NON-DESTRUCTIVE ON-LINE MONITORING OF MIC

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ABSTRACT

The formation of microbial biofilms on metal surfaces with the subsequent increase in heat transfer resistance and the induction of microbially influenced corrosion (MIC) is being increasingly recognized as an extremely important economic and safety problem for industrial water systems. The development of sufficiently rugged and accurate monitoring devices by which biofilm formation and activity of microbial biofilms can be monitored non-destructively, directly in water systems is the goal of this research. This on-line systems would allow the effective utilization of minimal levels of biocides and inhibitors as well as permit in situ testing of materials for MIC resistance. Several non-destructive technologies such as the quartz crystal microbalance (QCM), the attenuated total reflectance-Fourier transforming infrared spectrometer (ATR-FT/IR), and a genetically engineered bacterium containing the lux gene cassette in which its bioluminescence can be used to define

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*Operated by Martin Marietta Energy Systems, Inc. under contract DE-AC05-840R21400 with the U. S. Department of Energy

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its presence on coupons are on-line devices which accurately measure biofilm formation. These may be correlated to the open circuit potential (OCP), which under specific conditions correlates with the formation of microbial biofilms and is a sufficiently rugged electrode for *in situ* use. Corrosion activity can be estimated by electrochemical impedance spectroscopy (EIS) which is non-destructive, correlates to microbial biofilm activity, is an accurate monitor of corrosion, can indicate localized (pitting) corrosion, and is also sufficiently rugged for *in situ* monitoring.

INTRODUCTION

Metal surfaces are critical to many industrial processes. Unfortunately, metals are susceptible to biofilm formation, biofouling, and microbially influenced corrosion (MIC). Biofouling and MIC are being increasingly recognized as serious problems when surfaces are exposed to natural waters[1]. MIC can often be recognized as corrosion associated with tubercles, slimes, discolorations, odors of anaerobic metabolism, or sludges. The microbially mediated "under deposit corrosion" is often associated with a localized pitting process. Specific attack at weldments, heat affected zones (HAZ), and areas of stagnation (dependent areas where water collects) also are characteristics of MIC. Certain metallographic "signatures" can suggest MIC. Specific attack of austenitic or ferritic components of welds[2] or "tunnel" pitting of mild steel[3] suggest microbial involvement. Biofouling increases drag resistance in transmission tubing and can seriously depress heat transfer efficiency [4].

It has been an ideal to design a probe that could non-destructively monitor the formation, stability and activity of microbial biofilms *in situ* for on-line control of mechanical and chemical countermeasures. If the probe could also non-destructively measure corrosion activity then it would be possible to monitor MIC. Our laboratory has developed several on-line, non-destructive methods for the detection of biofilm formation and persistence . The quartz crystal microbalance (QCM) utilizes an AT cut quartz crystal in an extremely sensitive mass/area device which has been widely utilized to detect thin film deposits in vacuum systems with sensitivities up to 18 ng/cm^2-Hz. We have adapted the system to a flow through system and have shown that it can detect bacterial colonization non-destructively. The attenuated total reflectance-Fourier transforming infrared spectrometer (ATR-FT/IR) determines the spectrum of adsorbent materials attached to the surface of a

germanium crystal that fall within the envelop of the evanescent wave and can be used to detect microbial biofilms [5]. This system provides both biomass estimates and evidence that under specific conditions the relative concentrations of components can change[6]. It is possible to genetically engineer bacteria like those involved in MIC to contain the lux cassette of genes so they are bioluminescent under specific conditions[7]. Bioluminescence can then be detected non-destructively as an on-line "reporter" for the presence and specific activity of that microbe as it forms an adherent biofilm. The colonization of a metal electrode by bacteria results in a 300 to 500 mv more negative potential relative to a standard calomel electrode[8]. In a flowing, continuous culture apparatus the increasingly negative open circuit potential (OCP) paralleled the increase in bacterial colonization of the electrode[9]. Corrosion rates of metals in terms of the corrosion current density can be determined by the polarization resistance that is traditionally measured by perturbing the system above and below the corrosion potential. Linear polarization measurements assume there are no capacitive effects and unfortunately irreversibly damage microbial biofilms. Electrochemical impedance spectroscopy (EIS) if performed as 5 mV (rms) sinusoidal potential perturbation over a frequency range of 10 KHz to 3 mHz does no detectable damage to the biofilm[10], provides insight into both resistive and capacitive aspects of surface electrochemical activity, and gives indications of localized pitting corrosion by shifts of the maximum phase angle between applied potential and induced current to lower frequencies[8,11]. EIS provides a non-destructive measure of the average corrosion rate based on polarization resistance as well as indications of localized corrosion.

EXPERIMENTAL PROCEDURES

Quartz Crystal Microbalance

The QCM was built utilizing 5 MHz AT cut quartz crystals 25 mm in diameter on which keyhole gold electrodes were deposited. The apparatus was placed in a holder with o-rings in a temperature regulated (23.00 C) environment with a 2 ml flow cell allowing the solution to contact one side of the crystal. The solution is pumped at a rate of 0.5 ml/min through the flow cell. The flow cell needs to be enclosed in a Faraday cage and thermostated as carefully as possible. An alternating voltage is applied to the electrodes with a broad band oscillator to cause the crystal to vibrate in the thickness shear mode. The decrease in the frequency of the oscillation with deposition on the crystal is then determined using a suitable frequency counter (Hewlett Packard 5385A) and collected through a general purpose interface bus (GPIB) into an AT computer controlled with software

written in ASYST language. The experiments utilize Caulobacter crescentus supplied by J. Smit of the University of British Columbia grown in defined medium. Cell counts were determined by acridine orange direct counts (AODC) after fixation in 3% formaldehyde[6]. The flow cells were sterilized with ethylene oxide[12].

Attenuated Total Reflectance- Fourier Transform infrared Spectrometer

The IR spectra were collected with a Nicolet 60SX FT/IR using a liquid nitrogen cooled MCT detector using zero-filled and apodized interferograms by the Haap-Gebzek function prior to FT. Biofilms of C. crescentus were generated in a flow cell on a 50x10x2mm germanium crystal with an angle of incidence of 45° as previously described[5]. Depth of penetration in the experiments reported varied between 300 and 600 nm depending on the wavelength[5].

