375

Studies of the Reproducible Pitting of 304 stainless Steel by a Consortium Containing Sulphate-Reducing Bacteria.

Peter Angell
Center for Environmental Biotechnology
10515 Research Drive, Suite 300
Knoxville, TN 37932, USA.

Juinn-Shyong Luo
Center for Environmental Biotechnology
10515 Research Drive, Suite 300
Knoxville, TN 37932, USA.

David C. White
Center for Environmental Biotechnology
University of Tennessee/Oak Ridge National Laboratory, ESD
10515 Research Drive, Suite 300
Knoxville, TN 37932, USA.

Abstract

The pitting of stainless steels by sulphate-reducing bacteria (SRB) has been notoriously difficult to reproduce in the laboratory. A system has been developed in which pitting can be artificially induced by the passage of a current of 11 $\mu A\ cm^{-2}$ to a small anode (0.031 cm^2). The anode is concentric to, and separated from, the cathode (4.87 cm^2) by a PTFE spacer. The current is applied for seventy-two hours either during or after colonisation of the electrode by the test bacteria. Once the applied current is removed the resultant galvanic current flowing between the anode and the cathode can be monitored by a zero resistance ammeter.

It has been demonstrated that a current is maintained only in the presence of a mixed consortium containing an SRB (*Desulfovibrio vulgaris*) and a *Vibrio* sp. No current was maintained with either of the axenic cultures nor with the sterile control. It was further seen that a high cathodic charge transfer resistance (Rct) (>100 $K\Omega \cdot cm^2$) was obtained only when SRB were present either as an axenic or mixed culture. However, a low Rct (<1 $K\Omega \cdot cm^2$) was obtained only when the anode contained both SRB and the *Vibrio* sp. as a mixed culture. The microbially influenced corrosion (MIC) pitting process has been separated into three phases with the use of this method: 1) initiation, 2) maintenance & 3) propagation. The action of SRB in the microbial pitting of stainless steels will be explored in relation to all three phases. In should be noted that this system does not seek to mimic any natural situation: Rather it provides a technique by which MIC can be studied.

Key Words: SRB, MIC, stainless steel, mixed cultures, *Vibrio*.

Introduction

The bulk of the literature on the study of the mechanism of MIC relate to the pitting of mild steel by SRB. A couple of mechanisms have been put forward as reviewed by Hamilton[1], both of which have experimental evidence to support them. These are:

1) Cathodic depolarisation: Sulphate-reducing bacteria contain the enzyme hydrogenase which will oxidise the molecular hydrogen that is formed as a result of the cathodic reaction in corrosion. The molecular hydrogen polarises the cathode and prevents the further production of hydrogen limiting the corrosion process.

2) Production of cathodic sulphides: Although the ability of SRB to oxidise cathodic hydrogen has been demonstrated, it is generally considered that the major factor affecting the rate and extent of corrosion is the presence of iron sulphide. SRB utilize sulphate as the terminal electron acceptor, reducing it to sulphide which is released from the cell. This reaction is inhibited by the presence of oxygen.

More recently Crolet[2] has suggested that SRB are able to manipulate the local pH around the cell and produce areas of low pH which will become anodic to other areas with a high pH. From mass balance equations he has suggested that SRB grown on thiosulphate should be more aggressive than those grown on sulphate due to the differing pKa values of the two reactions. To date there is no experimental evidence to back up these theoretic predictions, although Campaignolle *et al.*[3] were able to show that SRB grown on concentric electrodes in the presence of thiosulphate were capable of maintaining an applied galvanic current after it had been removed.

The above mechanisms and laboratory systems have been developed using mild steel. There are few reports of reproducible pitting of stainless steel in laboratory systems. The main reason for this is that stainless steels are more resistant to corrosion than is mild steel. It is generally considered that this resistance is due to the formation of a passive film on the stainless steels. In stainless it is generally noted that failures due to pitting occur at sites were the integrity of the metal has been compromised by such activities as welding and crevices[4]. Many reports exist showing pitting of stainless steels which has been attributed to the action of SRB, but few if any of these deal with laboratory systems. Most of them follow exposure of coupons to natural waters.

In line with the above observation that failures normally occur at the weldments a system has recently been described by Angell *et al.*[5] in which pitting of stainless steel is artificially induced in the laboratory system by the passage of a current to a small anode concentric to a large cathode. It was noted that a galvanic current was maintained only in the system when a mixed culture of an SRB and a *Vibrio* sp., were grown together on the concentric electrode. This system is similar to that described above by Campaignolle *et al.*,[3] except that 304 stainless steel was used in place of the mild steel.

A wealth of research has been carried out into the metabolic pathways and specific inhibitors of SRB[6]. Most of it however, is in relation to their role in the sulphur cycle and biodegradation in stagnant, aqueous systems. An application of this knowledge of the metabolic pathways and various specific inhibitors should be useful to the studies of microbial corrosion. Close attention needs to paid to the reports, however, to check the details. For instance it is widely reported that a number of SRB are capable of utilizing nitrate as their terminal electron acceptor. It is difficult to ascertain whether or not this can occur in the absence of sulphate. Using strains of SRB that could utilize nitrate in the absence of sulphate would allow the action of sulphide production in MIC to be studied. Also, using specific inhibitors of the various hydrogenases (periplasmic and cytoplasmic) allows for the action of the hydrogenase to be determined.

