Effect of bacterial biofilms on carbon steel pit propagation in phosphate containing medium

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ABSTRACT

Chemical corrosion inhibitors are commonly added to aqueous systems to minimize the oxidation of steel. The efficacy of certain inhibitors may be reduced by bacteria. In the present study, the effect of microbial biofilms on carbon steel corrosion inhibition by phosphate was analyzed, using the scanning vibrating electrode technique (SVET) and monitoring of open circuit potential. Previous results have shown that in sterile medium containing 0.2 mM phosphate, as well as 1.0 mM chloride and 0.2 mM sulfate, carbon steel samples showed small anodic sites which subsequently became inactive. Whereas, in the presence of bacteria pits failed to become inactive and continued to propagate. In the present study, comparisons between viable bacteria and formaldehyde fixed bacteria on the pit propagation was analyzed. The results indicated that biofilms containing viable bacteria and fixed bacteria could cause pits to propagate. The time required for pit propagation was dependent on the concentration of bacteria as well as the viability of the bacteria. The results indicate that bacterial biofilms can cause pits, initiated by chemical corrosion, to continue to propagate either by reducing the efficacy of phosphate as a corrosion inhibitor or by maintaining the aggressive environment within pits.

INTRODUCTION

The scanning vibrating electrode technique (SVET), used for current density mapping over corroding metal surfaces, has been useful in understanding mechanisms of localized corrosion (1,2,3,4,5). This technique has recently been applied to the study of microbial influenced corrosion of carbon steel (6,7). In a stirred sterile microbiological medium containing 1 mM chloride, 0.2 mM sulfate, and 0.2 mM phosphate, as well as a number of trace minerals, the carbon steel electrodes had high impedances and remained shiny for as many as nine days of exposure (8), due to the corrosion inhibition by the phosphate (9). However, analysis by the SVET revealed that carbon steel in this medium was not passive throughout the exposure. In fact, the samples showed small active anodic sites which subsequently became inactive (7). In the presence of active bacteria, propagation of pits was favored over this pit repassivation (7). The physical presence of bacteria was required for the pits to propagate, since spent microbial medium did not result in pit propagation. From those results we proposed that the microbial biofilm prevented active pits formed by chemical corrosion from repassivating.

Pit propagation is dependent on the ability to maintain aggressive ions inside the pit (3). The dissolution current results in an increased concentration of ions, including chloride ions, inside a pit, and the hydrolysis of ferrous ions results in localized lowering of the pH. Diffusion or convection decreases

the concentration of aggressive ions in pits. Microbial biofilm formed on metal surfaces may act as a membrane which either reduces the flow of aggressive ions by diffusion or convection from the pits, or impedes the migration of inhibiting agent, in this case phosphate, from coming in contact with the metal. This mechanism is supported by the observation that in this same sterile medium which was not stirred pit propagation was favored over repassivation (11).

Many bacteria produce extracellular polymer which acts to bind the bacteria to surface. In fact much of biofilms consist of this exopolymer material. Geesey et al. (10) have demonstrated copper dissolution in the presence of bacterial exopolymer. And Nivens et al. have shown a correlation between corrosion rate and bacterial exopolymer production on stainless steel (11). In attempting to isolate extracellular polymer from killed bacteria used in this study we found, by FT-IR analysis, that most of the polymer remains associated with the bacteria (unpublished data). Therefore, in the present study, we tested the "bacterial membrane" hypothesis by exposing the carbon steel to formaldehyde killed bacteria rather than isolated exopolymer. The scanning vibrating electrode technique (SVET) and monitoring of open circuit potential (OCP) were used as indicators of pit propagation in the presence of viable and formaldehyde fixed bacteria.

EXPERIMENTAL METHODS

Bacteria and media. The bacteria used in this study were the same as those used in the previous study of pit propagation (7). They were originally isolated from a corrosion tubercle and have been identified as a Pseudomonas sp. based upon their membrane fatty acids. The bacteria were grown in a medium containing basal salts (in mg/l, NH_4Cl, 50; $MgSO_4 \cdot 7H_2O$, 50; $CaCl_2 \cdot H_2O$, 5; KH_2PO_4, 27) 1 ml/l trace mineral solution (7), glucose 1 g/l, and MOPS buffer 0.5 g/l, to an optical density of 0.2 at 660 nm.

The bacteria to be used in the formaldehyde fixed experiments were killed by the addition of formaldehyde at a final concentration of 4% for 0.5 hour. The formaldehyde and the spent bacterial growth medium were removed by centrifuging the bacteria and pouring off the supernatant. The cell pellet was then resuspended in fresh medium, defined above, with the exception that the glucose and the MOPS concentrations were reduced to 50 mg/l. The cells were centrifuged and resuspending in fresh medium three times to remove the remaining formaldehyde. The viable bacteria were also washed three times and resuspended in fresh medium. The final concentration of bacteria was adjusted in the fresh medium to yield an optical density of 0.03 or 0.2 at 660 nm, approximately 2×10^7 and 2×10^8 cells/ml as determined by acridine orange direct counting, respectively. The bacteria or sterile medium, 500 ml, were then added to flasks

containing carbon steel electrodes and standard calomel electrodes, connected to the solution via salt bridges. The carbon steel electrodes were epoxy embedded carbon steel coupons containing electrical leads soldered to the backs of the coupons. The edge between the metal and the epoxy was painted with a lacquer coating. In some experiments, the carbon steel electrodes were exposed to sterile medium prior to exposure to bacteria. In those experiments, the bacteria were concentrated to an O.D. of 0.8 and enough of this solution was added to flasks containing sterile medium to yield final optical densities of 0.03 or 0.2. Sterile medium was added to the sterile controls to control for the changes in volume.

