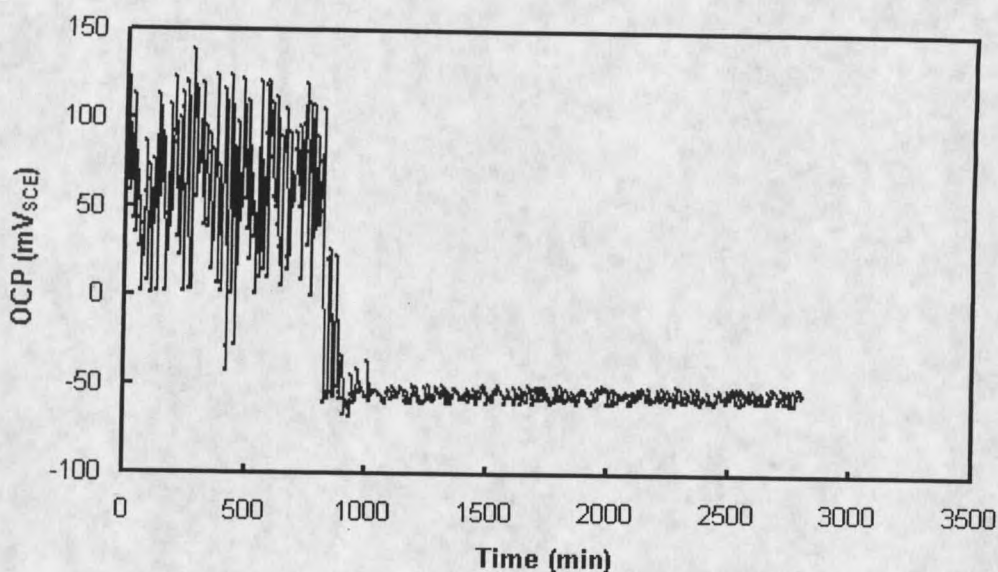


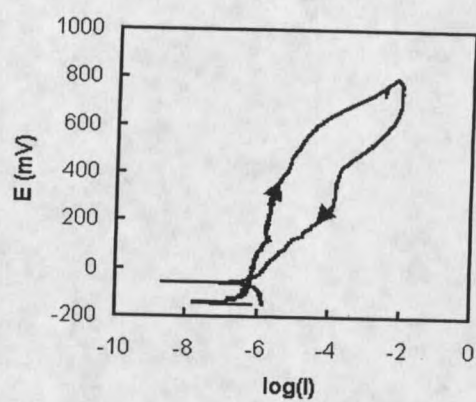
Figure 2.15. The variation of OCP by time. When an ennobled coupon was exposed to 0.2 M NaCl solution, the potential became unstable and then dropped sharply (after ~900 min) and remained more or less constant ~ -60 mV. The ennoblement potential was $364 \text{ mV}_{\text{SCE}}$.

Samples of cyclic polarization curves using coupons: as received and polished (group 2), biofilm treated (group V), and ennobled (group 1), are shown in Figure 2.16, and the summary of the results is in Table 2.4. Each measured value reported in Table 2.4 is an average of at least four repeated measurements. As seen from Figure 2.16A and 2.16 B, the cyclic polarization curves of as received and biofilm treated coupons were similar, with pitting potentials $573 \pm 67 \text{ mV}_{\text{SCE}}$ and $497 \pm 61 \text{ mV}_{\text{SCE}}$, respectively (approximately 70 mV difference). It is not clear whether this difference is caused by the presence of the biofilms.

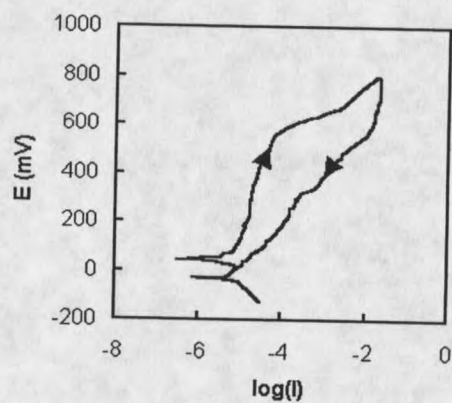
Figure 2.16C does show active metal behavior consistent with corrosion of the ennoblement coupon. Since the ennobled 316L stainless steel coupons pitted in 0.2 M NaCl, we conclude that ennoblement via manganese biomineralization lowers the pitting potentials and increases coupons' susceptibility to pitting corrosion.

Table 2.4. Pitting potentials of stainless steel coupons in 0.2 M NaCl.

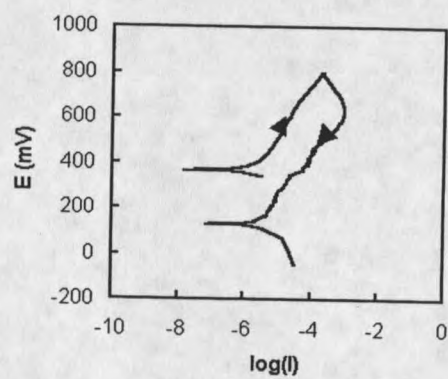
	As received (group II)	Biofilm treated (group V)	Ennobled (group I)
Pitting potential (mV _{SCE})	573 ± 67	497 ± 61	Pitted in 0.2 M NaCl



a



b



c

Figure 2.16. The cyclic polarization curves of as received-polished coupon (a), biofilm treated coupon (b) and ennobled coupon (c) in 0.2 M NaCl. Scan rate is 5mV/sec.

Conclusions

Using XPS and ToFSIMS, it is apparent that the quality of passive oxide layers on the ennobled coupons are thinner than those on control coupons. The ennobled coupons pitted when immersed in 0.2 M NaCl, even though the potential did not reach the pitting potentials determined for coupons covered with biofilm but not ennobled. Apparently, biomineralized manganese degrades the quality of passive oxide layers and lowers the pitting potential of 316L stainless steel.

CHAPTER 3

MULTIPLE SUBSTRATE GROWTH KINETICS OF

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Abstract

Growth parameters of *Leptothrix discophora* SP-6 were quantified based on steady state concentrations and utilization rates of pyruvate, dissolved oxygen, and concentration of microorganisms in a chemostat operated at 25 °C, pH of 7.2, and an agitation rate of 350 rpm. The results showed that the microbial growth was limited by both pyruvate and dissolved oxygen. A combined growth kinetics model using Monod growth kinetics for pyruvate and Tessier growth kinetics for oxygen showed the best agreement with the experimental data when analyzed using an interactive multiple substrate model. The growth kinetic parameters, and the respective confidence limits, estimated using Monte Carlo simulation, were: $\mu_{\max} = 0.576 \pm 0.021 \text{ h}^{-1}$, $K_{sMp} = 38.81 \pm 4.24 \text{ mg L}^{-1}$, $K_{sTo} = 0.39 \pm 0.04 \text{ mg L}^{-1}$, $Y_{X/P} = 0.150 \text{ (mg microorganism (mg pyruvate)}^{-1})$, $Y_{X/O} = 1.24 \text{ (mg microorganism (mg oxygen)}^{-1})$ and maintenance factors for pyruvate $m_p = 0.129 \text{ (mg pyruvate consumed (mg microorganism)}^{-1} \text{ h}^{-1})$, and oxygen $m_o = 0.076 \text{ (mg oxygen consumed (mg microorganism)}^{-1} \text{ h}^{-1})$.

Introduction

Leptothrix discophora SP-6 is a member of the *Sphaerotilus Leptothrix* group, which are manganese and iron oxidizing sheathed bacteria that thrive in iron- and manganese-rich environments such as iron seeps, swamps, and springs (Emerson and Ghiorse, 1992). Although *Leptothrix discophora* are not pathogens, they are considered a nuisance in water distribution systems because they oxidize iron and manganese, cause color problems, and in extreme cases clog water distribution conduits. In our laboratory, we have been quantifying the metabolic activity of biofilms of manganese oxidizing bacteria and their effect on microbially influenced corrosion (MIC), specifically on pitting corrosion of stainless steels (Dickinson and Lewandowski, 1996; Dickinson et al., 1996; Olesen et al., 2000a; Olesen et al., 2000b) using *Leptothrix discophora* SP-6, which forms biofilms and oxidizes Mn^{2+} to manganese oxides, as a model microorganism. To construct mathematical models of MIC caused by manganese oxidizing bacteria we attempt to evaluate two factors: (1) growth kinetics of the microorganisms involved and (2) rate of manganese oxide deposition in biofilms deposited on metal surfaces. This paper deals with the first factor, the growth kinetics of the model manganese oxidizing bacteria, *Leptothrix discophora* SP-6.

The goal of this study was to represent the growth rate of *Leptothrix discophora* SP-6 in form of a suitable mathematical model, and to calculate biokinetic parameters associated with this model. To simplify calculating growth parameters

from the chemostat data, researchers often use a single substrate limited growth model. However, it is well known that the microbial growth is often limited by more than one substrate (Machado et al., 1988; Beyenal and Tanyolac, 1997; Neeleman et al., 2001). An example of such situation is microbial growth that is limited by the concentrations of the electron donor (e.g. organics) and electron acceptor (e.g. oxygen). To accommodate such a possibility, we operated a chemostat and quantified the steady state growth parameters based on the mass balances of pyruvate, dissolved oxygen, and microorganisms, and their respective concentrations and used MATLAB[®] programming to estimate biokinetic parameters by testing single and double substrate growth models.

Materials and methods

Microorganisms

Frozen stock culture of *Leptothrix discophora* SP-6 (ATCC no. 51168) was obtained from the American Type Culture Collection (ATCC) and the stock cultures of our own were prepared by following procedures described in the ATCC Catalogue. As soon as frozen vial was received, it was placed in a deep freezer (-70 °C). For propagation, the vial was thawed rapidly by agitation in a 37°C water bath. Then the microorganisms were transferred aseptically into a flask containing 10 mL of the sterile ATCC # 1917 broth (Table 3.1). The flask was incubated at 20°C for 2-4 days, and after the microbial growth has been detected, the content of the flask was transferred to 100 mL of a fresh broth. The cells were harvested in the early stationary

phase of growth (30 – 40 hours) and pelleted by centrifugation using two sterile centrifuge tubes (50 mL each). The supernatant was removed, and the pellet resuspended in 100 mL of 5 % sterile broth + 20 % sterile glycerol, and 1 mL aliquots of the suspension were dispensed into small sterile vials. The vials were stored at -70°C. When needed, they were removed, thawed in a 37°C water bath, and inoculated into fresh ATCC # 1917 broth medium.

The inoculum were prepared as follows; 1 mL frozen stock culture of *Leptothrix discophora* SP-6 (ATCC no. 51168) was poured into a flask containing 100 mL of sterile growth medium (A and B) prepared as described above. The microorganisms were grown in the growth medium placed in Erlenmeyer flasks, without stirring, for 30-40 hours at room temperature, and the chemostat was inoculated with 200 mL of this culture.

Chemostat

The microorganisms were grown in a New Brunswick (BioFlo 2000) chemostat with a working volume of 2 L, equipped with pH, agitation, and temperature controllers. The sensitivities of the control units for pH, temperature, and agitation rate were 0.1 unit, 0.1 °C, and ± 1 rpm, respectively. Prior to use, the chemostat and the medium A (see Table 3.1) were autoclaved for 30 minutes at 121°C. Then the medium B (see Table 3.1) was aseptically syringed into medium A using the 0.2-micron filters (Corning, 431219). The pH was controlled by pumping solutions of 0.2 N NaOH and 0.2 N H₂SO₄ when needed. The solution in the

chemostat was stirred by a blade type double impeller at 350 rpm, and the stirring rate was optimized (results not shown) using specific oxygen uptake rate as the criteria. We compared specific oxygen uptake rates measured at a fixed dilution rate (0.1 h^{-1}) and at different agitation rates (150, 250, 350, 450 and 550 rpm). We found that optimum agitation rate as 350 rpm at which specific oxygen uptake rate did not change with agitation rate. At low agitation rates *Leptothrix discophora* SP-6 aggregated, and at higher agitation rates it was mechanically damaged. Dissolved oxygen concentrations measured at different steady states were between 0.1 mg L^{-1} and 7.8 mg L^{-1} . The dissolved oxygen concentration was controlled by sparging filtered air, or filtered mixtures of air + pure oxygen or air + nitrogen in various proportions, depending on the needs, at the flow rates between 1 L h^{-1} and 5 L h^{-1} . The chemostat was operated at $25 \pm 0.1 \text{ }^{\circ}\text{C}$.

Table 3.1. ATCC Culture 1917 MSVP for *Leptothrix discophora* SP-6 (American Type Culture Collection Catalogue, 1992).

Medium A

(NH ₄) ₂ SO ₄	0.24 g
MgSO ₄ ·7H ₂ O	0.06 g
CaCl ₂ ·2H ₂ O	0.06 g
KH ₂ PO ₄	0.02 g
Na ₂ HPO ₄ ·7H ₂ O	0.06 g
HEPES buffer	2.383 g
Distilled water to	984 mL

The pH of the medium A was adjusted to 7.2 with NaOH or H₂SO₄, autoclaved at 121 °C for 15 minutes (for 1 L volume of the solution); after cooling it to approximately 50 °C, the following filter-sterilized (0.2 micron sterile syringe filter, Corning, 431219) medium B solutions were added into the autoclaved medium A aseptically. HEPES is N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

Medium B

20 % Sodium pyruvate	5 mL
FeSO ₄ 10 mM	1 mL
Vitamin solution	1 mL

Vitamin solution

Biotin	20.0 mg
Folic acid	20.0 mg
Thiamine HCl	50.0 mg
D-(+) -calcium pantothenate	50.0 mg
Vitamin B12	1.0 mg
Riboflavin	50.0 mg
Nicotinic acid	50.0 mg
Pyridoxine HCl	100.0 mg
p-Aminobenzoic acid	50.0 mg
Distilled water to	1.0 L

The chemostat was inoculated with microorganisms using 10 % (volume of inoculum/total working volume of the chemostat) inoculum harvested during the exponential growth phase from a batch culture flask. Initially, the chemostat was run in a batch mode, and was operated in a continuous flow mode after the culture had reached the exponential growth phase (usually after 30 to 40h). To reach the steady state, the chemostat was operated for six or seven retention times, and the existence of the steady state was verified by checking that the absolute differences in effluent substrate concentrations differed by less than 3 % in consecutive measurements of corresponding retention time apart. The following parameters were measured or continuously recorded at steady state: dissolved oxygen and microorganism concentrations, influent and effluent pyruvate concentrations, oxygen consumption rate, dilution rate, pH, and temperature. New steady states were established by gradually increasing the dilution rates to the desired levels up to the washout point. The microbial culture was periodically examined for contamination by plating on agar plates. We continuously monitored biofilm formation on the reactor walls, and when it was necessary the reactor was shut off to clean the biofilm and restarted again.