In this paper the concentric system will be discussed in relation to its use as a laboratory system which provides reproducible pitting of 304 stainless steel. The system does not seek to represent any naturally occurring situation as the pitting is artificially induced. The reasons for pit initiation in natural systems is not fully understood except as noted above pitting normally occurs at sites of some recognizable "defect" in the material. In this system the pitting is artificially initiated by the applied current but as will be clearly demonstrated is only fixed and maintained in the presence of a mixed consortium of bacteria. Some instances where this system as been used to gain further insight into the corrosion process will also be discussed.

Materials and Methods

Bacteria and medium

Bacteria used in this study were sulphate-reducing bacteria (SRB): *Desulfovibrio vulgaris* and *Desulfovibrio desulfuricans*, as well as an unidentified *Vibrio* sp. and *Vibrio vulgaris*. All the bacteria were either anaerobic or facutatively anaerobic and capable of growth in marine systems. The medium used was based on ASTM sea water and has been described previously[5], except that for growth of *Shewenella putrefaciens* the sodium sulphate was replaced with either sodium thiosulphate or sodium nitrate.

Concentric electrodes

Concentric electrodes were fabricated as described previously [5] and in summary were machined from a 304 stainless steel rod to give an anode of 0.031 cm^2 concentric to a cathode 4.87 cm^2 separated by a Teflon spacer. The whole electrode was then mounted in a cold cure epoxy and attached to a glass tube through which electrical wires were passed that had been soldered to the back of the anode and cathode. During the initial stages of the experiments a galvanostat could be connected to the electrode to provide a current density of 11 $\mu A\ cm^{-2}$ at the anode, maintaining the anode as an anode and the cathode as a cathode. The galvanic current was applied for seventy-two hours, after it had been removed zero-resistance ammeters were connected to the electrodes and the resultant galvanic current was monitored and logged.

Electrodes were positioned in reaction vessels with a working volume of 600 ml with facility for medium addition and removal. Anaerobic conditions were maintained by sparging with 95 % nitrogen and 5 % hydrogen. Inoculations were provided three times over a seventy-two hour period when static conditions were maintained to allow bacterial colonisation of the electrodes. Various protocols were used for inoculation and current induction as described later in results section, always using the methods described above. A saturated Calomel reference electrode and titanium counter electrode were also positioned in the vessel to allow both electrochemical impedance spectroscopy (EIS) and dc polarisation scans carried out as described previously[7] in summary EIS was carried out at frequencies between 5 mHz and 10 Khz with a n amplitude of 5 mV and five frequencies examined per decade. DC polarisation were carried out 30 mV either side of the rest potential at a scan rate of 0.17 $mV\ sec^{-1}$.

Bacterial enumeration was carried out at the end of the experiment when bacteria were swabbed from the surface and resuspended in 10 ml of acetate/lactate medium[7] and either counted by MPN tubes or plate counts using the methods described previously[5].

Results and Discussion

System validation

Initially the system was run with inoculation and current application carried out simultaneously for the initial seventy-two hours, using a mixed culture of the *Desulfovibrio vulgaris* and the *Vibrio* sp., axenic cultures of each of the bacteria and a sterile control. As can be seen from the current density plot shown in figure 1 the only system which was capable of maintaining a galvanic current (3 $\mu A\ cm^2$) was the mixed culture, all the others rapidly dropping to zero current. Figure 2a & b show the Bode plots for the mixed culture anodes and cathodes respectively, the calculated charge transfer resistance (Rct) values were 0.439 $K\Omega\ cm^2$ and 184 $K\Omega\ cm^2$. Figure 3a & b shows similar plots for the axenic SRB culture where Rct values of 56 $K\Omega\ cm^2$ and 120 $K\Omega\ cm^2$ were calculated for the anode and cathode respectively. The other systems had Rct values of below 100 $K\Omega\ cm^2$ for the cathodes and above 10 $K\Omega\ cm^2$ for the anodes. In each case examined a current was never maintained by an axenic culture of the *Desulfovibrio vulgaris* but it was noted the numbers recovered from the anodes were in the range of 10^3 cfu ml^{-1} in the axenic systems but above 10^7 cfu ml^{-1} when a current was maintained in the mixed system. It is therefore suggested that the failure of the axenic culture to maintain a current is due to a lower level of colonisation and therefore that one of the roles of the *Vibrio* might be to aid biofilm formation of SRB.

Further evidence of the importance of the number of SRB attaching to the anode was seen in an experiment in which the current was not maintain in a mixed system but showed a very slow decline to zero in contrast to the rapid drop seen in the axenic and sterile controls (figure 4). Enumeration of the bacteria at the end of the experiment showed that 10^4 cfu ml^{-1} SRB were isolated from the anode. In the same experiment a mixed culture system showed no maintenance of the current, again enumeration at the end of the experiment showed that there were no SRB recovered from the cathode. From this set of data is was concluded that a certain level of SRB colonisation was necessary at the anode and that SRB were also necessary on the cathode but at lower numbers. The Rct values presented above, that a high Rct value is present with both the axenic SRB and mixed culture, would suggest that SRB are solely responsible for the elevation of the cathodic charge transfer resistance.

