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USE OF 2-D VIBRATING ELECTRODE TECHNIQUE IN MIC STUDIES

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ABSTRACT

The role of bacteria in microbial-associated corrosion of steels has been well documented. Recently, bacteria have been shown to be involved in the freshwater pitting corrosion of copper water pipes. However, there is still some controversy over how the pitting is actually initiated. This is due in part to the electrochemical methods traditionally employed in studying microbial corrosion. These methods are of very low resolution and provide information averaged over a relatively large area compared to the pits, that can be less than one millimeter in diameter. The scanning vibrating electrode technique (SVET) has been shown to be capable of detecting the localized anodic regions associated with microbial corrosion processes. The systems, as described previously, have not provided information on the localization of the microbes thought to be involved in the process. The inclusion of a microscope and a photon detecting camera to a two-dimensional scanning vibrating electrode has allowed the location and activity of genetically engineered "reporter strains" of bacteria to be mapped congruently with the localized current densities. These reporter strains have the luxB gene, which produces bacterial luminescence, inserted into specific pathways of interest for example alginate production, such, that the production of alginate is accompanied by the production of visible light. It has been observed that the anodic regions correspond to the location of the bacteria; providing further evidence that bacteria can be involved in the initiation of the pitting process. Similar results have been seen using a solution of purified exopolymers produced by some of the bacteria tested. This suggests that the anodic regions are a result of differential aeration cells being set up by the bacteria. Work is currently in hand to help determine possible mechanisms by which bacteria may be involved in pit initiation.

Keywords: microbially induced corrosion, scanning vibrating electrode, reporter bacteria, pitting,

exopolymers.

INTRODUCTION

It has been suggested that bacteria and bacterial biofilms can be found associated with every non-toxic aqueous surface[1]. Recently, bacterial biofilms have even been isolated from the toxic surface of copper water pipes carrying both cold and warm waters[2] . It has been estimated that the economic cost of these bacterial biofilms runs into the billions of U.S. Dollars per year[3] . This cost is made up of various factors which include the reduction in efficiency of heat exchangers due to fouling and the failure of systems due to microbially induced corrosion (MIC).

The involvement of the sulphate reducing bacteria (SRB) in the pitting corrosion of steels[4] and copper[5] is now well established. The role of other microorganisms in the corrosion process is now being recognized, both in enhancing SRB corrosion and by inducing corrosion themselves. Luo *et al.*,[6] were able to demonstrate that the corrosion rate of mild steel as measured by D.C. polarization was higher in a mixed consortium of bacteria, including an SRB, than when the SRB were present in an axenic culture. Similar results were seen in a study using a genetically-engineered strain of *Pseudomonas fluorescens* (5RL) and the SRB *Desulfovibrio gigas*. It was observed that the mixed culture induced localized pitting of mild steel under aerobic conditions as indicated by a drop in the open circuit potential (OCP) with time and confirmed at the end of the experiment by visual examination[7] . From these results it was concluded that 5RL acted to form a biofilm on the surface of the coupon which provided the anaerobic conditions necessary for the SRB to grow.

The ennoblement of 316 stainless steel due to the presence of photosynthetic biofilms was demonstrated during periodic illumination of a biofilm comprising a photosynthetic cyanobacterium and *Delaya marina*. No such ennoblement was evident in either sterile controls or axenic biofilms of the non-photosynthetic *D. marina*[8] .

Angell and Chamberlain[9a,15] recently demonstrated that a group of three pseudomonads were responsible for a newly defined, but characteristic, form of pitting in copper that has been termed Type 1½ pitting, due to its intermediate nature having some of the characteristics of both Types 1 & 2 copper pitting[9b]. It was shown that the pitting was only reproduced in the laboratory in systems inoculated with bacteria isolated from failed pipes. A limited site survey also showed that the numbers of bacteria and presence of a biofilm composed of acidic exopolysaccharide were correlated with various corrosion indicators. There was a good correlation between high numbers of bacteria and the presence of pits; and a sooty form of copper (I) oxide, characteristic of this type of pitting, was only present on the samples containing the bacterial biofilms[10] .

