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SPATIAL AND TEMPORAL RELATIONSHIPS BETWEEN LOCALIZED MICROBIAL METABOLIC ACTIVITY AND ELECTROCHEMICAL ACTIVITY OF STEEL

Michael J. Franklin[1,2], James B. Guckert[2], David C. White[1,2,3], and Hugh S. Isaacs[4]

[1]Department of Microbiology
University of Tennessee
Knoxville, Tn. 37996

[2]Institute for Applied Microbiology
University of Tennessee
10515 Research Dr. Suite 300
Knoxville, Tn. 37932-2567

[3]Environmental Science Division
Oak Ridge National Laboratory
P.O. Box 2008
Oak Ridge, Tn. 37831-6036

[4]Department of Applied Science
Brookhaven National Laboratory
Upton, NY. 11973

ABSTRACT

The relationship between the localized microbial activity and localized electrochemical on corroding steel surfaces was investigated. The scanning vibrating electrode technique provided a sensitive means for defining local anodic and cathodic currents associated with corrosion. Localized bacterial metabolic activity was determined using autoradiography of bacterial incorporated ^{14}C-acetate into insoluble cell material. Microautoradiography of individual bacteria, with the incorporated acetate, was used to determine percentages and morphological types of active bacteria. The results showed a correlation between the location of anodic activity of the steel and the location of incorporated label. Although the results do not necessarily indicate a role for bacterial activity in the initiation of pitting, the results suggest that the actively metabolizing bacteria may exacerbate the propagation of pits by colonizing active anodic sites.

Keywords: Scanning vibrating electrode technique, localized corrosion, microbial activity, microbiologically influenced corrosion

INTRODUCTION

Much of the microbial influenced corrosion (MIC), both from field observations and from laboratory investigations is localized. Bacteria tend to colonize surfaces in a nonuniform manner as microcolonies (1). The metabolic activities of heterogeneously colonized bacterial biofilms on steel surfaces can result in differences in aeration or catalytic activity of the steel surface, leading to localized attack. In order to gain a better understanding of microbial influenced corrosion, and to develop rational countermeasures, studies contrasting localized microbial activity and localized corrosion of steels must be performed. One difficulty in the study has been the difficulty in resolving and in correlating events in time and space. Most electrochemical polarization techniques provide average information for the whole electrode. Also, techniques for studying microbial activities in complex environments often only provide a mean value for relatively large samples.

Scanning microelectrode techniques have been used to map potential fields over corroding or polarized metal surfaces (2). These techniques have provided useful information of localized electrochemical activities. A scanning vibrating electrode technique (SVET) was used to map potential fields over metal samples (3,4,5,6,7,8). The vibrating electrode technique converts the potential fields to AC signals. Using a lock-in amplifier all frequencies not associated with the frequency of vibration can be filtered, greatly reducing the noise inherent in the microelectrode techniques. The SVET has recently been applied to the study of MIC (9,10), and has shown that bacteria can assist in the propagation of pitting corrosion.

Techniques for studying microbial activity at localized sites are also being developed. Mittelman and White (11) have used autoradiography after bacterial incorporation of radiolabelled metabolic precursors to study activity of bacteria in differential fluid shear environments. In addition microautoradiographic techniques have been used to examine the activity of individual bacteria within complex communities (12,13).

In this work, the techniques for studying localized microbial metabolic activity were combined with the SVET to determine the relationship between microbial metabolic activity and electrochemical activity. The objectives of this work were 1) to determine if nonuniform microbial metabolic activity on steel samples during colonization could be resolved, 2) to determine the percentages and morphological types of the metabolically active bacteria, and 3) to determine the relationship between the microbial metabolic activity to the anodic or cathodic activity of corroding steel samples.