Long-term experiment

The experiments discussed above examined the maintained current for less than seventy hours. In order to determine that the current was not just a transient event a longer term study was undertaken in which the current was seen to be maintained for an excess of two-hundred hours (figure 5). This clearly showed that the current was sustainable and a real corrosion event. Further evidence for this was provided by the visualisation of real pits on the anodes at the termination of the experiment. These pits were up to 0.5 mm in diameter. No such pits were seen on the cathode or in any system that did not maintain a current, the latter systems showed that the application of the current had resulted in general dissolution of the anode causing the whole surface to be evenly recessed from the cathode and Teflon spacer.

Bacterial metabolism

In order to determine the mechanisms of MIC and the role of the bacteria in the pitting process a series of experiments were undertaken. Initially the role of sulphate in the medium was examined. Sulphate-reducing bacteria use sulphate as the terminal electron acceptor and in the process reduce the sulphate to sulphide. As discussed earlier sulphide has been shown to act as a cathode and drive the corrosion process. It has been reported that some SRB can use nitrate or nitrite in place of the sulphate. In the case of the *Desulfovibrio vulgaris* is was found that removal of sulphate not only resulted in the prevention of sulphide metabolism but also inhibited metabolism. In order to get SRB to grow on the electrode it was necessary to carry out the inoculation for seventy-two hours in medium containing sulphate prior to the application of the current for a further seventy-two hours. Inoculations were of *Desulfovibrio vulgaris* and *Vibrio natrigens* as a mixed culture and sterile controls. While the current was being applied sulphate was removed from the vessels by flushing the system with sulphate free medium. If no sulphate was present in the medium after the current was removed no current was maintained. However, if sulphate medium was added at the same time as the current was removed a current was maintained (Figure 6). After this current had been maintained for fifty hours the sulphate was again removed with no effect on the current. No effect was noted even when the medium feed had run out and the vessels were operated for a further fifty hours with no fresh medium.

In order to further clarify the effect of metabolism on the corrosion as detected by the maintenance of the galvanic current a series of experiments was conducted in which a current was established and maintained for forty-eight hours and then the vessels were treated with various inhibitors of SRB. It was seen that glutaralahyde, carbon monoxide and copper (II) chloride all had no effect on the current. The first inhibitor resulted in total cell death the latter two are known to inhibit the hydrogenase enzyme of SRB. Subsequent culture tests showed that they also inhibited growth and sulphide production. When sodium molybdate was added this resulted in the termination of the applied current. However it needs to be noted that molybdate is known to be an inhibitor of corrosion as well as SRB metabolism.

The above set of experiments were repeated except that the inhibitor was added at the same time as the applied current was stopped. in this case the glutaraldahyde and molybdate resulted in the current not being maintained, carbon monoxide was seen to have no effect and copper (II) chloride caused a mark decline in the current. In the case of the carbon monoxide and the glutaraldahyde viable cells were recovered in low numbers from the electrodes.

From the above runs it has been concluded that SRB metabolism is necessary at the initial period when the current is removed but not later. It is suggested that the bacteria are somehow involved in the process of fixing an electrochemical cell which once established is self sustaining and no longer requires the bacteria maintain it. This, however, is not to say that the bacteria are no longer involved in the process, but rather any effect they may be exerting serves only to enhance the corrosion process. They are clearly necessary in the early stages when the current is removed and any anodic/cathodic activity needs to be localised and maintained in a specific spot. This would be in line with the results of Franklin *et al*[8] who using a scanning vibrating electrode technique showed that bacteria were responsible for preventing the repassivation of naturally forming anodic regions on a mild steel coupon.

Studies using *Desulfovibrio desulfuricans*

A number of reports were found that suggested that certain strains of *Desulfovibrio desulfuricans* were capable of utilizing nitrate as their terminal electron acceptor in place of sulphate. However, close examination of the papers and culture experiments revealed that nitrate could be reduced at the same time as sulphate, in culture tests it was found that no growth occurred in sulphate free medium. However, a series of experiments was conducted to determine whether the maintenance of the current was unique to *Desulfovibrio vulgaris* or whether other SRB could produce the same effect.

A number of runs were conducted using *Desulfovibrio desulfuricans* and *Vibrio natrigens* in which the results were typically those shown in figure 7, which were similar to the results seen in figure 4, with a slow decline to zero with the mixed culture, in contrast to the sharp decline seen in the axenic cultures or sterile control. Counts performed at the end of the experiment again showed that low numbers of SRB were present on both the anode and cathode. It was therefore concluded that the lack of current maintenance was due to poor colonisation by this species compared to the *Desulfovibrio vulgaris*. However, an effect was seen in this slow decline indicating that given conditions allowing greater colonisation a current might have been maintained.

