Pit initiation on 316l stainless steel in the presence of bacteria leptothrix discophora

Authors: Michael J. Geiser, Recep Avci, and Zbigniew Lewandowski.

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Pit Initiation on 316L Stainless Steel in the Presence of Bacteria *Leptothrix discophora*

M. Geiser*, R. Avci** and Z. Lewandowski***.

* Center for Biofilm Engineering
**Department of Physics and Image and Chemical Analysis Laboratory
***Department of Civil Engineering and Center for Biofilm Engineering

Montana State University
Bozeman, MT 59717

ABSTRACT

Ennoblement of stainless steel by microbially deposited manganese oxides can cause pitting corrosion of the SS in natural waters at low chloride concentrations, leading to unexpected material failures. To study the effects of microbially deposited manganese oxides on pit initiation, we exposed 316L stainless steel to manganese oxidizing bacteria *Leptothrix discophora* under well-defined laboratory conditions. We then placed the ennobled coupons in a solution of low concentration sodium chloride until pitting initiated. The pits had different morphologies than those initiated by electrochemical polarization, hypothetically indicating a direct involvement of the bacteria in pit initiation.

Keywords: pitting, pitting of stainless steel, pit morphology, MIC, *Leptothrix discophora*, ennoblement

INTRODUCTION

This paper discusses the level of involvement of bacteria in the process of pit initiation on stainless steels. As a material to study pit initiation, we used 316L stainless steel in low sodium chloride concentrations, which is not expected to pit at room temperature (23°C) in water solutions with chloride concentrations not exceeding a typical sea water (30g salt/L) [1,10].

It is well known that ennoblement of stainless steels and other passive metals in natural waters (positive shift in the open circuit potential, OCP) occurs in the presence of microbes depositing manganese oxide (MnO_2) [1,4,8,11]. Because the microbially deposited manganese oxides are in electrical contact with the underlying metal, the OCP of the metal maintains the equilibrium potential of the oxides (+382 mV_{SCE} at a pH 7.2) [4,9]. As a result of ennoblement, the OCP of stainless steels in natural waters can raise from –200mV_{SCE} to over +320mV_{SCE} with as little as 6% surface coverage by manganese oxides [3] which is a 500mV increase.

The open circuit potential of the metal is determined from the equilibrium between manganese oxides deposited on the surface with manganese ions in the solution. The anticipated half reactions are:

$$MnO_{2(s)} + H^+ + e^- = MnOOH_{(s)} \qquad E^0 = +0.81\ V_{SCE} \qquad E'_{pH=7.2} = +0.383\ V_{SCE} \qquad [1]$$

$$MnOOH_{(s)} + 3H^+ + e^- = Mn^{2+} + 2H_2O \qquad E^0 = +1.26\ V_{SCE} \qquad E'_{pH=7.2} = +0.336\ V_{SCE} \qquad [2]$$

The overall reaction is:

$$MnO_{2(s)} + 4\ H^+ + 2e^- = Mn^{2+} + 2H_2O \qquad E^0 = +1.28\ V_{SCE} \qquad E'_{pH=7.2} = +0.360\ V_{SCE} \qquad [3]$$

Standard potentials (E^0) of reactions [1],[2] and [3] have been estimated based on free energies of formation: $\Delta G_f^0 Mn^{2+}$=-54.5 kcal/mole, ΔG_f^0 γ-MnOOH=-133.3 kcal/mole, and ΔG_f^0 γ-MnO_2=-109.1 kcal/mole[3,17]. Potentials at a pH of 7.2 are based on $[Mn^{2+}]$ of 10^{-6} M.

The idea that the manganese oxidizing bacteria causing ennoblement may also be directly involved in pit initiation on stainless steels originated when we noticed unusually shaped indentations on the surface of ennobled 316L stainless steel coupons, colonized by *Leptothrix discophora*, and immersed in a mild solution of sodium chloride. *Leptothrix discophora* is a manganese oxidizing bacterium, capable of ennobling stainless steels[5]. Based on the initial observations, we hypothesized that these indentations in the passive film were pits initiated by the bacteria. This is a different mechanism than that by which bacteria are electrostatically attracted to prior corrosion sites[10]. Consequently, we set the goals of this project to: 1) demonstrate whether these indentations in the passive film were not just preexisting flaws in the metal surface but were resulted from microbial colonization of the surface, and 2) demonstrate that the pits initiated in such way can propagate.

