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Spatial and Temporal Relationships Between Localised Corrosion and Bacterial Activity on Iron-Containing Substrata

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ABSTRACT
Pit propagation in carbon steel exposed to a phosphate-containing electrolyte required either stagnant conditions or microbial colonisation of anodic regions. A scanning vibrating electrode (SVE) was used to resolve formation and inactivation of anodic and cathodic sites on carbon steel. In a sterile continuously aerated medium, pits initiated and repassivated. However, in a medium that was not aerated, pits initiated and propagated. Pit propagation was also observed in a continuously aerated medium when a heterotrophic bacterium, originally isolated from a corrosion tubercle was inoculated into the medium. Autoradiography in combination with SVE analysis demonstrated that the sites of anodic activity coincided with sites of bacterial activity and that the bacteria were attached preferentially to the corrosion products over the anodic sites. Attraction to the anodic sites did not depend on the viability of the bacteria and was not specific for iron as a substratum.

1. Introduction

Microbiologically influenced corrosion (MIC) is localised corrosion. The presence and metabolic activities of bacteria heterogeneously colonising metal surfaces produce pitting, under-deposit corrosion, dealloying, enhanced galvanic corrosion, and enhanced turbulence-induced corrosion. The biological mechanisms that produce these localised phenomena cannot be easily discriminated from abiological mechanisms producing the same result. Most electrochemical techniques provide an average corrosion rate over an entire surface, making studies on localised corrosion, including MIC, difficult. Techniques for studying microbial activity in complex ecosystems such as biofilms often require large samples. In addition, it has been difficult to discriminate between microbiological events that produce electrochemical reactions and electrochemical reactions that influence microbial settlement and distribution. Little *et al.* [1,2] investigated spatial relationships between bacteria and anodic regions on carbon steels and stainless steels. Results demonstrated that bacteria were co-located with iron corrosion products. Their results could not be used to

conclude that bacteria induced the corrosion. An alternative explanation is that bacterial cells are preferentially attracted to corrosion products over well-established anodic regions. In the present study, a non-destructive approach was used to map the anodic and cathodic sites on corroding carbon steel. Sites of bacterial metabolic activity and attachment were determined by mapping incorporation of ^{14}C labelled precursors into bacterial cellular material.

2. Materials and Methods

2.1. Electrolyte

The medium/electrolyte contained (in mg L^{-1}) NH_4C1 50, $MgSO_4 \cdot 7H_2O$ 50, $CaCl_2 \cdot 2H_2O$ 5, KH_2PO_4 27, glucose 50, 2-morpholinoethane sulfonic acid (MOPS buffer) 50, and 1 mL L^{-1} trace minerals (in $\mu g\ L^{-1}$) $Na_2MoO_4\ 2H_2O$ 0. 1, $ZnSO_4 \cdot 7H_2O$ 50, $MnSO_4 \cdot H_2O$ 7.7. $CuSO_4 \cdot 5H_2O$ 2, $CoCl_2 \cdot H_2O$ 1, $NaB_4O_7 \cdot 10H_2$ 0 0.8, $NiSO_4$ 0.5. All solutions were sterilised by autoclaving for 20 min.

2.2. Inoculum

Pseudomonas sp. isolated from a corrosion tubercle on carbon steel and identified by membrane fatty acid profile [3] was used to inoculate media. Bacterial cells were grown to late exponential phase (24 h) in 100 mL of medium to a concentration of 10^6 mL^{-1}. Cells were centrifuged and spent medium removed. Bacteria were resuspended in 100 mL of fresh medium. Fifteen mL of the suspension was added to the electrochemical cell. Electrochemical cells were maintained at 15 mL of volume, with medium continuously pumped into the flow cell at 1 mL/min. Since the flow rate was more rapid than the growth rate, only bacteria that were attached to a surface were maintained in the electrochemical cell. During experiments where the medium was continuously aerated, sterile air was pumped into the flow cell using an aquarium pump.

2.3. Metal Samples and Electrochemical Cells

The electrochemical cell for scanning vibrating electrode (SVE) experiments had a working volume of 15 mL and contained a saturated calomel reference electrode (SCE) immersed in a salt bridge and a platinum wire for a counter electrode (Fig. 1a). Working electrodes (Metal Samples, Munford, AL) of C1020 carbon steel (mass% C 0.17, Mn 0.42, P 0.009, S 0.006, remainder Fe) with a spot-welded electrical connection, were embedded in epoxy and finished with 600-grit silicon carbide paper.

Thin (75 μm) pressure-sensitive tape (3M Co. No. 92) was used to insulate the sample except for an area (approx. 25 mm^2) in the centre of the coupon. Microshield lacquer (Pyramid Plastics, Inc., Hope, AR) was painted at the edge of the tape to reduce crevice corrosion. The working electrode and a salt bridge to a saturated calomel reference electrode were suspended in a beaker of medium. Media were stirred with a Teflon coated magnetic bar, controlled by a magnetic stirrer. Analog output of open circuit potential (OCP) from an electrometer was recorded by a strip chart recorder.

2.4. Current Density Maps

Current density maps over corroding metal samples were obtained using SVE. The vibration of the electrode converts the potential field associated with anodic and cathodic sites over corroding steel into an alternating current. The vibrating electrode was an insulated platinum/iridium wire attached to a piezoelectric reed [4]. The tip of the electrode was exposed to the solution approx. 100 μm over the metal sample. A 2 V alternating signal (152 Hz) was applied to the piezoelectric reed, causing the electrode to vibrate vertically over the metal sample with an amplitude of approximately 40 μm. The alternating signal generated by the vibrating electrode was analysed with a PAR model 124A lock-in amplifier with a model 116 plug-in unit. A computer-controlled data acquisition unit was used to collect data and to position the electrochemical cell underneath the vibrating electrode. Metal samples were scanned by moving the electrochemical cell underneath the vibrating electrode in 0.2 mm increments, using computer- controlled stepper motors (Fig. 1b). Output voltage from the lock-in amplifier was calibrated with a known uniform current density.