Conclusions

- Reproducible pitting of 304 stainless steel following artificial pit initiation is possible only with a mixed culture of SRB and a *Vibrio* sp. The action of the *Vibrio* sp. is thought to assist in biofilm formation allowing greater colonisation by SRB.
- The resultant current seems to be dependent on the level of colonisation of the anode in particular by SRB.
- Once a current has been established active SRB are no longer required, presumably due to the establishment of an active self sustaining electrochemical cell. However, bacterial activity is necessary during the establishment of the electrochemical cell immediately after the inducing current is removed.
- The maintenance of the current is not unique to *Desulfovibrio vulgaris* with *Desulfovibrio desulfuricans* showing an effect on the current, however, a large current was not maintained due to the lower level of coloniosation of by this bacteria.

Acknowledgments

This work was supported by the US Office of Naval Research under grant N0014-93-1-0326. The authors wish to thank Dr. T.J. Phelps for his useful advice regarding the growth and metabolism of SRB.

References

1. Hamilton, W.A. Annual Reviews in Microbiology, **39**, (1985), 195-217
2. J.L- Crolet, S. Daumas and M. Magot, pH regulation by sulfate-reducing bacteria, Corrosion 93, Paper #303 (Houston, TX, NACE,)
3. X. Campaignolle, J-S. Luo, J. Bullen, D.C. White, J. Guezennec and J.-L. Crolet, Stabilization of localized corrosion of carbon steel by sulfate-reducing bacteria, Corrosion 93, Paper #302 (Houston, TX, NACE,)

4. S.W. Borenstein, Materials Performance, (January 1991) 52-54.
5. P. Angell, J-S. Luo and D.C. White. Corrosion Science (in press)
6. N. Pfenning, Annual reviews in Microbiology, **47**, (1993), 1-29
7. J-S. Luo, X. campaignolle, D.C. White and I. Vance, Corrosion of mild steeel by thermophilic anaerobes. Corroison 93, Paper # 301 (Houston, TX, NACE)
8. M. J. Franklin, D.C. White and H.S. Isaacs, Corrosion Science, **32**, (1991), 945-952

Legends

Figure 1. Time plot of measured current flowing between anode and cathode for: (—) mixed culture of *Desulfovibrio vulgaris* and *Vibrio* sp. with a current being maintained after applied current is removed, (---) representative plot for axenic cultures and sterile control showing no current maintained after applied current is stopped after 72 hours.

Figure 2. Bode plots for anodes (🏳) & (◆) no current maintained, (□) & (✧) current maintained.

Figure 3. Bode plots for cathodes (🏳) & (◆) no current maintained, (□) & (✧) current maintained.

Figure 4. Time plot of measured current flowing between anode and cathode for:(—) mixed culture of *Desulfovibrio vulgaris* and *Vibrio* sp. with a slow decline of the current after applied current is removed, compared with sharp decline (---) for sterile control after applied current is stopped after 72 hours.

Figure 5. Extended time plot of measured current flowing between anode and cathode for: (—) and (····) mixed culture of *Desulfovibrio vulgaris* and *Vibrio* sp. with a current being maintained for over 200 hours after applied current is removed, (---) representative plot for axenic cultures and sterile control showing no current maintained after applied current is stopped after 72 hours.

Figure 6. Time plot of measured current flowing between anode and cathode for: (—) mixed culture of *Desulfovibrio vulgaris* and *Vibrio* sp. (---) sterile control where sulphate was added for initial period of colonisation for 72 hours while no current was applied. Sulphate was then removed while the current was applied for a further 72 hours, when the current was stopped sulphate was again added to the systems and later removed and then the experiment was continued with the flow stopped. The current was maintained in the mixed culture even after sulphate was removed and the medium flow was stopped.

Figure 7. Time plot of measured current flowing between anode and cathode for:(—) mixed culture of *Desulfovibrio desulfuricans* and *Vibrio vulgaris*. with a slow decline of the current after applied current is removed, compared with sharp decline (---) for sterile control after applied current is stopped after 72 hours. The slow decline is thought to be due to low numbers of SRB colonising the surface.