Detection of Attached Bioluminescent Bacteria

A genetically engineered Pseudomonas fluorescens was by transposon insertion of a promotorless lux cassette of genes into the nahG salicylate hydroxylase gene of a naphthylene catabolic plasmid[7]. The engineered strain produced light continuously in the presence of salicylate but was not able to utilize it as a carbon source. A complex medium containing yeast extract, polypeptone, and glucose was designed to promote bacterial adhesion. This medium was utilized at half strength for tests of substratum colonization in cell adhesion measurement modules (CAMM)[6]. The CAMMs are part of a continuous culture system[6] in which a shear force gradient was maintained between parallel plates, one of which was glass, to generate shear environments between 10 and 40 dynes/cm^2 (Figure 1). Bioluminescence was detected by moving a flexible liquid light cable and collimating beam probe 0.2 cm^2 (area) over the 10 cm diameter parallel plate. The photoelectric induced current was determined with an Oriel photomultiplier with a digital output. Data were corrected for the bioluminescence from the bulk phase bacteria (< 10% of the total) and the bacteria attached to the stainless steel plate parallel to the glass in the CAMM. AODC counts of bulk phase, or after sonic release from the glass or underlying stainless steel plate, as well as determinations of the rates of microbial lipid synthesis from ^{14}C-labeled acetate were performed as described[6].

Open Circuit Potential - Electrochemical Impedance Spectroscopy

OCP was measured using a multiple electrode holder with coupons (16 mm diameter) of AISI C1020 carbon steel finished to a 600 grit finish, sealed in epoxy, and any edges coated with

corrosion. The average corrosion rate correlates with the activity of the biofilm as indicated by the OCP [3,6].

Differential MIC with different biofilm microbial composition: With a flow through test system and non-destructive monitors it was possible to determine if specific combinations of bacteria could produce different MIC responses on coupons of AISI C1020 mild steel. Combinations of an absolute aerobe Bacillus sp., a facultative heterotroph Hafnia alvei and an obligately anaerobic sulfate-reducer Desulfovibrio gigas were inoculated and the effects on R_p^{-1} in an aerobic freshwater (0.5 mM sulfate) dilute medium determined (Figure 2). The combination of Hafnia + D. gigas induced the greatest MIC response. Bicultures of Bacillus + either D.gigas or Hafnia showed less MIC activity. The triculture showed essentially the same MIC activity as the Bacillus + D. gigas biculture. After 16-17 hours in the continuous culture system (Figure 1) the working electrodes were recovered and the biofilm examined by AODC and for PLFA patterns. There was no correspondence between the number of bacteria recovered (viable count) or detected by phospholipid ester-linked fatty acid (PLFA) and the MIC response (Figures 3,4) and as expected, the plot of PLFA vs visble counts recovered from the electrodes showed a linear response. The viable bacteria recovered from the electrodes showed ratios of Bacillus:Hafnia of 25:1, Bacillus:Hafnia:D. gigas of 20:300:1 and for the most corrosive combination of Hafnia:D. gigas of 6000:1. The total biofilm bacterial count of the most corrosive biculture was 8-fold lower than the triculture (Figure 3). The PLFA patterns of each bacteria is distinctive and PLFA analysis of the bacteria recovered from the electrodes showed the expected patterns--domination by Hafnia except in the Bacillus dominated biofilms. To determine the relationship between biofilms formed from these isolated bacteria to the real world, PLFA patterns of enrichments of aerobic, fermentative, and sulfate-reducing bacteria recovered from a corrosion tubercle were compared to that found in the manipulated tubercle. The tubercle PLFA pattern most closely clustered with the sulfate-reducing enrichment. MIC responses of combinations of these enrichments were control:aerobic+fermentative:aerobic+fermentative+SRB :0.6±0.07:1.4±0.3:3.1±0.3 $x10^{-3}$ mhos cm^{-2} which is close to those with the isolated components in the same combinations except that the triculture combination was about half as corrosive as the total enriched mixture. Ratios and total numbers of aerobic bacteria recovered from the coupons were similar to those with the isolated bacteria except for the ratio of aerobes:SRB which was 10^6:1. It is clear that specific combinations of bacteria in biofilms in specific proportions have greatly different MIC responses. Traces of SRB relative to the heterotrophs that create the anaerobic niches for their activity

Measurements were made every 10 minutes throughout the experiments. EIS analysis was performed using the Solartron 1286 (Schlumberger Technologies, Burlington, MA) electrochemical interface and 1250 frequency response analyzer controlled by a microcomputer. Sinusoidal potentials of 5 mV (rms) were applied between 3 mHz and 10 KHz at 5 steps/decade. Results were plotted as the imaginary impedance versus the real impedance in a Nyquist diagram or as the log of real impedance versus the log of the frequency in a Bode plot. The system was monitored with a dual channel oscilloscope to verify the waveform of the perturbations.

RESULTS

ON-LINE, NON-DESTRUCTIVE MONITORS FOR MICROBIAL BIOFILMS

The addition of the sterile medium to the QCM flow cell results in a marked decrease in the oscillating frequency (Figure 2, upper graph). The oscillating crystal frequency remains stable after the flow cell is filled with medium. When the medium is inoculated with C. crescentus the attached bacteria induce a slow decrease in the crystal oscillations (Figure 2, lower graph). After 20 hours, changing the pH of the medium from 7.2 to 5.2 (by mistake when replenishing the media reservoir) produced in a reversible shift in frequency that once corrected resulted in resumption of bacterial growth. At 40 hours a change in temperature in the room resulted in an estimated one degree change in the temperature in the QCM flow cell that produced a 25 Hz shift in frequency. Once re-equilibrated and stabilized, the bacterial biofilm continued to grow. When the biofilms attached to the QCM are of sufficiently low density (between 10^4 and 10^6 cells/cm^2) there is a linear relationship between the frequency shift and the AODC counts of attached bacteria (Figure 3). Utilizing the Sauerbrey equation to model the effect of the attached mass on the surface in which the change in mass (g)/area (cm^2) = -1.8×10^{-8} x change in frequency (Hz) and the data in Figure 2, one cell of C. crescentus weighs 1.6×10^{-12} gm (wet weight). The wet weight of the smaller bacterium Escherichia coli is estimated to be 0.95×10^{-12}. The QCM as currently operated has a sensitivity of about 10^3 attached C. crescentus per cm^2 per Hz.