2.5. Autoradiography

To identify sites of bacterial activity, biofilms attached to corroding steel samples were incubated with ^{14}C radiolabelled metabolic precursors. Following 1 h of bacterial incorporation of radiolabelled material into cellular material, biofilms were fixed and dehydrated using a procedure designed to preserve samples for electron microscopy. Uptake of labelled precursors was stopped by adding 2% glutaraldehyde in 0.1M cacodylate buffer. Unincorporated label was removed by five exchanges with cacodylate buffer containing 5 mg L^{-1} sodium acetate. Samples were dehydrated with increasing concentrations of ethanol (50%, 75%, 100%) and air dried. Dried biofilms were exposed to X-ray film.

Control experiments were performed to determine if unincorporated radiolabelled precursors preferentially adsorbed on corrosion products or anodic sites. For these experiments, corrosion was induced on steel samples abiotically by incubating the samples in electrolyte in the absence of aeration. OCP and SVE were used to monitor the formation of active pits. Once pits developed, sterile samples were incubated with the ^{14}C precursors. Individual experiments were conducted with (i) 5 $\mu Ci.mL^{-1}$ ^{14}C glucose, (ii) 0.5 $\mu Ci.mL^{-1}$ ^{14}C glucose, (iii) 0.5 $\mu Ci.\ mL^{-1}$ ^{14}C acetate, and (iv) 0.5 $\mu Ci.mL^{-1}$ ^{14}C acetate that had been preincubated with 5 mg L^{-1} cold acetate. Following 1 h incubation with the ^{14}C labelled compounds, samples were fixed, dehydrated, and exposed to X-ray film.

Biotic experiments were conducted under two sets of conditions. Steel samples were incubated under continuous flow conditions with constant aeration to allow development of biofilms. Formation and repassivation of pits were monitored using OCP and SVE. Following development of pits, bacteria were labelled *in situ* with ^{14}C labelled metabolic precursors. In other experiments, pits were allowed to develop abiotically by incubating the steel samples in sterile medium in the absence of aeration. Bacteria that had been prelabelled with ^{14}C acetate were added to the corrosion cells and incubated for 1 h. The samples were fixed, dehydrated, and exposed to X-ray film.

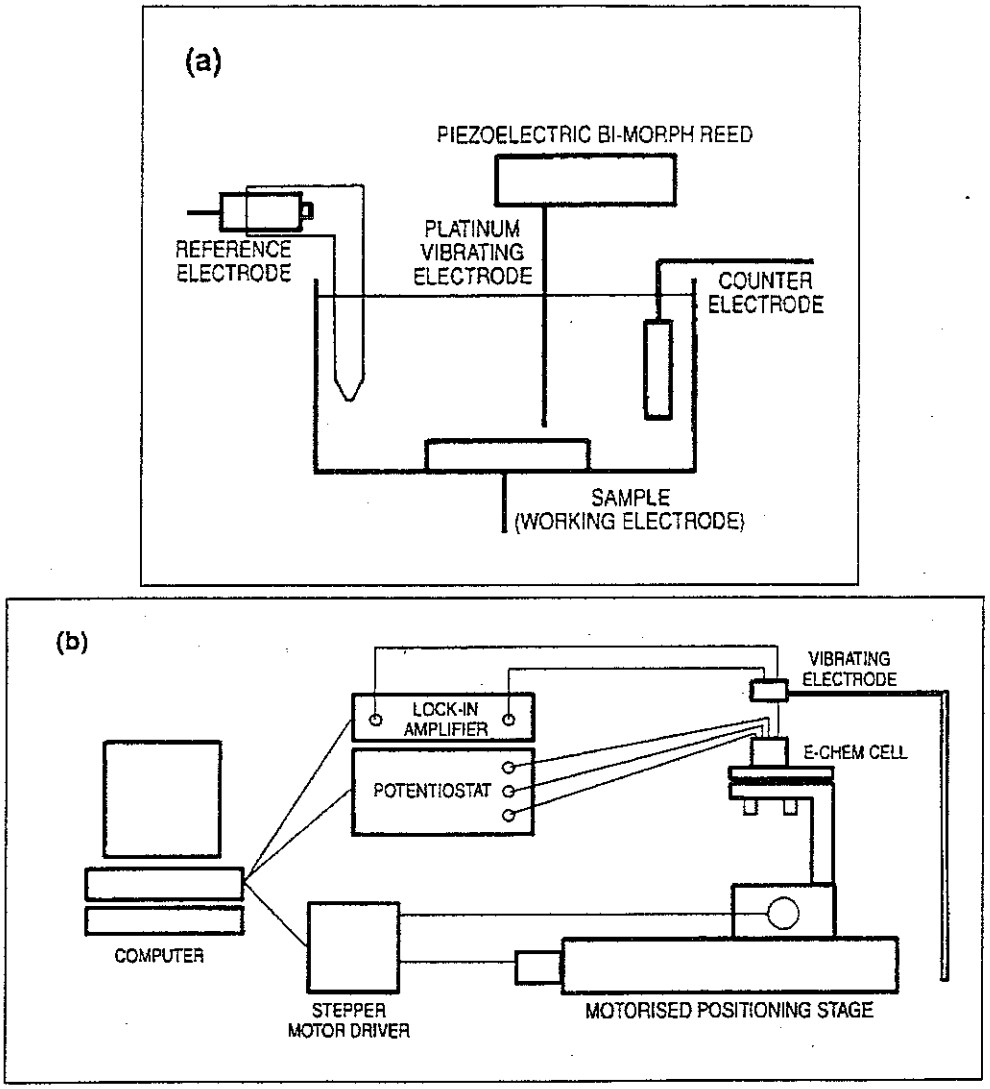


Fig. 1 Schematic diagrams of (a) the electrochemical cell used for SVE analyses of nonuniform potential fields and (b) the system used for SVE analyses.