MATERIALS AND METHODS

Bacteria and media. The bacteria used in this study were the same as those used in previous studies of MIC (9,10,14). They have been identified as a strictly aerobic Pseudomonas sp., a facultative aerobe Hafnia sp., and the sulfate reducing bacterium Desulfovibrio gigas. The Desulfovibrio gigas was cultivated on API sulfate reducer medium. The medium used for cultivation of the Pseudomonas sp. the Hafnia sp. and for the experiments contained basal salts (in mg/l NH_4Cl, 50; $MgSO_4 \cdot 7H_2O$, 50; $CaCl_2 \cdot 2H_2O$, 5; KH_2PO_4, 27; pH=7.2), 1ml/l mineral solution (10), and 50 mg/l of glucose, yeast extract, peptone, and sodium lactate.

During experiments, the medium was continuously flowed into a 500 ml flask at a rate of 0.1 h^{-1}. The flasks for the study of microbial activity of sessile bacteria in a continuous flow system were described previously 15). Epoxy embedded coupons, of 304 stainless steel and of C1020 carbon steel, abraded with 600 grit emery paper, were suspended in different flasks. The samples were removed aseptically over time.

Bacterial activity. After removal of the coupons from the flowing system, tubes with O-ring seals were clamped onto the coupons (16). Basal salts solution, 400 µl, containing 2.5 µCi ^{14}C-acetate was added to the tubes. The sessile bacteria on the coupons were incubated with this ^{14}C-acetate solution for 30 min. After the 30 min incubation, the bacteria were killed with 2% glutaraldehyde in a 0.1 M cacodylate buffer. The killed control samples were treated in the same way, with the exception that the biofilms were killed with the 500 µl glutaraldehyde solution for 15 min prior to exposure to the ^{14}C-acetate. Any unincorporated ^{14}C-acetate was removed by exchanging the solution five times with the glutaraldehyde solution. The samples were then dehydrated by increasing concentrations of acetone (50%, 75%, 100%, 100%). A 100 µl aliquot of the final acetone wash was placed in scintillation cocktail and counted using a scintillation counter to determine if unincorporated ^{14}C-acetate or labelled nonadhered bacterial cells remained in the solution. The samples were then air dried. A similar procedures was used in electron microscopy to maintain the spatial integrity of the samples (15). The samples were then exposed to X-ray film for seven days.

The sample preparation procedure for determining the activity of individual sessile bacteria was similar to the procedure described above. Steel samples were removed from the flasks and O-ring tubes were clamped onto the samples. Basal salts solution, 500 µl, containing 2.5 µCi of ^{14}C-acetate was added to the tubes. The bacteria were incubated with this solution for 30 min., then killed with the glutaraldehyde solution, described above. The glutaraldehyde solution was removed from the tubes and replaced with 500 µl of basal salts. The sessile bacteria on the steel surface were removed from the

surface by sonication for 5 sec. The bacteria removed from the surface were placed in 9.5 ml of basal salts solution. This sonication procedure was repeated twice. The basal salts solution containing the labelled bacteria was serially diluted in basal salts to obtain an appropriate concentration of cells for microscopic enumeration.

The bacteria were filtered with 0.2 μm average pore size membrane filters. The technique for microautoradiography has been extensively described (13). Briefly, microscope slides were dipped into photographic emulsion (Kodak NBT-2) diluted 2 to 1 with distilled water. The filters were placed on the emulsion, and allowed to incubate for seven days in the dark at a temperature of 4°C. The slides were then developed using Dectol developer. The bacteria were stained with acridine orange in a citrate buffer (pH 6.6). Destaining of the slides was done in citrate buffers of decreasing pH (6.6, 5.0, 5.0, 4.0,4.0, ten min each). The filters were removed from the slides and the bacteria remained immobilized in the photographic emulsion (13). Developed silver grains around the fluorescing bacterial cells were indicative of incorporation of the ^{14}C acetate.

Scanning vibrating electrode technique. The scanning vibrating electrode technique was used to obtain potential field maps as described previously (6). The vibrating electrode was positioned 100 μm over the surface of the metal, and was vibrated at a frequency of 154 Hz using a piezoelectric reed. The direction and the magnitude of the signal was determined using a E.G. and G. model 5210 lock-in amplifier. The scanning of the sample with the vibrating electrode was performed by stepping the sample underneath the electrode using computer controlled stepper motors. The steps were in 200 μm increments. Data from the lock-in amplifier was collected using a computer programmed in the ASYST language.