By utilizing the scanning vibrating electrode technique (SVET), Isaacs was able to demonstrate that the current density associated with a metal surface could by mapped, allowing both anodic and cathodic regions to be localized in time and space[11]. This technique is based on the principle that over corroding metal, the solution resistance produces potential fields between local anodic and cathodic sites. The current densities producing the fields can be detected with a scanning vibrating electrode. The SVET converts fields to an alternating signal. The sign and magnitude of this current can be determined by using a lock-in amplifier. The lock-in amplifier allows the frequency of vibration to be subtracted from the signal, resulting in a DC current which is proportional to the current density

measured at the probe tip. By using a computer-driven motion system, these currents can be plotted, allowing the spatial localization of anodic and cathodic regions. It has been demonstrated that by vibrating the electrode in two dimensions, i.e. vertically as well as horizontally (X and Y), and utilizing two-lock in amplifiers, the resolution of the system can be enhanced[12]. It was suggested that the major limitation to the lateral resolution with the 1-D system was the limit on the proximity of the probe to the surface. It was shown that this could be overcome by vibrating the probe in two dimensions.

Utilizing the 1D-SVET, Franklin *et al.*[13] were able to map local anodic regions which formed and repassivated on the surface of a mild steel coupon exposed to sterile medium. It was further shown on the addition of a *Pseudomonas sp.* that the repassivation of the anodic regions did not occur. This effect was only noted with the addition of live bacteria; similar effects were not seen with spent bacterial medium or killed bacterial cells. Davenport *et al.*[12] successfully applied the 2D-SVET to the study of corrosion inhibitors.

In this paper we describe a system which allows for the congruent mapping of bacterial activity utilizing the luminescent emission of a genetically engineered bacteria, with the current densities as measured by the 2D-SVET. The luminescence was measured with a photon-detecting camera with a resolution of better than 1 μm. Specially modified flow cells were developed, based on the design of Campbell[14], which allowed for the spatial localization of the bacteria and current densities as well as the more traditional electrochemical methods of EIS and OCP, thus allowing the comparison of all the methods in their ability to detect the early stages of pitting.

MATERIALS AND METHODS

Specimens.

Test specimens used were 16 mm diameter disks of AISI C1020 Carbon steel. These disks were mounted into epoxy resin casts and wet-polished in sequence with 240, 400 and 600 grit SiC paper, ultrasonically cleaned with distilled water, degreased with acetone and sterilized with 70% alcohol for 30 minutes.

Bacteria and exopolymers.

Two bacteria were used in this study. A genetically engineered strain of *Pseudomonas fluorescens* into which the lux gene had been inserted, hereafter referred to as 5RL. This strain was chosen due to its ability to bioluminescence when induced, its adhesive characteristics in liquid culture, and its involvement in localized corrosion of mild steel[7]. The second bacteria used was an environmental isolate from a corrosion tubercle of *Xanthomonas multiphillia* . This bacterial strain designated 046, was also genetically engineered to contain the lux gene, this time in the alginate production pathway. Bacterial cultures were grown on agar plates before being resuspended in fresh medium to cell densities between to 10^5 and 10^{10} cfu/ml, and 1 μl was spotted on the coupon, giving approximate cell concentrations on the coupon between 10^4 and 10^{10} cfu cm^{-2}. This spot was left for one hour, allowing it to dry slightly to aid attachment of the cells in a localized region. Crude exopolymer was isolated as described by Angell[15]. Drops of a 10% (w/v) solution were then applied to the test surface. Bacterial alginate drops were applied as a 1% solution to the metal surfaces.

Electrochemical Cell.

Figure 1 shows the electrochemical cells used which were modified from those described previously by Campbell[14]. The main body of the cell was made from a PTFE rod milled to form a tube with an internal diameter of 31 mm and an external diameter of 58 mm. Inlet and outlet ports were fabricated into the sides of the tube, to which a 2 mm internal diameter silicone tube was attached. The coupons were mounted in one end of the cell and sealed with a silicone rubber o-ring. The back of the mount was tapped, and a 4 BA brass bolt was inserted to provide electrical contact with the coupon. For analysis using the 2D-SVET, the other end of the cell was left open to allow access for the probe and platinum reference electrodes. For the other electrochemical analysis, an Ag/AgCl reference electrode was mounted into a conductive resin mount which was inserted opposite the mounted coupon in a similar fashion. The conductive mount was tapped and a 4 BA brass bolt inserted to allowing its use as the counter electrode.