A major technical difficulty associated with the project was to find exactly the same locations on the metal surface before and after microbial colonization. Only then could the indentations in the metal surface found after microbial colonization be associated with the microbial colonization. To accomplish this task we polished 316L stainless steel coupons and marked small squares on their surfaces (200x200 μm) using ion milling[15]. We used Atomic Force Microscope (AFM) imaging to register the topography of the surface within these squares, to determine any flaws prior microbial colonization. The coupons were then ennobled in a batch reactor with manganese oxidizing bacteria (*Leptothrix discophora*) until their potentials exceeded +300mV_{SCE}. The ennobled coupons were then removed from the reactor and exposed to 0.2M solution of sodium chloride (NaCl). Their OCP was monitored. Once the OCP decreased to approximately 0mV_{SCE}, as a result of pit initiation, the coupons were removed, washed from the remaining solution of sodium chloride, and the biofouling deposits were gently removed to expose the surface. The surface topography within the squares etched by ion milling was imaged using AFM and SEM to quantify the dimensions of any indentations in the passive layer that were created as a result of the described procedure.

MATERIALS AND METHODS

Stainless Steel Coupon Preparation

Stainless steel coupons with a 1.6cm diameter were cut out from 1mm thick sheet of type 316L (Ryerson in Spokane, WA). Nine coupons were mounted in polycarbonate holders and held in place with a silicon gel[4]. The holders were hollow tubes (10cm long) designed to hold the corrosion coupons with an inner and outer diameter of 9mm and 19mm (Figure 1).

Once the silicon gel solidified, the coupons were polished using Buehler-Met II metallographic grinding discs with varying grit sizes made of silicon carbide and Buehler Microcloths. The coupons were first wet polished using 120 grit-sized sandpaper, then rinsed with tap water, and then subjected to a series of wet polishing and rinsing cycles using progressively smaller grit sizes of 240, 360, 400 and 600. For fine polishing we used Buehler Aluminum Oxide Powder and Buehler micropolish II powder suspended in water and applied to the polishing cloths. We started with a 5 micron aluminum oxide powder with a water rinse in between cycles, followed with a 0.5 micron micropolish and a final rinse with deionized water. To achieve the mirror finish, we finished polishing using a 0.05 micron micropolish followed by rinsing with deionized water.

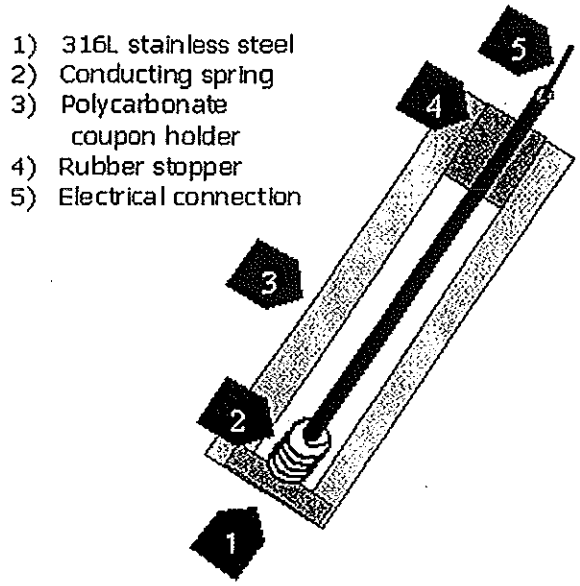

Figure 1 – Schematic of a coupon holder. The holder was constructed as described in reference 13.

Near the center of each coupon, we etched two squares (200x200 μm) with identification numbers in the upper left corners using a focused Ga^+ ion beam of a Time of Flight-Secondary Ion Mass Spectrometer (ToF-SIMS) for seven minutes at ~1.5 μA ion current at 22 KeV impact energy[15]. The etching produced a trench approximately 100nm deep on the surface of the polished coupon (Figure 2).