The samples for the SVET analysis were carbon steel coupons. A small area of the coupon, approximately 135 mm^2, was exposed to solution, by masking most of the coupon with thin pressure sensitive tape. The edge of the tap and the metal was coated with a lacquer coating, to help prevent crevice corrosion.

RESULTS

Bacterial activity on stainless steel. The autoradiograms in Figure 1 show the localization of bacterial activity associated with the 304 stainless steel sample. After six hours of exposure, little activity of bacteria was seen associated with the stainless steel samples (not shown). Little activity is seen associated with the killed controls, showing that the radiation from the sample is not due to unincorporated acetate. This can be see from Figure 1A and 1B where there is little activity of the glutaraldehyde fixed controls after three and four days. Figures 1C and 1D show the activity of the bacteria on the

stainless steel after two and three days of exposure, respectively. Figure 1C shows that the activity of the bacteria was greater on parts of the sample than on other areas after two days of exposure. This nonuniform microbial activity could result in differential aeration of the sample. After three days of exposure the activity of the bacteria on the surface is relatively uniform over the surface of the sample.

Activity of individual bacteria. Figure 2A shows the activity of individual bacteria removed from the surface after two days of exposure, and Figure 2B shows the killed control. Developed silver grains can be seen in association with the bacteria, indicating uptake of the ^{14}C-acetate. No silver grains can be seen associated with the glutaraldehyde fixed bacteria, suggesting that the ^{14}C-acetate is incorporated into cellular material and not adsorbed onto the bacteria. The incorporation of ^{14}C-acetate is not uniform for all the bacteria in the biofilm, and not all bacteria show incorporation. This indicates that the 30 min incubation time may be insufficient for the diffusion of the acetate to all levels of the biofilm, or that bacteria within the biofilm have varying growth rates. In addition, the smaller aerobic bacteria are label preferentially over the larger Desulfovibrio. This preferential labeling does not indicate that the aerobic bacteria are more active in the biofilm, but only that they are more capable of incorporating the acetate under the experimental conditions used. The number of surface bound bacteria and the number of bacteria with associated silver grains is shown in Table 1.

Bacterial activity on carbon steel samples. The distribution of bacteria with incorporated ^{14}C-acetate on the carbon steel surfaces is shown in Figure 3. Little silver development was observed for the glutaraldehyde fixed controls (Figure 3A,B,C,D). In some experiments using longer times of exposure to the X-ray film, some development of silver grains was observed for the killed controls, suggesting that some unincorporated label was associated with either the metal or adsorbed to the bacteria. In future experiments, unlabelled acetate will be added to the glutaraldehyde solution to help remove the unincorporated label.

Nonuniform activity was seen on the samples after six hours and after one day of exposure (Figure 3A and 3B). After two days of incubation in the flowing system, most of the uptake activity is seen in one spot near the middle of the sample (Figure 3C). This activity corresponded to a small tubercle on the surface of the steel. After three days of exposure, several localized areas of activity could be seen (Figure 3D). Again these sites of activity were associated with small tubercles on the surface of the metal.

The number of bacteria metabolizing the acetate and the total number of bacteria associated with the surface of the carbon steel is shown in Table 1. As was seen in the

autoradiogram, the cell counts reveal that the carbon steel was colonized more rapidly than the stainless steel. Also, not all the cells showed uptake of the acetate.

Relationship between local electrochemical activity and microbial activity. The localized electrochemical activity obtained by the SVET of carbon steel exposed to bacteria is shown in Figure 4. Figure 4A shows several areas of anodic activity after two days of exposure to bacteria. Figure 4B shows a contour map of the same data. Figure 4C is an autoradiogram of the sample after one hour of exposure to ^{14}C-glucose and development as described in the materials and methods. Spatial relationship between the microbial incorporation of the glucose and the active anodic sites can be seen from these figures.