2.6. Confocal Scanning Laser Microscopy

Iron and zinc filings (1 g) were allowed to corrode overnight in individual drops of sterilised medium. A 50 µL inoculum of viable and fixed cells was added to individual drops. After 1 and 24 h, 10 µL aliquots were removed, fixed in glutaraldehyde, and examined with a Molecular Dynamics Sarastro 2000 confocal laser scanning microscope (CLSM). Autofluorescing cells, due to glutaraldehyde fixation, were imaged with an argon/krypton laser at 488 nm using a 10% attenuation filter and a 100 µm aperture.

3. Results

3.1. Potential vs Time

Potential vs time plots for carbon steel exposed to sterile media, and media with fixed and viable cells indicated differing electrochemical activities (Fig. 2). When exposed to sterile media, the potential of the carbon steel remained between –0.15 and –0.25 V(SCE) for the duration of the experiment (90 h). When viable bacteria were inoculated into aerated medium and biofilms developed under continuous flow conditions, the OCP showed initial noisy behaviour due to initiation and repassivation of the pits. However, after approximately 20 h, OCP dropped and stabilised as pits propagated. When the experiments were performed with glutaraldehyde-fixed cells, pits initiated, repassivated, and stabilised after approximately 48 h.

3.2. SVE Analysis

Prior to experiments using biofilms, control experiments were performed to identify the conditions for nonspecific adsorption of the radiolabelled precursors. Corrosion was induced by abiotically incubating samples in electrolyte in the absence of aeration. Initiation and propagation of corrosion pits was monitored by OCP. The SVE was used to detect pits (anodic sites). Once pits initiated and stabilised, the sample was exposed to the ^{14}C labelled metabolic precursors. Figure 3(a) is an SVE analysis of a carbon steel sample exposed to sterile medium for which the OCP dropped to –440 mV(SCE). The SVE showed the site of anodic activity associated with pitting in the bottom left corner of the sample (anodic activity indicated by the positive Z-direction).

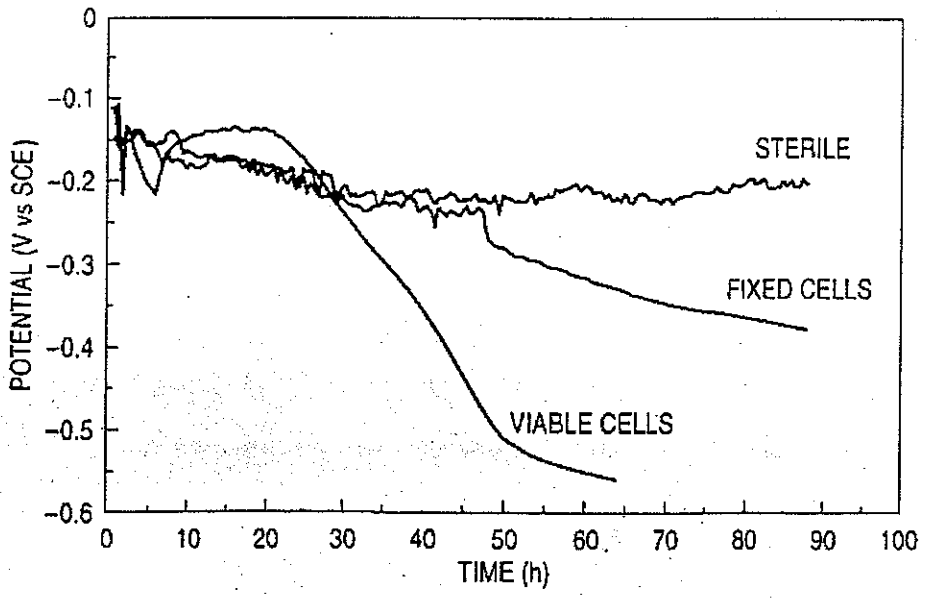


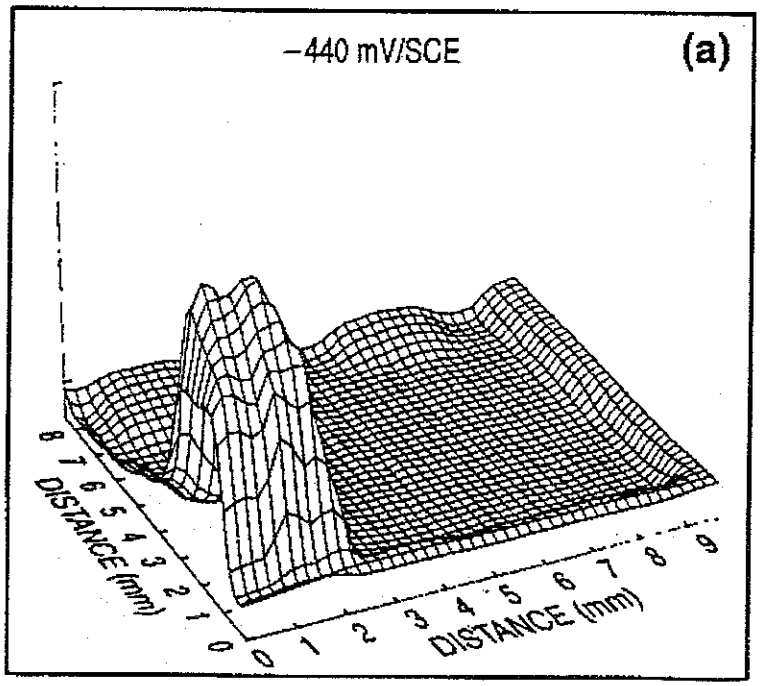
Fig. 2 OCP measurements, carbon steel sample exposed to sterile medium, fixed cells, and viable cells.