Analytical Methods

Pyruvate concentration was determined using a Dionex (Sunnyvale, CA) ion chromatograph (Model DX 300) equipped with an autosampler (25 μ L sample loop), IonPAC AS10 analytical column (Dionex P/N 43118), ion suppresser, conductivity

detector and Peaknet[®] software. The eluant was 3.5 mM potassium tetraborate with a flow rate of 1.0 mL min⁻¹. The retention time for pyruvate was 5.5 minutes.

The column was washed for 3 minutes with 100 mM potassium tetraborate and returned to initial conditions before the next injection.

The microorganism concentration was determined using the Standard Volatile Solids Method (Standard Methods for the examination of Water and Wastewater, # 2540E) (Eaton et al., 1995).

Dissolved oxygen concentration was monitored by the Ingold[®] dissolved oxygen probe and pH was monitored the Mettler Toledo[®] pH electrodes, all integrated with the chemostat (New Brunswick, BioFlo 2000).

The oxygen uptake rate was estimated using a procedure described by Bandyopdhyay et al. (1967). Before each measurement, the aeration was stopped, and the gas space in the chemostat was flushed with nitrogen to remove oxygen and prevent reaeration. Immediately after, the dissolved oxygen concentration in the reactor was recorded versus time. The rate of oxygen concentration decrease, dS_O/dt , determined at the linear region, was equivalent to the oxygen uptake rate. The specific oxygen uptake rate (SOUR) has been determined as the oxygen uptake rate divided by the biomass concentration ($dS_O/dt/X$). We also tested if oxygen transfer from the liquid to the gas phase influences SOUR by replacing air with nitrogen in the headspace of the chemostat at different gas flow rates (zero and 10 L min⁻¹), and found that this effect was negligible.

Selecting The Growth Model and Estimating the Kinetic Constants

According to Bailey and Ollis, 1986, when microbial growth is limited by more than one substrate, three forms of multiple substrate growth kinetics can be considered:

(1) An interactive or multiplicative form,

$$\mu/\mu_{\max} = [\mu(S_1)] [\mu(S_2)] \dots \dots [\mu(S_i)] \quad (3.1)$$

(2) An additive form,

$$\mu/\mu_{\max} = [\mu(S_1) + \mu(S_2) + \dots \dots + \mu(S_i)]/i \quad (3.2)$$

(3) A non interactive form,

$$\mu/\mu_{\max} = \mu(S_1) \text{ or } \mu(S_2) \text{ or } \dots \dots \text{ or } \mu(S_i) \quad (3.3)$$

Based on the preliminary results, we selected pyruvate and oxygen as substrates limiting microbial growth in our chemostat (see results and discussion sections). The additive form (Equation 3.2) was not applicable because it implied that the microorganisms would grow on one of the limiting substrates only, pyruvate or oxygen, which was not the case as we demonstrated that microbial growth in the chemostat was negligible in the absence of either oxygen or pyruvate. Many mathematical models describe microbial growth rate as a function of single substrate concentration (see Bailey and Ollis, 1986 for details). To develop multiple substrate growth kinetics, these individual microbial growth models are combined in a manner described by Equations (3.1)–(3.3) (Shuler and Kargi, 1992). In this study, we considered the following single substrate growth models:

$$\text{Monod} \quad \mu = \mu_{\max} \frac{S_i}{K_{sMi} + S_i} \quad (\text{Monod, 1949}) \quad (3.4)$$

$$\text{Tessier} \quad \mu = \mu_{\max} (1 - e^{-S_i / K_{sTi}}) \quad (\text{Tessier, 1942}) \quad (3.5)$$

$$\text{Moser} \quad \mu = \mu_{\max} (1 + K_{sMZi} S_i^{-\lambda_i})^{-1} \quad (\text{Moser, 1958}) \quad (3.6)$$

$$\text{Contois} \quad \mu = \mu_{\max} \frac{S_i}{B_i X + S_i} \quad (\text{Contois, 1959}) \quad (3.7)$$

For each steady state, the specific growth rate, μ , was calculated as equal to dilution rate:

$$\mu = D = Q/V \quad (3.8)$$

Maintenance and Yield Factors

The maintenance factor, m_i , and the yield factor, $Y_{X/i}$, were calculated from mass balances on the respective substrates (pyruvate or oxygen) by rearranging Equation (3.9a).

Equation (3.9b) is a rearranged form of Equation (3.9a) and it allows plotting $(S_{fp} - S_p)/X$ versus $1/D$, from which the slope of the line gives m_p and the intercept gives $1/Y_{X/P}$. Equation (3.9c) is another rearranged form of Equation (3.9a) and it allows for plotting $SOUR/D$ versus $1/D$, from which plot the slope of the line gives m_o and the intercept gives $1/Y_{X/O}$.

$$D(S_{fi} - S_{ei}) = \frac{\mu_i X}{Y_{X/i}} + m_i X \quad (3.9a)$$

$$\frac{(S_{fp} - S_p)}{X} = \frac{m_p}{D} + \frac{1}{Y_{X/P}} \quad (3.9b)$$

$$\frac{SOUR}{D} = \frac{m_o}{D} + \frac{1}{Y_{X/O}} \quad (3.9c)$$

Non-linear Regression to Calculate Biokinetic Parameters

We combined all biokinetic expressions (25 combinations, see Table 3.2), and used the direct search method (Lagarias, et al., 1998), which requires minimizing a predefined objective function to estimate the biokinetic parameters from the experimental data. We have defined the objective function (Equation 3.10) as the sum of squares of differences (SSD) between the experimentally measured specific growth rates and the specific growth rates calculated by the mathematical model.

$$SSD = \sum_{i=1}^N (\mu_{\text{experimental}} - \mu_{\text{model}})^2 \quad (3.10)$$

Specific growth rates used in the models were calculated from different combinations of Equations (3.4)-(3.7) and Equations (3.1) and (3.3), as shown in Table 3.2. SSD values calculated for each combined model are given in Table 3.2.

Table 3.2. Combined and single growth models were used to find the best model, and the corresponding biokinetic coefficients.

Model #	Multiple model eqn #	Single model eqn # for pyruvate	Single model eqn # for oxygen
1	3	-	-
2	3	-	4
3	3	-	5
4	3	-	6
5	3	-	7
6	3	4	-
7	1	4	4
8	1	4	5
9	1	4	6
10	1	4	7
11	3	5	-
12	1	5	4
13	1	5	5
14	1	5	6
15	1	5	7
16	3	6	-
17	1	6	4
18	1	6	5
19	1	6	6
20	1	6	7
21	3	7	-
22	1	7	4
23	1	7	5
24	1	7	6
25	1	7	7

The direct search method (Lagarias, et al., 1998) is available with the MATLAB[®] software, and we used the MATLAB programming to calculate the biokinetic parameters. The MATLAB program to calculate the best model and the biokinetic coefficients, is given in Appendix B. As a general rule the following restrictions were applied in the search: 1) Any model that produced non-realistic

biokinetic coefficients (values that were negative, extremely high or significantly out of range of biokinetic constants reported in the literature) were discarded. 2) If the model was not stable (i.e., it produced different biokinetic parameters for very close initial estimates), it was ignored as ill-conditioned. To test if the model solution depends on the initial estimates, we ran the program at least 20 times using, randomly generated initial estimates and found that the solutions did not depend on initial estimates. All experimental results from the chemostat experiments, collected in Table 3.3, were subjected to this procedure.

Selecting the Best Model and Running Sensitivity Analysis

The best multiple substrate growth model was selected from among different combinations of Equations (3.4)-(3.7) (Table 3.2). Since several models yielded very close SSD values, to determine the most suitable one Monte Carlo simulation was used to determine confidence limits for the growth parameters, then the best growth model was determined as the one that provided the narrowest confidence limits, thereby optimizing reliability of the procedure.

To estimate the effect of measurement errors on the calculated growth parameters, random data sets (similar to Table 3.3) were generated based on the actual data (boot strapping Monte Carlo, Press et al, 1992). The data for this procedure were generated by randomly changing the measured pyruvate concentration by $\pm 5\%$, dissolved oxygen concentration by $\pm 1\%$ and the measured microorganism concentration by $\pm 10\%$. The deviations were considered as

reasonable estimates of the experimental error in measuring the pyruvate, dissolved oxygen and microorganism concentrations. MATLAB's random function was used to calculate these randomized errors from the actual data set (Table 3.3). To find the confidence limits, one thousand different randomly generated hypothetical data sets were used. Increasing the number of the simulations above one thousand did not change significantly (up to four significant figures) the calculated average parameters and standard deviation values.

Results and Discussion

The experimental data in Table 3.3 were used to estimate growth parameters of *Leptothrix discophora* SP-6. It was also verified experimentally that the growth of *Leptothrix discophora* SP-6 in the absence of oxygen or pyruvate was negligible (results not shown) which was the basis for rejecting Equation (3.2) as a combined growth model and that changing NH_4^+ concentration in the feed did not affect the effluent concentrations of pyruvate, oxygen, and microorganism (results not shown). The results in Table 3.3 demonstrate that both pyruvate and oxygen concentrations influenced the growth kinetics of *Leptothrix discophora* SP-6. Hence, the growth of *Leptothrix discophora* SP-6 should be represented by a kinetic model taking into account double substrate limitation: pyruvate and oxygen. The data in Table 3.3 show that for the same dilution rate, 0.1 h^{-1} , the steady state microorganism and pyruvate concentrations depended on oxygen concentration in the chemostat.

Table 3.3. The data collected at various steady states. The chemostat was operated at temperature = 25°C, pH = 7.2, agitation rate = 350 rpm, $S_{fp} = 1000 \text{ mg L}^{-1}$, $S_{fn} = 65 \text{ mg NH}_4^+ \text{ L}^{-1}$.

D (h^{-1})	X (mg L^{-1})	S_p (mg L^{-1})	S_o (mg L^{-1})	SOUR ($\text{mg oxygen (mg microorganism)}^{-1} \text{ h}^{-1}$)
0.029	87	2	5.7	0.0935
0.052	113	3.6	5.6	0.11
0.071	131	5.2	3.8	0.137
0.081	110	26.9	0.2	0.16
0.129	111	12.8	1.3	0.174
0.163	97	40.7	0.4	0.22
0.24	143	28.9	2.2	0.378
0.48	106	225	6.8	0.425
0.118	134	10.4	2.1	0.156
0.1	104	7.7	3.3	0.151
0.08	175	10.2	0.9	0.152
0.1	109	470	0.1	0.162
0.1	125	8.1	5.1	0.163
0.1	175	10.4	6	0.151
0.047	114	3.4	7.6	0.124
0.055	121	4.5	7.7	0.112
0.03	98	1.9	7.8	0.105
0.11	119	8.1	7.7	0.139
0.188	132	17.5	5.5	0.21
0.5	99	325	6.8	na
0.3	103	34.1	1.6	0.27
0.35	121	74	0.9	0.31
0.43	113	234	0.65	0.435

Modeling Growth Kinetics

Table 3.4 shows calculated biokinetic parameters and SSD (in descending order) for the possible realistic growth models using interactive and non-interactive (only for oxygen or pyruvate or none of them) combinations of Equations (3.4)-(3.7). Model 8 had the minimum SSD, but models 13 and 23 gave low SSD values too. To select the best growth model, Monte Carlo simulation was used to determine the confidence limits of the calculated parameters (Table 3.5). Table 3.5 shows biokinetic parameters for these 3 models (#8, #13 and #23), the SSD for the data, the average SSD and the standard deviations for the simulated data. The average SSD for the model #23 is 0.132 which is higher than the SSD calculated from the experimental data (0.0058), indicating that the model #23 was prone to experimental errors, and this model was rejected. Models #8 and #13 showed smaller average SSD values than model #23, and therefore were considered a better representations of the data. Because the average SSD for model #8 (0.0183) and the standard deviation for the simulated data (0.0069) were smaller than those for the model #13, we have selected model #8, Monod growth kinetics for pyruvate and Tessier growth kinetics for oxygen, as the one that best described growth of *Leptothrix discophora* SP-6 in the chemostat:

$$\mu = \mu_{\max} \frac{S_p}{K_{sMp} + S_p} \left(1 - e^{-\frac{S_o}{K_{sTo}}}\right) \quad (3.11)$$

For the selected model, the biokinetic parameters are: $\mu_{\max} = 0.576 \pm 0.021 \text{ h}^{-1}$, $K_{sMp} = 38.81 \pm 4.24 \text{ mg L}^{-1}$, $K_{sTo} = 0.390 \pm 0.040 \text{ mg L}^{-1}$. Figure 3.1 shows the

specific growth rates – measured, and predicted from Equation 3.11. The high correlation coefficient ($R^2=0.97$) demonstrates that the selected growth model accurately represents the growth of *Leptothrix discophora* SP-6.

Although, our analysis shows that model #8, had the lowest SSD value, model #13 was not poor and it can also be used to describe the growth of *Leptothrix discophora* SP-6. Model #23 had a very high average SSD value, and was rejected, while models 12, 15, 7, 22, 10, 25 and 17 had high SSDs and did not fit the data in Table 3.3 as good as models # 8 or #13.

Table 3.4. Growth models, biokinetic parameters, and SSD. First column shows the type of the model used in the simulation (See Equations 3.1-3.3). The third and fourth columns show the equations we combined to assemble the double substrate kinetic expressions. The results are presented in ascending order of SSD. K(p), column 6, refers to K_{sMp} , K_{sTp} , K_{sMZp} and K(o), column 7, refers to K_{sMo} , K_{sTo} , K_{sMZo} .

1	2	3	4	5	6	7	8	9	10	11	12
Multiple model eqn #	Model #	Single model eqn # for pyruvate	Single model eqn # for oxygen	μ_{max} (h^{-1})	K(p) (mg/L)	K(o) (mg/L)	λ_p	λ_o	B_p (mg pyruvate/mg microorganism)	B_o (mg oxygen/mg microorganism)	SSD
1	8	4	5	0.5763	38.9829	0.3944					0.0054
1	13	5	5	0.5007	40.6310	0.4269					0.0056
1	23	7	5	0.5622		0.4106			0.2995		0.0058
1	12	5	4	0.5321	38.0799	0.2967					0.0110
1	15	5	7	0.5316	37.4906					0.0027	0.0121
1	7	4	4	0.6057	36.2021	0.2731					0.0142
1	22	7	4	0.5929		0.2874			0.2781		0.0145
1	10	4	7	0.6031	35.3772					0.0025	0.0152
1	25	7	7	0.5894					0.2739	0.0026	0.0172
1	17	6	4	0.5442	192.2085	0.3283	34.5957				0.0203
3	11	5	-	0.3742	34.4625						0.1404
3	6	4	-	0.4055	26.6233						0.1473
3	21	7	-	0.3964					0.2089		0.1509
1	14	5	6	0.4900	37.4601	67.4615		23.4690			0.3582
1	9	4	6	0.5587	35.8746	96.2865		24.5351			0.3585
1	24	7	6	0.5464		162.5678		214.3115	0.2862		0.3587
1	19	6	6	0.5418	43.3277	53.7801	1.0818	68.3582			0.3587
3	3	-	5	0.1738		0.1422					0.4526
1	18	6	5	0.1738	8.2754	0.1422	35.6069				0.4526
3	5	-	7	0.1735						0.0003	0.4592
1	20	6	7	0.1735	177.0147		128.8711			0.0003	0.4592
3	2	-	4	0.1728		0.0303					0.4596
3	1	-	-	0.1676							0.4622
3	16	6	-	0.1676	72.6072		47.9024				0.4622
3	4	-	6	0.1558		24.5984		42.5332			0.6948

Table 3.5. The models, which give the best SSD. The biokinetic parameters were calculated for actual data, and for simulated data sets.