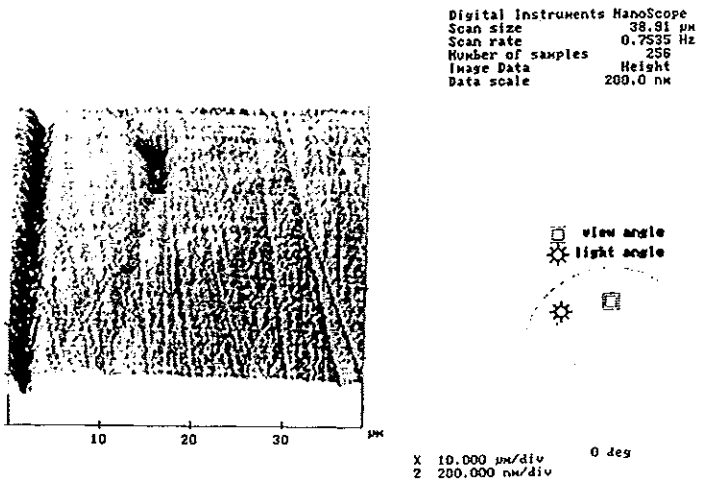

Figure 2 – An AFM image of a section of a square etched by ion milling. Notice the number '1' etched in the upper left corner for identification. The entire square was 200x200 μm.

Biofilm Reactor

To grow biofilms, we used a batch reactor, which was a polycarbonate cylinder 4 inches (10.16 cm) tall and 4 3/8 inches (11.11 cm) in diameter (Figure 3). The etched coupons, secured in the polycarbonate holders (Figure 1), were mounted to the top of the reactor. The assembly is the same as described by Olesen[13] with the exception of the reference electrode, which was submerged in the solution instead of being connected by an additional salt bridge as in Olesen's reactor. This modification prevented microbial contamination of the reactor and stabilized the measured potential. The reactor had ports for air inlet and outlet equipped with Pall-Gelman bacterial air vents. Mixing was accomplished by a magnetic stir bar. The reactor was sealed with silicon gel to prevent microbial contamination. Before use, the assembled reactor was autoclaved for 30 minutes at 123°C and 17.5 psi (1.2 atm) on dry setting.

The growth media was 600ml of ATCC Culture Medium 1917 MSPV. The media was autoclaved at 123°C, 17.5 psi (1.2 atm) for 20 minutes on gravity setting. After sterilization, 1ml of a filtered vitamin solution called for in the MSPV media, 4ml of 50mM filtered manganese sulfate solution, and 5ml of a 20% filtered sodium pyruvate solution were added to the media. All chemicals were from Fisher Scientific.

The bacterial stock cultures, *Leptothrix discophora* SP-6 (ATCC 51168), were kept refrigerated at -70°C before use. Prior to inoculation of the reactor, *Leptothrix discophora* was grown for 2 days in a shake flask containing 150ml sterile MSPV media. Vitamins, manganese sulfate and pyruvate added to the media had the same final concentrations as those in the reactor. After two days, 50ml of the bacterial culture was aseptically added to the reactor. The SCE reference electrode was sterilized by soaking for 1 hour in 95% ethanol, prior to being placing in the reactor. The reference electrode and nine stainless steel coupons were connected to a computer via a Hewlett Packard 34970A Data Acquisition/ Switch Unit (multiplexer) to monitor the potential of the coupons.