A minimal medium, modified from that described by Franklin *et al.*, was used which was designed to provide sufficient nutrients to induce the luminescence of 5RL and contained (in g/l) ammonium nitrate 0.05, magnesium sulphate 0.05, calcium chloride 0.005, potassium orthophosphate 0.027, sodium salicylate 0.05, glucose 0.05 and TRIS buffer (10 Mm) 10 ml. TRIS buffer was used in place of MOPS buffer as the latter was found to adversely affect the capacitance of the platinum black coating the tip of the probe. The use of any buffer in corrosion studies is considered to be a questionable practice, in this case it was thought to be beneficial in reducing the level of general corrosion that would otherwise have occurred on the mild steel coupons, therefore masking the effect of the bacteria. The flow cells were operated at ambient temperature (23-25 °C).

The test cells were sterilized using 70% ethanol and the tubing and medium were sterilized by autoclaving at 120 °C and 15 PSI for 20 minutes. Aseptic conditions were maintained in the SVET by blowing a stream of filter-sterilized air over the open end of the flow cell.

Electrochemical Analysis

A two-dimensional scanning vibrating electrode microscope (2D-SVEM) utilizing a Model 200 2D lock-in amplifier system was used. A four-axis motion system allowed movement of the specimen in the X, and Y plane, the probe in the Z plane, and the microscope[i] focusing, also in the Z plane. The microscope allowed visual images to be collected via a photon-detecting camera[ii]. This camera also allowed luminescence data to be collected, and all images were processed by the Argus 10 image analyzer[ii] before being sent to the computer. The whole system was operated under the control of a personal computer using VP software. Stainless steel probes coated with platinum black were used for all 2D-SVEM experiments with areas approximately 5 mm x 5 mm being scanned.

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[i] Zeiss Axioplan, Hanover, MD.

[ii] Hamamatsu C2400-47, Brigewater, N.J.

RESULTS AND DISCUSSION

Sterile controls were run in order to determine whether any anodic regions were produced in the absence of either bacteria or polymers. Franklin[13] had suggested that anodic regions had formed but were subsequently repassivated. In our studies, no such occurrences were seen, possibly due to a lack of active aeration of the system described here, as opposed to the systems described by Franklin in which the medium directly above the coupon was aerated. The lack of aeration in this study was felt to be appropriate in order to minimize the general corrosion of the mild steel. Further studies by Franklin[16] also suggested that minor changes in the medium affected the formation of anodic regions, which again could result in the variations seen in the different studies.

Initial studies were carried out using a 1% (w/v) alginate solution that was spotted on the center of the test coupons. When scanned using the 2D-SVEM, large anodic regions were detected, which correlated with the position of the alginate (Figure 2). The anodic current was highest at the center of the spot. The samples were then scanned regularly, and it was noted that the anodic current decreased until it was the same as the surrounding area. This took from between four to ten hours. On a few samples, it was noted that after a further six to ten hours, the area of the alginate appeared cathodic to the surrounding area. When the experiments were terminated after forty-eight hours, any corrosion products on the surface were carefully removed and the samples examined for pitting attack. In none of the samples run was a pit seen which correlated to the area of the alginate. In order to show that this anodic region was caused by a flow of ions involving the metal and was not a result of ions moving between the alginate and the bulk solution, a sample of alginate was placed on an epoxy blank. In this case no such anodic regions were observed. It was therefore concluded that the current was a result of an interaction between the polymer and the metal coupon.

The method of inoculation forming pseudo-colonies on the coupon surface was adopted to provide a degree of control and information on the location and number of bacteria present at the start of the experiment. Franklin[16] had suggested that 10^8 cfu ml^{-1} of bacteria were necessary in the bulk phase in order to produce pitting. No information was provided on the number of bacteria attached to the surface. It is, however, recognized that there was an error in the method adopted, as not all the bacteria would stick. This method was thought to be acceptable as it gave a fair approximation of the numbers and allowed for the initial localization of the bacteria, although they were seen to colonize the whole surface during the course of the experiment, and the highest concentration of bacteria was always at the initial point of inoculation.