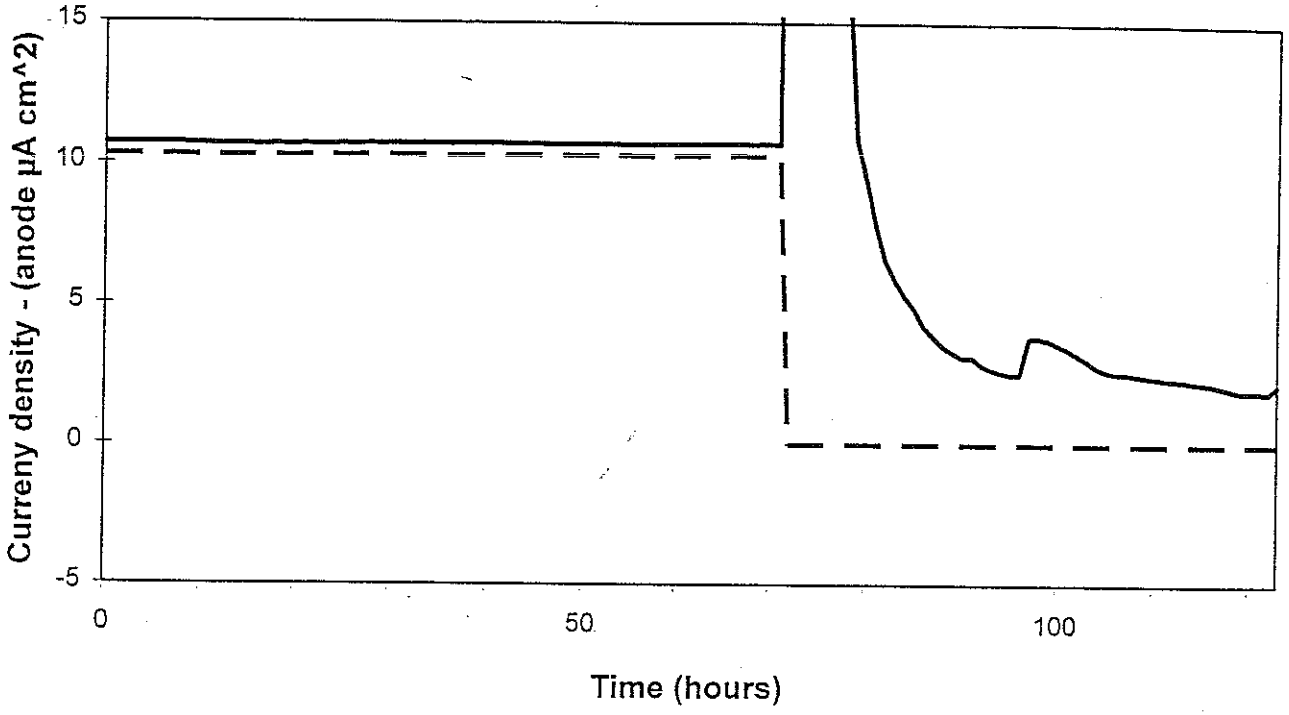
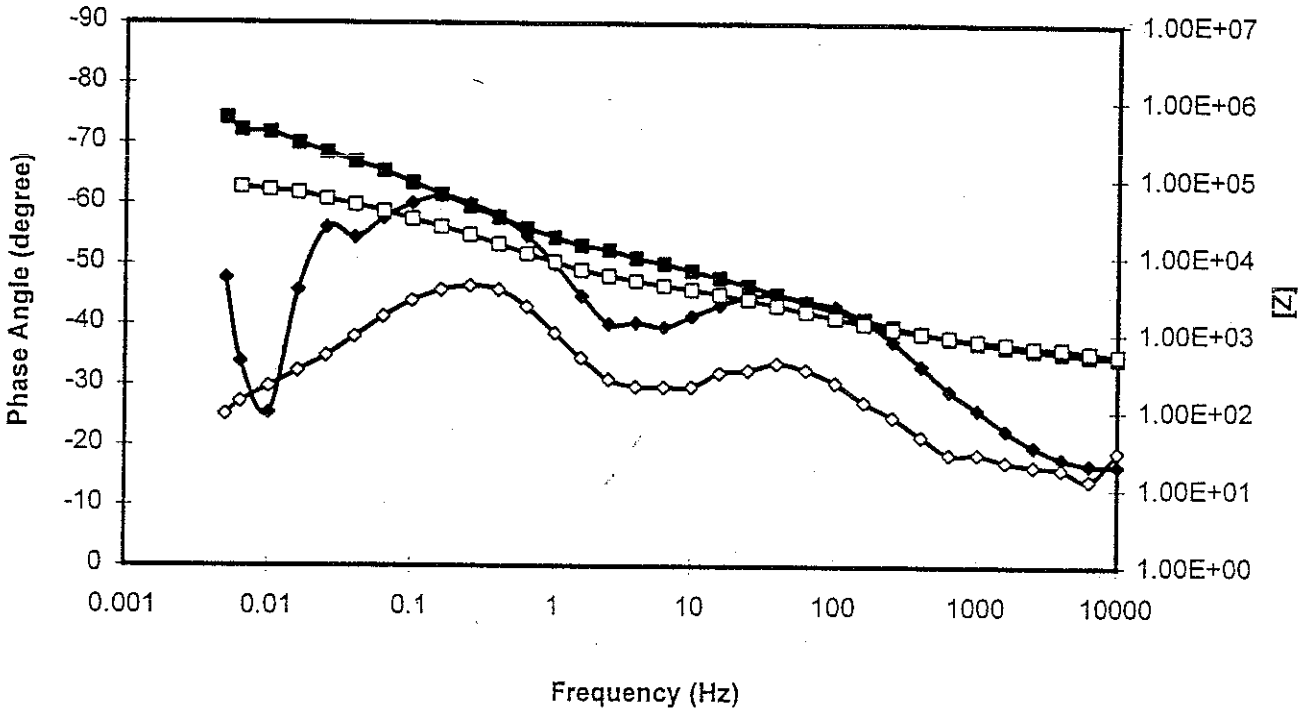