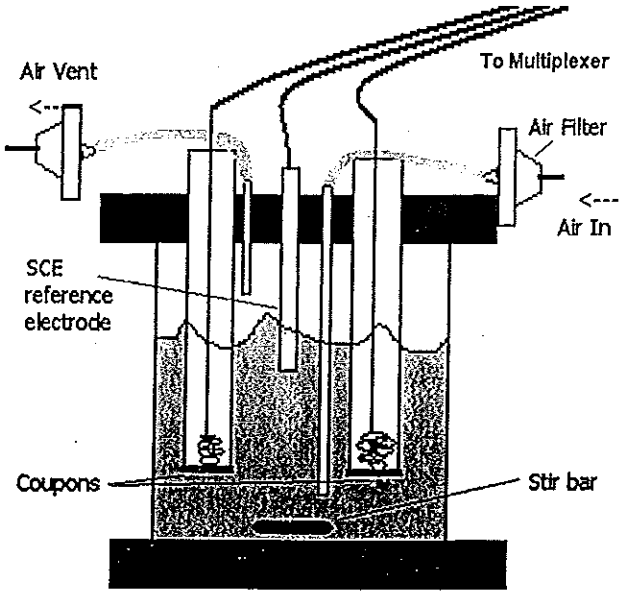

Figure 3 – The reactor configuration.

The reactor was operated until the coupons were ennobled, i.e. when the potentials of the coupons exceeded +200mV; (see Table 1 and Figure 4), which usually lasted 5 days. Two coupon holders with ennobled coupons were then removed from the reactor, thoroughly rinsed with deionized water to remove biofouling deposits, and air-dried. They served as a control for the surface analysis. Remaining ennobled coupons were removed from the reactor and immersed in a 0.2M sodium chloride solution to initiate pitting. Evolution of their OCP was monitored and the exposure interrupted when the potential decreased to approximately 0 mV. During the time of exposure to the saline solution we noticed quick drops and subsequent raises in the OCP, possibly from initiation and healing of pits in the surface of the stainless steel [2,12].

Table 1 – Final potential of the corrosion coupons exposed to *Leptothrix discophora* in the batch reactor. Seven coupons reached potentials above 200 mV, which fulfilled our criterion for ennoblement. Coupon #12 did not ennoble because the coupon holder was not properly sealed and the solution leaked to the inner part of the holder. Coupon #34 ennobled only to 165 mV for unknown reasons.

Sample ID	12	61	23	24	45	56	35	34	13
Potential (mV)	Leaked	224.0	301.5	282.4	299.5	251.5	267.5	165.0	217.0

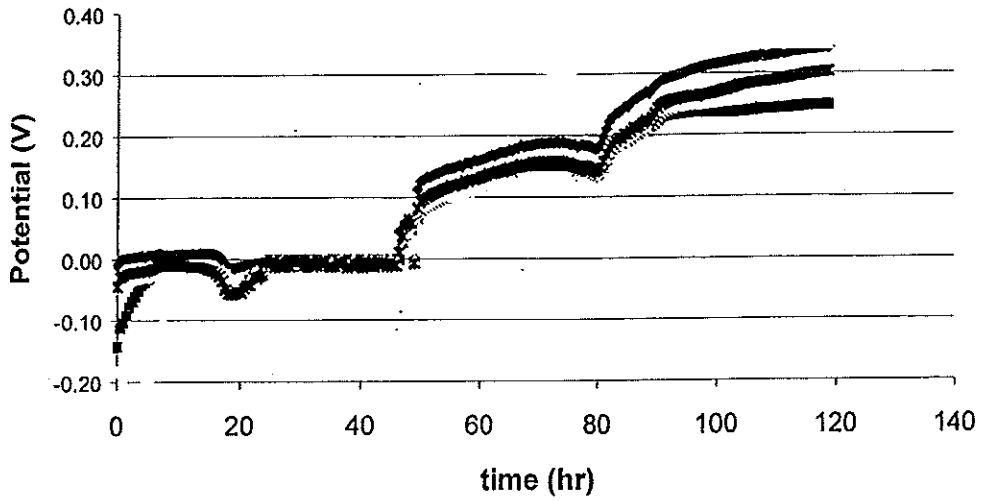

Figure 4 – Potential of the coupons in the presence of manganese oxidizing bacteria, (*Leptothrix discophora)* gradually increased indicating ennoblement.

For the SEM analysis, the coupons were carefully removed from their holders and the manganese oxide deposits were wiped off using acetone and lab tissue paper. Care was exercised to prevent damaging the surface. Any remaining silicon gel on the surface was coated with colloidal graphite from Ted Pella, Inc. to minimize charging in the SEM.