Open circuit potential. The open circuit potential was monitored over time using a Solatron digital multimeter. The multimeter was connected to a Kiethly scanner and a computer data acquisition unit, so that measurements of several different electrodes could be made at once. Readings were taken every 0.5 hour and stored in a Lotus 123 spreadsheet.

Current density mapping. Current density maps were obtained using a scanning vibrating electrode as described previously (4). The vibrating electrode is an insulated platinum wire which is positioned 100 μm over the surface of the metal. The electrode is attached to a piezoelectric reed which vibrates the electrode when an AC signal is applied to the reed. In these studies, the

vibration frequency was 154 Hz. The vibration of the electrode converts the potential fields in solution over the steel to an alternating signal. An E.G.and G. model 5210 lock-in amplifier was used to amplify the alternating signal and to filter out signal except that associated with the frequency of vibration. The scanning of the sample with the vibrating electrode was performed by stepping the sample underneath the electrode using stepper motors. The steps were in 200 μm increments. Both the lock-in amplifier and the stepper motors were controlled and data was acquired using a computer data acquisition unit programmed in ASYST language. Thirty mm^2 of the carbon steel was exposed to solution the remaining coupon was masked by pressure sensitive tape. The edge of the tap and the metal was coated with a Lacquer coating, to help prevent crevice corrosion.

Scanning electron microscopy. Following exposure of some metal samples to bacteria, the coupons were removed and fixed in 0.01 M cacodylate buffer containing 2% glutaraldehyde. The samples were then dehydrated in increasing concentrations of acetone (50%, 75%, and 100%). The samples were then critical point dried, sputter coated with gold/palladium, and examined with an ETEC autoscan scanning electron microscope.

Acridine orange direct counts. At the end of the experiments where the OCP was monitored, the metal samples were removed, and an O-ring extractor was clamped onto the sample

(12). Basal salts, 500 μl, was added to the extractor, and the samples were sonicated for five seconds. The cells removed from the surface by sonication were added to 10 ml of basal salts. The sonication procedure was repeated twice. The cells were then serially diluted in basal salts, filtered using Nucleopore filters, stained with acridine orange, and counted using epiflourescence microscopy.

EXPERIMENTAL RESULTS

Biofilm formation. Colonization of viable bacteria to the carbon steel surface was observed by scanning electron microscopy. Figure 1a shows the colonization of the corrosion products by the isolate. Dehydrated bacterial exopolymer can be seen in association with the corrosion products. The presence of bacterial exopolymer was also observed by Fourier transform infrared spectroscopy (FT-IR) (data not shown). In FT-IR analysis considerable material which absorbed at 1080 cm^{-1} was observed relative to the absorbance at 1648 cm^{-1}. The absorbance at 1080 cm^{-1} has been correlated to exopolymer material and the absorbance at 1648 cm^{-1} has been correlated to bacterial protein (13). Figure 1b shows the association of the bacteria to the metal surface after the corrosion products were removed. In addition to colonization of the corrosion products, bacteria also colonized the metal surface to form a biofilm.

Current density mapping. Figure 2 shows the current density maps obtained by SVET analysis for a sample expose to sterile medium, then to formaldehyde fixed cells, O.D. 0.2. Figures 2 a,b,c show the current density maps for the carbon steel exposed to sterile medium over 16 hours. The positive Z-axis represents anodic current densities, whereas the negative Z-axis represents cathodic current densities. Note that the magnitude of the Z-axis scale is increase ten fold in the last four frames. Figure 2a shows two small active sites adjacent to the lacquer coating after 3 hours of exposure to the sterile medium. After 4 hours of exposure (fig 2b), one of the active sites increased in magnitude and two additional anodic sites were observed. Figure 2c shows the same sample after 16 hours. The two active sites which were present after three hours became inactive, and three additional sites formed. The OCP of the sample was monitored during these experiments. The OCP showed potential fluctuation, apparently due to the formation and repassivation of active anodic sites (5,7).

After 17 hours the medium was removed and replaced by medium containing formaldehyde fixed bacteria (0.2 O.D.). At 21 hours, it was observed that two of the active sites seen in fig 2c remained active and increased in magnitude. Those sites remained active until 30 hours (fig 2e,f), when the experiment was terminated. The OCP showed a steady drop during the remainder of the experiment, as the magnitude of the local anodic sites increased.

Effect of bacteria on carbon steel. In this study (fig 2) as well as with other studies using the SVET, correlation between pit propagation and a steady drop in OCP has been observed (3,5,7,9). The drop in OCP was used to indicate irreversible pit propagation in the presence of bacteria, under several different conditions. Figure 3a compares the effect of bacteria under growing conditions at different concentrations with sterile control. The potential of the steel in the sterile flask remained high compared to the steel exposed to bacteria. Fluctuations in potential, characteristic of pitting and repassivation (5), were also observed. The potential of the steel exposed to bacteria initially was lower than that of the sterile control, and the potential gradually rose to -200 mV/SCE. After 12 hours in the solution containing the higher concentration of bacteria and 22 hours in the solution containing the lower concentration of bacteria, the potential gradually dropped to -600 mV/SCE. Loosely adhered orange corrosion products were observed on the steel samples exposed to bacteria. Virtually identical results were obtained upon replication of this experiment.