				$\mu_{\max}$ (h ⁻¹)	K_{sMp} (mg/L)	K_{sTp} (mg/L)	K_{sTo} (mg/L)	B_P (mg pyruvate/mg microorganism)	SSD
Model #	8								
Pyruvate:	4	Oxygen:	5						
Parameters from original data				0.5763	38.9829		0.3944		0.0054
Average from simulated data				0.5758	38.8105		0.3903		0.0183
Standard deviation from simulated data				0.0212	4.4241		0.0390		0.0061
Model #	13								
Pyruvate:	5	Oxygen:	5						
Parameters from original data				0.5007		40.6310	0.4269		0.0056
Average from simulated data				0.4988		40.7680	0.4189		0.0194
Standard deviation from simulated data				0.0072		3.9905	0.0424		0.0063
Model #	23								
Pyruvate:	7	Oxygen:	5						
Parameters from original data				0.5622			0.4106	1.2201	0.0058
Average from simulated data				0.4796			0.3389	1.1303	0.1320
Standard deviation from simulated data				0.0790			0.0996	0.5624	0.0600

Tessier's Growth Model and Half Rate Constant for Oxygen

The growth models #8, #13 and #23 showed a good agreement with the Tessier growth kinetics for oxygen (see Table 3.4 and 3.5). However, the K_{sT} in the Tessier equation should not be confused with the half rate coefficient K_{sM} in the Monod Equation. Tessier (1942) developed his equation hypothesizing that the dependence of specific growth rate on the substrate concentration was proportional to the difference between μ and $\mu_{\max}$:

$$\frac{d\mu}{dS_i} = \frac{1}{K_{sT}}(\mu_{\max} - \mu) \quad (3.12)$$

Equation 3.12, when integrated, gives the well-known form of the Tessier Equation (Equation 3.5). To compare this model with Monod's model, the half rate constant (substrate concentration for which $\mu = \mu_{\max}/2$) can be estimated from this equation, and is equal to $K_{sT} \log 2$ (Powell 1974). In our model $K_{sTo} = 0.390 \text{ mg L}^{-1}$, which corresponds to the half rate constant for oxygen in Monod equation equal to $K_{sMo} = 0.39 \times \log 2 = 0.237 \text{ mg L}^{-1}$.

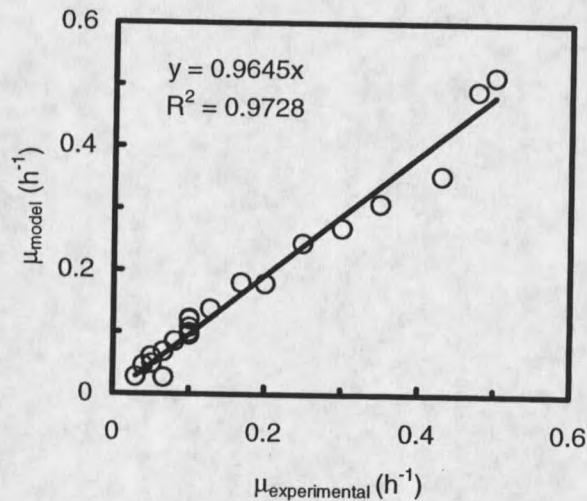


Figure 3.1. The specific growth rates, predicted from Equation 3.11 versus experimentally measured.

Maintenance Coefficients and Yield Factors

The maintenance and yield factors for pyruvate and oxygen were calculated from Figures 3.2 and 3.3 and Equations 3.9b and 3.9c: $Y_{X/P} = 0.150$ mg microorganism (mg pyruvate) $^{-1}$, $Y_{X/O} = 1.24$ mg microorganism/mg oxygen, $m_p = 0.129$ mg pyruvate consumed (mg microorganism) $^{-1} h^{-1}$, and $m_o = 0.076$ mg oxygen consumed (mg microorganism) $^{-1} h^{-1}$.

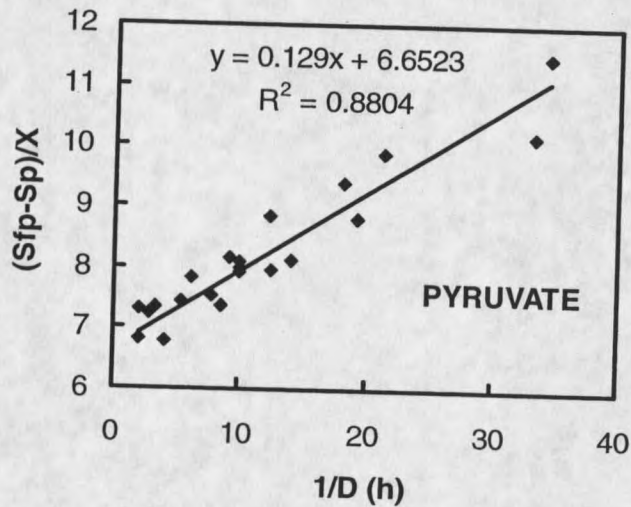


Figure 3.2. $(S_{fp} - S_p)/X$ versus $1/D$ for pyruvate. The slope of the line gives m_p and the intercept gives $1/Y_{X/P}$.

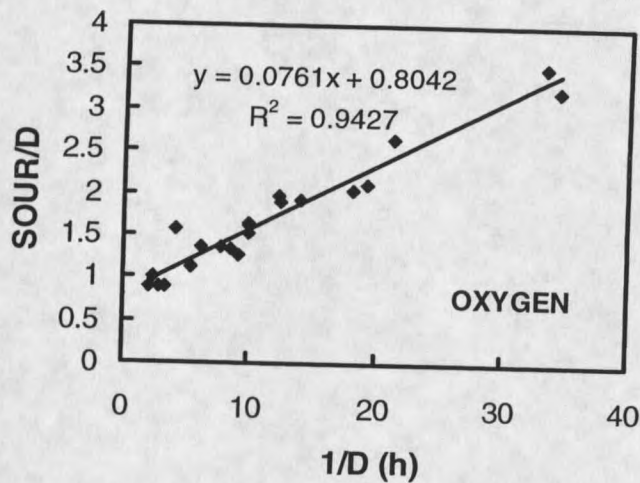


Figure 3.3. SOUR/D versus 1/D. The slope of the line gives m_0 and the intercept gives $1/Y_{X/O}$.

Emerson and Ghiorse (1992) grew *Leptothrix discophora* SP-6 in MSVP and in PYG (peptone, yeast extract, glucose) media shaken at 150 rpm at 25 °C, and reported the maximum specific growth rate of 0.5 h^{-1} in both media which is close to the maximum specific growth rate, 0.576 h^{-1} , estimated using our procedure. We could not find the yield coefficients for *Leptothrix discophora* SP-6 in the literature. However, the measured yield coefficients for pyruvate ($Y_{X/P} = 0.150$) and oxygen ($Y_{X/O} = 1.24$) are close to the limits reported in the literature for other microorganisms grown on different carbon sources between 0.18 and 1.01 g g^{-1} and that between 0.17 and 1.5 g/g for oxygen (Bailey and Ollis, 1986; Shuler and Kargi, 1992).

Conclusions

A double-substrate model, using Monod growth kinetics for pyruvate and Tessier growth kinetics for oxygen is in good agreement with the chemostat data. Using this model we calculated the following growth parameters for *Leptothrix discophora* SP-6: $\mu_{\max} = 0.576 \pm 0.021 \text{ h}^{-1}$, $K_{sMp} = 38.81 \pm 4.24 \text{ mg L}^{-1}$, $K_{sTo} = 0.390 \pm 0.040 \text{ mg L}^{-1}$, $Y_{X/P} = 0.150 \text{ (mg microorganism/mg pyruvate)}$, $Y_{X/O} = 1.24 \text{ (mg microorganism (mg oxygen)}^{-1})$, $m_P = 0.129 \text{ mg pyruvate consumed (mg microorganism)}^{-1} \text{ h}^{-1}$, and $m_O = 0.076 \text{ mg oxygen consumed (mg microorganism)}^{-1} \text{ h}^{-1}$.

In summary, we have developed an algorithm to estimate biokinetic parameters. This algorithm can be modified and used to predict biokinetic parameters from variety of microbial species grown in chemostats. We hope that making the algorithm available may stimulate more frequent use of multiple microbial growth kinetics to describe growth of different microorganisms.

CHAPTER 4

BIOKINETIC PARAMETERS FOR MICROBIAL GROWTH IN BIOFILMS
QUANTIFIED FROM SUBSTRATE CONCENTRATION PROFILES

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Abstract

It is a common practice to use growth parameters measured in planktonic cultures to predict substrate concentration profiles in biofilms. To obtain true biofilm biokinetic parameters, a reliable method is needed to compute biokinetic parameters from substrate concentration profiles measured directly in the biofilms. We have developed such a method, and demonstrated its utility by evaluating biokinetic parameters from oxygen concentration profiles measured in biofilms of manganese oxidizing bacteria *Leptothrix discophora* SP-6. Confidence limits of the evaluated parameters were estimated using the experimentally measured concentration profiles. We found that the Monod model represented growth of *Leptothrix discophora* SP-6 biofilms better than the Tessier model. The Monod half rate coefficient was 0.333 ± 0.077 mg/L and the dimensionless maintenance constant was close to zero (6×10^{-4}). Biofilms of *Leptothrix discophora* SP-6 had higher Monod half rate constant than planktonic type growth. The maintenance coefficients calculated in this study (for a biofilm) were compared to the maintenance coefficient measured for planktonic growth of this microorganism in our previous work, we found that *Leptothrix discophora* SP-6 biofilms uses oxygen primarily for growth rather than maintenance.

Introduction

Microbial growth kinetic model quantifies the relationship between the specific growth rate (μ) of a microorganism and the substrate concentration (S). The constants in the model are termed biokinetic parameters, as an example, in Monod equation they are termed half saturation constant and maximum specific growth rate. Biokinetic parameters that fit various planktonic growth models of different microorganisms on various substrates have been measured and reported in the literature for many microorganisms that can grow in biofilms. To model microbial growth in biofilms, biokinetic parameters obtained from planktonic culture are commonly used (Kreft and Wimpenny 2001; Eberl et al., 2000; Noguera et al., 1999; Beyenal et al., 1997; Bakke et al., 1989). Few authors calculated growth parameters in biofilms from the overall biofilm growth rate and the rate of substrate removal (Bakke et al., 1984, Samb et al., 1998 and Reifler et al., 1998). However, it is well known that both electron donors and electron acceptors form concentration profiles in biofilms and it is not clear which one is the growth limiting substrate as their concentrations decrease toward the bottom because of combined effects of mass transfer limitations and microbial consumption. Therefore changes in the bulk substrate concentrations do not represent the substrate concentrations in biofilms, and should not be used to quantify the biokinetic parameters in biofilms.

Lewandowski et al., (1991) noticed this problem and developed a method to calculate biokinetic parameters for biofilms from dissolved oxygen concentration

profiles measured across a biofilm. Their technique, however, was applicable only to Monod kinetics and the computational procedures employed performed well only at low dissolved oxygen concentrations, near the half saturation concentration for oxygen.

In other report addressing similar problems, Berg et al., (1998) developed a method of predicting substrate concentration profiles from the reaction rates in different zones in the sediments. By increasing the number of the reaction zones, they successfully predicted concentration profiles of NO_3^- and O_2 using Nelder and Mead's (1965) down hill simplex method. Even though the application of this method to biofilms for quantifying biokinetic parameters is difficult, the authors showed that the simplex method could, in principle, be used to predict substrate concentration in biofilms. Similarly Robinson (1985) argued that using the simplex algorithm is superior to using Gaussian methods when determining microbial growth kinetic parameters from nonlinear regression analysis. Despite that mostly used technique, the Levenberg-Marquart, is modified versions of the Gaussian method (Robinson 1985).

Visser et al., (2002), at the Center for Biofilm Engineering, used the Levenberg-Marquart algorithm to determine biokinetic parameters in biofilms by minimizing the squared differences between experimental data and the model prediction. To determine growth parameters, these authors used the MATLAB's *leastq routine* (in version 6.1 it is called *lsqnonlin*), which requires good guesses of the initial values, and for that reason they had to use a separate method to select the initial values that reduce the number of iterations. We are addressing this problem

again, mostly because their method does not allow predictions of concentrations at *exactly* the same location as obtained by microsensor measurement, and such predictions are needed to compare the experimental concentration with the predicted concentration.

In the Biofilms Structure and Function Group, at the Center for Biofilm Engineering, we have been studying metabolic activity of manganese oxidizing bacteria *Leptothrix discophora* SP-6 biofilms and their effect on microbially influenced corrosion (Dickinson and Lewandowski, 1996; Olesen et al., 2000), for that reason we selected *Leptothrix discophora* SP-6 as a model biofilm microorganism in this study. In our previous study, we found that *Leptothrix discophora* SP-6 planktonic growth was limited by pyruvate and oxygen in the solution, and that could be represented by combined Monod or Tessier growth kinetics with maintenance substrate consumption (Yurt et al., 2002). Therefore, to determine biokinetic parameters in *Leptothrix discophora* SP-6 biofilms, we decided to examine Monod and Tessier growth kinetics with maintenance substrate consumption. To our best knowledge, none of the previous works examining biofilms considered both Monod and Tessier growth models, with maintenance substrate consumption, as only Monod kinetics without maintenance was tested.