An essential part of the work was to quantify the morphology of the pits initiated on ennobled coupons exposed to the solution of sodium chloride. The working hypothesis was that the microorganisms were involved in this process. To verify this hypothesis, we compared the morphology of pits generated in the presence of manganese oxidizing bacteria with the morphology of pits generated in the absence of microorganisms i.e., (by anodic polarization of the coupons in sterile media).

Anodic polarization of stainless steel was performed using an EG&G Princeton Applied Research Potentiostat/Galvanostat, (Model 273A) by increasing the potential at a rate of 10 V/hr from –0.5 V_{SCE} to +0.8 V_{SCE} using a graphite counter electrode. Care was exercised to ensure that crevice corrosion did not occur[7]. The stainless steel coupons were polished as described and placed in an electrochemical cell with the MSPV media, solutions of vitamins, sodium pyruvate, and manganese sulfate. Sodium chloride was added to make a 0.1M solution.

Surface Analysis

Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM) were used to image the surface topography before and after exposure. The coupons were searched for surface features with dimensions consistent with the size of the microorganisms colonizing the surface. We used a Jeol JSM-6100 Scanning Electron Microscope with a beam voltage set to 15KV. The features of interest were photographed using a Polaroid camera (type 665) at a working distance of approximately 11mm. The surface features identified by the SEM were then imaged using an Atomic Force Microscope (AFM). We used a Digital Instruments Dimension 3100 Scanning Probe Microscope in contact mode.

RESULTS AND DISCUSSION

Pit identification

To resolve whether the pits initiated in the ennobled coupons were due to bacterial action or flaws in the material, that existed prior to microbial colonization, we used SEM and AFM to monitor the surface topography (before and after microbial colonization) within the squares etched by ion milling on coupon surfaces. Areas that had any observable discoloration were analyzed, as well as any flaws in the material from sanding. Figure 2 shows typical SEM and AFM images of a clean surface.

The pits we identified and analyzed were all within the etched squares or just outside of the squares. Figure 5 shows a representative result of such analysis.

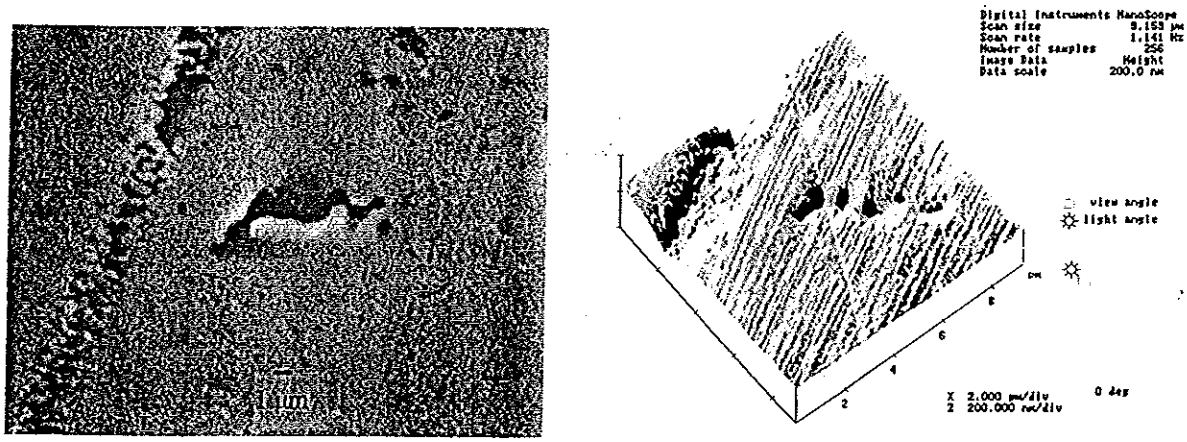

Figure 5 – The SEM image (left) and the AFM image (right). The heavy line on the left corner is part of the square etching by ion milling. The indentation in the surface was detected after the coupon was microbially ennobled. It was not there before microbial colonization.