Figure 3b shows the effect of formaldehyde fixed bacteria on the OCP of the steel. The potential of the sterile control remains high and fluctuations in potential were observed. The potential for one of the samples exposed to 0.03 O.D. cells is similar to the sterile control for the first 50 hours. Following this time the potential gradually dropped. The other sample

exposed to 0.03 O.D. cells also showed a gradual decrease in OCP after 50 hours. The samples exposed to higher concentrations of fixed bacteria showed a more rapid drop in OCP to a final value of -600 mV/SCE at 90 hours. After 90 hours of exposure, 0.1 ml of solution was removed from the flask and plated on agar medium containing glucose, yeast extract, and peptone, 100 mg each per liter of basal salts, to check for viable bacteria and contamination. No growth was observed on the agar medium after one week of incubation.

After termination of the experiments the number of bacteria associated with the electrode surfaces and with the corrosion products was determined (Table 1). The numbers of cells associated with the surface in the flasks containing viable bacteria at 0.03 O.D. was greater than 10^8 cells/cm^2 and was three to 16 times greater than the number of surface associated bacteria in the formaldehyde fixed flasks. The numbers of surface associated bacteria in the 0.2 O.D. flasks were similar for the viable and formaldehyde fixed bacteria, at approximately 10^9 cells/cm^2.

Effect of bacteria on pretreated carbon steel. Figure 4 shows the effect of viable and formaldehyde fixed bacteria on carbon steel samples pretreated with sterile medium. Figure 4a shows the effect of viable bacteria at different concentrations on the carbon steel OCP. Prior to the addition of bacteria, the OCP is high and shows fluctuations, apparently due to pitting and

repassivation. Following the addition of bacteria the potential drops to -650 mV/SCE in the 0.2 O.D. flask and to -250 mV/SCE in the 0.03 O.D. flask. These drops may be due to the consumption of oxygen by the bacteria, since these drops were not observed in the steel exposed to formaldehyde fixed bacteria (fig 4b). After this initial rapid drop in potential, the potential rose to -200 mV/SCE for several hours, then showed a more gradual decline, apparently due to the propagation of anodic sites. The steel exposed to formaldehyde fixed bacteria also shows a high potential prior to addition of bacteria, and a slower drop in potential. These experiments were also duplicated, and similar results were obtained for each sample. At the end of this experiment the number of surface associated bacteria were enumerated by AODC (Table 1). The steel surfaces exposed to 0.03 viable bacteria was approximately 10^9 cells/cm^2, this value was an order of magnitude greater than the steel exposed to fixed bacteria. The steel exposed to 0.2 O.D. viable bacteria was $2x10^9$ cells/cm^2, and was slightly greater than the 0.2 O.D. fixed bacteria.

DISCUSSION

The "bacterial membrane" hypothesis proposed here, assumes that the killed bacteria can adhere to the surface of the metal to form a film. AODC values showed that high numbers of killed bacteria (although not as high as experiments with viable

bacteria) were found in association with the metal surface and the corrosion products. The bacteria used in this study were originally isolated from a biofilm associated with a steel pipe tubercle. Under laboratory conditions the bacteria formed biofilms containing extracellular polymer (fig 1). The dehydrated extracellular polymer seen in figure 1 is a stringy substance, however hydrated bacterial extracellular polymer is thought to be a viscous gel. In addition to the bacteria, the bacterial extracellular polymer likely adds to the formation of the film.

SVET analyses in Fig 2 show that the addition of a high concentration of killed bacteria did cause the pits to propagate. While the steel was exposed to sterile medium small pits initiated and subsequently became inactive. The results observed here are in agreement with previously reported data (7). After the killed bacteria were added, two of the initiated pits failed to repassivate, and continued to propagate. These results as well as previously reported results show pit propagation results in a steady drop in the OCP (3,5,7,9). Therefore, in the remaining experiments, pit propagation was indicated by a drop in the OCP. In this way samples under a variety of conditions could be tested simultaneously.

The effect of viable and fixed bacteria on carbon steel corrosion, without prior without exposure to any solution, was determined in the first set of experiments. Cells at 0.03 O.D. were chosen because that is the approximate final optical density

bacteria used in the previous study (7). As in fig 2, upon exposure to the formaldehyde fixed bacteria the potential dropped, corresponding to propagation of local anodic sites. Fixed cells at 0.03 O.D. showed pit propagation after 50 hours of exposure. Experiments were also performed with cells at 0.003 O.D. The results of those experiments were similar to the sterile control (data not shown). Few bacteria were attached to the surface in the 0.003 O.D. solution. These results suggest that a minimum number of cells are required to form a film and cause pit propagation. In addition, the time required for pit propagation is dependent on the concentration of cells.

The results with viable bacteria (fig 3b) are similar to previously reported results (7). The bacteria resulted in pit propagation after hours of exposure. Again the time required for propagation was dependent on the concentration of bacteria. An interesting note is that the viable bacteria caused pits to propagate more rapidly than the fixed bacteria (fig 3). This indicates that 1) the viable bacteria formed a film more rapidly than the fixed bacteria or 2) bacterial metabolic activity, either synthesis of addition cellular or extracellular material or some other metabolic activity contribute to pit propagation. In order to test these hypotheses, the effect of localized metabolic activity on the localized corrosion of steel is being tested (13).

The results for steel samples which were exposed to the sterile inhibiting medium prior to exposure to bacteria showed

similar results to the steel exposed immediately to bacteria. Prior to exposure to bacteria the potential fluctuations indicated pitting and repassivation (5,7). Following exposure to bacteria the potential dropped as pits propagated. Again the time required for pit propagation was dependent on the cell concentration, and also on the viability of the bacteria, as viable bacteria resulted in more rapid pit propagation than killed bacteria. At the end of the experiment, high concentration of bacteria were found associated with the surface and/or the corrosion products of the steel, indicating that films of bacteria did form on the metal in both the viable bacteria and the killed bacteria experiments.