The formation of an attached microbial biofilm can be readily monitored by the IR absorbance using the ATR-FT/IR (Figure 4). This has the advantage of showing shifts in chemical composition non-destructively. Mittelman et al. [6] showed shifts in the carbohydrate:protein ratio with shear force in attached monocultures of Pseudomonas atlantica by utilizing the FT/IR.

If a flow cell is constructed in which bioluminescence can

be monitored, it is possible to detect attached cells non-destructively. The bioluminescence produced by attached P. fluorescens induced by the presence of 1.25 mM sodium salicylate is linearly related to the number of bacteria attached to the glass surface as determined by AODC (Figure 5). The power of this non-destructive, on-line "reporter" technology is clearly demonstrated by showing the relationship between shear force and attachment of the bacteria (Figure 6). The metabolic activity as measured by biofilm lipid synthesis is directly proportional to the bioluminescence and cell numbers (data not shown).

Numerous observations of biofilm densities on electrodes and the shifts in the OCP led to the idea that OCP could be a non-destructive monitor of biofilm formation. Changes in the bulk phase conditions such as the flow rates or stirring resulted in changes in the OCP that could be related to changes in the microbial biofilm activity[12]. The OCP of an aerobic/anaerobic consortium of fermenters, slime producers, acidogenic bacteria and the sulfate reducing bacteria Desulfovibrio desurfuricans show a clear relationship between the change in OCP and the production of bulk phase acetate and butyrate (measured as pH) from glucose (Figure 7). Increases in biofilm metabolic activities as evidenced by corrosion rates also correlate with the shift in OCP[9, 12].

ON-LINE, NON-DESTRUCTIVE MONITORS OF MIC

The intensity of MIC clearly correlates to biofilm microbial metabolic activity[8,9,10,12]. This can be readily demonstrated by correlating the polarization resistance determined by EIS to the in a biofilm with the production of acetate + butyrate by the bacteria (Figure 8). In these experiments the bulk phase microbes contributed very little acid to the medium. The bacterial consortium consisting of aerobes and anaerobes formed a biofilm in a continuous culture accelerated test system[12]. the biofilm induced an initial rapid and reproducible 10^4 increase in the average corrosion rate of a 316 weldment coupon (308 filler) that was followed by passivation and a subsequent steady increase in corrosion rate. The initial rapid increase in corrosion correlates with the rapid formation of the biofilm (Figure 7) and the metabolic activity as indicated by the total volatile acid production.

EIS provides a second advantage for on-line, non-destructive monitoring of MIC. The perturbation response technology particularly at slow sweep frequencies allows the detection of localized processes[8]. Pits or cracks can act as small areas of high conductivity in an insulating (resistive) plane at low frequencies whereas at high frequencies the pits and the

relatively passive surface act as a more uniform conductor based on the capacitance of the double layer[11]. Decreased polarization resistance and shifts in the maximum phase angle towards lower frequency. In an anaerobic system in which the corrosion of pipeline steel in the presence of biofilm consortium formed from an acetogen Eubacterium limosum and two sulfate-reducing bacteria Desulfobacter sp and Desulfovibrio desulfuricans produced an average corrosion rate of 5.3 mils/year, a monoculture of the acetogen alone produced an average corrosion rate of 0.3 mils/year (measured by EIS). The triculture produced extensive pitting that was not seen in the system containing the acetogen alone. The frequency corresponding to the maximum phase angle in the Bode plot of the triculture biofilm was at 0. 2 Hz in contrast to the acetogen monoculture maximum phase angle which was at 10 Hz. Both were determined by EIS.

DISCUSSION

ON-LINE, NON-DESTRUCTIVE DEMONSTRATION OF BIOFILM FORMATION

This paper documents the relationships between formation of an attached microbial biofilm and the lowering of the frequency of a vibrating quartz crystal in the QCM, the increase in the IR adsorption of proteins and carbohydrates in the ATR-FT/IR, the increase in the bioluminescence of attaching bacteria with a bioluminescent "reporter" gene sequence that is turned on, and with the decreasing (increasingly negative) OCP. All these technologies provide a non-destructive means of monitoring the formation and maintenance of a microbial biofilm. All have shown under the conditions described a direct correlation with the numbers of attached bacteria and/or the biofilm microbial activities. These technologies can be utilized together to monitor several facets of biofilm composition and activity simultaneously. Their utilization in a flow-through system should provide a powerful new technology for understanding the interactive dynamics of multicomponent microbial biofilms.

NONDESTRUCTIVE MONITORING OF MIC

The OCP provides a convenient,non-destructive means to follow the maturation and status of biofilms on electrodes that can be correlated with the QCM, ATR-FT/IR, and if the proper organisms can be used the bioluminescent detection technology.

The EIS offers a non-destructive means to follow the average corrosion rate as well give indications of localized corrosion. Non-destructive measurements using electrochemical techniques such as EIS, OCP, and small amplitude cyclic voltametry (SACV) have been shown to work effectively in the on-line monitoring of

MIC [8]. The EIS analysis allows determination of both the solution resistance and the polarization resistance[8]. Knowing the polarization resistance or charge transfer resistance, the average corrosion rate can be determined. Comparison of I_{corr} from the anodic Tafel slope and R_p (determined with EIS), and DC polarization analyses showed equivalence between the two measures [8,10]. The DC polarization measurements destroy the biofilm. Repeated measurements of R_p by EIS on noble electrodes with biofilms show no evidence of damage to the biofilms.

An additional advantage of EIS measurements is that response to effects of surface inhomogeneities in resistance and capacitance is accentuated at lower scan frequencies. This has been interpreted as evidence for more localized activity [8, 11,12].