The goal of this study was to develop an algorithm and a computational procedure to determine biokinetic parameters from substrate concentration profiles in biofilms. As an example, we used biofilms of *Leptothrix discophora* SP-6, because we have determined their biokinetic parameters for planktonic growth. Experimentally we measured dissolved oxygen concentration profiles in *Leptothrix*

discophora SP-6 biofilms. Our approach is based on developing an optimization technique that minimizes the squared differences between the oxygen concentrations measured in the biofilm, and the oxygen concentrations predicted by the model, at the same locations. All model equations were written in dimensionless form for generalized solutions. An explicit Runge-Kutta (4,5) formula, the Dormand-Prince pairs, was used to solve the differential equations to calculate oxygen concentrations at the location of microsensor measurements. We used Nelder and Mead's (1965) simplex algorithm to calculate the biokinetic parameters. To calculate the confidence limits of the predicted parameters, we used the oxygen concentration profiles measured in biofilms. All computations were performed using MATLAB.

Materials and methods

Biofilms

We measured oxygen concentration profiles in a colony biofilm of *Leptothrix discophora* SP-6. Colony biofilms grow on solid media, and are used to study antibiotic penetration (Anderl et al., 2000). Their physical structure is similar to biofilms grown in water but they are uniform structurally which, was an important feature for our purpose. Because homogeneous structure of the colony biofilms was needed to satisfy the assumptions of the biofilm model. Colony biofilms of *Leptothrix discophora* SP-6 were grown on membrane filters placed on the top of agar layers, and dissolved oxygen concentration profiles were measured across these biofilms (Figure 4.1).

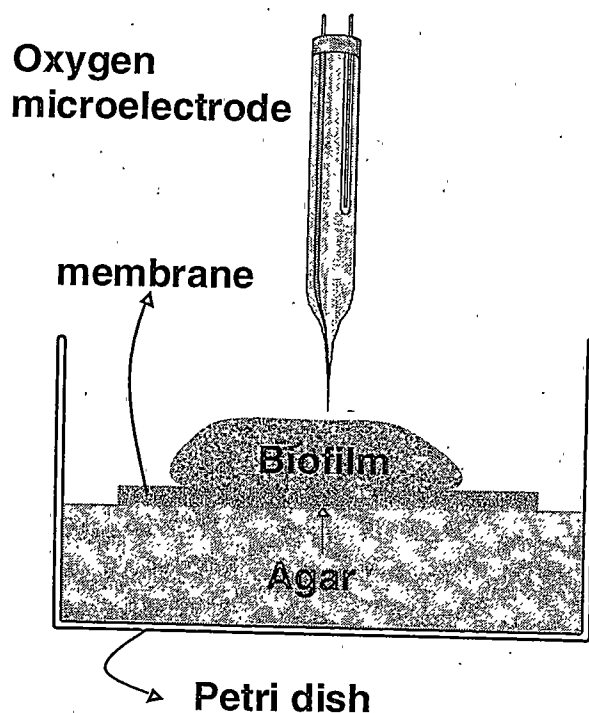


Figure 4.1. A colony biofilm grown on the top of a membrane filter. The growth substrate is supplied by the agar, and is transferred through the polycarbonate membrane to the biofilm. The microorganisms receive oxygen from the air.

The colony biofilms of *Leptothrix discophora* SP-6 were prepared according to the procedure described by Anderl et al., (2000). Forty – eight hours old cultures of *Leptothrix discophora* were diluted to an optical density of 0.02 at 600 nm (using 1-cm path length) in growth medium specified below. Ten μL of diluted culture was used to inoculate one sterile, polycarbonate membrane filter (25 mm ID, 0.2 micron pore size, Poretics Corporation, Livermore, CA, Cat. No. 11013) placed on an agar plate, prior to inoculation each side of the membrane filters was sterilized by the UV

light for 15 minutes. Inoculated Petri plates were kept at room temperature and the membrane filters with the biofilm were transferred to fresh agar plates every 24 h. The growth substrates were supplied by the agar and transferred through the membrane to the colony biofilm (Figure 4.1), while the oxygen required for growth was delivered from the air.

To grow *Leptothrix discophora* SP-6 we used ATCC Culture 1917 MSVP (American Type Culture Collection Catalogue, 1992). It consisted of 0.24 g $(\text{NH}_4)_2\text{SO}_4$, 0.06 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.06 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.02 g KH_2PO_4 , 0.06 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, and 2.383 g HEPES buffer in 950 mL distilled water. The pH of this medium was adjusted to 7.2 with NaOH or H_2SO_4 , as needed. We added 15 g Agar Noble (Difco 214230), and autoclaved this solution at 121 °C for 15 minutes per 1 L of the solution. Then, after cooling the solution to approximately 50 °C, we added 1 mL vitamin solution (composition given below), 1 mL FeSO_4 (10 mM) and 48 mL of pyruvate solution (at various concentrations) aseptically by filter-sterilizing (0.2 micron sterile syringe filter, Corning, 431219). One liter of vitamin solution consisted of 20 mg Biotin, 20 mg Folic acid, 50 mg Thiamine HCl, 50 mg D-(+) -calcium pantothenate, 1 mg Vitamin B12, 50 mg Riboflavin, 50 mg Nicotinic acid, 50 mg Pyridoxine HCl, 50 mg p-Aminobenzoic acid. The solution was poured into sterilized petri dishes and kept in a cold place.

Microelectrode Measurements

We measured dissolved oxygen concentration profiles vertically, through the biofilm, using Clark type microelectrodes with guard cathodes, as described by

Revsbech, (1986) (Figure 4.1). The microelectrodes were made with tip diameters less than 10 μm to prevent damaging the biofilm structure during measurements. Local oxygen concentrations were measured at 5 μm intervals, and the response time of the microelectrodes was less than 0.3 s. Before and after the measurements the microelectrodes were calibrated using air and pure nitrogen gases.

To measure the oxygen concentration profiles, the microelectrode was mounted on a micromanipulator (Model M3301L, World Precision Instruments, New Haven, CT) equipped with a stepper motor (Model 18503, Oriel, Stratford, CT) controlled by the Oriel Model 20010 interface. Then, tip of the microelectrode was introduced to the biofilm from the top, perpendicularly to the biofilm using the micromanipulator in manual mode. The stepper motor was then interfaced with a computer, and the microelectrode movement for the remaining time was handled by a controller (CTC-283-3, Micro Kinetics) with a positioning precision of 0.1 μm . Custom software was used to control and to coordinate microelectrode movement, the data acquisition, and to display the concentration profiles in real time. The position of the microelectrode was monitored during the measurement using a stereo microscope (Leica Stereo Zoom 7) with 40 – 70 times magnification.

An Example of the Measured Profiles

Figure 4.2 shows a typical dissolved oxygen concentration profile measured in a 120 hours old colony biofilm of *Leptothrix discophora* SP-6. The continuous line shows the location of the biofilm surface, as selected using the microscope, the biofilm thickness (L_f) at the location was 150 micrometers and the oxygen surface

concentration (S_s) was 5.18 mg/L. The detection of the correct surface location in our biofilms was quite difficult. Any point, which is close to surface in the biofilm, can be used as surface point in the solution algorithm. As a general rule we selected a surface location 20 μm below from the bulk. From microscope observations we are sure that at this point the tip of the microelectrode is located in the biofilm. The symbols S^* ($=S/S_s$) and x^* ($=x/L_f$) designate the dimensionless concentration of oxygen and the dimensionless distance from the bottom. Since we could not see the exact location of the microelectrodes near the bottom, we measured the oxygen profile across the biofilm until at least two consecutive measurements showed the normalized oxygen concentrations lower than 0.01. The dissolved oxygen concentration did not change in the biofilm and was constant at the saturation level in killed biofilms (no biofilm activity).

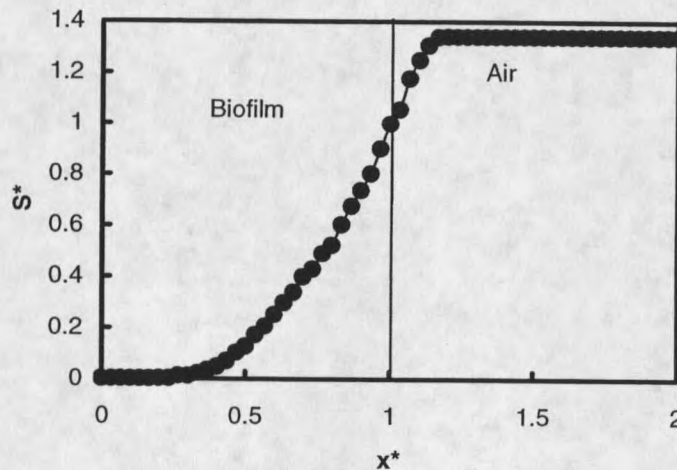


Figure 4.2. The normalized dissolved oxygen concentration (S^*) is plotted versus the normalized distance (x^*) from the bottom in a colony biofilm of *Leptothrix discophora* SP-6.

Mathematical Model

Microbial Growth and Substrate Consumption

Monod, Tessier, Moser, and Contois models are popular kinetic expressions describing microbial growth (Bailey and Ollis 1986; Shuler and Kargi, 1992). In our previous chemostat study (Yurt et al., 2002), we demonstrated that Monod and Tessier models adequately describe the growth of *Leptothrix discophora* SP-6, these growth models are examined in these study.

$$\text{Monod} \quad \mu = \mu_{\max} \frac{S}{K_{sM} + S} \quad (\text{Monod, 1949}) \quad (4.1)$$

$$\text{Tessier} \quad \mu = \mu_{\max} (1 - e^{-S/K_{sr}}) \quad (\text{Tessier, 1942}) \quad (4.2)$$

Since there is no clear notion how important the substrate consumption rate for maintenance consumption is in the biofilms, it is also considered (Powell 1974; Pirt 1975);

$$-\frac{dS}{dt} = \frac{\mu}{Y_{x/o}} X + mX \quad (4.3)$$

Thus, the one-dimensional steady state diffusion-reaction equation is;

Monod:

$$D_{\text{eff}} \frac{d^2S}{dx^2} = \frac{\mu_{\max} SX_f}{Y_{x/o} (K_{sM} + S)} + m_M X_f \quad (4.4)$$

Tessier:

$$D_{\text{eff}} \frac{d^2S}{dx^2} = \frac{\mu_{\text{max}} (1 - e^{-\frac{s}{K_{st}}})}{Y_{x/o}} + m_T X_f \quad (4.5)$$

Searching for the best-fit model, we expected that the predicted Monod or Tessier biokinetic might have different maintenance factors. To take this into account, we used two different symbols, m_M and m_T , for the maintenance factors in Equations 4.4 and 4.5.

The following assumptions were made with respect to the models described by Equations 4.4 and 4.5, and they are discussed and verified in the Results and Discussion section.

1. *The growth limiting substrate is oxygen.*
2. *The biofilm is uniform.*
3. *The substrates in the biofilm are transported only by diffusion.*
4. *Biofilm density and diffusivity can be represented by their average values.*
5. *Transfer of oxygen is one-dimensional.*
6. *The biofilm is at pseudo steady state (with respect to the oxygen consumption rate).*

Equations 4.4 and 4.5 cannot be solved analytically, and numerical methods have to be applied. To improve the stability and efficiency of the numerical procedures employed, we converted equations 4.4 and 4.5 to dimensionless forms:

Monod kinetics (Equation 4.4)

$$\frac{d^2 S^*}{dx^{*2}} = \Phi_M^2 \frac{S^*}{(\beta_M + S^*)} + M_M \quad (4.6)$$

where;

$$\Phi_M = \sqrt{\frac{\mu_{\max} L_f^2 X_f}{Y_{x/o} D_{\text{eff}} S_s}} \quad (4.7)$$

$$\beta_M = \frac{K_{sM}}{S_s} \quad (4.8)$$

$$M_M = \frac{m_M L_f^2 X_f}{D_{\text{eff}} S_s} \quad (4.9)$$

Tessier kinetics (Equation 4.5)

$$\frac{d^2 S^*}{dx^{*2}} = \Phi_T^2 \left(1 - e^{-\frac{S^*}{\beta_T}} \right) + M_T \quad (4.10)$$

where;

$$\Phi_T = \sqrt{\frac{\mu_{\max} L_f^2 X_f}{Y_{x/o} D_{\text{eff}} S_s}} \quad (4.11)$$

$$\beta_T = \frac{K_{sT}}{S_s} \quad (4.12)$$

$$M_T = \frac{m_T L_f^2 X_f}{D_{\text{eff}} S_s} \quad (4.13)$$

The boundary conditions for the solution of Equations 4.6 and 4.10 are;

$$@ \quad x^*=0 \quad S^*=S^*_{\text{bottom}} \quad (4.14)$$

$$@ \quad x^*=0 \quad dS^*/dx^*=0 \quad (4.15)$$

We use the subscripts M and T to refer to Monod kinetics (Equations 4.6 – 4.9) and to Tessier kinetics (Equations 4.10 – 4.13). Equations 4.7 and 4.11 really refer to the same Φ . Since we used different growth kinetics in search of the best-fit model, the predicted Φ values could be different, and we have used two different symbols; Φ_M and Φ_T . We use the subscript “i” when referring to the conditions that apply to both M and T.

If the parameters, Φ_i , β_i and M_i are known, two boundary conditions are enough to solve Equations 4.6 and 4.10. The meaning of these boundary conditions are examined by inspecting Figure 4.2: Equation 4.14 is the dimensionless oxygen concentration at the bottom of the biofilm while Equation 4.15 states that there is no flux of oxygen through the bottom i.e. the concentration at the bottom of the colony biofilm is constant.

Terms “*biokinetic parameters*” refer to Φ_i , β_i and M_i . From β_M we can calculate K_s , and from β_T we can calculate K_{sT} . However, we cannot calculate the maximum specific growth rate and the yield coefficient because these parameters are lumped into Φ_i .

Solution Algorithm

The algorithm we developed to compute the biokinetic parameters is shown in Figure 4.3 and we follow this algorithm solve either equation 4.6 or 4.10.