DISCUSSION

Jack (14) has demonstrated that changing consortia of bacteria within a biofilm, changed the corrosion rates of carbon steel. In particular, the presence of sulfate reducers caused an increased corrosion rates, even though the total biomass of the biofilm was less than consortia without sulfate reducers. This indicates that in addition to microbial biomass, the metabolic activities of particular bacteria within biofilms were important to microbial influenced corrosion. Understanding the activities of individual bacteria within complex biofilm consortia is important in understanding mechanisms of corrosion facilitation by key organisms such as sulfate reducers, and in establishing countermeasures against corrosion by the key organisms.

In this study, we demonstrated that the biosynthetic activities of individual bacteria within a biofilm consortia could be analyzed, using ^{14}C-acetate as a bacterial metabolic precursor. Since, acetate is used as a precursor in bacterial lipid synthesis, acetate should be incorporated into the cellular material of all bacteria. However, acetate was not equally incorporated into all of the bacteria on the steel surfaces. This suggests that the bacteria within the biofilm either had different growth rates or were exposed to nutrient limiting conditions within the biofilm. The large sulfate reducers showed little incorporation of the acetate. This is likely due to the incubation conditions rather than the lack of metabolic activity of the sulfate reducers. The samples were removed from the flask and exposed to air prior to incubation with the acetate. This removal may have inhibited the activity of the strictly anaerobic sulfate reducers. In future studies, the labelled precursors will be added directly to the flask to gain a better understanding of sulfate reducer activity under in situ conditions.

With the biofilm consortium used here, it was possible to distinguish between the large sulfate reducers and the small aerobic bacteria. However in more complex systems, these

morphological distinctions can not be made. Nucleic acid probes have been used to identify bacteria in complex communities (17). The nucleic acid probes in combination with autoradiographic techniques should provide further information regarding the activity of specific bacteria in complex communities such as biofilms.

The autoradiographic techniques in conjunction with scanning microelectrode methods allowed better understand of the relationship between localized microbial metabolic activity and localized electrochemical activity. The biosynthetic activity indicated by the developed silver grains of the autoradiograms corresponded most strongly with tubercles formed on the carbon steel, and with anodic activity observed by the SVET. Several explanations can account for this increased ^{14}C at these anodic sites. First, the unincorporated acetate may have formed an insoluble abiotic precipitate with the oxidized iron. As mentioned, in some cases under long exposure times, exposure of the film was seen in the killed controls. Therefore some of the acetate may have formed precipitates with the oxidized iron. However, the amounts of acetate bound to the corrosion products was small compared to that incorporated into the cellular material (Figure 1 and 3). Second, the nonuniformly metabolizing bacteria may have caused differential polarization of the sample and resulted in active anodic sites. Third, the actively metabolizing bacteria may have preferentially colonized the corrosion products of the corroding steel. Using these techniques it was not possible to distinguish between the second and third possibilities. In order to address this difficulty, our laboratory is currently developing reporter technology, where location of actively metabolizing luminescent bacteria can be established prior to the onset of corrosion (18).

Whether the differential metabolic activity of the bacteria resulted in initiation of localized anodic sites or was caused by bacterial colonization of the corrosion products is an area for future investigation. However, either case could result in the acceleration of corrosion. In a previous study, the physical presence of microbial biofilms, whether viable or nonviable, caused initiated pits to propagate rather than repassivate (19). We proposed that the microbial biofilm, containing bacteria and extracellular polymer, impeded the flow of inhibiting ions to the surface, or impeded the flow of aggressive ions away from the pits. Propagation of pits was more rapid when viable bacteria were present than in the presence of killed bacteria. Therefore, in addition to the microbial biofilm, some metabolic activity of the bacteria contributed to the propagation of the pits. Here we demonstrated that most of the biosynthetic activity of bacteria occurred over the anodic sites. Therefore, the rapid pit propagation in the presence of the viable bacteria may be due to the synthesis of additional biological membrane over the active anodic sites.