Effect of bacterial numbers using 046

The number of bacteria was found to be important in determining the resultant effect on the anodic current. When a high number of bacteria above 10^8 cfu ml^{-2} were added, a large anodic region was seen that covered the whole of the area on which the bacteria had been placed as shown in figure 3. This region was maintained for eighteen hours over which time it was seen to spread as the bacteria grew and colonized more of the metal coupon. This result was similar regardless of the bacteria used. When a colony of 10^6 cfu ml^{-2} was formed, (Figure 4) it was found that a large anodic current was created which was only over a small percentage of the area to which the bacteria were applied. It is suggested that clumping of the bacteria had occurred with a number present in this area leading to the high anodic region. This anodic region was maintained for twenty-four hours. When 10^4 cfu ml^{-2} were

added (figure 5), only very small anodic regions were formed which were very short lived lasting only for one hour. In all cases the anodic regions were no longer detectable after twenty-four hours despite fresh medium being added.

Correlation with light production

With the 5RL similar, results regarding the effect of bacterial numbers were seen as described above with 046. Due to the ease with which light production can be initiated in 5RL. The addition of salicylate, this bacteria was initially used to validate the system. Light production was not seen until twelve hours after inoculation of the coupon. This light was still detectable after twenty hours, which revealed that oxygen levels were not limiting to the bacteria, as light production is an oxidative process which requires the presence of oxygen. It was also noted that after this time period light, above background levels, was detected from the entire coupon surface, although there was a far higher level of light being emitted from the original area of inoculation. This would suggest that colonization of the surrounding area, as well as growth in the original area, had taken place.

These results would suggest that the bacteria are still active and growing on the surface. If the anodic region was a result of a metabolic product such as an organic acid, it would be difficult to account for the decrease in the anodic region with time. The other major mechanism for the anodic region would be due to the setting up of an oxygen differential cell with the bacterial cells or their polymer acting as a diffusion barrier to oxygen. This also provides a problem in determining the reason for the decrease in the anodic currents detected. The luminescence data and the change in the medium would suggest that it was not a result of oxygen depletion in the medium, which would have resulted in the loss of an oxygen differential between the medium and the metal surface. The colonization of the surrounding area by the bacteria could in part explain the decrease, as this would lead to a decrease in oxygen to these areas removing the potential cathodic regions.

However, this theory is further complicated by the observation that an old culture of 5RL failed to produce an anodic region when placed on the coupon in high numbers, which should have served as a diffusion barrier to oxygen. It is possible that the growth of the bacteria on a plate prior to inoculation allows for the accumulation of a metabolic product which reacts with the metal over the first few hours until it is depleted.

CONCLUSION

It has been shown that the vibrating electrode technique is a useful method for looking at the early stages of microbially induced corrosion, in that anodic regions can be seen associated with pseudo-colonies of bacteria. The mechanism for the production of these anodic regions is currently unclear, although work is in hand to try to answer these questions. Of concern are the short-lived nature of these regions and their relevance in natural environments. It is, however, recognized that although low levels of nutrients were added to the medium, these are not representative of environmental conditions found in waters. These nutrient conditions will lead to faster colonization of the surface, possibly speeding up the decline of anodic regions seen in the experiments.

Two possible mechanisms are currently being considered for the production of these anodic regions. First, due to the bacteria/exopolymeric material creating a barrier to oxygen diffusion, an

oxygen differential cell could be established. A second method would employ the production of a metabolic product by the bacteria. This could be either a low molecular weight compound such as an organic acid or amino acid, or a high molecular weight compound such as an exopolysaccharide containing acidic groups. At present, there is evidence supporting either mechanism as being responsible for the production of anodic regions. It needs to be borne in mind that MIC is not a simple process involving just one bacteria working by one mechanism, as would appear to be the case in the above reported experiments.