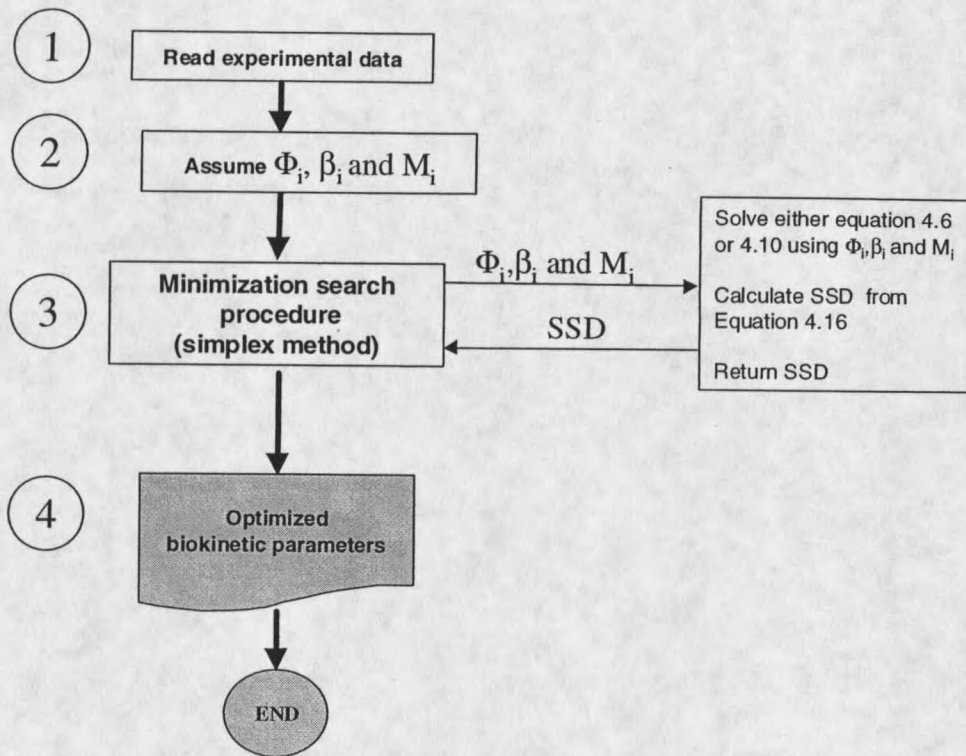


Figure 4.3. The algorithm we developed to compute biokinetic parameters in biofilms.

The procedures in the algorithm (Figure 4.3) are best explained using a pseudo – code.

1. Read the sequence of pairs: dimensionless distance and dimensionless oxygen concentration.

2. Assume Φ_i , β_i and M_i values, and solve Equation 4.6 or 4.10, using boundary conditions 4.14 and 4.15, as an initial value problem, using Runge-Kutta Dormand pairs techniques (MATLAB[®] function *ode45*).
3. Calculate the predicted concentrations of oxygen, at the same locations where they were measured, and calculate the sum of squared differences (SSD) for the pairs of predicted and measured values as:

$$SSD = \sum_{n=1}^{n=n_{\text{of data}}} (S_{\text{predicted}}^* - S_{\text{experimentally_measured}}^*)^2 \quad (4.16)$$

4. Find Φ_i , β_i and M_i values that minimize Equation 4.16; Equation 4.16 is considered the objective function in the search for optimum Φ_i , β_i and M_i values.

To minimize SSD and to calculate optimum Φ_i , β_i and M_i values, we used the direct search method (Lagarias, et al., 1998) integrated with the MATLAB's *fminsearch* function. The *fminsearch* is referred to as unconstrained nonlinear optimization in MATLAB's help menu and in Lagarias, et al., (1998). The *fminsearch* starts searching from the assumed Φ_i , β_i and M_i values, and calculates respective SSD from the solution of Equations 4.6 or 4.10. Then, the *fminsearch*, using the Nelder and Mead's (1965) simplex algorithm, finds the optimum Φ_i , β_i and M_i values for the next iteration. The iteration stops when the optimum Φ_i , β_i and M_i values are found i. e. when parameters from two consecutive iteration differ by less than 0.00001. We relied on the MATLAB's default criteria of convergence.

To facilitate computing the biokinetic parameters, we integrated the algorithm (Figure 4.3) into a MATLAB program using MATLAB version 12.1, and run it on a PC with a Pentium 3 processor using Windows operating system. This algorithm will be available in our web side, <http://www.erc.montana.edu>, prior to publication of the paper.

Calculation of Confidence Limits

To calculate confidence limits of K_{sM} and K_{sT} , we performed 21 concentration profiles measurements similar to those in Figure 4.2. Eight freshly prepared colony biofilms of *Leptothrix discophora* SP-6 was used for at least 2 to 3 profiles measurement from each colony. The concentration profiles were measured at the center of the biofilm colonies but also 100 μm away from the center. From each concentration profile, we calculated biokinetic parameters Φ_i , β_i , and M_i . However, the confidence limits were only calculated for K_{sM} and K_{sT} .

Results and discussion

Assumptions in The Model

1. The growth limiting substrate is oxygen:

Depending on experimental conditions the biofilm growth can be limited by the organic substrate or by oxygen. To determine the conditions for which the growth of the colony biofilms is limited by oxygen, we grew biofilms for 120 h on agar plates

consisting of 1, 5, 10, 20 and 30 g/L pyruvate. Measuring oxygen concentration profiles in these biofilms we concluded that when pyruvate concentrations exceeds 10 g/L the oxygen profiles do not change much, indicating that oxygen limits the growth (Figure 4.4). Therefore, in subsequent measurements, we used 20 g/L pyruvate in agar.

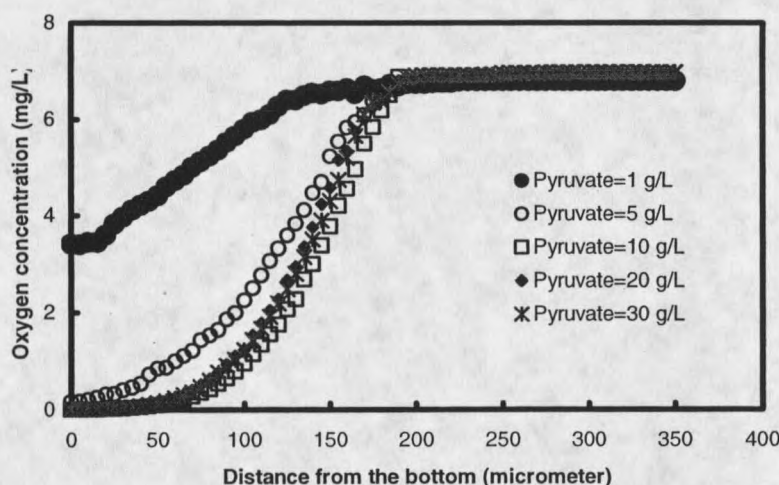


Figure 4.4. Oxygen concentration profiles measured in 120 hours old colony biofilms grown on agar plates containing 1, 5, 10, 20 and 30 g/L pyruvate. When pyruvate concentrations exceeds 10 g/L, the oxygen profiles do not change much, indicating that pyruvate concentration is in excess and for these conditions the growth is limited by oxygen.

2. The biofilm is uniform:

The main reason we used a colony biofilm, and not a biofilm grown in water solution, is that colony biofilms are homogeneous. To test this condition we measured vertical oxygen profiles at different locations in a microcolony and found

that those profiles were almost identical (Figure 4.5). The distances between the locations were approximately 100 micrometers. Location 1 is the center of the colony, and other locations are at the vertexes of a plus sign around location 1. The profiles are similar (the average K_{sM} for all 5 profiles was 0.33 ± 0.03 mg/L), showing that the biofilm had a uniform structure.

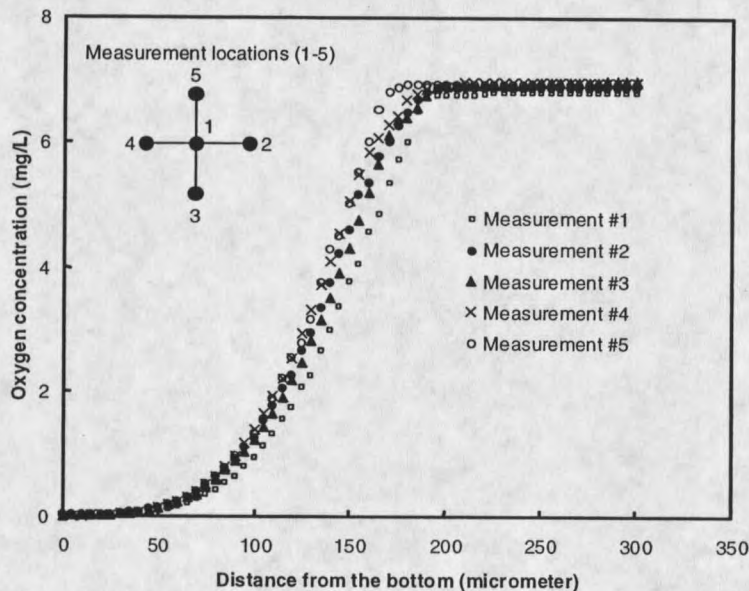


Figure 4.5. Oxygen concentration profiles measured at different locations (1-5) in the colony biofilm. Location 1 was at the center of the microcolony and other locations were approximately 100 micrometers apart.

3. The substrates in the biofilm are transported only by diffusion:

We do not have doubt that the internal mass transport is by diffusion because there was no convective flow within the microcolony and all substrates must have been transported by diffusion. The external mass transport can be controlled by diffusion

or convection. During the microelectrode measurements, when the reactor was opened to the atmosphere, we took precautions to avoid convectonal flow of air. Therefore, we assumed that the substrate diffusion in biofilm was adequately described by Fick's law and convection was ignored.

4. Biofilm density and diffusivity can be represented by their average values:

We are aware of the fact that biofilm density and effective diffusivity vary across the biofilm (Beyenal and Lewandowski 2000). However, since we could not measure density or diffusivity variations across the biofilm, we have assumed that biofilm density and effective diffusivity can be represented by their average values. In our models, biofilm density and effective diffusivity are lumped into the dimensionless module (Φ_i), which represents their average value across the biofilm, and is therefore consistent with the assumptions.

5. Transfer of oxygen is one-dimensional:

We measured oxygen profiles at four additional locations (Figure 4.5) and found that the profiles are similar. If they were significantly different, the mass transfer could not be considered as one-dimensional, as it is in the case of heterogeneous biofilms (Beyenal and Lewandowski, 2001; De Beer et al., 1994). In our biofilm, the colony diameter (3000 micrometers) was 20 times larger than the biofilm thickness (150 micrometers). Therefore, we assumed that the substrate transport in radial and angular directions can be neglected at the center of the colony, and that the lateral component of oxygen mass transfer is negligible.

6. The biofilm is at pseudo steady state (with respect to the oxygen consumption rate):

To verify this assumption we measured oxygen concentration profiles at the same location in a 120 and 144 hours old colony biofilm. The profiles have not changed much and, therefore, the oxygen consumption can be considered to be at pseudo steady state.

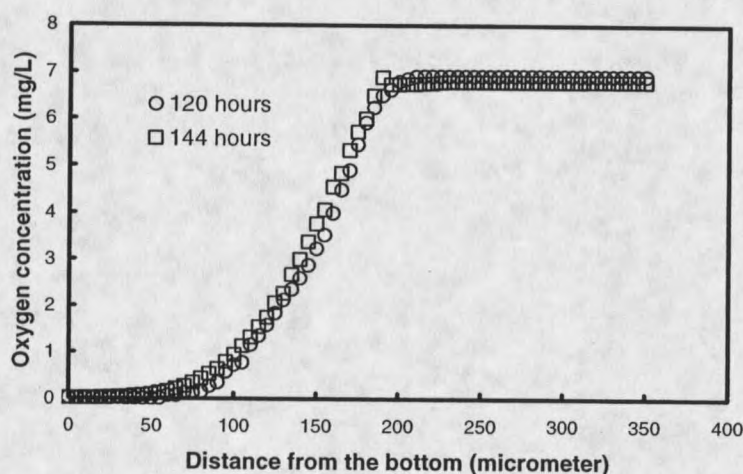


Figure 4.6. Similar oxygen concentration profiles are measured in 120 and 144 hours old colony biofilms verify that oxygen consumption is at pseudo steady state.

Predictions of Φ_i and β_i and M_i

As an example, Equation 4.6 was solved for the data in Figure 4.2 and for this set of data, we calculated $\Phi_M = 4.7401$, $\beta_M = 0.0645$, $M_M \approx 0$ and $SSD = 0.0064$ using initial estimates of $\Phi_M = 1$; $\beta_M = 1$, and $M_M = 1$. To test how sensitive is the computational procedure to the initial estimates, we ran the program for randomly

generated initial estimates; Φ_M between 0 and 1000, β_M between 0 and 100 and, M_M between 0 and 10. Even though we used different initial estimates, the same optimal fit values of Φ_M , β_M and M_M were calculated repeatedly. We also repeated this procedure using the Tessier model (Equation 4.10), and again the calculated biokinetic parameters were independent of the initial estimates. Therefore our technique seems to be immune to changes in initial estimates, within the tested limits. This can be caused by various reasons, and we believe that the following two are important; (1) we use dimensionless equations which increases sensitivity and lowers round-off errors and (2) because the simplex method is an efficient algorithm to find global minimum values.

Biokinetic Parameters and Confidence Limits

Table 1 shows the biokinetic parameters, standard deviations for a biofilm of *Leptothrix discophora* SP-6. We also introduce a new parameter: ratio of maintenance to maximum specific growth, ζ_M . These values were determined from 21 independent measurements in the different biofilms and at the different locations. The listed parameters for planktonic growth in Table 1 were imported from our previous study (Yurt et al., 2002). According to Table 1, the Monod model (Equation 4.6) represents the growth kinetics of *Leptothrix discophora* SP-6 biofilms better than the Tessier model, as it has a slightly lower SSD value than that calculated for Tessier model. For planktonic growth, growth parameters were: $\mu_{\max} = 0.576 \pm 0.021 \text{ h}^{-1}$, K_{sM}

$=0.286 \pm 0.012$ mg/L, and maintenance for oxygen, $m_o=0.761$ (mg oxygen consumed/mg microorganism.h) (Yurt et al., 2002).

Table 4. 1. Biokinetic parameters and confidence limits for biofilm and planktonic growth of *Leptothrix discophora* SP-6. Note the data for planktonic growth is exported from our previous study (Yurt et al., 2002).

	Parameters	Monod (Equation 4.6)	Tessier (Equation 4.10)
Biofilm	K_{sM} (mg/L)	0.333 ± 0.077	na
	K_{sT} (mg/L)	na	0.338 ± 0.141
	SSD	0.099 ± 0.095	0.121 ± 0.114
	M_M	0.0006333 ± 0.000616	na
	M_T	na	0.000849 ± 0.000584
	ζ_M	0.000341	na
planktonic	K_{sM} (mg/L)	0.286 ± 0.012	na
	K_{sT} (mg/L)	na	0.390 ± 0.039
	ζ_M	0.164	na

na : Not applicable

Monod half rate constants for *Leptothrix discophora* SP-6 biofilms and planktonic growth were approximately 15 % different, $K_{sM} = 0.333 \pm 0.077$ mg/L

and 0.286 ± 0.012 mg/L respectively. We used t-test to test if this 15 percent difference between mean values is statistically significant, (Moore and McCabe 1993) and found that two-tailed P value was less than 0.0001. This difference by conventional criteria is considered to be statistically significant. Therefore, we concluded that *Leptothrix discophora* SP-6 have different Monod half rate constant for biofilm and for planktonic growth.