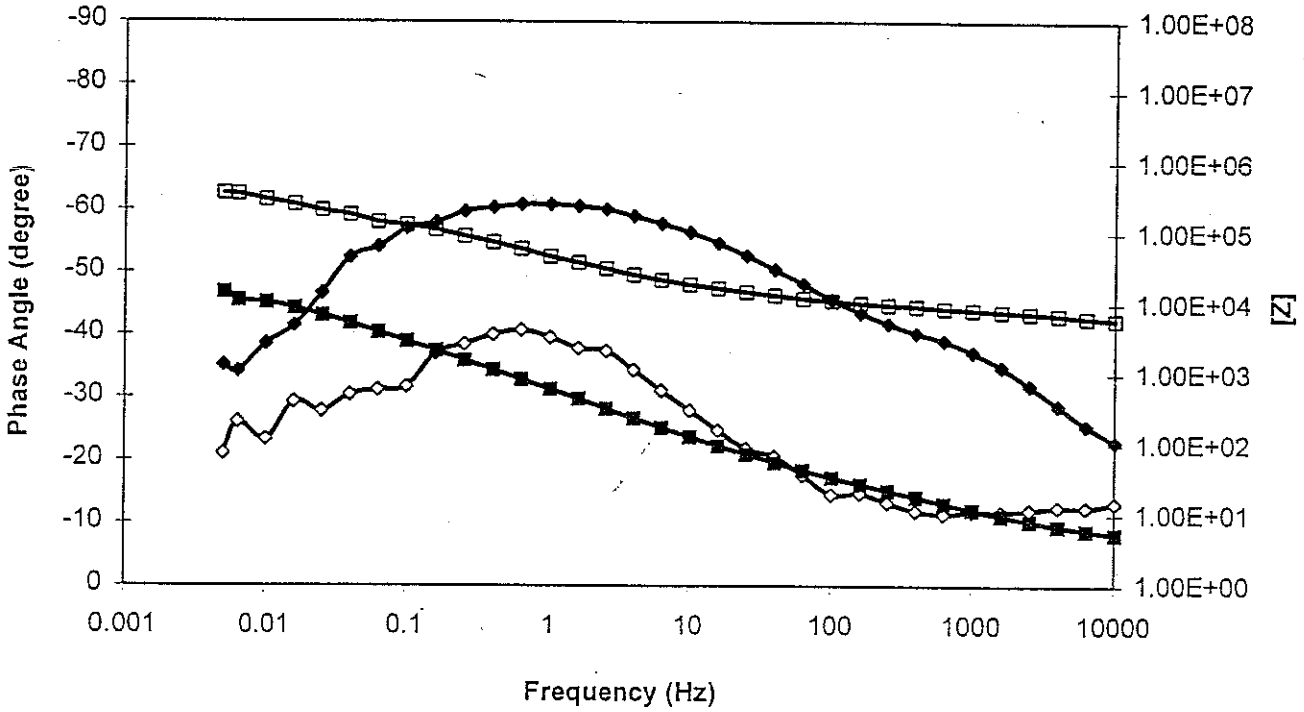
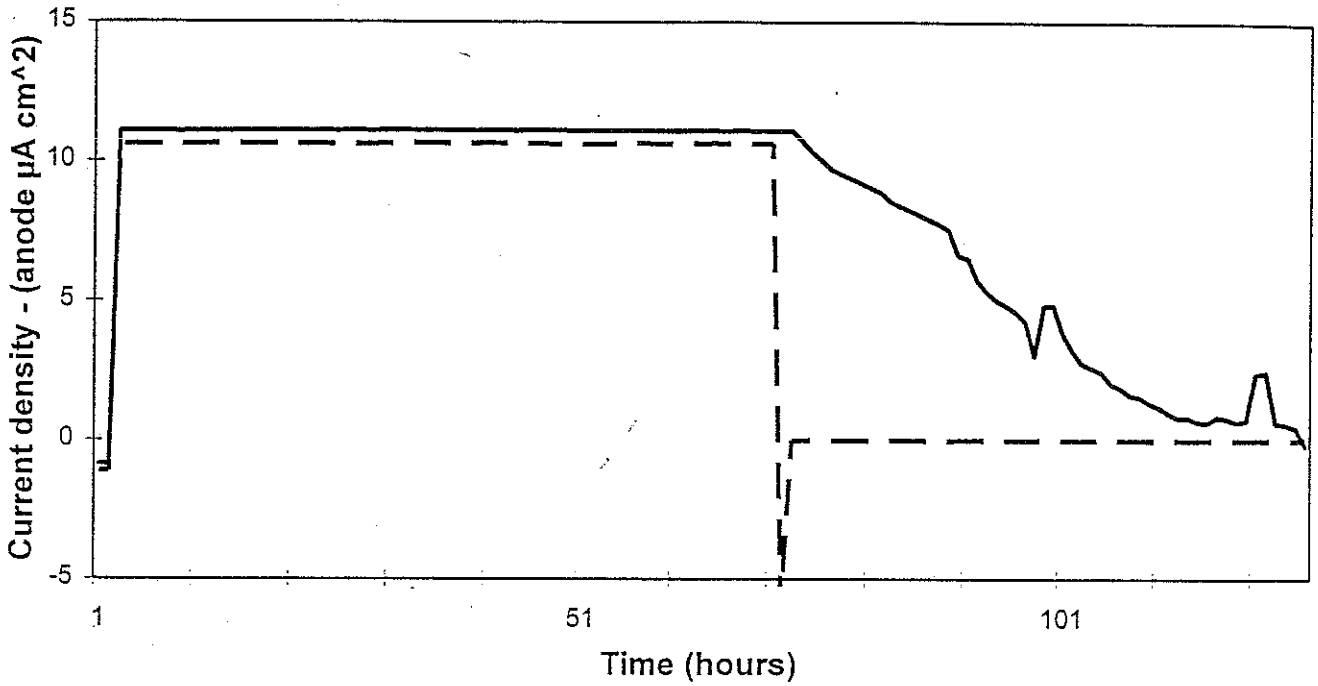