ACKNOWLEDGMENTS

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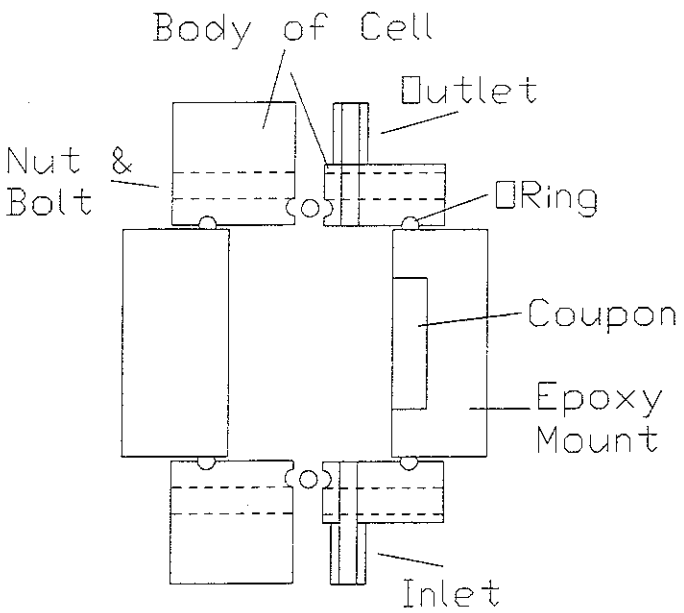

Figure 1. Diagram of flow cell design.

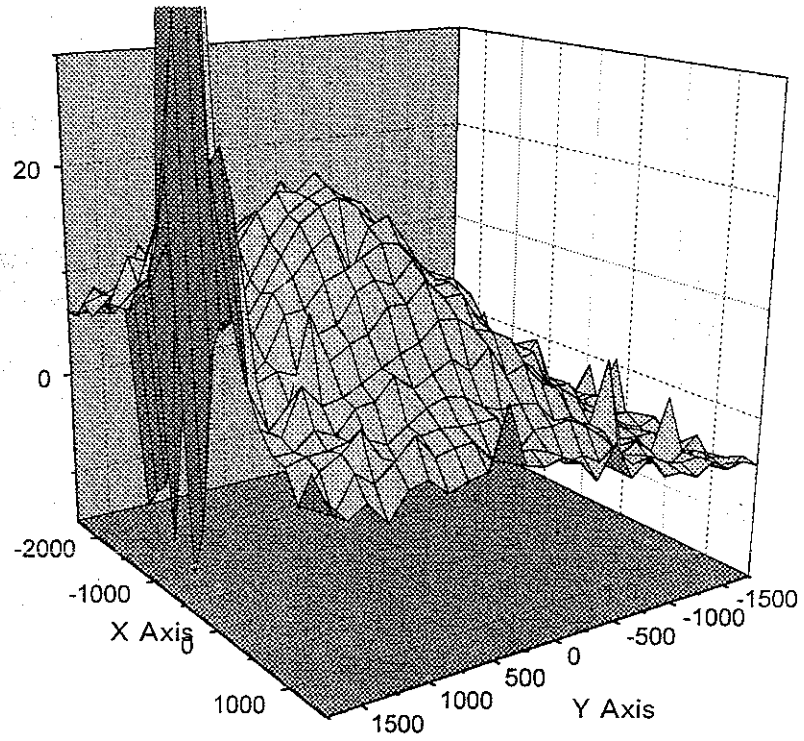

Figure 2. Current density (Z axis) scan resulting from a drop of 1% Alginate on mild steel.

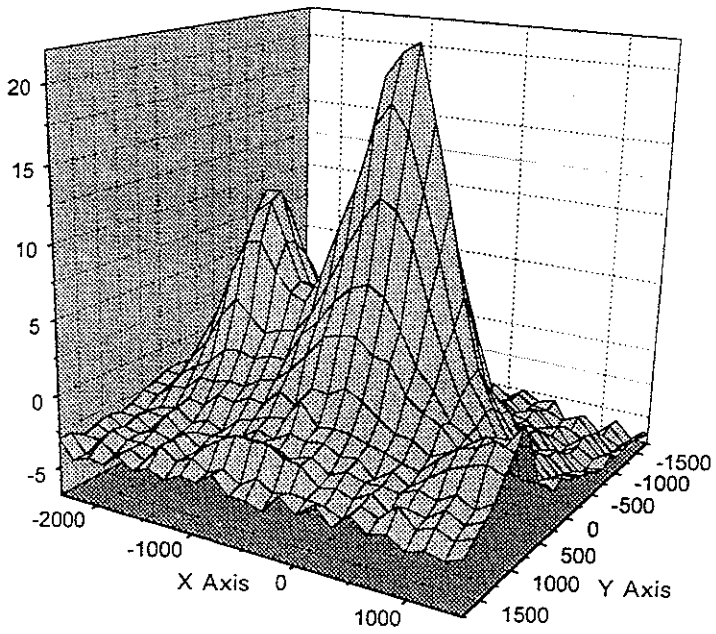

Figure 3a.