Pit morphology

a. Pits initiated by anodic polarization

The pits generated by anodic polarization of corrosion coupons were bowl shape, sometimes with a thin metal sheath[6] partially covering the pit (Figure 6). SEM images of such pits typically show a lighter surface with a black hole in the middle. These pits vary in size depending on the time they were exposed to noble potentials, the magnitude of the applied potential, and the chemistry of the solution.

b. Pits initiated spontaneously in the presence of *L. discophora* in MSPV media

Pits initiated by exposing the microbially ennobled stainless steel coupons to a solution of sodium chloride look like a cluster of highly organized small indentations (0.5 to 2 microns wide by 3 to 20 microns) long in a long narrow row (Figure 7). SEM shows these pits as lighter with a white halo around the pit and a black hole in the center. White halos are likely due to organic deposits; they do not extend above the surface of the metal, but are flush with the surface. Organic substances appear white in SEM due to charge accumulation -- holding electrons instead of conducting them through the metal to an electron sink.

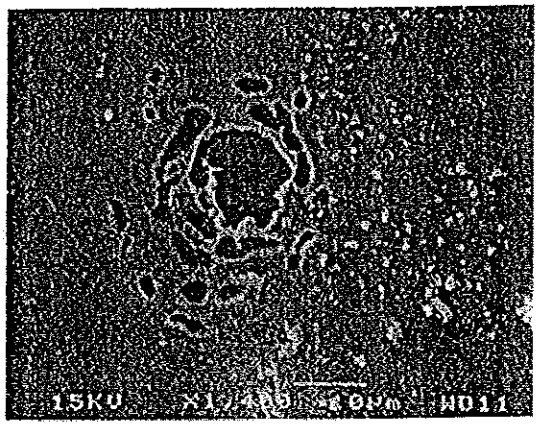

Figure 6 – SEM image of a typical pit initiated in 316L stainless steel using anodic polarization. Notice the thin sheath covering the pit and several little holes surrounding the large hole in the center.

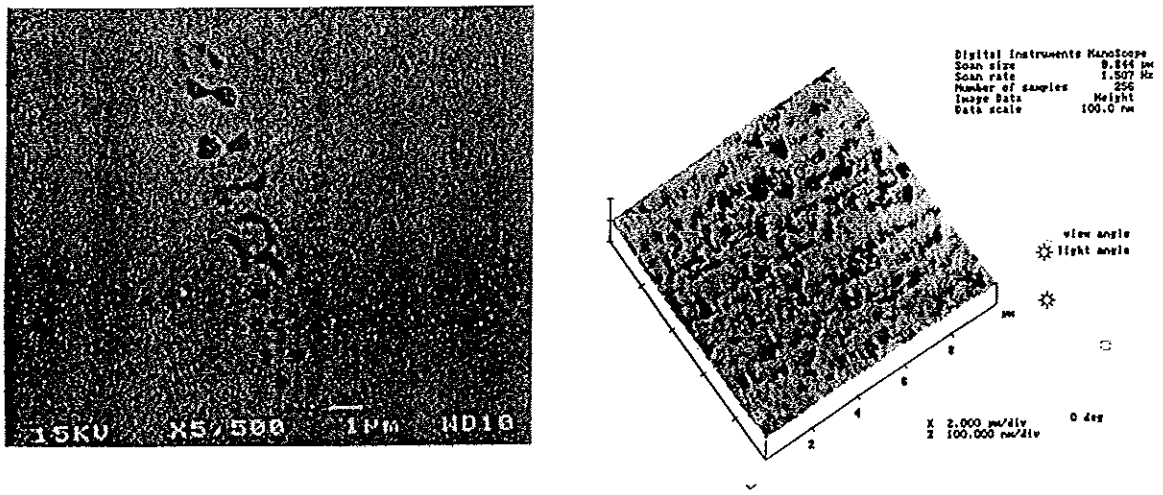

Figure 7 – SEM (left) and AFM (right) images of pits initiated in the presence of *L. discophora* in MSPV media. Material: 316L stainless steel.