The results of this study indicate that bacteria, whether viable or killed and presumably bacterial exopolymer can form films on the surface of carbon steel and prevent initiated pits from repassivating. The propagation of pits is presumably due to the formation of a biofilm, which helps maintain an aggressive environment within the pits or inhibits the flow of inhibiting ions coming into contact with the surface. The results also indicate that viable bacteria result in a faster rate of pit propagation, suggesting that additional mechanisms involving microbial metabolic activity may be involved in pit propagation.

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Table 1
Numbers of surface associated cells

Samples from figure 3

Treatment	cell concentration[a]	AODC range[b]
viable	0.03	1.7(±0.8) - 6.7(±3.2)
formaldehyde fixed	0.03	0.4(±0.1) - 0.5(±0.3)
viable	0.2	15 (±6.2) - 27 (±6.4)
formaldehyde fixed	0.2	12 (±3.7) - 12 (±6.6)

Samples from figure 4

Treatment	cell concentration	AODC range
viable	0.03	9.2(±3.2) - 16 (±8.3)
formaldehyde fixed	0.03	1.0(±0.4) - 1.6(±0.5)
viable	0.2	26 (±6.5) - 29 (±6.7)
formaldehyde fixed	0.2	8.5(±3.0) - 19 (±7.3)

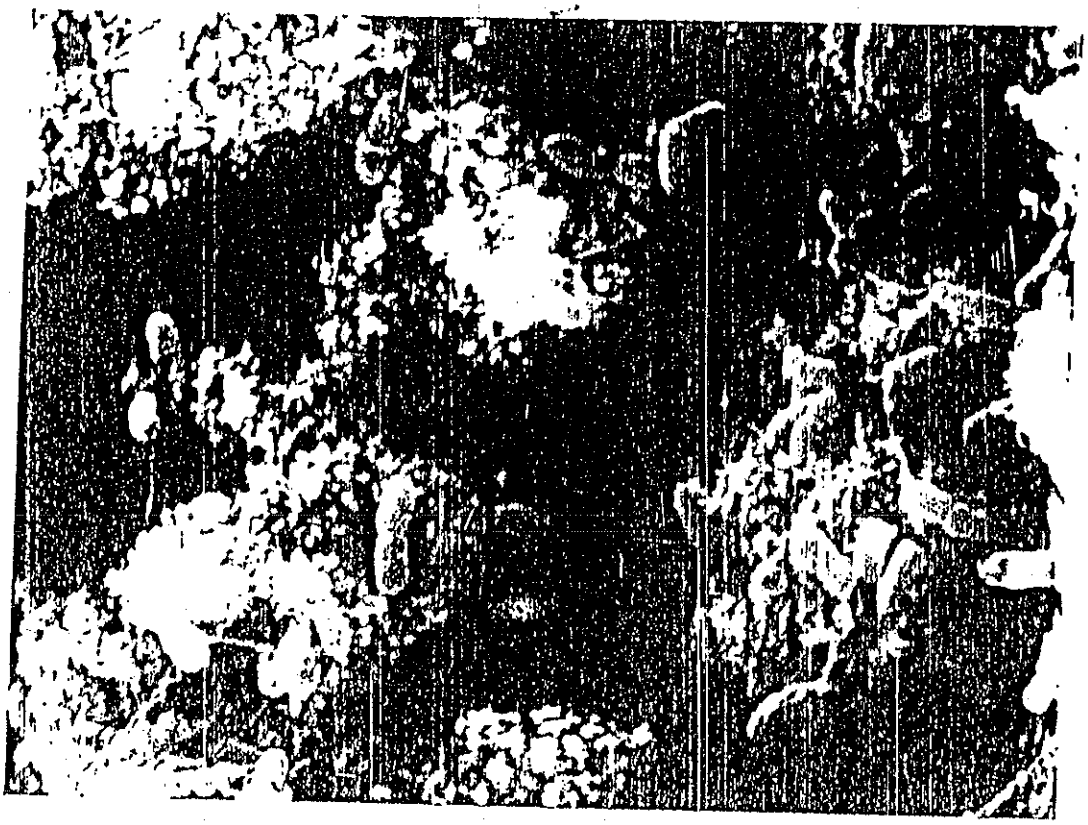
[a] cell density at 660 nm at the begining of the experiment.
[b] Value time 10^8 cells/cm^2. The range is for two samples. The values represent the means and standard deviations for 10 fields counted.