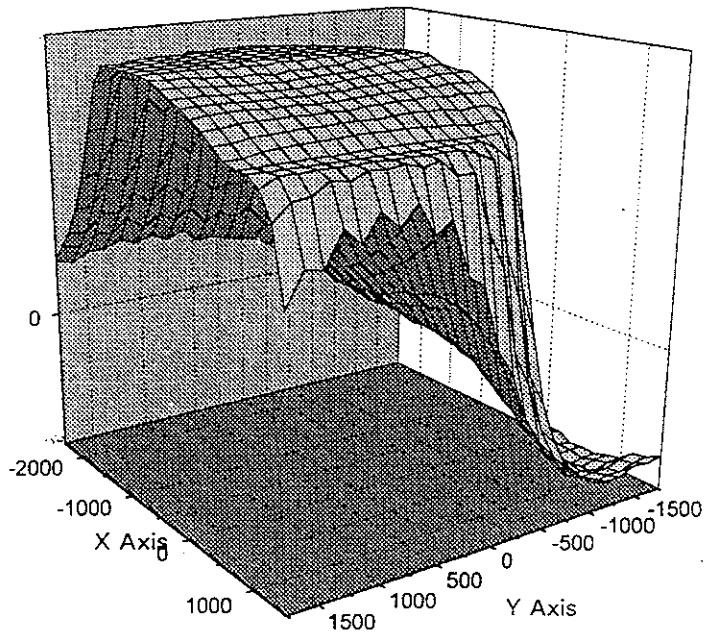

Figure 3b.

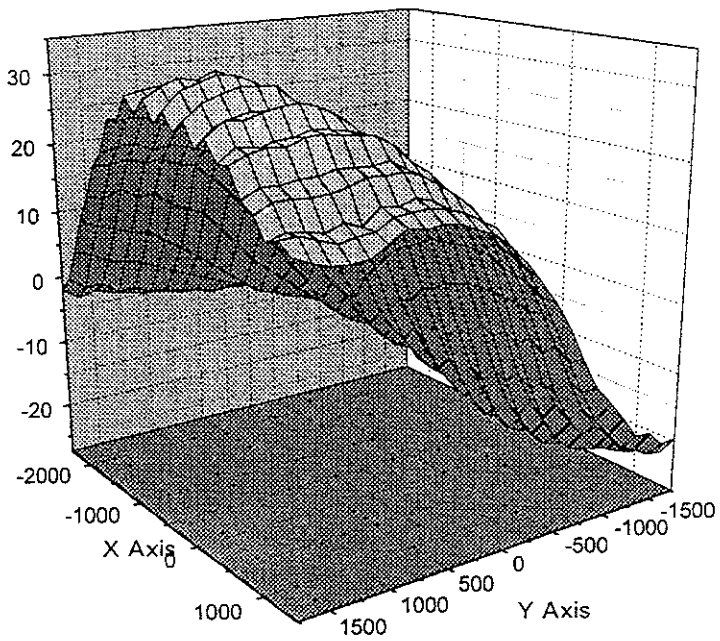

Figure 3c.

Figure 3. Current density (Z axis) scan resulting from a "pseudo colony" of bacteria (046) with an initial concentration of 10e8 cfu/ml on mild steel: a) 1 hour exposure; b) 4 hour exposure; c) 8 hour exposure.