CONCLUSIONS

1) The formation, maintenance and activity of an attached microbial biofilm can be monitored non-destructively by the increase in areal density with the QCM, by the increased IR absorbance of microbial components by ATR-FT/IR, by the increased bioluminescence of genetically engineered bacterial reporter strains, and by a lowering of the OCP.

2) EIS provides a non-destructive, on-line capability of monitoring both the average corrosion rate and gives indications of localization of MIC.

ACKNOWLEDGEMENTS

This research was partially supported by grants CHE 8718-057 from the National Science Foundation, N00014-86-K-0275 and N00014-87-K-0012 from the Office of Naval Research, F49620-89-C-0023 from the U.S. Air Force, Department of Defense, Water Research grant 14-08-001-G1482 from the U.S. Geological Survey, contracts 5088-260-1747 and 5087-253-1490 from the Gas Research Institute, RP-3015-1 from the Electric Power Research Institute, Waste Management Research and Education Institute, Science Alliance, University of Tennessee, IT Corporation, Environmental Science Division of Oak Ridge National Laboratory, and represents the initiation of research under RP-8011-2, from the Electric Power Research Institute on genetic ecology of biofilms.

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DISCLAIMER

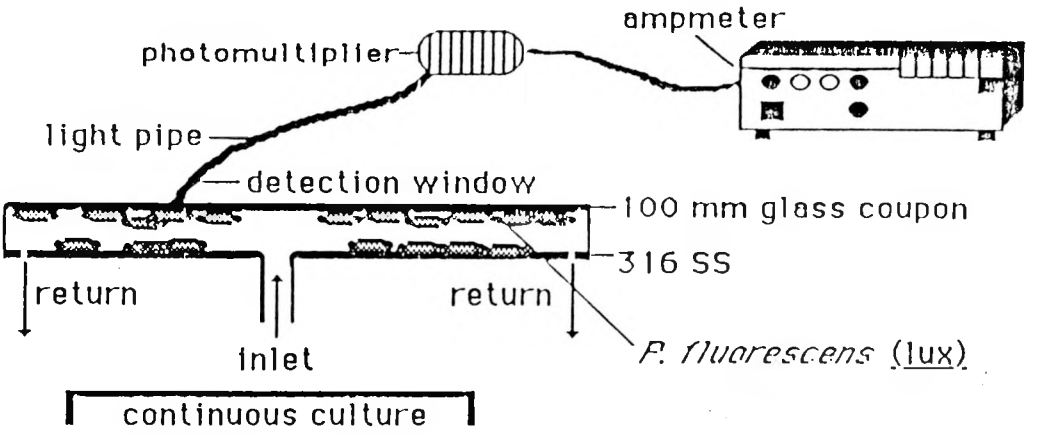

Figure 1. Schematic of <u>in situ</u> CAMM biofilm monitoring system in which the shear force is directly proportional to the flow rate and inversely proportional to the radius of the interaction. A continuous culture system supplies media and cells at a constant flow and dilution rate to the CAMM. The bioluminescence is monitored through the glass plate at the top.