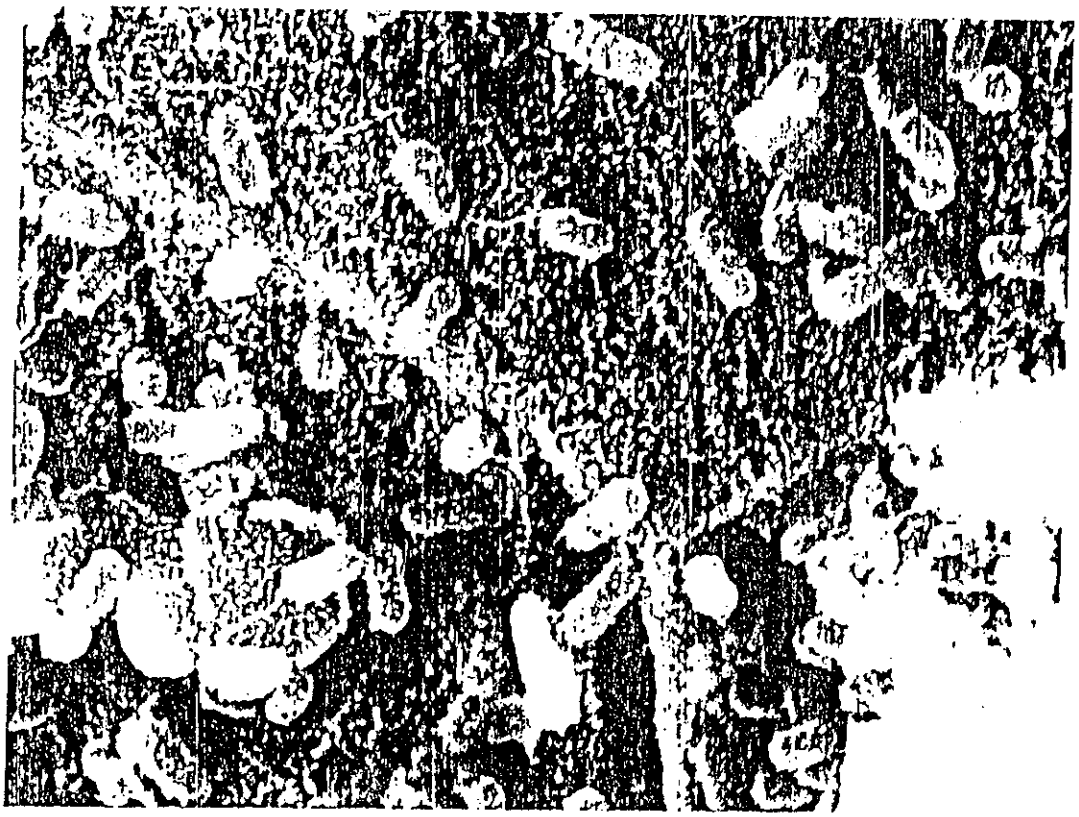
Figure 1. Bacteria and bacterial extacellular polymer associated with the corrosion products of a carbon steel sample. B) Carbon steel sample which had the corrosion products removed. Bacteria are attached to the surface of the carbon steel sample.

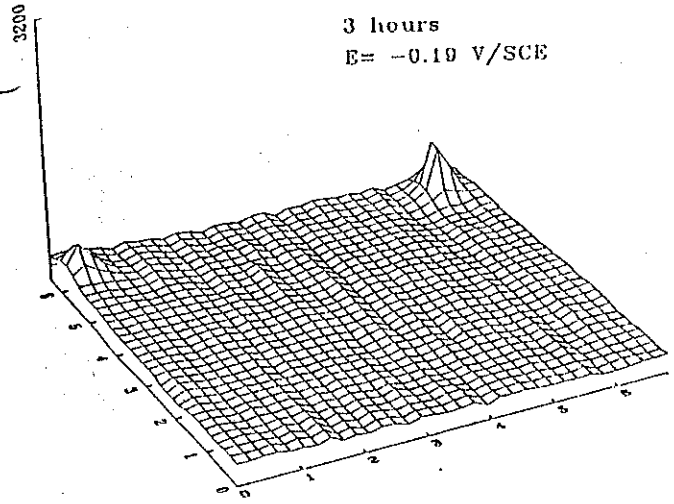
Figure 2. Current density maps over carbon steel sample exposed to sterile medium (fig 2a,b,c), then exposed to approximately 10^8 formaldehyde killed bacteria/ml (fig 2d,e,f).

Figure 3. Carbon steel open circuit potential exposed to bacteria without prior exposure to sterile medium. A) OCP of steel exposed to viable bacteria at the optical density indicated. B) OCP of steel exposed to formaldehyde killed bacteria, at the optical density indicated.

Figure 4. Open circuit potential of carbon steel exposed to sterile medium, followed by exposure to bacteria. A) OCP of steel exposed to viable bacteria. B) OCP of steel exposed to formaldehyde killed bacteria.