A fairly uniform cathodic activity was observed over the remainder of the samp (in the negative Z-direction). Figure 3 (b) shows a contour map representing th same data. The sample was exposed to 5 $\mu Ci.mL^{-1}$ ^{14}C glucose and incubated for 1 The autoradiograph (Fig. 3c) shows that glucose adsorbed preferentially to the anod site (or to the corrosion products associated with the anodic site). A similar resu was obtained with 0.5 $\mu Ci.mL^{-1}$ ^{14}C glucose and 0.5 $\mu Ci.\ mL^{-1}$ ^{14}C acetate (data n shown).

To eliminate abiotic adsorption of ^{14}C acetate to the anodic sites, corroding sampl were pre-incubated with 5 mgL^{-1} cold acetate prior to incubation of the sampl with ^{14}C acetate. When ^{14}C acetate was added to the sample, following initi incubation of the sample with cold acetate, no detectable radiolabel was foun associated with the anodic sites or with the corrosion products (data not shown).

3.3. Electrochemical Activity vs Bacterial Metabolic Activity

When steel samples were inoculated and maintained under continuous flo conditions with constant aeration, biofilms developed and pit propagation wa observed (Fig. 4a). The current density map shows the site of anodic activity in th bottom left corner of the sample (Fig. 4a and b, contour map). Following developmen of a well-defined anodic region and an OCP of –540 mV(SCE), bacteria wer pre-incubated with cold acetate and then exposed to 0.5 $\mu Ci.mL^{-1}$ ^{14}C acetate. Result indicated that incorporation of radiolabel into the cellular material occurre preferentially at the site of active anodic activity in the bottom left corner of th



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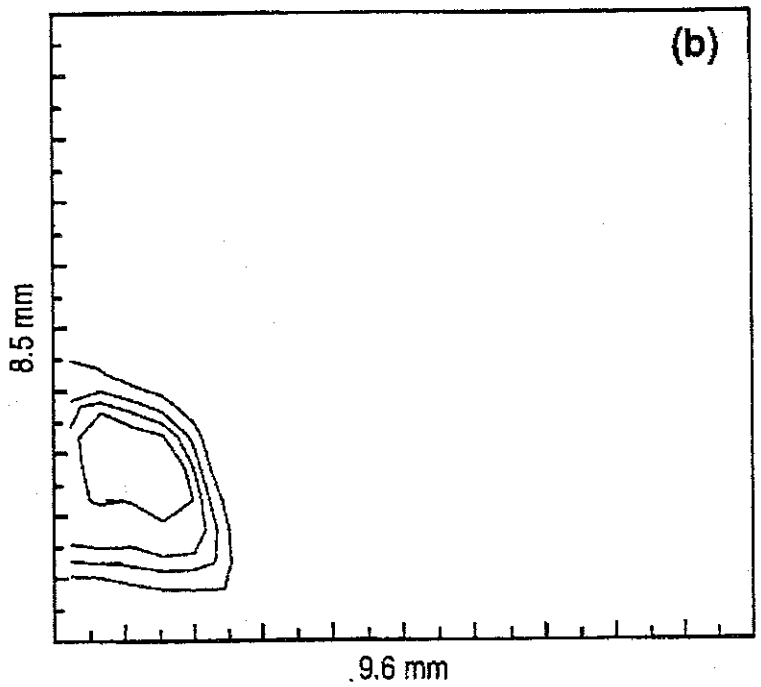