CONCLUSIONS

1) Autoradiography in conjunction with the scanning vibrating electrode technique can be used to determine relationships between localized microbial metabolic activity and localized electrochemical activity.

2) Microautoradiography was used to determine the nature of the individual actively metabolizing bacterial cells, and to study the effect of those bacteria on the corrosion.

3) Since the autoradiographic techniques are destructive to the bacteria on the sample, these techniques could not necessarily provide a cause and effect relationship between microbial metabolic activity and the onset of localized corrosion. However, these techniques do indicate that bacterial metabolism is involved in the propagation of anodic sites. Nondestructive techniques, currently being developed, should provide information regarding the effect of localized microbial activity on the onset of pitting.

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Table 1
Numbers of bacteria and active bacteria on metal surfaces

Steel	Time	Total cells[a]	Active cells	Total cells (killed control)
304	6 hours	0.4 ± 0.2	0.2 ± 0.1	0.6 ± 0.3
C1020	6 hours	1.4 ± 0.4	0.4 ± 0.1	1.0 ± 0.4
304	2 days	5.7 ± 1.9	1.9 ± 0.8	4.8 ± 2.2
C1020	2 days	10 ± 3.0	3.6 ± 1.0	7.3 ± 3.1
304	3 days	25 ± 5.0	10 ± 2.0	ND[b]
C1020	3 days	22 ± 4.0	10 ± 3.0	5.0 ± 3.2

[a] All cell numbers are times 10^6 cells/cm^2.
[b] Not done.

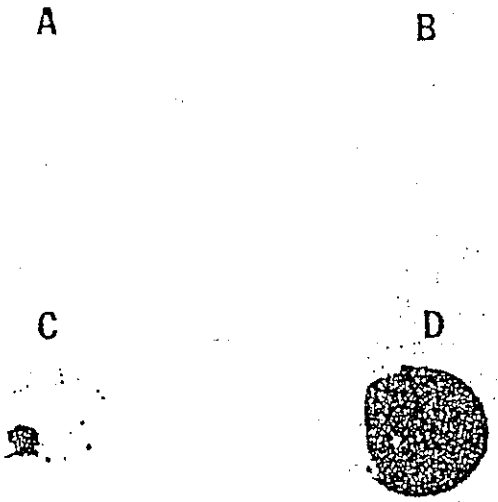

Figure 1. Autoradiograms of 304 stainless steel samples exposed to bacteria in a flowing system then incubated for 30 min with 2.5 μCi ^{14}C-acetate. A) Sample exposed to bacteria for 2 days then killed prior to exposure to acetate. B) Same as 2A but exposed to bacteria for 3 days. C and D) ^{14}C-acetate incorporation activity of bacteria exposed to flowing system for two and three days, respectively.