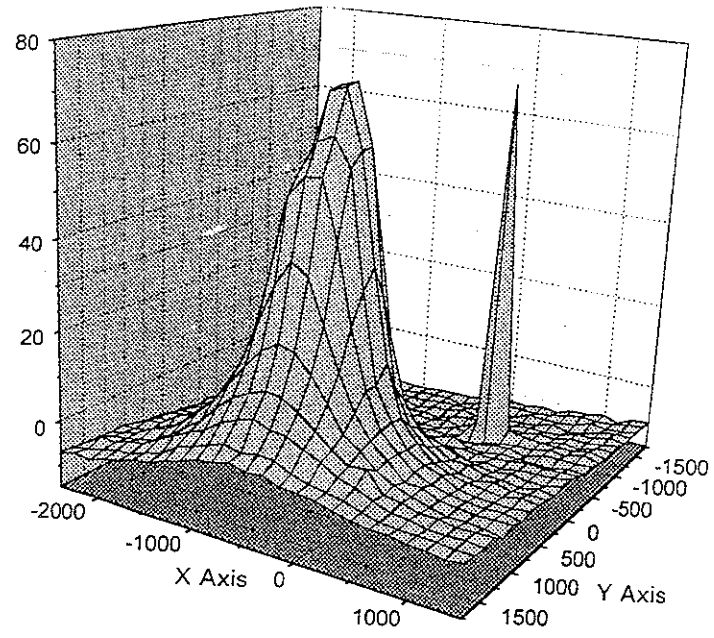

Figure 4a.

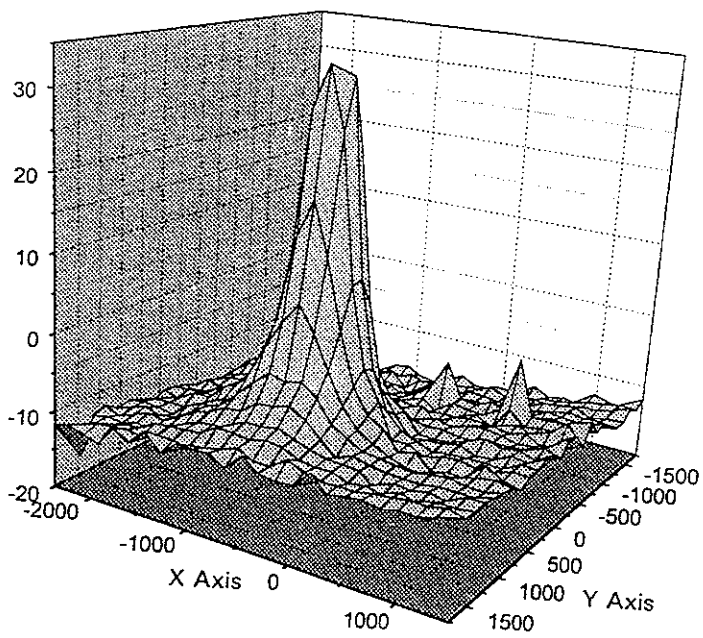

Figure 4b.

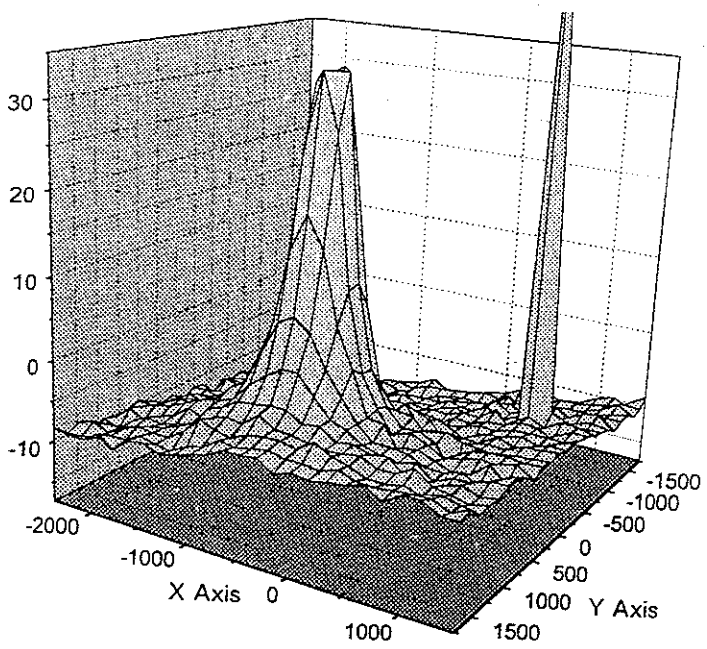

Figure 4c.