Fig 1


-90
-80
-70
-60
-50
-40
-30
-20
-10
0
Phase Angle (degree)
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
[Z]
0.001
0.01
0.1
1
10
100
1000
10000
Frequency (Hz)


-90
-80
-70
-60
-50
-40
-30
-20
-10
0
Phase Angle (degree)
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
|Z|
0.001
0.01
0.1
1
10
100
1000
10000
Frequency (Hz)


15
10
5
0
-5
Current density - (anode µA cm^2)
1
51
101
Time (hours)

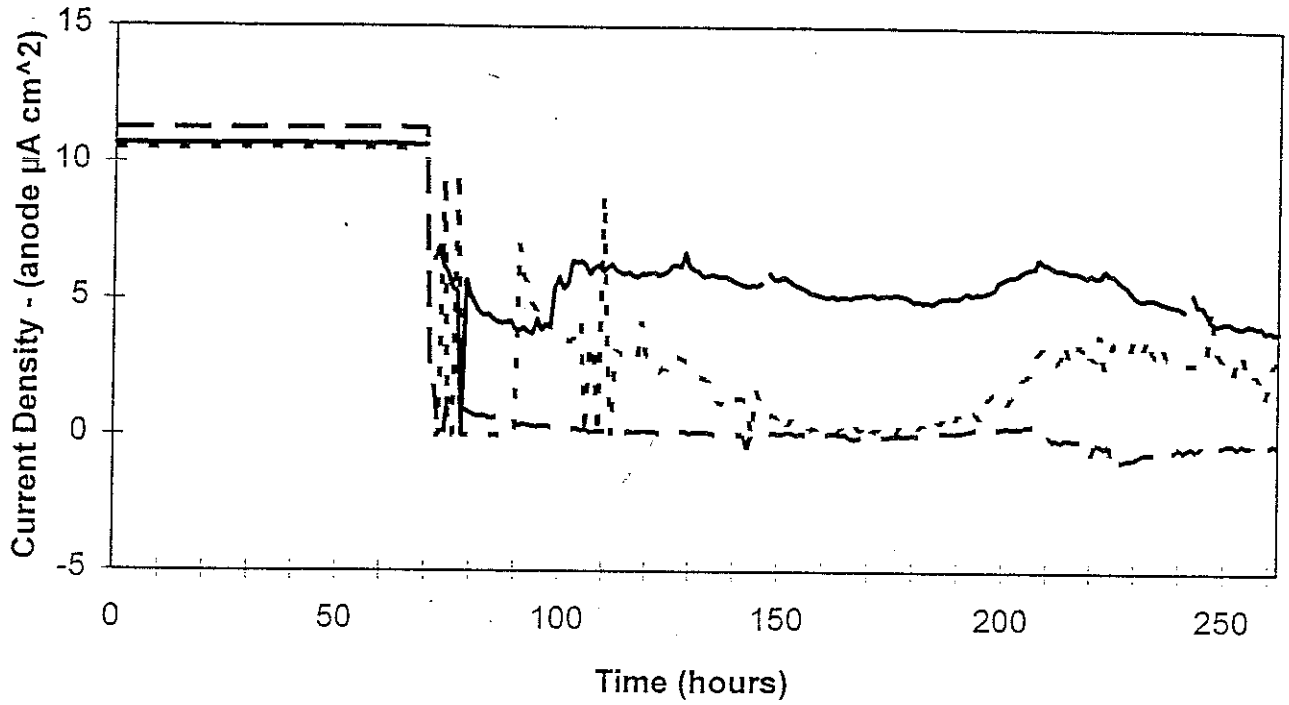

Current Density - (anode µA cm^2)
15
10
5
0
-5
0
50
100
150
200
250
Time (hours)