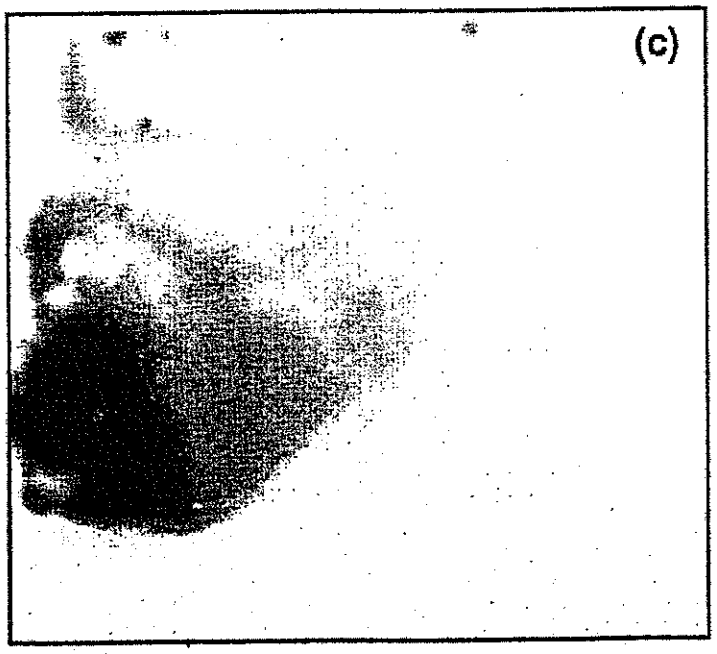
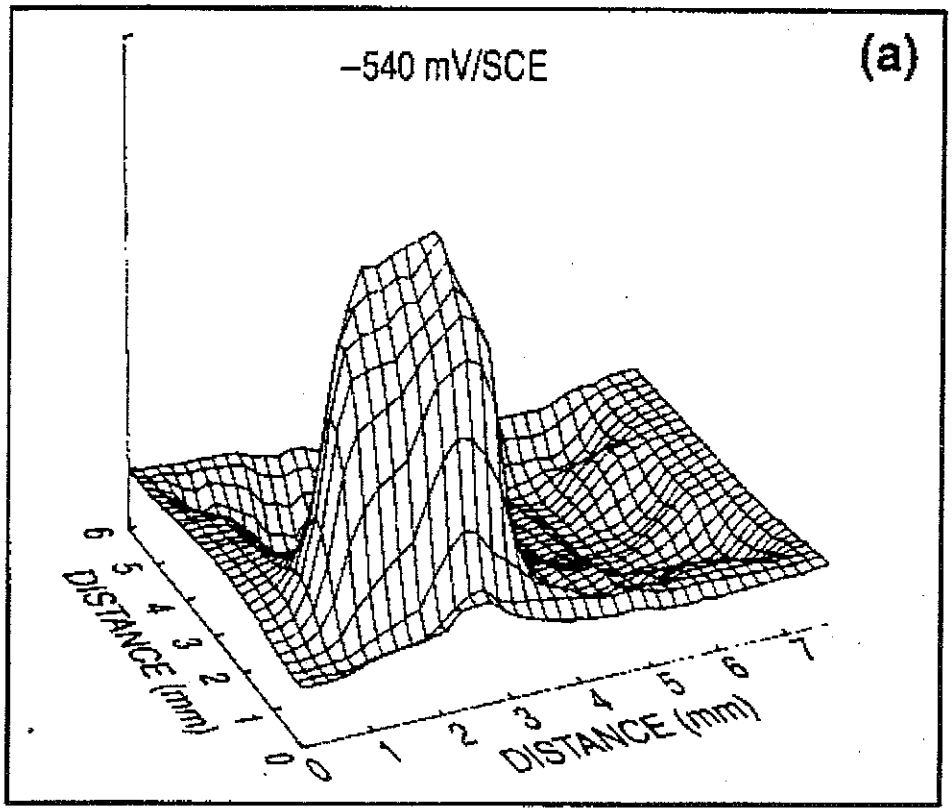
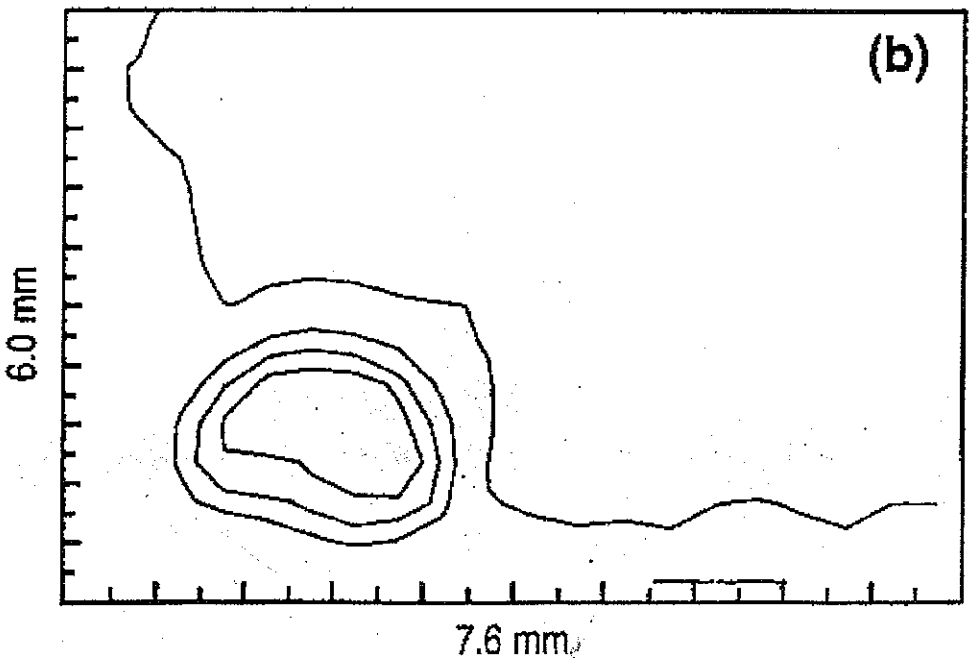


Fig. 3 Sample with well-developed anodic region exposed to 5 μCi.ml^{-1} ^{14}C glucose. (a, opposite) Potential field map over steel sample, maximum potential –440 mV/SCE. (b) Contour map of potential fields. (c) Autoradiograph of sample after exposure to ^{14}C glucose.