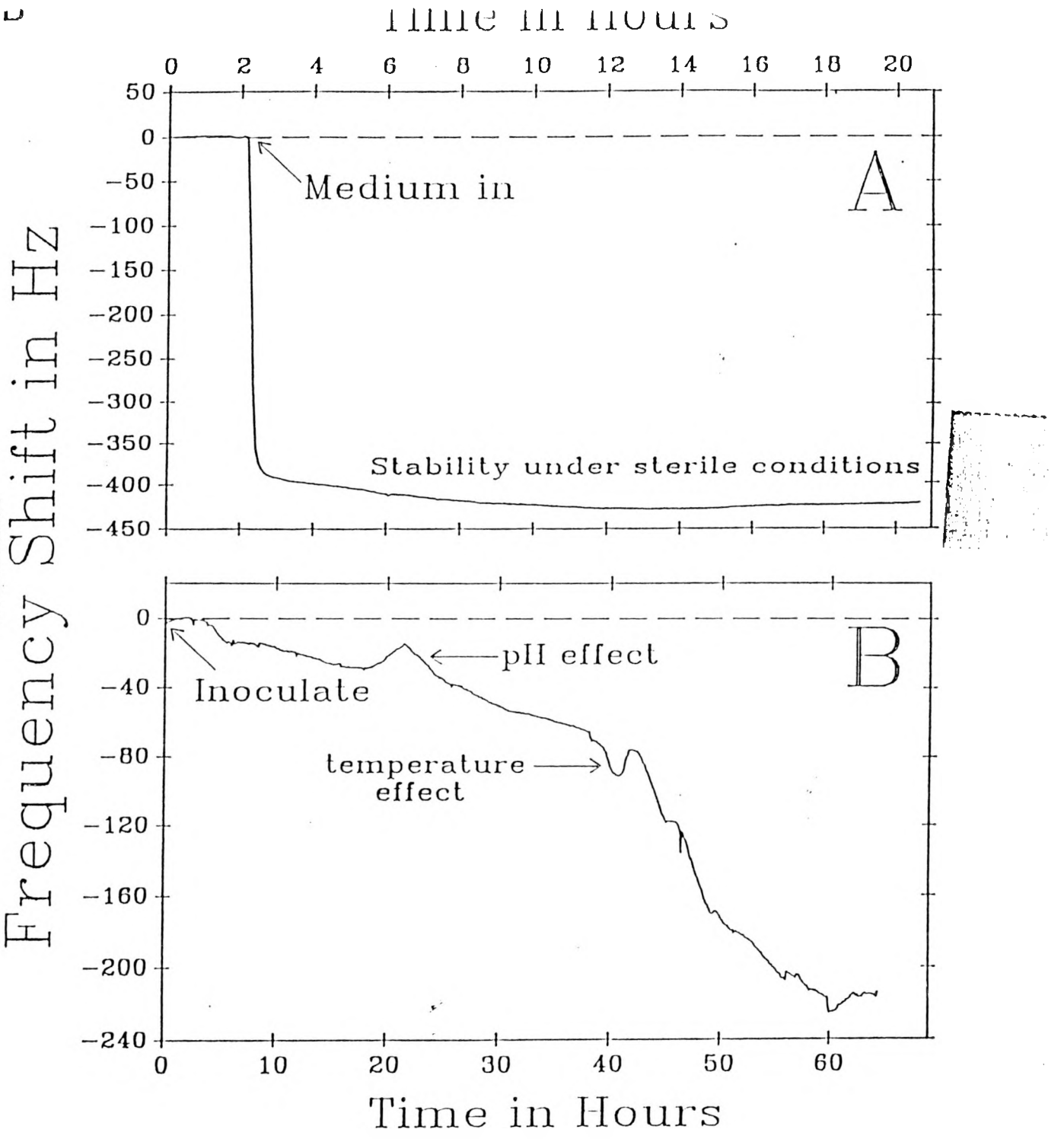

Figure 2. Effect of addition of sterile medium to the Quartz Crystal Microbalance (QCM) and the stability under sterile conditions (upper figure). Growth of the attached biofilm of C. crescentus showing perturbations resulting from a shift in pH of the input medium (10 hours) and a temperature fluctuation (40 hours (lower figure).

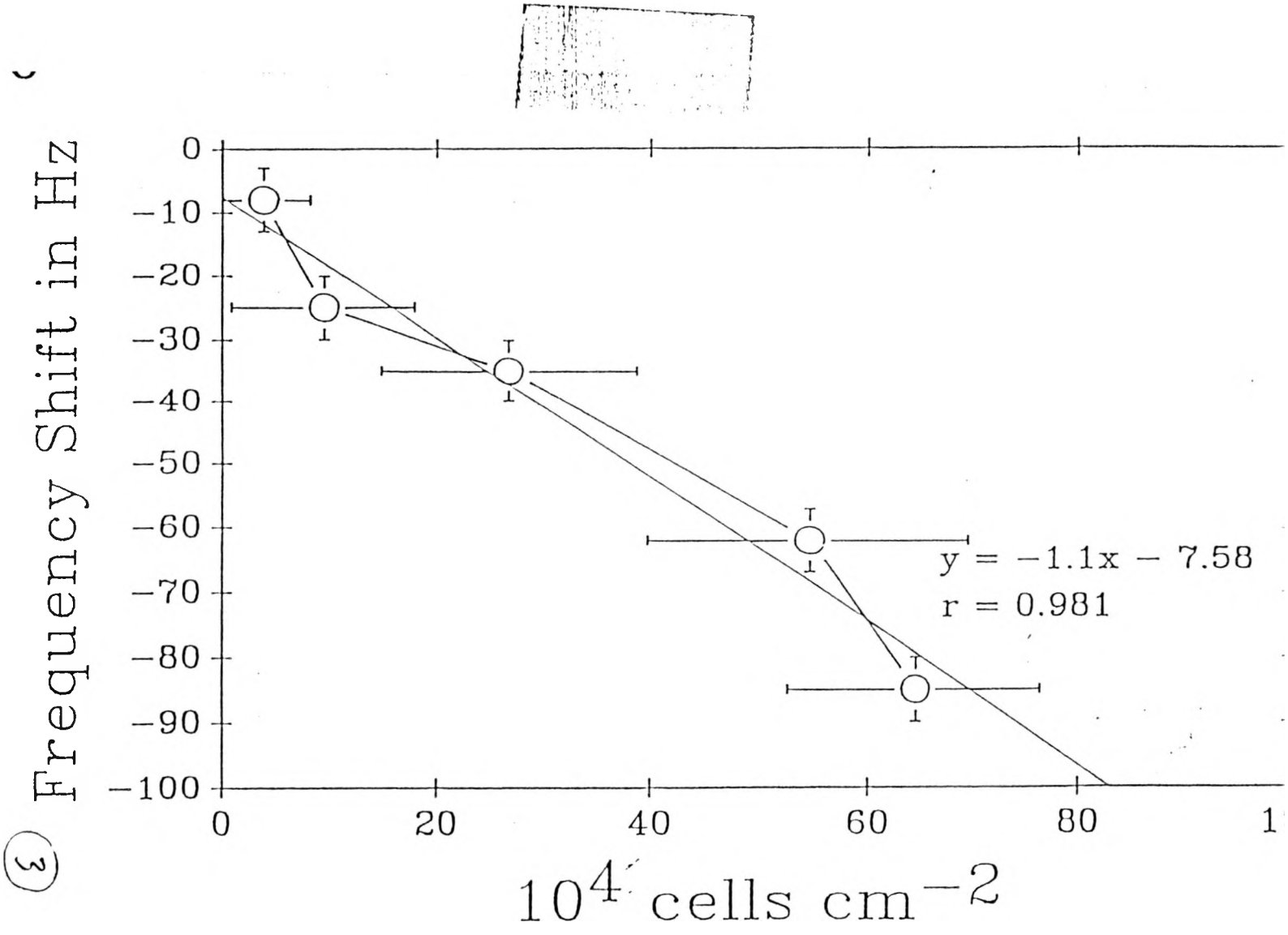

Figure 3. Linearity of low density biofilms of C. crescentus between numbers of cells and the decrease in the frequency of quartz crystal oscillation in the QCM flow cell.