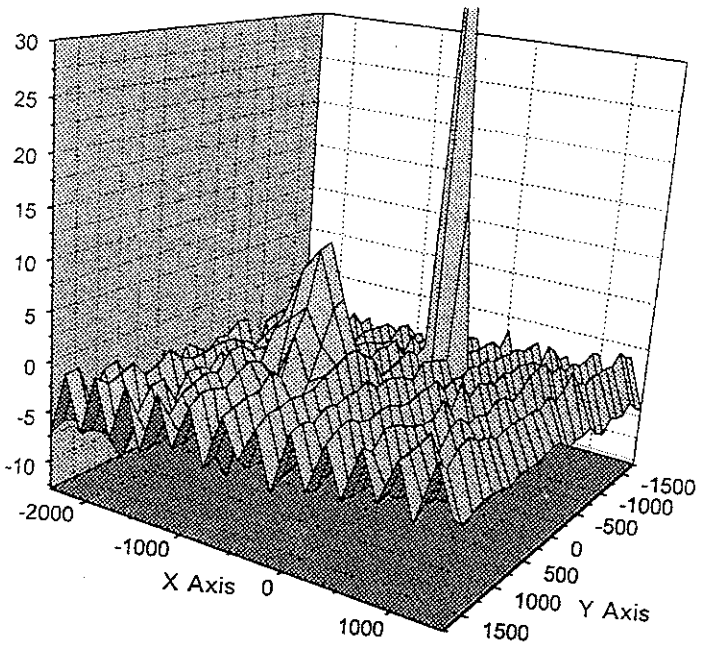

Figure 4d.

Figure 4. Current density (Z axis) scan resulting from a "pseudo colony" of bacteria (046) with an initial inoculation of 10e6 cfu/ml on mild steel: a) 2 hour exposure; b) 6 hour exposure; c) 8 hour exposure; d) 18 hour exposure.

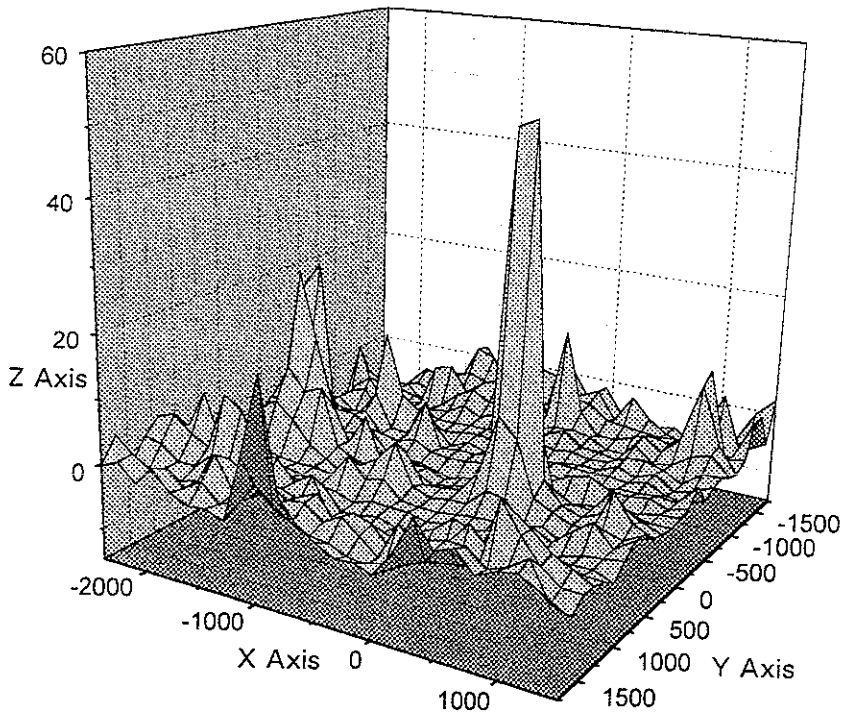

Figure 5. Current density (Z axis) scan resulting from a "pseudo colony" of bacteria (046) with an initial inoculation of 10e4 cfu/ml on mild steel after 1 hour exposure.