The standard deviations of Monod half rate constants for colony biofilms is 6.4 times higher than that for planktonic cultures (0.077 and 0.012). In two recent review papers written by Kovarova-Kovar and Egli (1998) and Frenci (1998), the authors concluded that the physiological state and culture history played an important role in the variability of half rate coefficient. For our data, we believe these differences demonstrate that the physiological state and culture histories are different in biofilms than in the planktonic growth.

A New Parameter: Ratio of Maintenance to Maximum Specific Growth ($\zeta = mY/\mu_{\max}$)

We could not calculate separately the maximum specific growth rate ($\mu_{\max}$), yield coefficient ($Y_{x/o}$) and the maintenance factor (m). However from the equation (4.17), we can calculate another parameter ζ :

$$\zeta_M = \frac{M_M}{\Phi_M^2} = \frac{Y_{x/o} m_M}{\mu_{\max}} \quad (4.17)$$

As seen from the Table 1, ζ_M is significantly lower in biofilms than it is in planktonic growth; the ratio of ζ_M for planktonic growth to ζ_M for biofilm growth is

480. Although, $\mu_{\max}$ and $Y_{x/o}$ for a biofilm remains unknown, since the calculated dimensionless maintenance values were close to zero for biofilms, we can expect m_M to be close to zero in biofilms. To test this expectation we assume; $D_{\text{eff}} = 2 \times 10^{-5}$ cm^2/s , $X_f = 50 \text{ mg}/\text{cm}^3$, $S_s = 5 \times 10^{-3} \text{ mg}/\text{cm}^3$, $L_f = 5 \times 10^{-3} \text{ cm}$ and by solving Equation 4.9, we calculate the maintenance coefficient m_M as;

$$m_M = \frac{M_M D_{\text{eff}} S_s}{L_f^2 X_f} \quad (18)$$

where: S_s and L_f are average values measured experimentally. From the literature we know that X_f and D_{eff} may vary between $5 \text{ mg}/\text{cm}^3$ - $200 \text{ mg}/\text{cm}^3$, and between $0.1 \times 10^{-5} \text{ cm}^2/\text{s}$ - $2.1 \times 10^{-5} \text{ cm}^2/\text{s}$ (Fan et al., 1990). The m_M is calculated from the Equation 4.18 as $0.17 \times 10^{-5} \text{ mg oxygen consumed}/\text{mg microorganism.h}$ for *Leptothrix discophora* SP-6 biofilms, using assumed parameters above. For planktonic growth m_M is $0.761 \text{ mg oxygen consumed}/\text{mg microorganism.h}$. The maintenance coefficient calculated for planktonic growth is approximately 4400 times higher than the maintenance coefficient calculated for biofilms. Although we used the assumed values to calculate maintenance coefficient for biofilms, we do not expect in reality m_M to be different by more than one or two orders of magnitude (depending on the range for D_{eff} and X_f). This analysis shows that the maintenance coefficient in biofilms is close to zero.

Conclusions

We have developed an improved method to calculate biokinetic parameters for Monod and Tessier microbial growth kinetics including maintenance consumption, from oxygen concentration profiles measured in biofilms, and demonstrated its utility by calculating biokinetic parameters for microbial growth of *Leptothrix discophora* SP-6 in a biofilm and, we concluded that;

1. The algorithm presented in the paper successfully calculated biokinetic parameters.
2. The Monod model represented growth of *Leptothrix discophora* SP-6 biofilms better than the Tessier model.
3. *Leptothrix discophora* SP-6 biofilms uses oxygen for growth rather than for maintenance.
4. Monod half rate coefficients for biofilms and planktonic growth were 0.333 ± 0.077 mg/L and 0.286 ± 0.012 mg/L, respectively, and they were statistically different.

CHAPTER 5

CONCLUSIONS

Hypothesis 1: *Manganese oxidizing activity of Leptothrix discophora SP-6 in a biofilm leads to ennoblement of stainless steel, causes changes in the passive film chemistry of the steel.*

This hypothesis was found correct.

The oxide layers on the ennobled coupons are significantly thinner than those on the control coupons.

Hypothesis 2: *The presence of biomineralized manganese oxides on the surface of 316L stainless steel initiates pitting corrosion in a low-chloride aqueous environment.*

This hypothesis was found correct.

The ennobled samples pitted in 0.2 M NaCl solution, and pitting potentials of biofilm covered (non-ennobled) and clean coupons were 497 ± 64 and 573 ± 67 mV_{SCE}, respectively.

Hypothesis-3: *The optimum model, statistically, to represent Leptothrix discophora SP-6 growth in the planktonic form can be determined by an algorithm which optimizes parameters for the four major kinetic models.*

This hypothesis was found correct with following conclusions.

A double-substrate model, using Monod growth kinetics for pyruvate and Tessier growth kinetics for oxygen, is in agreement with the chemostat data.

Using this combination of growth kinetics, the following growth parameters for *Leptothrix discophora* SP-6 were determined; $\mu_{\max} = 0.576 \pm 0.021 \text{ h}^{-1}$, $K_{sMp} = 38.81 \pm 4.24 \text{ mg/L}$, $K_{sTo} = 0.390 \pm 0.040 \text{ mg/L}$, $Y_{X/P} = 0.150$ (mg microorganism/mg pyruvate), $Y_{X/O} = 1.24$ (mg microorganism/mg oxygen), $m_P = 0.129$ mg pyruvate consumed/mg microorganism.h, and $m_O = 0.076$ mg oxygen consumed/mg microorganism.h.

Hypothesis 4: *The optimum model, statistically, to represent Leptothrix discophora SP-6 growth in the biofilm form can be determined by an algorithm which optimizes parameters.*

This hypothesis was found correct.

The algorithm presented in the thesis successfully calculated biokinetic parameters.

Hypothesis 5: *Leptothrix discophora* SP-6 has different growth and substrate consumption kinetics in planktonic and biofilm form.

This hypothesis was found correct.

Leptothrix discophora SP-6 biofilms uses oxygen for growth rather than for maintenance.

Monod half rate coefficients for biofilms and planktonic growth were 0.333 ± 0.077 mg/L and 0.286 ± 0.012 mg/L, respectively, and they were statistically different.

APPENDICES

APPENDIX A

EFFECT OF BIOMINERALIZED MANGANESE ON PITTING CORROSION OF
TYPE 304L STAINLESS STEEL

EFFECT OF BIOMINERALIZED MANGANESE ON PITTING CORROSION OF TYPE 304L STAINLESS STEEL

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British summary

During the past few years, biomineralized manganese has been shown to cause ennoblement of various stainless steels to open circuit potentials of 300 – 400 mVSCE. We have demonstrated that ennoblement, caused by biologically deposited manganese minerals, along with a relatively low stainless steel pitting potential, caused by the presence of chloride, is sufficient to initiate and drive active pitting corrosion. Stainless steel samples (type 304L), chemically or microbiologically ennobled with manganese dioxide, were exposed to a 0.35% w/v NaCl solution; an environment otherwise not corrosive against the 304L stainless steel. In the first case, steel samples were ennobled by electroplating the sample with a thin film of manganese dioxide, except for a small anodic area. In the latter case, the manganese dioxide was deposited on the steel within biofilms of the manganese oxidizing bacterium *Leptothrix discophora* SP-6. After 24 hours exposure to the chloride solution the samples were investigated by atomic force microscopy (AFM). Both types of ennobled samples were found severely pitted, whereas reference samples (w/o manganese minerals) had remained intact.

Keywords - 304L stainless steel, pitting corrosion, microbially influenced corrosion (MIC), manganese dioxide, biomineralization, *Leptothrix discophora* SP-6, atomic force microscopy (AFM)

Introduction

The area of Microbiologically Influenced Corrosion (MIC) that involves pitting of stainless steels is generally associated with the activity of sulfate reducing bacteria (SRB) [^{i, ii}]. The influence of SRB on corrosion processes has been extensively studied during the past decades revealing a great deal about their activity [^{iii, iv}]. There are, however, other areas of MIC that can be important in relation to stainless steel pitting. Recently, the field of MIC was expanded to reflect an increasing general interest in biomineralization [^v]. Particularly the effect of microbially deposited manganese minerals on the electrochemical behavior of metals is being targeted by current studies [^{vi-xi}]. Though still poorly understood, manganese biomineralization is a common phenomenon in natural waters as well as in industrial systems and water distribution system, where the manganese minerals often are found as black/brownish deposits [^{xii, xiii}]. Until recently, problems regarding manganese mineral fouling were mostly owed to unwanted coloration of water, particularly drinking water, through manganese biofouling and sloughing within the drinking water distribution system. However new discoveries shows that manganese dioxide (MnO_2), and maybe other manganese minerals too, if deposited on passive metals changes the electrochemical properties of the metal by increasing the open circuit potential (OCP), known as ennoblement [^{xiv-xviii}]. Potential ennoblement of passive metals, particularly in marine environments, has been and is still frequently observed and studied [^{xix-xxii}]. Consequently, there exist numerous theoretical explanations to

the ennoblement behavior. Deposition of biomineralized manganese, however, is one of the firsts to have a documented effect on the OCP of metals.

During studies of potential ennoblement of 316L stainless steel in a fresh water environment, a connection between biological deposition of manganese oxides on the surface and the OCP of the steel was discovered. Furthermore it was shown, that the presence of biomineralized manganese oxides solely caused ennobled potentials of the steel in the range of 350-400 mV_{SCE}. Similar effects were obtained by partially covering the steel surface with chemically prepared hydrous MnO₂. The organic materials constituting the biofilm did not itself cause any changes in the electrochemical properties of the metal.

Investigations of severe pitting failure of CrNi134 steel runner blades in a Dutch hydroelectric power plant, revealed large amounts of manganese rich minerals within corrosion products and biofilms on the surface. Analysis of the mineral deposit revealed a mixture of MnO₂ and MnOOH. OCP of isolated deposit was measured as 570 mV_{SCE} at pH 7.5, 20°C, and 0.1 mg/L Mn²⁺. Similar mixtures have been identified in later electrochemical studies of manganese rich corrosion products [xxiii].

In another case elevated amounts of manganese minerals were found at sites of pitting corrosion in a pipeline transferring cooling water from the river Rhine, Germany [xxiv]. The corrosion attacks were, as often reported in cases of MIC, located around welds. Based on these findings the importance of manganese minerals and manganese depositing bacteria among other influences on stainless steel failure was discussed [xxv].

Another paper reported pitting corrosion of type 304 stainless steel in a potable water treatment plant nine months after startup [^{xxvi}]. The corrosion was most probably influenced by microbiological activity. A number of metal-oxidizing bacterial strains were isolated from black/brown deposits around the corrosion sites. Furthermore the well water contained high amounts of manganese and, following treatment, low concentrations of chlorine. The incident was concluded to be a combined effect of the high concentration of manganese, the deposition of manganese minerals, and the low concentration of chlorine.

Studies of the electrochemical properties of manganese minerals on 316L stainless steel showed that MnO_2 at neutral pH is reduced to divalent manganese through manganese oxyhydroxide. Under those conditions the MnOOH was not stable and there were no net accumulation of this compound at the steel surface. At low pH there were no intermediate products and at high pH only insoluble manganese compounds were thermodynamically stable. It was also shown that at least one strain of manganese oxidizing bacteria, *Leptothrix discophora* SP-6, mainly deposits manganese as MnO_2 . The cathodic activity of MnO_2 , through reduction to divalent manganese, was proposed to increase the probability of active pitting and to increase the corrosion rate of active pits. It has previously been stated that the elevated potentials of manganese ennobled stainless steel could reach the pitting potential of the steel, which in the presence of high amounts of chloride can be as low as zero mV_{SCE} .

This paper addresses the question: "Can the presence of biomineralized MnO_2 on the surface of stainless steel initiate pitting corrosion in a chlorinated environment otherwise resistive to pitting?" Initially the pitting potential of the chosen steel type was determined in different concentrations of chloride, and compared to OCPs of MnO_2 ennobled samples of the same steel type. Secondly, a chloride concentration, which gave pitting potentials below the potentials of ennobled samples, was chosen. Ennobled samples were then exposed to solutions of this concentration, and the surfaces were examined for localized corrosive attacks.

Materials and methods

Type 304L stainless steel coupons ($\varnothing$ 16.8mm) were punched from 16 gauge sheet metal. The elemental composition of the steel as provided by the vendor, J&L Specialty Steel, Inc., is shown in table 1.

Coupons were epoxy embedded in polycarbonate rings (height 3mm, OD 19.1mm, ID 16.8/10.0mm) using a slow hardening epoxy (Buehler Epoxide). Figure 1 illustrates a mounted steel coupon. Copper wires were positioned through 1mm holes in the sides of the polycarbonate rings and glued to the backsides of the coupons, using a conductive glue (Nickel Print, GC Electronics). The backsides of the rings were filled with the slow hardening epoxy to secure and insulate the electrical connection. Each application of epoxy/glue was allowed 24 hours to harden. The mounted coupons were polished first on a series of sandpaper's ranging from 120 to 600 grit then on polishing mats with 5.0, 0.3, and 0.05 micron aluminum powder. Preparing the samples this way enabled the use of various surface analysis

techniques without removing the sample from the holder. These coupons, without further processing, are referred to as type I samples.

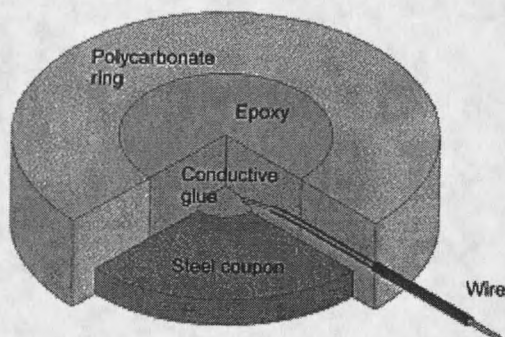


Figure 1. Illustration of an embedded steel coupon. Only the down facing side of the coupon was exposed.

Electrochemical experiments were conducted using a potentiostat/galvanostat (EG&G Princeton Applied Research, model 273A) with a platinum mesh auxiliary electrode (30cm^2 surface area) and a saturated calomel electrode (SCE) as reference. OCPs were measured versus the SCE reference electrode using a handheld multi meter (Wavetek DM23XT, internal resistance $10\text{m}\Omega$).

Pitting potentials of the 304L steel (type I samples) were obtained through potentiodynamic polarization. Scans were initiated 50mV below the OCP and scanned, using a scan rate of 5mV/sec , to $1.2V_{\text{SCE}}$ or 1mA , whichever was reached first. Experiments were conducted at room temperature in $0.1\text{M Na}_2\text{SO}_4$ solutions with 0, 0.0035, 0.035, 0.35, and 3.5% w/v NaCl.