−540 mV/SCE
(a)
DISTANCE (mm)
DISTANCE (mm)
0
1
2
3
4
5
6
7


(b)
6.0 mm
7.6 mm

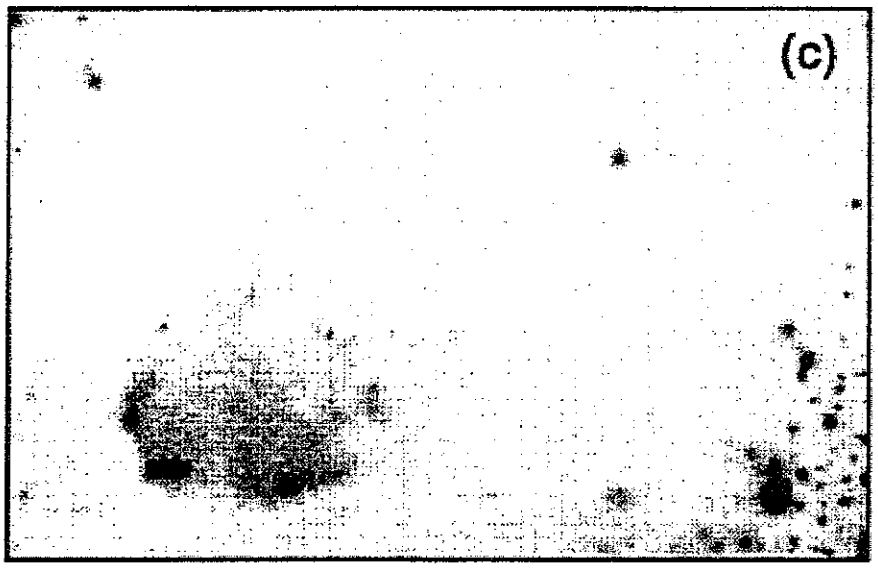
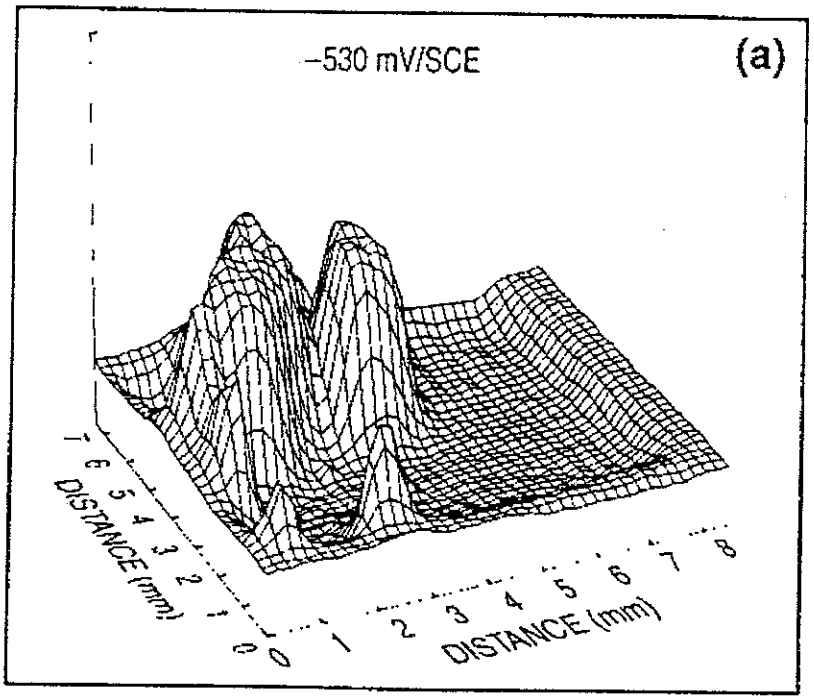


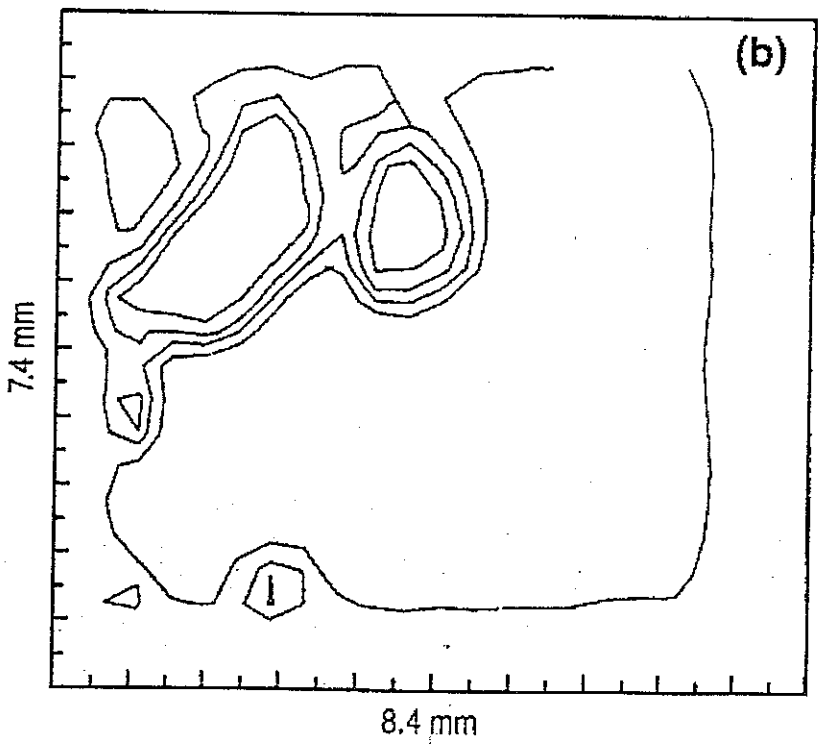
Fig. 4 Sample exposed to Pseudomonas *sp. for 2 days in continuous flow system. Bacteria labelled after settlement at anodic sites. (a, opposite) Potential field map over steel sample, maximum potential –540 mV/SCE. (b, opposite) Contour map of potential fields. (c, above) Autoradiograph after exposure to ^{14}C acetate.*

autoradiograph. Labelled bacteria were also observed in association with the small, inactive pits that had initiated and repassivated (bottom right corner). Little to no silver grain development was observed in similar experiments where the bacteria were fixed with glutaraldehyde prior to exposure of the bacteria to ^{14}C acetate (data not shown). The results suggested that the bacterial biosynthetic activity was primarily associated with anodic sites of the steel, and that bacteria may either initiate the anodic activity or preferentially bind to the corrosion products associated with the anodic sites.

To determine if the bacteria attached preferentially to corrosion products associated with the anodic sites, corrosion was induced on steel samples abiotically under non-aerated conditions. The OCP was monitored and the samples were analysed by the SVE to identify anodic and cathodic sites. Once well-defined corrosion pits were established and the OCP reached –530 mV(SCE), the samples were incubated for 1 h with bacteria that had been pre-labelled with ^{14}C acetate. The results in Fig. 5(a) and (b) demonstrated the development of the anodic areas in the upper left corner of the sample. Autoradiography of the labelled bacteria showed preferential binding of pre-labelled bacteria to the anodic sites (Fig. 5c).