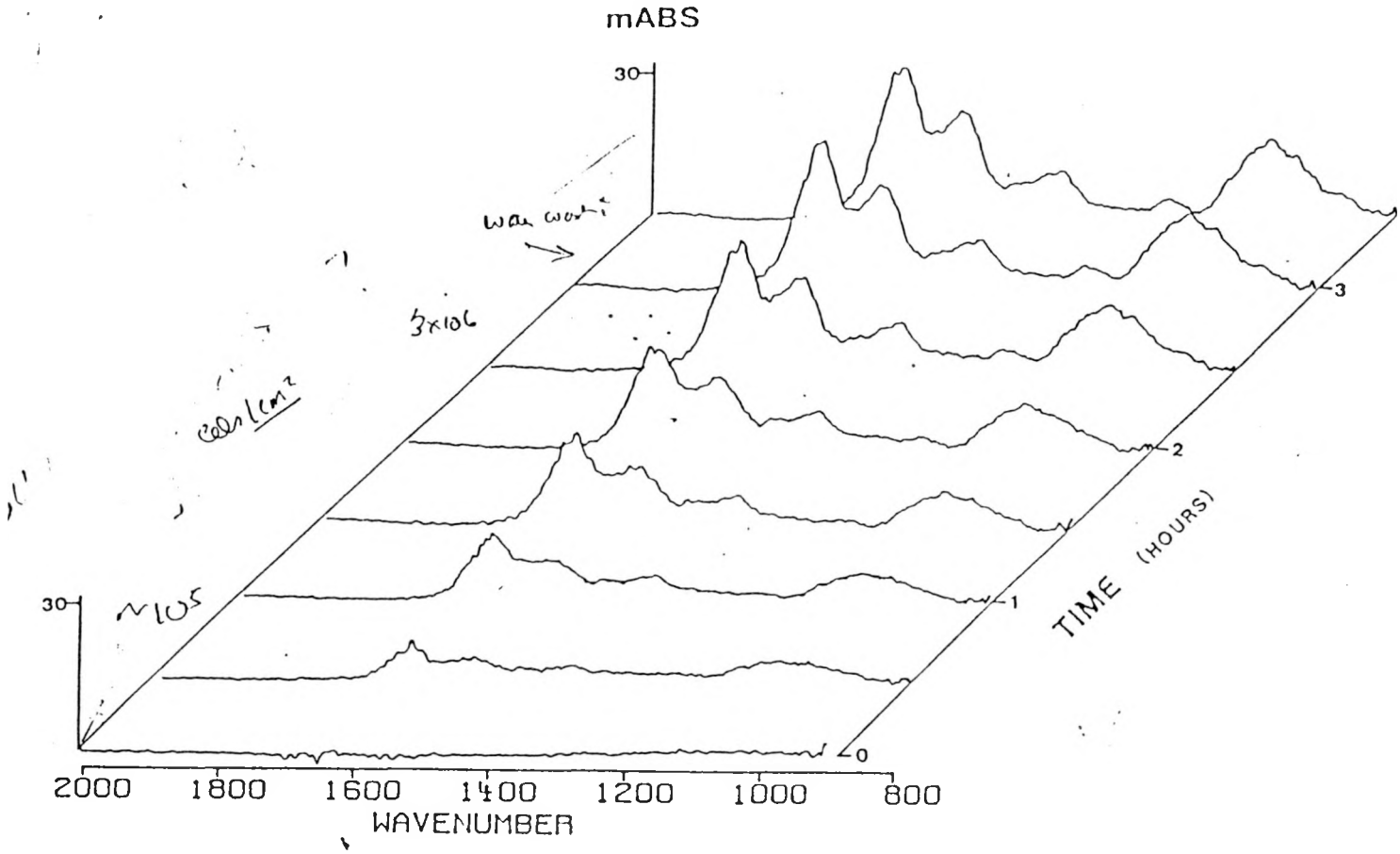

Figure 4. Formation of biofilm of C. crescentus on the surface of a germanium crystal measured with ATR-FT/IR. The bacterial proteins (amide I, amide II ~1650 cm^{-1}, ~1550 cm^{-1}) and carbohydrates (C-O stretch, ~1090 cm^{-1})[5] increase as the attached bacteria form the biofilm. At 0.5 hours the bacterial density was ~5 x $10^5 cm^{-1}$ after 3 hours 3 x 10^6 cm^2.