Quantifying the differences between pits initiated spontaneously in the microbially ennobled coupons and those initiated by anodic polarization in sterile media.

Pits initiated by anodic polarization of sterile coupons in the MSVP growth media enriched in sodium chloride and those initiated by exposing ennobled coupons to a chloride solution have different shapes. To quantify the differences in pit morphologies, the average length and width of the pits were divided to give an aspect ratio. The 15 pits initiated by anodic polarization had an aspect ratio (length divided by width) of 1.28 ± 0.27. In contrast, 71 pits initiated in the presence of manganese oxidizing bacteria had an aspect ratio of 9.97 ± 5.50. Pits initiated by anodic polarization were circular, while pits initiated in the presence of bacteria were about 10 times longer then they were wide. The standard deviation for the pits initiated in the ennobled coupons is large, signifying that there is a large range in length to width ratios, but the aspect ratio is always at least 4 times larger then that calculated for pits initiated by anodic polarization. The pits initiated by anodic polarization averaged 60.7μm long by 50.2μm wide. The pits spontaneously initiated in ennobled coupons were on the average 12μm long and 1.4μm wide.

AFM scans show that average depth of 25 pits initiated in the presence of bacteria was 44.15 ± 20.51 nm. The depth of the pits initiated by anodic polarization was at least equal their diameters, thus too deep to be measured by the AFM (the maximum depth we could measure by AFM was 6μm).

Correlation between the size of the microorganisms and the sizes of the pits

We observed a close correlation between the dimensions of the indentations in the passive layers created in the presence of bacteria and the physical dimensions of the bacteria. *Leptothrix discophora* is a cylindrical bacterium that connects end to end with its neighbors, and the entire assembly of linked bacteria is covered by a protein sheath[5]. The dimensions of these groups of bacteria are approximately 10 microns long by 1 micron wide (see Figure 8). Twenty groups of bacteria we measured had an aspect ratio (length divided by width) of 10.0 ± 3.7. The average value of the aspect ratio is then very close to the aspect ratio of the pits initiated in the presence of these bacteria, (9.97 ± 5.50), although the standard deviations in both cases are large. The pits initiated in the microbially ennobled corrosion coupons had the size, shape, and aspect ratio similar to that of the bacteria we used to colonize the surface. This fact indicates a possibility that the microbes were attached to the surface before the pits initiated and that the pits initiated exactly at the sites where the microbes were attached.

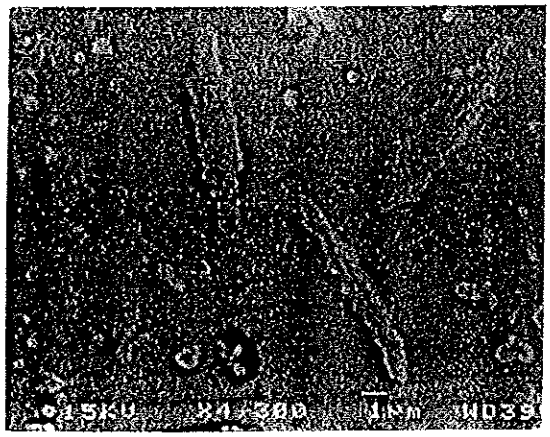

Figure 8 – SEM image of *Leptothrix discophora* on stainless steel.

CONCLUSION

- Pits initiated by exposing microbially ennobled coupons to a solution of sodium chloride had different morphologies than pits initiated by anodic polarization.
- Pits initiated by exposing microbially ennobled coupons to a solution of sodium chloride were approximately 10 times longer than they were wide, was much smaller than those created by electrochemical means, and not nearly as deep.
- Pits initiated by exposing microbially ennobled coupons to a solution of sodium chloride have almost identical sizes and aspect ratios as the sizes and aspect ratios of the manganese oxidizing bacteria (*Leptothrix discophora*), which was used to ennoble the coupons.
- Similarity between dimensions of the bacterial cells attached to the surface and the dimensions of corrosion pits initiated at the surface indicate a possibility that the pits were initiated at the sites where the microbes were attached.

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