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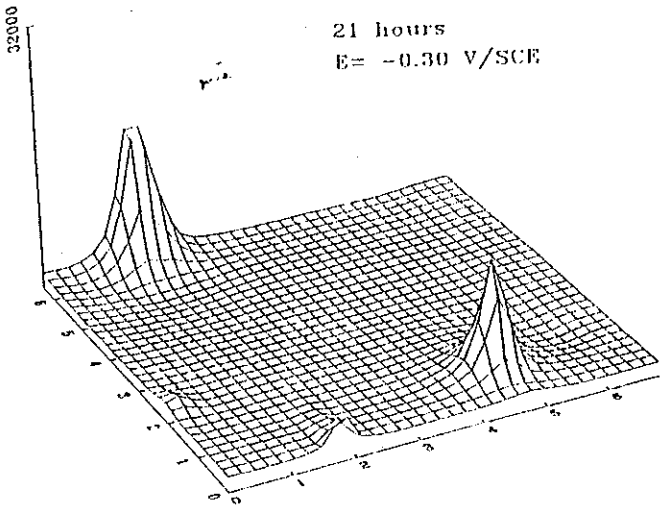
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21 hours
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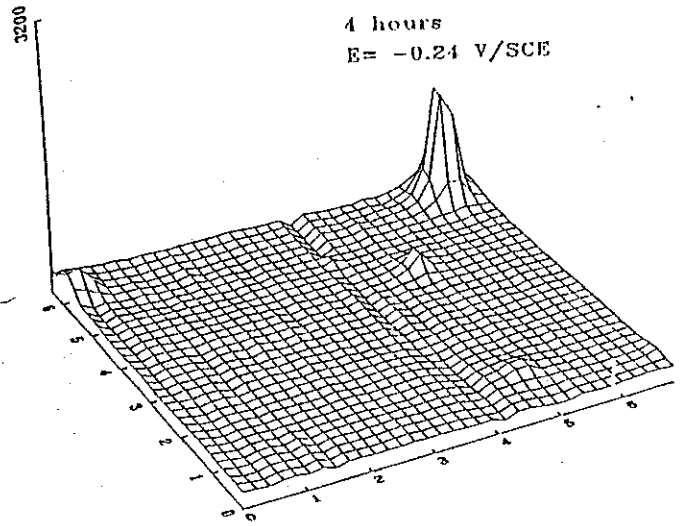
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4 hours
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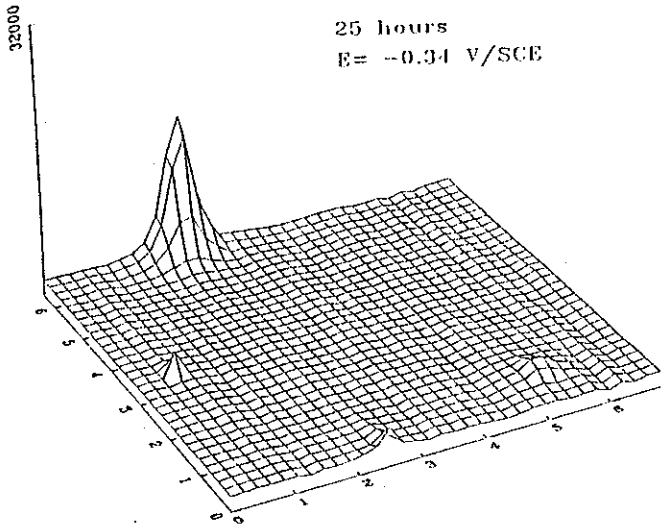
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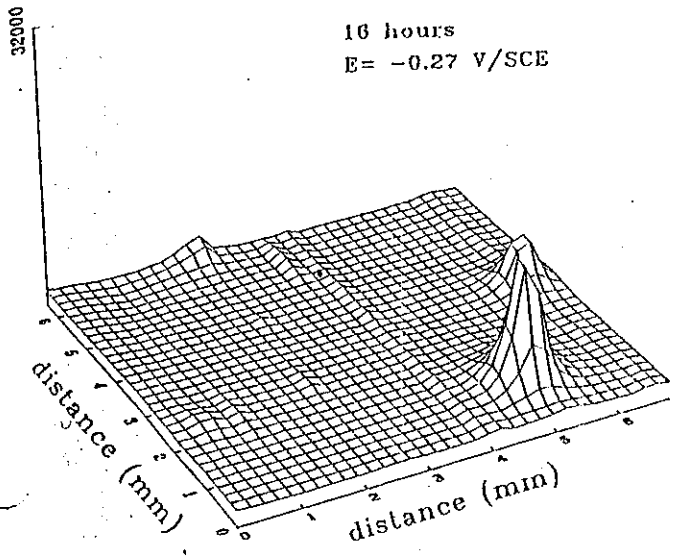
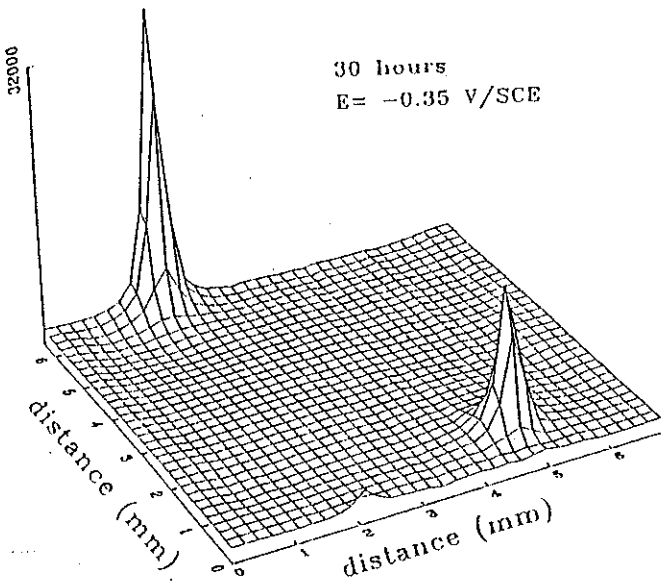