A number of samples (type I) were electro plated with a thin film of MnO_2 as described previously. Briefly, samples were immersed in an aqueous electrolyte containing 0.1M Na_2SO_4 and 5mM MnSO_4 at pH 6.5. A total of $30\text{mC}/\text{cm}^2$ was transferred per sample at a rate of $2\mu\text{A}/\text{cm}^2$ corresponding to $13.5\mu\text{g}/\text{cm}^2$ or a 270 nm thick film of solid MnO_2 . Prior to electroplating, a small drop of immersion oil (a few hundred microns in diameter) was placed in the center of the coupon. The oil drop served as an insulator preventing plating of the underlying steel. Following plating, the oil drop was removed with acetone, having no effect on the composition of the mineral film or the potential of the ennobled coupons. Following plating, the samples were allowed at least one hour to equilibrate in 0.1M Na_2SO_4 . These samples had a large cathodic area covered with MnO_2 and a small anodic area with bare steel. The cathode to anode ratio was about 50,000:1. The composition of the deposited mineral was analyzed by XPS, as described elsewhere, revealing only MnO_2 . Electroplated coupons are referred to as type II samples. Type I samples served as reference for the type II samples.

Other steel samples (type I) were fouled with a biofilm of the manganese oxidizing bacterium *Leptothrix discophora* SP-6, within which manganese minerals were deposited onto the steel surface. Cultures of *Leptothrix discophora* SP-6^{xxvii} obtained from American Type Culture Collection (ATCC no. 51168) were grown in sterile mineral-salt-pyruvate-vitamin (MSPV) medium (ATCC no. 1917). Subcultures were preserved by freezing at -70°C [xxviii]. Prior to each experiment a frozen culture was thawed and incubated for 48 hours in 100 ml MSPV medium. Five ml of this culture was used to inoculate the experimental reactor. The

construction and operation of the experimental reactor has been described elsewhere. Briefly a reactor with eight type I steel samples suspended from the lid was filled with 500 ml MSPV containing $200\ \mu\text{M Mn}^{2+}$ as MnSO_4 , inoculated and run in batch mode for 48 hours. Hereafter, the reactor was feed continuously for up to two weeks (1 hour retention time) with similar, but five times concentrated, medium and filter sterilized dilution water in a 1:5 ratio. A Saturated Calomel Electrode (SCE), connected to the media through a 1% agar salt bridge containing 1 mM Na_2SO_4 , enabled OCP measurements of the steel samples. Biofouled samples prepared this way are referred to as type III samples. As a reference for the type III samples, similar coupons were prepared without added manganese in the growth media. These samples, fouled with a biofilm containing no manganese, are referred to as type IV samples.

Samples of all four types (eight of each type - bare, electroplated, biofouled with Mn, and biofouled without Mn) were exposed to 0.1M Na_2SO_4 containing 0.35% w/v NaCl for 24 hours. During the exposure, the OCP of every sample was measured hourly versus the SCE.

Topographical imaging of selected samples was conducted using contact mode Atomic Force Microscopy (AFM) analyzing an area of until $80 \times 80\ \mu\text{m}$. Prior to exposure, type II samples were mapped within the anodic areas, whereas the type I samples were mapped at randomly chosen sites. Type III and IV samples were mapped as type I prior to fouling. Similar analyses were performed after exposure, searching for changes in the surface topography caused by corrosion attacks. Images were collected at corrosion sites, revealing the depth, width and shape of the attack.

Except for light polishing streaks, no unusual topography was found prior to the exposure to chloride.

Results

Polarization curves obtained for 304L stainless steel in 0.1M Na_2SO_4 solutions with 0, 0.0035, 0.035, 0.35, and 3.5% w/v NaCl are shown in figure 2. In both the reference case, without NaCl, and the case with 0.0035% NaCl, the pitting potential was not reached within the stability of water. In 0.035% NaCl the pitting potential was poorly defined between 500 and 700 mV_{SCE}. The polarization curves showed several peaks of metastable pitting and repassivation in that potential range and the significant current increase could have been caused by decomposition of water. In 0.35% NaCl the pitting potential, at the point where the current rapidly increased, was more precisely determined to be $+300 \pm 50 \text{ mV}_{\text{SCE}}$. Few of these curves showed repassivation peaks. Curves obtained in 3.5% NaCl, corresponding to a general seawater level, were flattened with a higher overall current and a poorly defined pitting potential around 0mV_{SCE}. This could have been an indication that at least metastable pitting already happened at OCP.

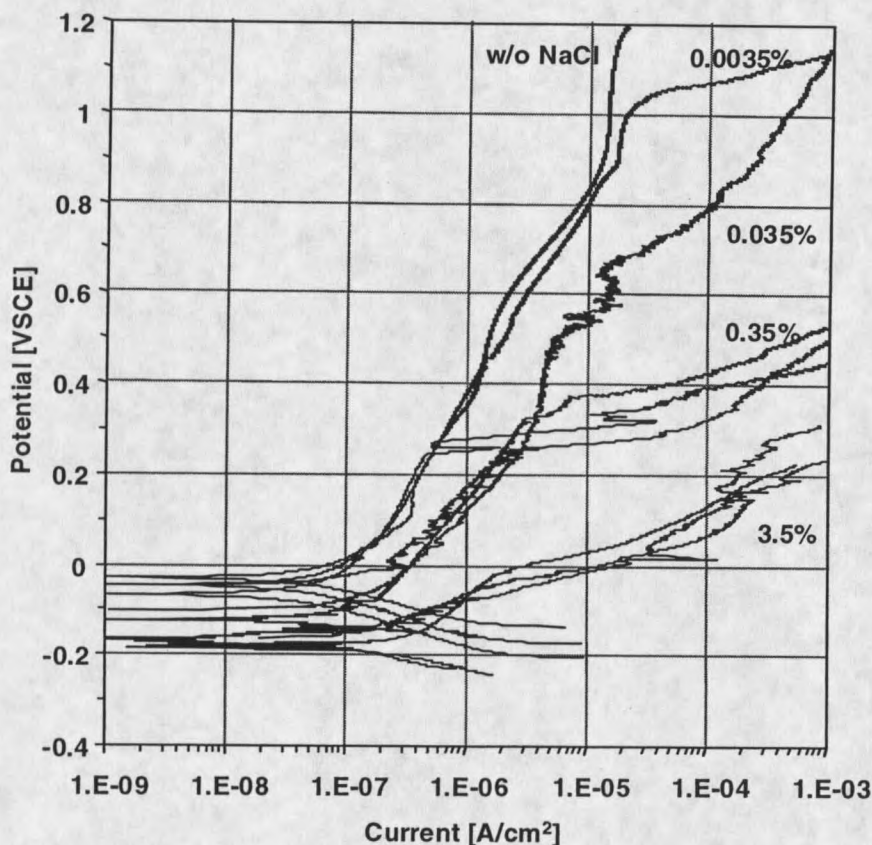


Figure 2. Polarization curves for 304L stainless steel in 0.1 M Na_2SO_4 solutions with 0, 0.0035, 0.035, 0.35 and 3.5 % w/v NaCl.

The OCP of stainless steel samples, ennobled by electroplating with MnO_2 , was initially between 550 and 650 mV_{SCE}, but stabilized at $+500 \pm 25$ mV_{SCE} within one hour in 0.1M Na_2SO_4 . The values correspond well with previously published potential data for MnO_2 plated stainless. Anodic areas ranged in size from 20-100 μm in diameter. Figure 3 shows an AFM topographical image of an anodic spot on a type II sample.

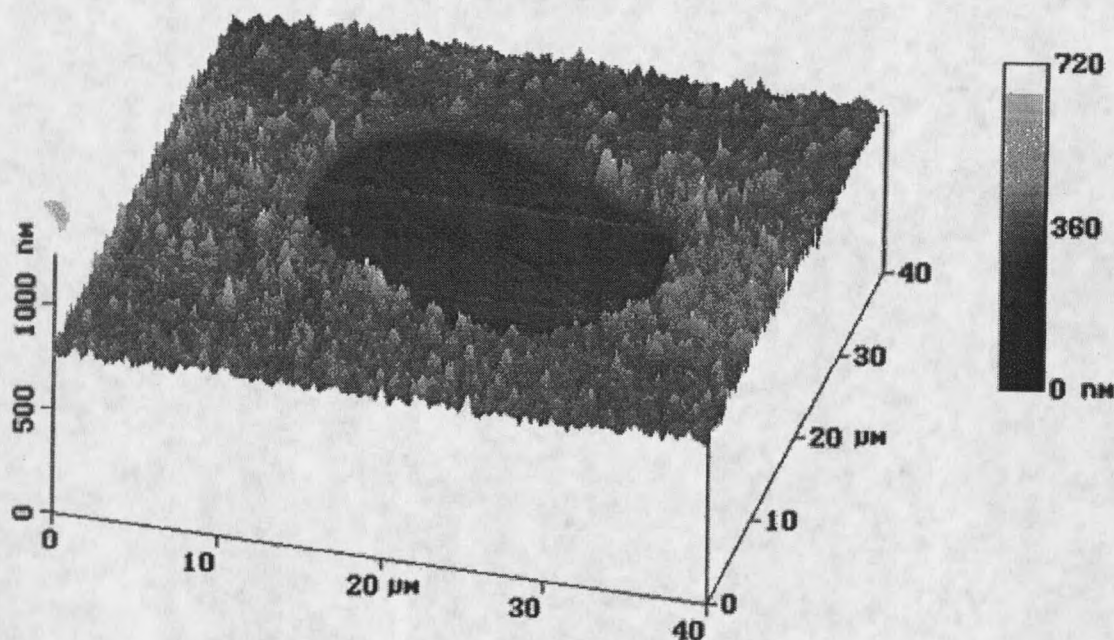


Figure 3. AFM topographical image of an anodic hole in the cathodic MnO_2 film on an electroplated sample.

Samples ennobled through manganese biofouling with *L. discophora* SP-6, reached stable OCPs of $390 \pm 20 \text{ mV}_{\text{SCE}}$ within one week following inoculation. The potentials remained stable for an additional week until the experiment was terminated.

Based on the OCPs of ennobled samples, a NaCl concentration of 0.35% was chosen for exposure of the ennobled samples. At 0.35% NaCl the pitting potential was about $300 \text{ mV}_{\text{SCE}}$ and OCP of the ennobled samples were around 400 and 500 mV_{SCE} for biofouled and plated samples respectively. The combination of this concentration and either of the ennobled samples could theoretically lead to active pitting corrosion.

Steel coupons of type I, II, III, and IV were exposed to the 0.35% NaCl solution for 24 hours. Biofilms and corrosion products were removed from the coupons by sonication in distilled water for 2 minutes followed by drying in nitrogen gas. OCPs, collected during exposure to the NaCl solution, were stable between -200 and $-100\text{mV}_{\text{SCE}}$ for the reference (type I and IV) samples. The potential for the ennobled (type II and III) samples remained initially at $300\text{--}500\text{mV}_{\text{SCE}}$, but dropped after 1-3 hours of exposure to values similar to the references.

Reflected light microscopy ($100\times$) revealed spots of discoloration on the type II and III samples, whereas the type I and IV samples seemed unaffected. Contact mode AFM showed a large single pit within the anodic areas on the type II samples, and small pits located at cracks in the mineral film within the cathodic area. Figure 4 shows an AFM topographical image of a pit within the anodic area of a type II sample. After the 24 hours exposure, there were still films of manganese minerals covering the cathodic areas. The thickness of the film, however, was reduced compared to the originally plated film (figure 3). Pits on the type II samples ranged from $5\text{--}15\mu\text{m}$ in diameter and $0.5\text{--}5\mu\text{m}$ in depth. In two cases, the pit depth exceeded the $6\mu\text{m}$ limit of the AFM apparatus. No pits were found on the type I samples. In a single case, however, a crevice between the steel coupon and the polycarbonate holder had initiated corrosion. Images of type III samples showed randomly distributed clusters of smaller irregular pits. Figure 5 shows a top view and a cross section of such a cluster. Few of the pits on these samples exceeded $1\mu\text{m}$ in depth. The majority of pits were $0\text{--}0.5\mu\text{m}$ deep and a few microns in diameter. Type IV

samples (the biofouled samples without manganese minerals) showed no sign of corrosion.

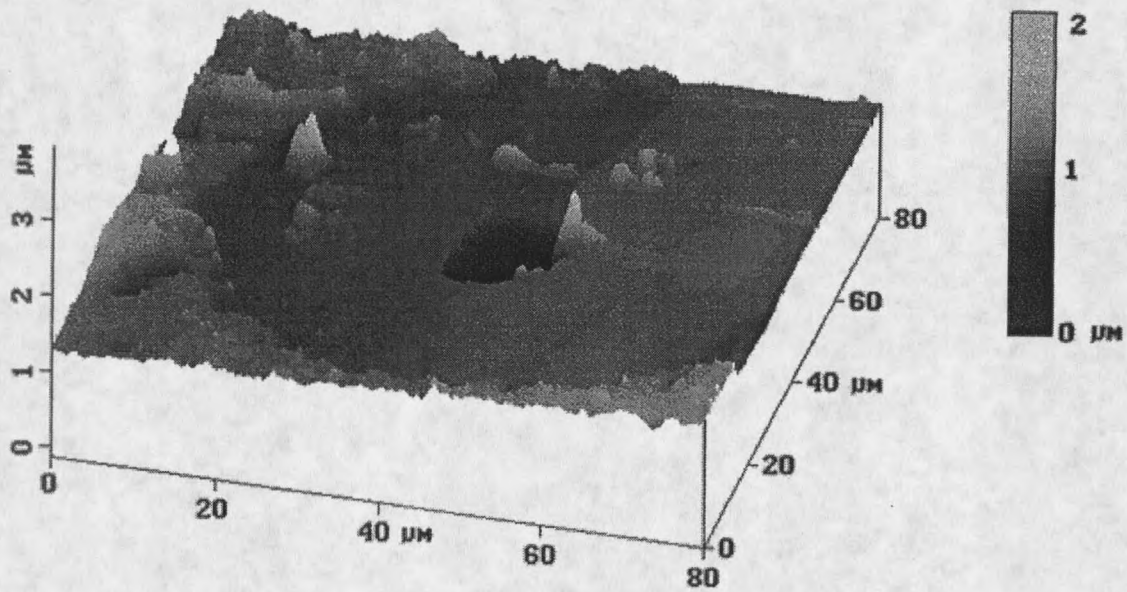


Figure 4. AFM topographical image of a pit in the anodic area of a MnO_2 electroplated sample.