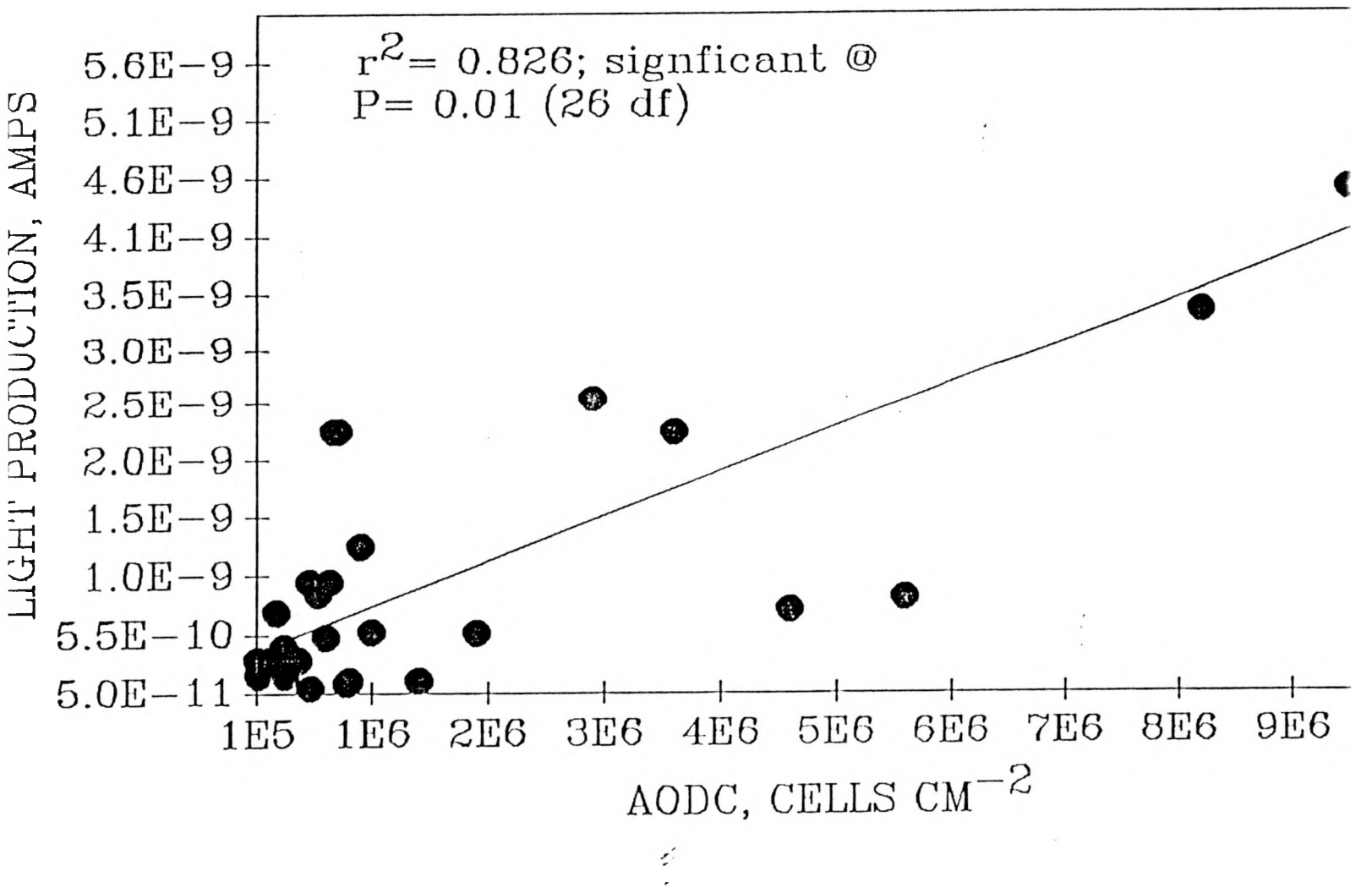

Figure 5. Relationship between bioluminescence and AODC of induced P. fluorescens attached to glass in a flow through continuous culture system.

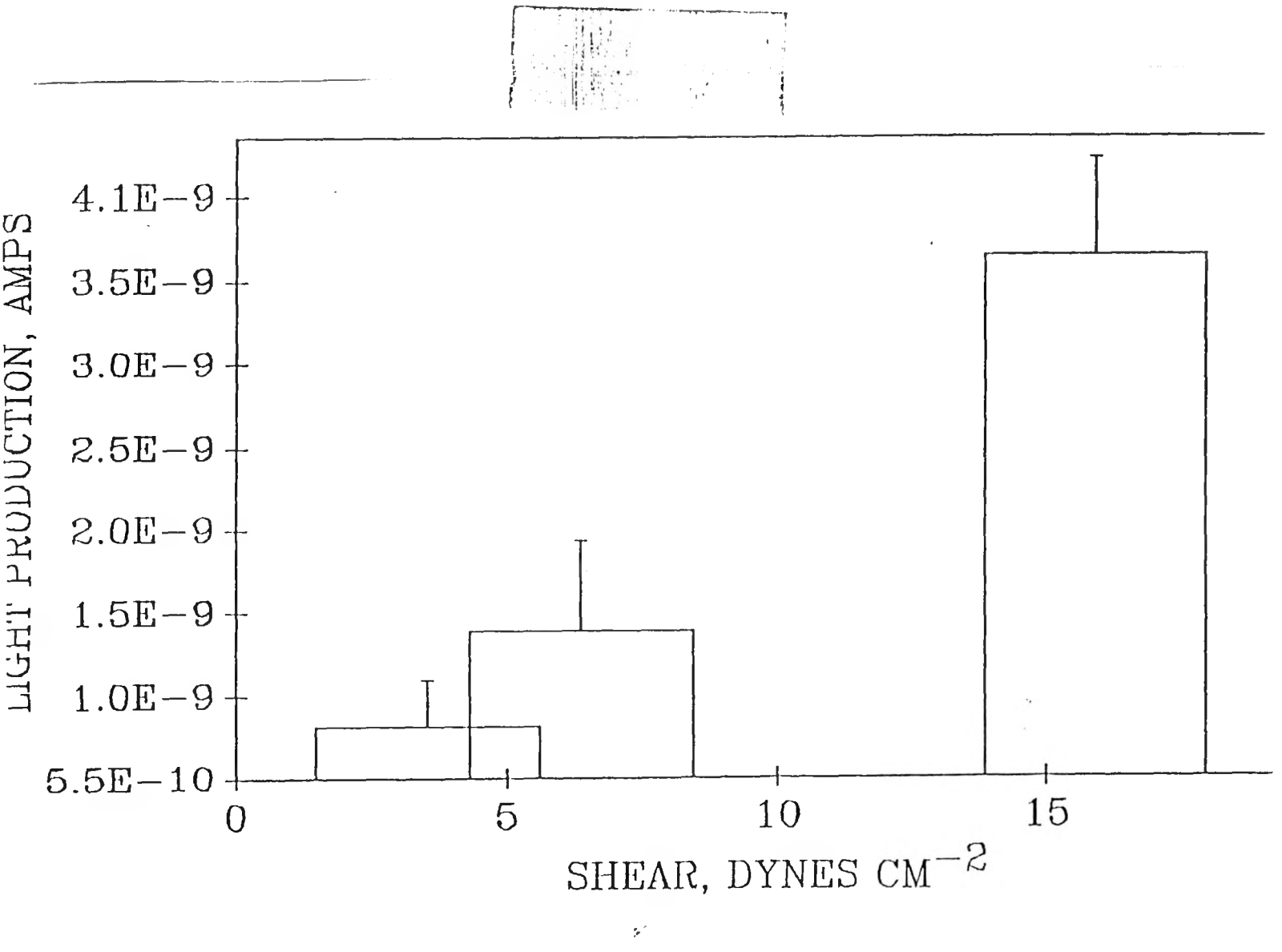

Figure 6. Relationship between the bioluminescence of engineered P. fluorescens and the attachment to glass in a defined shear force gradient.