32000
16 hours
E= -0.27 V/SCE
distance (mm)
distance (mm)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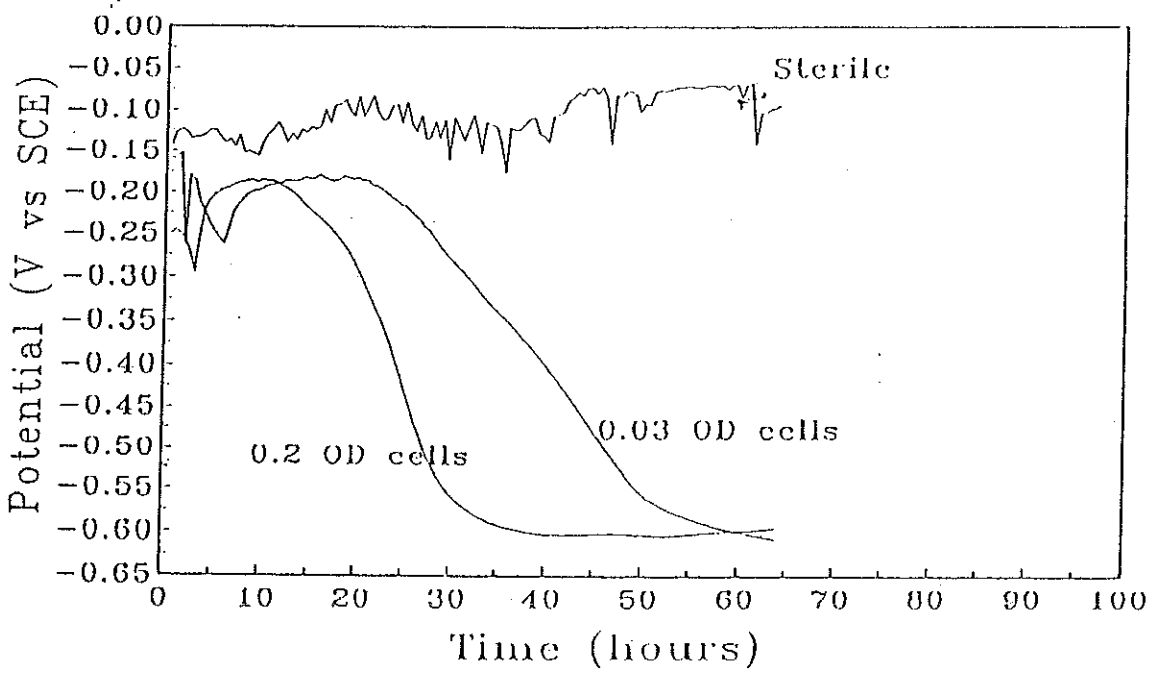
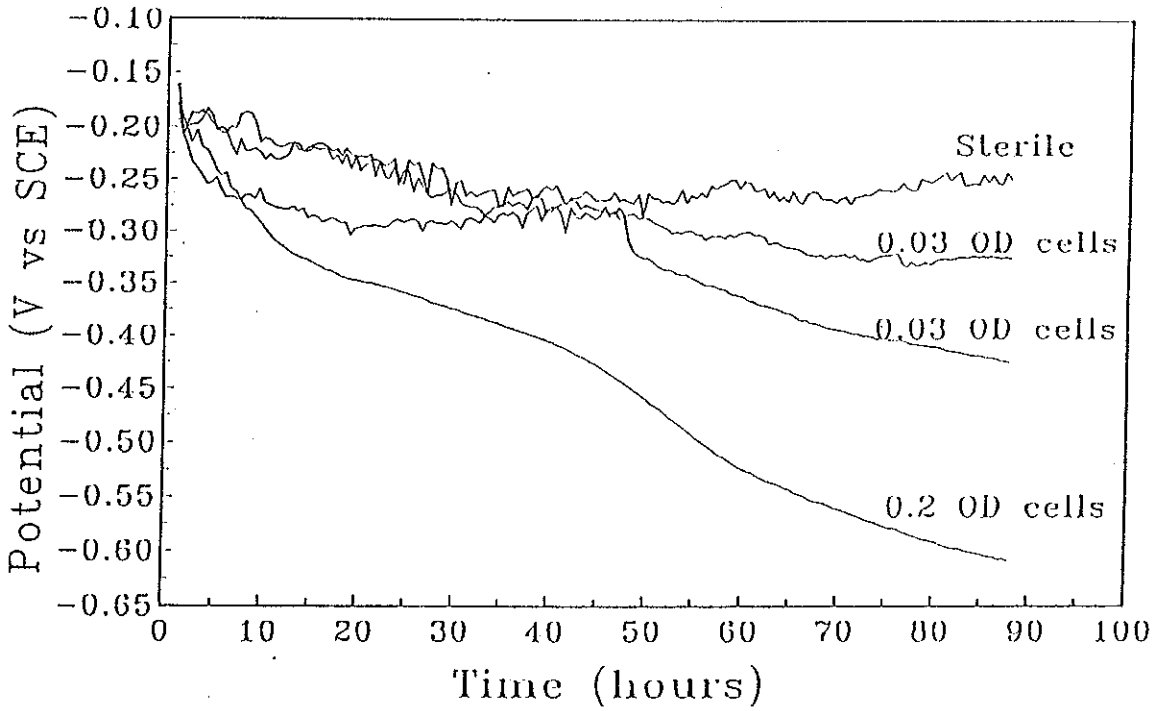
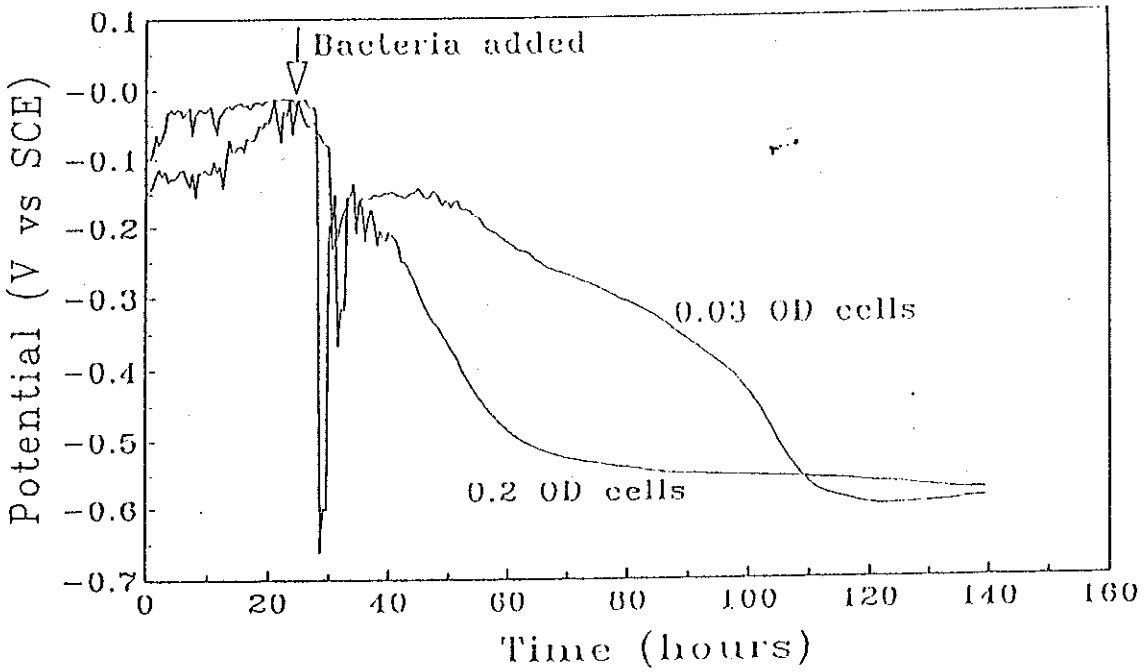
30 hours
E= -0.35 V/SCE
distance (mm)
distance (mm)