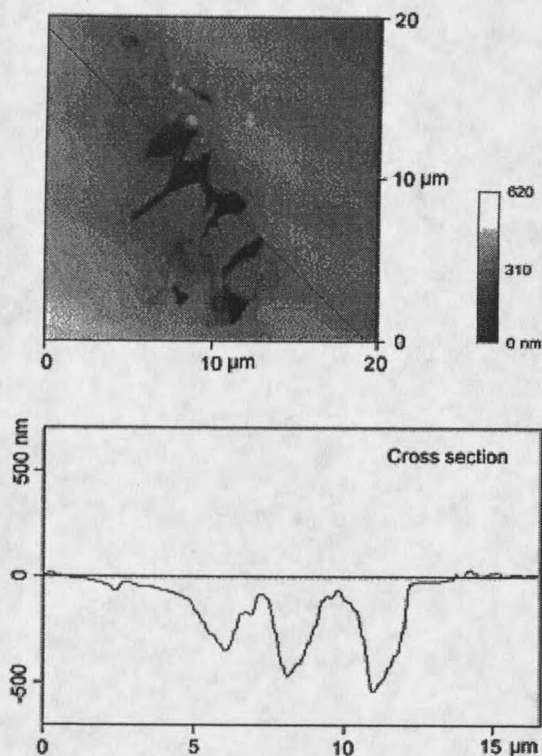


Figure 5. AFM topographical image and cross section of a group of pits from MnO_2 biofouled steel sample.

Discussion

From a comparison between the behavior of the references and the ennobled samples, when exposed to chloride, it is clear that the deposited MnO_2 mineral lead directly to pitting corrosion of both types of ennobled samples. These findings prove, at least for 304L stainless steel, our previous, that the cathodic activity of MnO_2 is sufficient to initiate pitting of stainless steels in a chlorinated environment. Previous observations of severe pitting corrosion at sites with low chloride concentrations but large amounts of manganese rich minerals corresponds to this behavior.

The onset of pitting corrosion of otherwise passive metals usually results in a sudden drop in the OCP. Our OCP measurements, though not continuous, showed such a drop during exposure of the ennobled samples. Elsewhere published results on the effect of manganese ennoblement on pitting have shown that OCP of ennobled samples dropped when chloride was added. As this behavior was taken as a sign of pitting corrosion, it agrees well with our findings.

It has been stated before [xxix] that in order to have any effect on the electrochemical behavior of the underlying metal, the manganese minerals have to be in direct electrical contact with the metal. This is one of the basic differences between the two types of ennobled samples. We have previously documented that the electrochemical reduction of manganese dioxide on a metal surface takes place at the mineral/water interface. The type II samples were electrochemically plated with a solid film of manganese dioxide. Though not perfectly smooth, as seen in figure 3, each molecule in the film was in electrical contact with the underlying metal, and thus potentially available for electrochemical reduction. In the case of the microbially ennobled samples, the deposition of minerals took place within a structurally heterogeneous biofilm creating patches or clusters of minerals. It is unlikely that all minerals within a biofilm of tenths or hundreds of microns can be in electrical contact with the substratum, thus only a fraction of these minerals were available for electrochemical reduction. Nevertheless, this fraction has several times been proven sufficient to fully ennobel stainless steel and now also to initiate pitting corrosion in the presence of chloride.

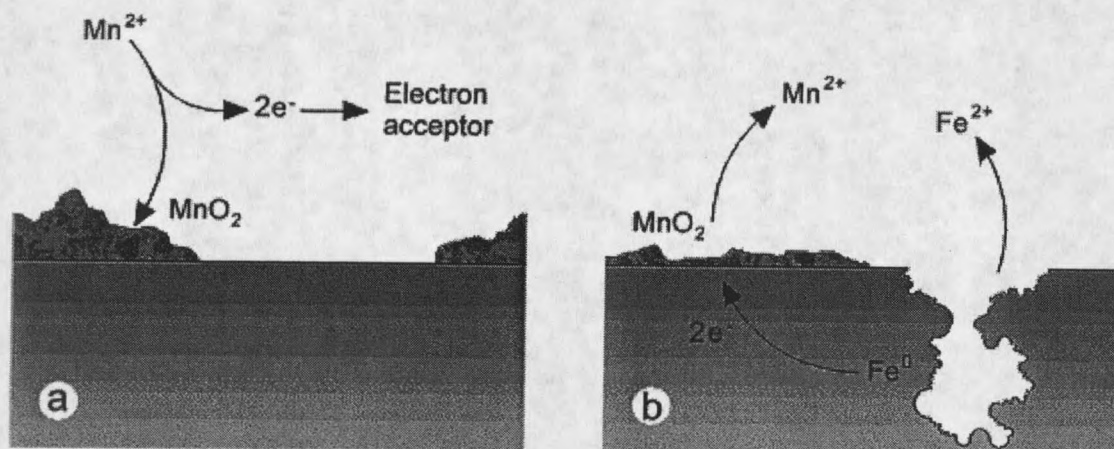


Figure 6. Schematic illustration of a) the microbial oxidation and deposition of MnO_2 , and b) the cathodic reduction of MnO_2 in a corrosion process.

Figure 6 summarizes the basic processes involved in ennoblement and pitting corrosion of stainless steel through deposition of manganese dioxide. A number of microorganisms, including *L. discophora*, are capable of oxidizing manganese (Mn^{2+}) to insoluble minerals like MnO_2 . For *L. discophora* SP-6 the minerals are mainly deposited in the form of MnO_2 . The microbial oxidation process uses different electron acceptors to place the excess electrons. When deposited on the stainless steel, the MnO_2 changes the chemical potential of the surface. Being far more reactive than the passive layer on the steel, it only takes a few percent coverage to change the overall potential of the steel to that of the. Depending on pH and temperature, the potential-determining redox reaction can be one of the following:



$$E_0 = 1.315 \text{ V}$$



$$E_0 = 1.189 \text{ V}$$

The specific reaction influences the redox potential and thus the overall OCP of the metal, however since the reaction (2) is followed by a further reduction of manganese oxyhydroxide (3) the end product will be the same: divalent manganese.



$$E_0 = 1.442 \text{ V}$$

It has previously been demonstrated that chloride ions can break the passive film on stainless steel [xxx, xxxi]. Depending on the steel type, the potential of the steel, and the temperature, it will take a certain chloride concentration to do so, thus the ennoblement itself increases the risk of pitting initiation. Assuming that the pit has been initiated, iron will be dissolved in the pit, generating two excess electrons for each iron atom within the metal matrix. With no cathodic reaction to remove the electrons from the matrix, the dissolution of iron will soon come to an end, due to the build up of electrons, and the pit will repassivate again. Usually the reduction of oxygen or the production of hydrogen gas (depending on whether the environment is aerobic or not) will supply the cathodic reaction. The rate of iron dissolution will then be determined from the total free energy released through either of the cathodic reactions and the anodic dissolution. We have shown earlier that the free energy released through the reduction of MnO_2 to either MnOOH or Mn^{2+} is far greater, coupled to the dissolution of iron, than the reduction of oxygen or the production of

hydrogen gas. Thus, due to the thermodynamic properties of MnO_2 , the processes (pictured in figure 6) will lead not only to an increased probability of pitting corrosion but also to a higher corrosion rate of active pits.

Conclusions

The pitting potential of 304L stainless steel in 0.35% w/v NaCl was found to be $+300 \pm 50 \text{ mV}_{\text{SCE}}$.

OCP of 304L stainless steel samples electroplated with MnO_2 were stable at $500 \pm 25 \text{ mV}_{\text{SCE}}$. Steel samples fouled with a biofilm of *Leptothrix discophora* SP-6, depositing MnO_2 , stabilized at $390 \pm 20 \text{ mV}_{\text{SCE}}$.

Combining the low pitting potential in 0.35% w/v NaCl with either of the two types of ennobled steel samples resulted in pitting corrosion of the specimens. Reference samples without manganese minerals, either clean or fouled, did not show any signs of pitting following exposure to the 0.35% NaCl solution.

On electroplated samples, pits were formed within specified anodic areas. At these sites, the steel was not plated, but the potential was raised to $500 \pm 25 \text{ mV}_{\text{SCE}}$ by the surrounding plated cathodic areas. The position of the pits within the anodic areas was closely related to the geometry of the area.

Structural and chemical heterogeneity within the biofilm on the fouled samples created similar anodic and cathodic areas on the steel surface that was responsible for the observed randomly distributed clusters of corrosion pits.

Acknowledgement

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APPENDIX B
THE MATLAB® CODE

The MATLAB® code

The MATLAB® code is presented below. It consisted of two files. The MATLAB *script file* is used to test all models presented in Table 3.1, and to calculate biokinetic parameters for these models. To calculate biokinetic parameters for a model we used MATLAB's random function generator to produce initial estimates (line 34). Also, we added a loop to run the program for different initial estimates (line 33). The optimum biokinetic parameters which give minimum SSD (Equation 3.10) is calculated using *fminsearch* function (line 37) by selecting parameters (biokinetic coefficients) and sending them to the *double_engine* function. The *double_engine* function uses these parameters and calculate SSD (Equation 3.10). Depending on the SSD values, the *fminsearch* procedure simulates biokinetic parameters until it finds biokinetic parameters, which give the minimum SSD (Lagarias, et al., 1998). We found the default MATLAB values satisfactory to limit convergence of the predicted parameters. Although we have numbered lines to document the code, it is not necessary for the MATLAB programs.

MATLAB SCRIPT FILE

```

1. % Biokinetic parameter finder
2. % This is MATLAB script file to find biokinetic parameters
3. %
4. % Double_engine is the function which
5. % calculate sum of loss squares (loss)
6. % returns loss value
7. %

8. % global parameters used by engine function
9. global Dilution; % dilution rate
10. global Microorganism; % microorganism concentration
11. global Pyruvate; % pyruvate concentration
12. global Oxygen; % oxygen concentration
13. global model_p; % models for pyruvate
14. global model_o; % models for oxygen
15. global n_of_data; % number of data points

16. % *****
17. % read experimental data
18. % to run using different data just change the data here
19. % *****

20. % dilution rate
21. Dilution=[0.029;0.052;0.071;0.081;0.129;0.163;0.24;0.48;0.118;0.1;0.08;0.1;0.1; 0.1; 0.047;
0.055;0.03;0.11;0.188;0.5;0.3;0.35;0.43];

22. % microorganism concentration
23. Microorganism=[87;113;131;110;111;97;143;106;134;104;175;109;125;175;114;121;98;119;
132;99;103;121;113];

24. % pyruvate concentration
25. Pyruvate=[2;3.6;5.2;26.9;12.8;40.7;28.9;225;10.4;7.7;10.2;470;8.1;10.4;3.4;4.5;1.9;8.1;17.5;3
25;34.1;74;234];

26. % oxygen concentration
27. Oxygen=[5.7;5.6;3.8;0.2;1.3;0.4;2.2;6.8;2.1;3.3;0.9;0.1;5.1;6;7.6;7.7;7.8;7.7;5.5;6.8;1.6;0.9;0.
65];

28. n_of_data=size(Dilution); % calculate number of data points
29. % this data file will be located same directory where MATLAB runs
30. fid = fopen('dataout.txt','w');

31. for model_p=0:4 % models for pyruvate
32. for model_o=0:4 % models for oxygen
33. for iteration=1:1 % number of runs for different initial gauss
34. for i=1:7 % maximum 7 parameters to calculate
a. x(i)=rand(1)*100; % generate random initial values
35. end % for i
36. % Run minimum search algorithms for biokinetic parameters
37. [xx,fval]=fminsearch(@double_engine,[x(1),x(2),x(3),x(4),x(5),x(6),x(7)])
38. % xx.calculated parameters matrix- returned from fminsearch

```

```

39. % ignore any unused parameter values as an example if the model requires
40. % use of X(1) and X(2) you will see X(3), X(4).... in output
41. % however they will not be used anywhere

42. % print results to data file
43. fprintf(fid,'%6.5f\t',model_p, model_o, xx,fval);
44. fprintf(fid,'\n'); % new line
45. % to see which model is currently running at runtime
46. disp(model_p); disp(model_o);
47. xx % see calculated parameter on screen
48. fval % see loss
49. end % iteration
50. end % model_o
51. end %for loop

52. fclose(fid) % close file

```

FUNCTION DOUBLE ENGINE

```

1. % funtion double_engine
2. %
3. % Copyright
4. % Center for Biofilm Engineering
5. % Biofilm Structure and Function Research Group
6. % Montana State University
7. % Bozeman, MT, USA
8. %
9. %
10. % calculates loss values for model and experimental data

11. function f=double_engine(xx)

12. % global data
13. global Dilution; % Dilution rate
14. global Microorganism; % Microorganism concentration
15. global Pyruvate; % Pyruvate concentration
16. global Oxygen; % Oxygen concentration
17. global model_p; % specific growth rate for pyruvate
18. global model_o; % specific growth rate for oxygen
19. global n_of_data; % number of experimental point

20. % -----
21. % the following values controlled by fminsearch
22. % fminsearch automatically changes their values
23. % and double_engine function returns loss value
24. % so fminsearch finds minumum loss and corresponding biokinetic parameters

25. % passed by function
26. Mmax=xx(1); % maximum specific growth rate
27. Kpyr=xx(2); % Monod or Tessier coefficeint for pyruvate
28. KO=xx(3); % Monod or Tessier coefficeint for oxygen
29. Lpyr=xx(4); % Moser coefficient for pyruvate

```

```

30. LO=xx(5);    % Moser coefficient for oxygen
31. Bpyr=xx(6);  % Constant in Contois model for pyruvate
32. BO=xx(7);    % Constant in Contois model for oxygen
33. % -----

34. loss=0; % initialize it to zero

35. for i = 1:n_of_data

36. % for pyruvate
37. if model_p==0 mu_p=1; end % noting
38. if model_p==1 mu_p=Pyruvate(i)/(Pyruvate(i)+Kpyr); end % Monod
39. if model_p==2 mu_p=1-exp(-Pyruvate(i)/Kpyr); end % Tessier
40. if model_p==3 mu_p=(1+Kpyr*Pyruvate(i)^(-Lpyr))^-1; end % Moser
41. if model_p==4 mu_p=Pyruvate(i)/(Pyruvate(i)+Bpyr*Microorganism(i)); end % Contois

42. % for oxygen
43. if model_o==0 mu_o=1; end % noting
44. if model_o==1 mu_o=Oxygen(i)/(Oxygen(i)+KO); end % Monod
45. if model_o==2 mu_o=1-exp(-Oxygen(i)/KO); end % Tessier
46. if model_o==3 mu_o=(1+KO*Oxygen(i)^(-LO))^-1; end % Moser
47. if model_o==4 mu_o=Oxygen(i)/(Oxygen(i)+BO*Microorganism(i)); end % Contois

48. mu=Mmax*mu_p*mu_o; % calculate overall specific growth rate
49. loss=loss+(Dilution(i)-mu)^2; % calculate SSD

50. end % end of for loop

51. f=loss; % this is return value
52. return

```

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