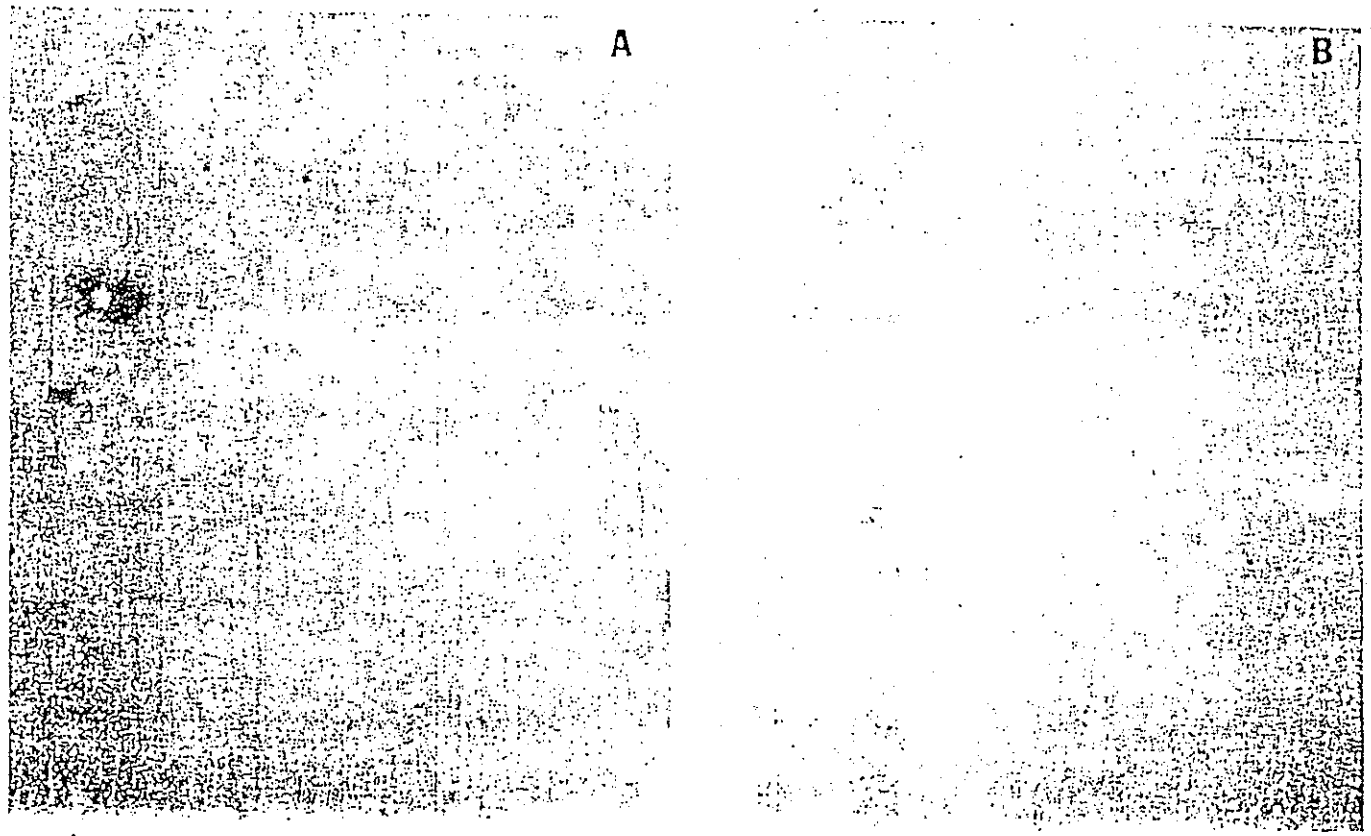

Figure 2. Autoradiograms of sessile bacteria after exposure to flowing system for two days. A) Bacteria exposed to 2.5 μCi ^{14}C-acetate for 30 min. B) Bacteria killed with glutaraldehyde prior to exposure to ^{14}C-acetate.