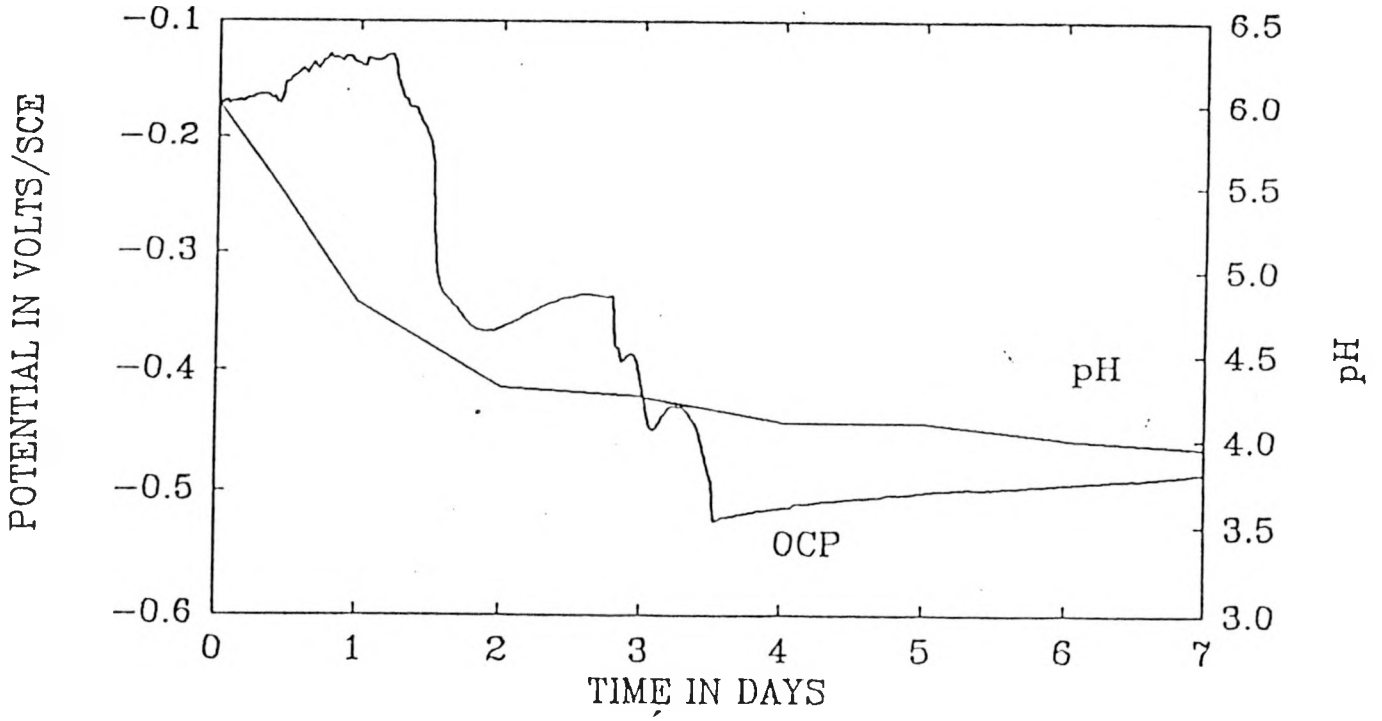

Figure 7. Relationship between the OCP, and the production of acetate and butyrate measured as the pH, by an attached aerobic/anaerobic bacterial consortium on a 316 stainless steel weldment.

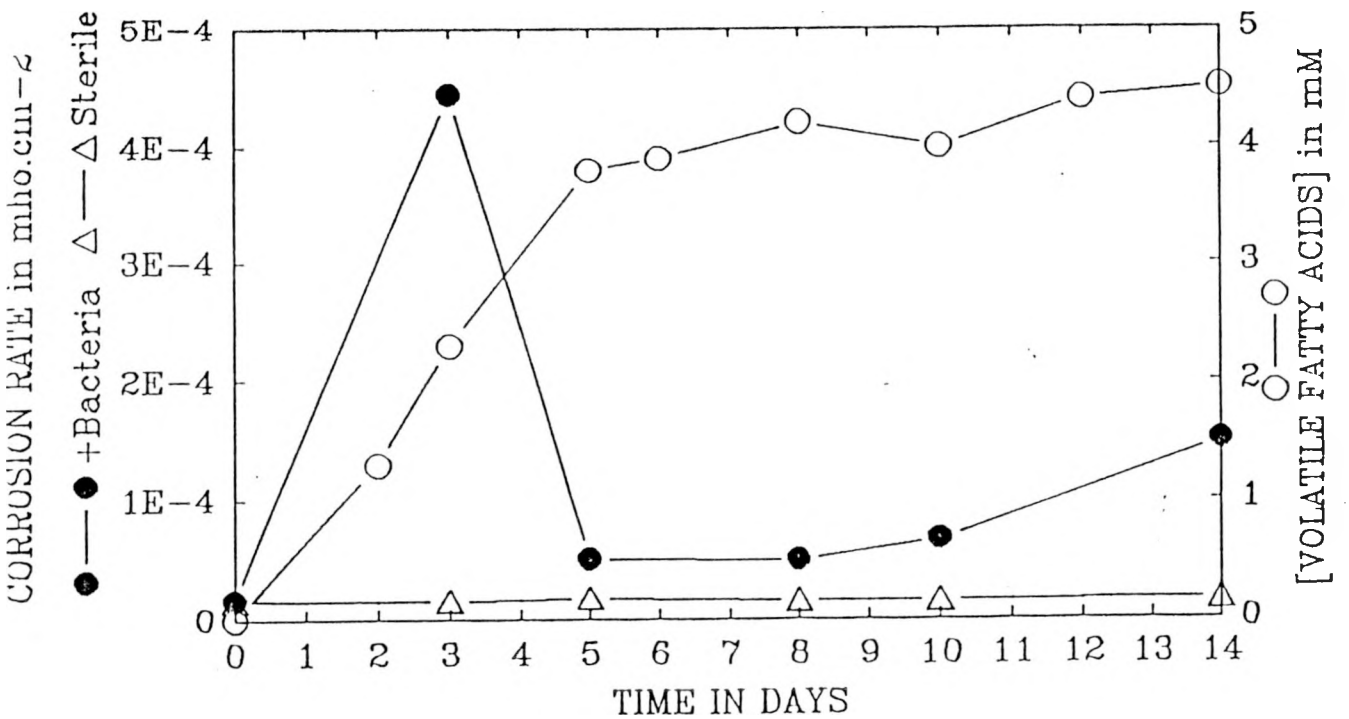

Figure 8. Relationship between EIS (measured as polarization potential) of a sterile control (open triangles) and a bacterial consortium containing aerobic and anaerobic organisms recovered from a corrosion tubercle (solid dots) on 316 weldment with 308 filler in a continuous flow system with the total volatile fatty acids (acetate and butyrate measured by gas chromatography) as an indicator of bacterial metabolic activity (open circles).