Potential (V vs SCE)
0.00
−0.05
−0.10
−0.15
−0.20
−0.25
−0.30
−0.35
−0.40
−0.45
−0.50
−0.55
−0.60
−0.65
0
10
20
30
40
50
60
70
80
90
100
Time (hours)
Sterile
0.03 OD cells
0.2 OD cells


Potential (V vs SCE)
−0.10
−0.15
−0.20
−0.25
−0.30
−0.35
−0.40
−0.45
−0.50
−0.55
−0.60
−0.65
0
10
20
30
40
50
60
70
80
90
100
Time (hours)
Sterile
0.03 OD cells
0.03 OD cells
0.2 OD cells


Bacteria added
0.03 OD cells
0.2 OD cells
Potential (V vs SCE)
Time (hours)
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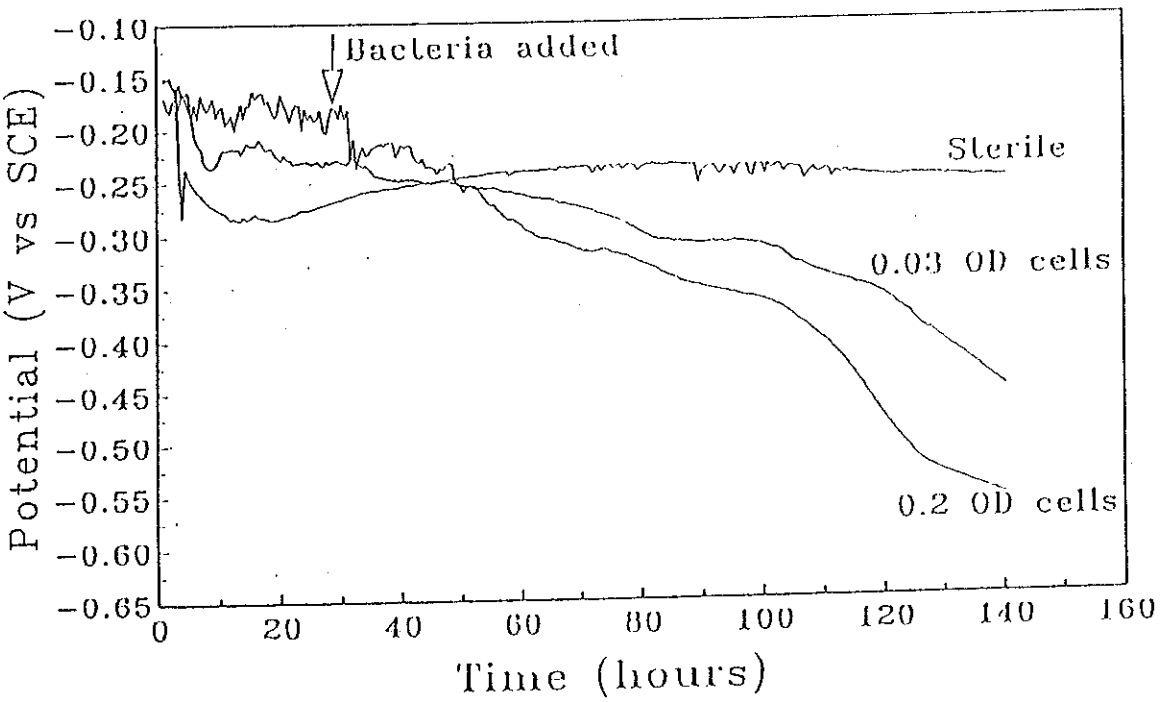
−0.0
−0.1
−0.2
−0.3
−0.4
−0.5
−0.6
−0.7
0
20
40
60
80
100
120
140
160


Bacteria added
Sterile
0.03 OD cells
0.2 OD cells
Potential (V vs SCE)
Time (hours)
−0.10
−0.15
−0.20
−0.25
−0.30
−0.35
−0.40
−0.45
−0.50
−0.55
−0.60
−0.65
0
20
40
60
80
100
120
140
160