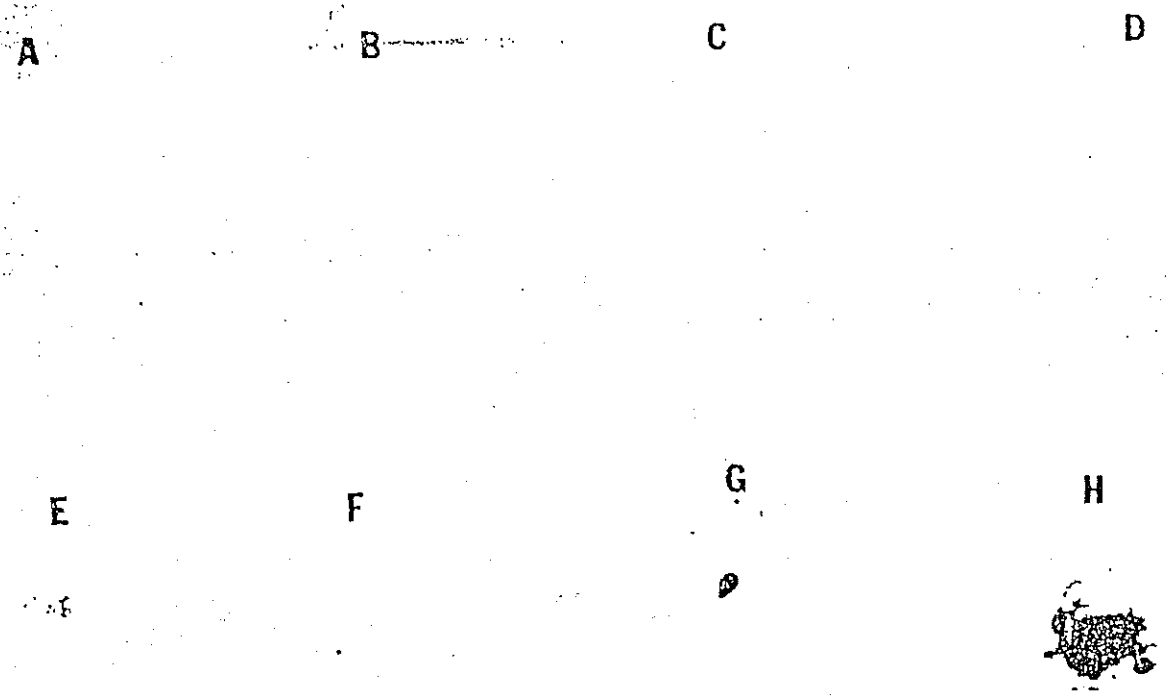

Figure 3. Autoradiograms of C1020 carbon steel samples exposed to bacteria in a flowing system, then incubated for 30 min with 2.5 μCi ^{14}C-acetate for 30 min. A,B,C,D) Killed controls after exposure to the flowing system for six hours, one day, two days, and three days, respectively. E,F,G,H) Samples exposed to ^{14}C-acetate after exposure to the flowing system for six hours, one day, two days, three days, respectively.

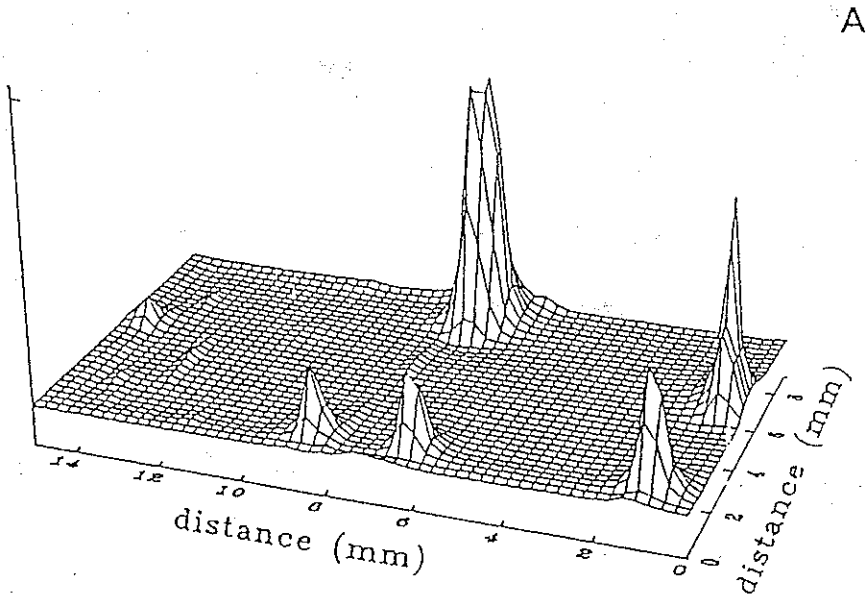

Figure 4. Current density map obtained by the SVET of carbon steel sample after two days of exposure to bacteria. B) Contour map of the data of 4A. C) Autoradiogram of sessile bacteria on sample seen in 4A, incubated with ^{14}C-glucose for 1 h.