Fig 5

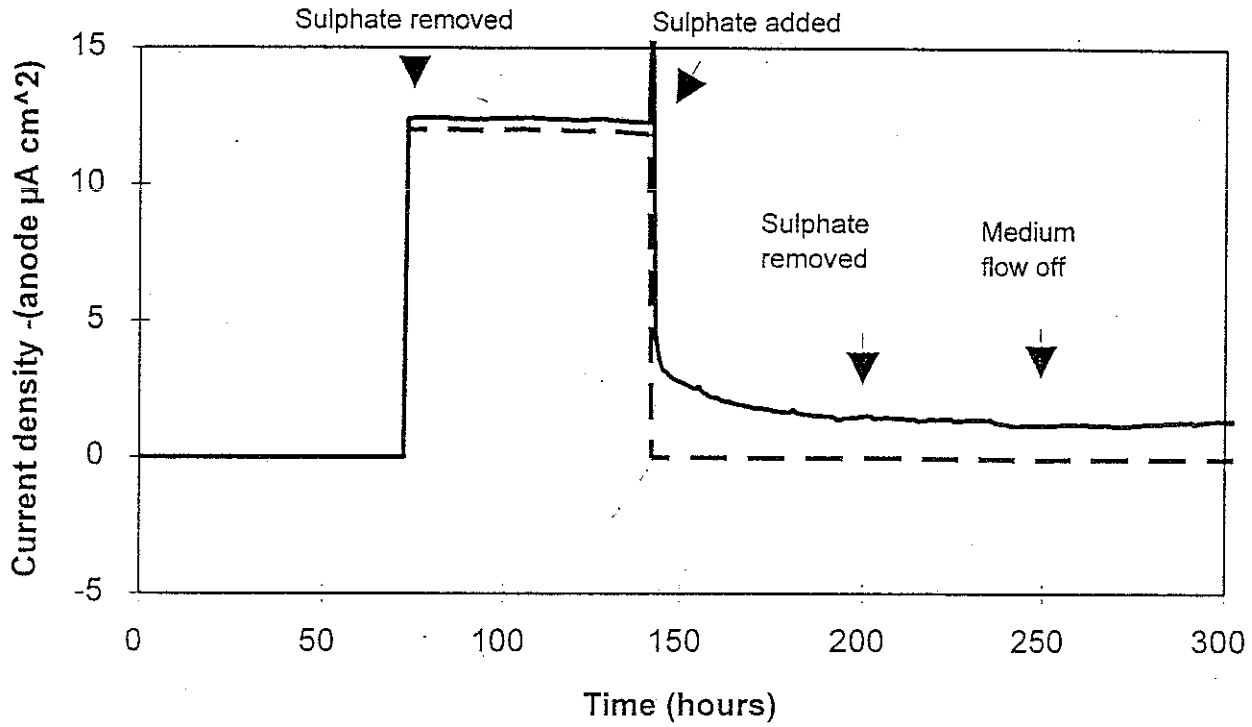

Sulphate removed
Sulphate added
Sulphate removed
Medium flow off
Current density -(anode µA cm^2)
15
10
5
0
-5
0
50
100
150
200
250
300
Time (hours)

Fig 6

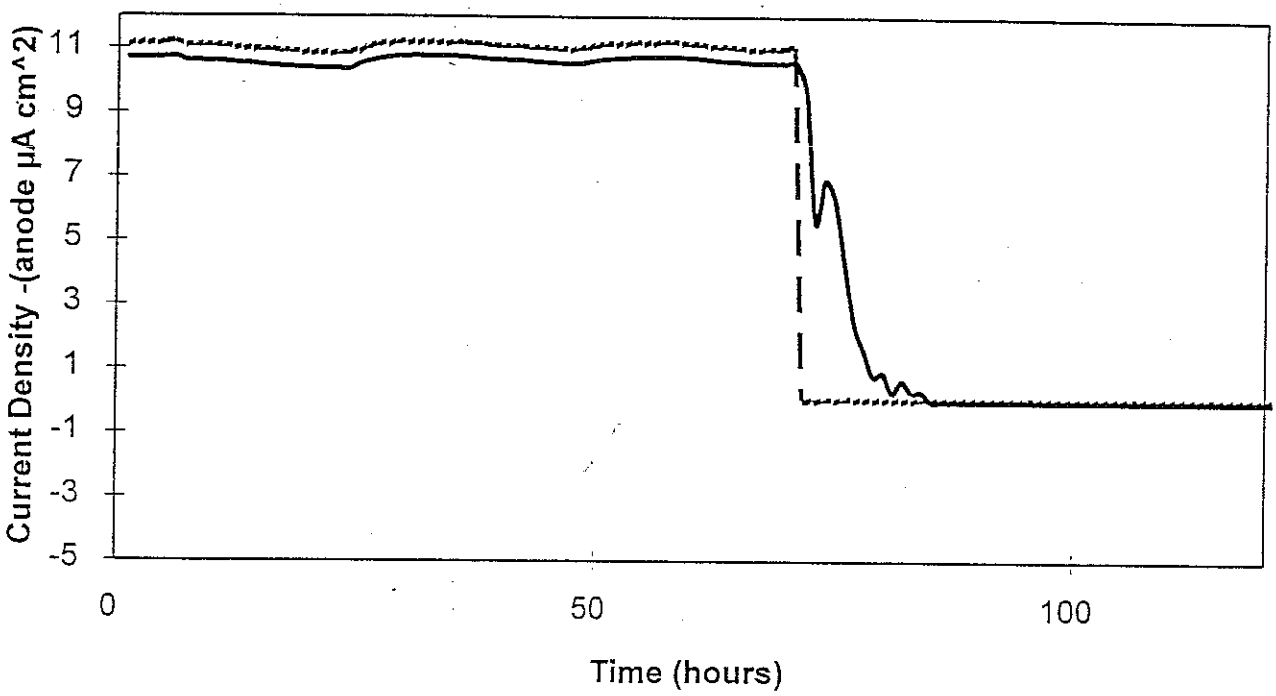


Fig 7