CLSM was used to characterise the association of bacteria with iron and zinc corrosion products. Figure 6(a) indicates the position of corrosion products on iron filings. The highest concentration of bacteria was observed directly in contact with corrosion products (Fig. 6b). Similar results were observed with glutaraldehyde-fixed cells, suggesting that the bacteria may become preferentially bound to iron corrosion products in the absence of biosynthetic activity. Analysis by CLSM showed the same binding to zinc corrosion products.


–530 mV/SCE
(a)
DISTANCE (mm)
DISTANCE (mm)


(b)
7.4 mm
8.4 mm

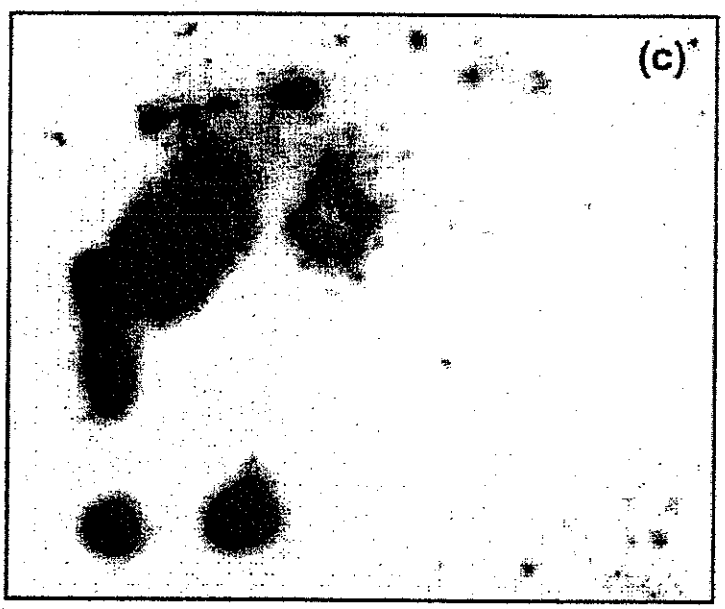


Fig. 5 Bacteria labelled by incubating with ^{14}C acetate prior to exposure of cells to corroding sample. Bacteria associated primarily with anodic sites, suggesting bacteria preferentially bound to corrosion products. (a, opposite) Potential field map over steel sample, maximum potential – 530 mV/SCE. (b, opposite) Contour map of potential fields. (c, above) Autoradiograph.

4. Discussion

It has been established that the most destructive MIC takes place in the presence of microbial consortia in which many physiological types of bacteria, including slime-producing bacteria such as *Pseudomonas* sp.; sulfate-reducing bacteria; acid-producing bacteria; metal-oxidising bacteria, and metal-reducing bacteria, interact in complex ways within the structure of biofilms. *Pseudomonas* sp. used in the experiments described in this paper does not grow anaerobically and does not produce detectable levels of volatile fatty acids when grown on glucose. The bacterium produces extracellular polysaccharide when attached to a steel surface, as demonstrated by Fourier transform infrared spectroscopy and electron microscopy [5]. *Pseudomonas* spp. are commonly associated with biofilms, and other investigators have demonstrated that this organism can increase the corrosion rate of carbon steel compared to sterile controls. A mechanism for the increase had not been established.

Measurement of corrosion influenced by bacteria, as well as evaluation of individual mechanisms, has been difficult due to the destructive nature of most corrosion measurement techniques. Direct current techniques require high applied currents that can alter biofilms. Scanning vibrating electrode techniques provide a non-destructive means to define the magnitude and sign of current densities in solution over freely corroding metals. When localised corrosion of metal occurs, ionic currents flow in solution between the local anodic and the local cathodic sites. Potential fields are established perpendicular to the flow of ionic current that can be mapped by converting potential fields to a.c. signals using scanning microelectrode

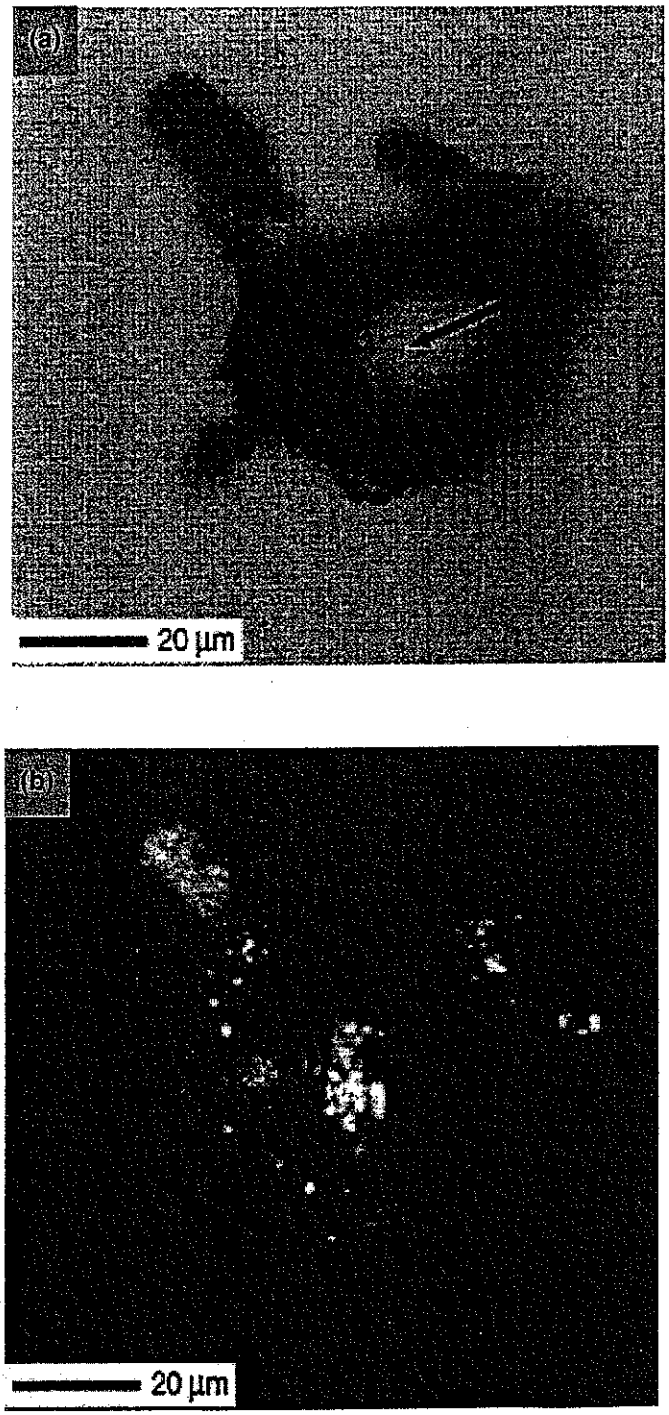


Fig. 6 (a) Confocal Scanning Laser micrograph of corroding iron filing. Arrow indicates area of active corrosion. (b) CLSM micrograph of bacteria preferentially attached at corrosion site.

techniques [4]. Current density maps have been used to define defects in vapour deposited aluminium [6]; pitting of iron in dilute chloride and sulfate solutions [7]; localised anodic and cathodic activities of iron and stainless steel [8], galvanic corrosion of soldered copper [9], and corrosion inhibition by phosphate and organic buffers [4].

Pit initiation and passivation can be followed using OCP measurements [10]. For short transients of a few minutes, changes in potential are dominated by capacitance of a passive surface. After pits initiate, the OCP drops and capacitance is discharged producing the cathodic current equal to the anodic pitting current. If pits repassivate, then OCP gradually rises due to recharging of the passive surface. If pits continue to propagate, OCP continues to drop. The cathodic current equivalent is supplied by the slow oxygen reduction reaction. When the cathodic oxygen reduction reaction is sufficiently rapid, the rate of potential decrease slows, and the contribution from capacitance discharge is negligible. Conditions in the sterile medium used in these experiments favoured repassivation. The medium contained chloride, sulfate, and phosphate ions at concentrations of approximately 1, 0.2 and 0.2mM, respectively. Air was continuously bubbled through the media. An independent investigation demonstrated that phosphate with aeration or stirring produced repassivation and inhibited pit propagation [10]. In contrast, the presence or the metabolic activity of aerobic heterotrophic bacteria had a marked effect on the corrosion of the carbon steel in a system containing the same medium. The OCP, rather than remaining above –200 mV (SCE) as in the sterile control, slowly dropped to a value below –550 mV(SCE). The drop in OCP was not likely due to depletion of oxygen by bacterial respiration, since the medium was continually bubbled with air. Bacterial growth was also limited by the supply of glucose, 0.28mM. These conditions should not lead to an anaerobic environment.

Autoradiography after bacterial incorporation of radiolabelled metabolic precursors has been used to study activity of bacteria in differential fluid shear environments and to examine activity of individual bacteria within complex communities [11,12]. Since acetate is a precursor in bacterial lipid synthesis, acetate should be incorporated into the cellular material of all bacteria. In the present study, 5 mgL^{-1} cold acetate followed by 0.5 $\mu Ci.mL^{-1}$ ^{14}C acetate was used to label the bacteria growing on corrosion products after demonstrating that the combination did not become preferentially incorporated in iron corrosion products. The biosynthetic activity indicated by developed silver grains of the autoradiographs corresponded most strongly with tubercles formed on carbon steel and with anodic activity observed by the SVE. Results of the continuous flow experiments with *Pseudomonas* sp. exposed to carbon steel clearly demonstrate that metabolically active bacteria were associated with anodic sites. The data cannot be unambiguously interpreted as to whether bacteria initiated the anodic site or were attracted to corrosion products. However, in experiments in which bacteria were labelled prior to exposure to established anodic areas, metabolically active cells were preferentially bound to abiotically generated corrosion products.

Pit propagation requires maintenance of a critical level of aggressive ions inside a pit. Dissolution current acts to increase the concentration of ions inside pits, and hydrolysis of ferrous ions maintains a low pH as demonstrated previously using pH microelectrodes. Diffusion of aggressive ions from pits reduces their concentration.

Bacterial biofilms over anodic sites may inhibit diffusion of aggressive ions from pits and/or diffusion of passivating ions, such as phosphate into pits. Rapid pit propagation in the presence of the viable bacteria may be due to the synthesis of cellular material and exopolymers over active anodic sites. Sequeira *et al.* [13] stressed the membrane properties of polymeric substances produced in a biofilm. They validated the physico–chemical properties of biologically produced membranes, their impact on ionic transport, and the significance to corrosion of copper tubes.

The role of microorganisms in causing pitting corrosion has traditionally been defined as one of initiation, i.e. the presence and activities of the organisms initiate an oxygen concentration cell and pit propagation is controlled by hydrolysis reactions. These experiments indicate that the sequence of events is subtly different and that the role of the microorganisms may be related to pit propagation.

5. Conclusions

The scanning vibrating electrode technique combined with autoradiography can be used to resolve causal relationships between localised corrosion and bacteria. Spatial and temporal relationships between *Pseudomonas* sp. and localised corrosion of carbon steel in a phosphate-containing medium can be summarised as follows:

(1) anodic sites form and repassivate in the absence of bacterial cells;

(2) bacterial cells are attracted to anodic regions formed simultaneously with their growth or to established anodic sites; and

(3) once bacteria become associated with an anodic region, repassivation is unlikely.

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