NON-DESTRUCTIVE MONITORING OF MICROBIAL BIOFILMS AT SOLID-LIQUID INTERFACE USING ON-LINE DEVICES

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Abstract - Corrosion, biofouling, and related problems have been an impetus for investigating interactions between microorganisms and solid surfaces. In recent years, a number of studies have been performed to assess the damages caused by microbially influenced corrosion (MIC). In a number of these studies, electrochemical techniques have monitored the performance of metal surfaces exposed to bacteria. However, most of these methods can only indirectly detect the presence of biofilms. In this paper, two non-destructive on-line monitoring technologies, attenuated total reflection Fourier transform infrared spectroscopy (ATR-FT/IR) and the quartz crystal microbalance (QCM) were used to directly monitor biofilm formation. Devices based on these technologies have been developed to study the initial fouling process and subsequent biofilm development and not merely the effects of the living film on the host material. The ATR-FT/IR technique provides information about biomass, extracellular polymer production, and the nutritional status of microbial biofilms. The QCM provides a direct measure of biomass. ATR-FT/IR and QCM techniques detected 10^6 and 10^4 Caulobacter crescentus cells/cm^2, respectively. Both techniques can be coupled with electrochemical methods for deeper insight into mechanisms of MIC.

INTRODUCTION

Studies of microbially influenced corrosion (MIC) have involved electrochemical measurements such as open circuit potential, linear polarization scans (potentiodynamic), small amplitude cyclic voltametry, and/or impedance spectroscopy to examine corrosion processes occurring at the solution/metal interfaces[1,2]. These techniques have monitored the performance of metal surfaces, but they can only indirectly detect the presence of microorganisms. In order to better understand the mechanisms of MIC, techniques that provide estimates of biomass, activity and the nutritional status of microbial biofilms attached to metal surfaces are needed. Unfortunately, most biochemical and microscopic procedures that provide data on these biological parameters require the removal of the samples from their original environments[3]. Two techniques which circumvent this difficulty by providing on-line and non-destructive analyses of surface phenomenon are attenuated total reflection Fourier transform infrared spectroscopy (ATR-FT/IR) and quartz crystal microgravimetry. Both technologies have been coupled with electrochemical techniques[4,5]. The long term goal of this research is to develop and evaluate these technologies to determine if they can be used to build devices that can monitor microbial biofilms and material performance in industrial settings.

In the ATR-FT/IR technique, light from a infrared (IR) source impinges on an IR transparent material (such as germanium). The beam is reflected at a number of points on the inside surfaces of the crystal[6]. At each reflection site some of the radiation penetrates out of the crystal and subsequently returns. This penetrating radiation is termed an evanescent wave because the radiation

is a standing wave normal to the surface and its intensity exponentially decays to zero with distance from the surface. ATR-FT/IR studies provide infrared absorption spectra of the components located within this evanescent wave (approximately a micrometer from the surface). These spectra can provide detailed chemical information about the organic accumulations in this region[6]. Thin films of metals, such as copper, have been deposited on these crystals and the ATR-FT/IR technique has been used to monitor the corrosion of these films[7]. In this paper, data presented shows that this technique can be used to nondestructively monitor on-line the nutritional status of a bacterial biofilm and the direct interactions between living cells, their polymers and a germanium substratum.

A quartz crystal microbalance (QCM) is an extremely sensitive mass sensing device which employs an AT-cut quartz crystal, an oscillator circuit, and a frequency counter to achieve a mass measurement[5]. The oscillator circuit, which is connected to the crystal by metal films, provides the potential stimulus to force the quartz crystal to oscillate at a given frequency. The frequency is measured directly by a frequency counter. When one side of a quartz crystal is exposed to a solution, the QCM can be used to monitor mass changes occurring at the solution and metal film interface. As material is adsorbed onto the metal film, the frequency of the oscillation will decrease and this frequency shift is directly proportional to the mass of the attached material. This frequency/mass relationship was discovered by Sauerbrey[8]. Sauerbrey's theory assumes that the attached film does not undergo any shear deformations during oscillation. The Sauerbrey equation for an AT-cut 5 MHz quartz crystals simplifies to the following:

$$\Delta f = \Delta m \ (-1.8 \times 10^{-8} g/cm^2\text{-}Hz) \qquad (1)$$

where Δf is the frequency shift in Hertz and Δm is the mass change in grams. This equation yields excellent results for frequency to mass conversions of thin rigid films. For thicker nonrigid films, Z-matching theory can be used to calculate mass from the frequency shift data[9]. This theory takes into account the acoustic impedance of the film, and requires that the shear modulus and density be known. The QCM will also respond to density and viscosity changes at the surface[10,11]. In this paper, the data presented demonstrate that the QCM can be used to monitor the formation of microbial biofilms on gold surfaces.

MATERIAL AND METHODS

Bacteria. Caulobacter crescentus was used as a test organism for both the ATR-FT/IR and QCM experiments. C. crescentus was chosen as a test organism because it is found in many freshwater environments, flourishes under oligotrophic conditions, and is able to attach to most solid surfaces. Caulobacter sp.[12] have a biphasic life cycle (swimmer and attached state) and generate an appendage that is used for attachment. These organisms secure themselves to a surface with a holdfast organelle located at the distal end of their appendage.

Media. The medium contained the following ingredients:
175 $mg \cdot L^{-1}$ KH_2PO_4 (1 mM), 20 $mg \cdot L^{-1}$ glucose, 5 $mg \cdot L^{-1}$ NH_4Cl, 5 $mg \cdot L^{-1}$ $MgSO_4$, 1 $mg \cdot L^{-1}$ $CaCl_2$ and 0.25 $mL \cdot L^{-1}$ Wolfe's mineral solution[13].
A 5X medium was used in the QCM experiments. The "low $[NH_4^+]$" medium decreased the amount of NH_4Cl from 5 $mg \cdot L^{-1}$ to 2 $mg \cdot L^{-1}$. All media were prepared with 18 Mohm·cm deionized water and titrated to pH 7.2. All chemicals used were reagent grade.

Flow System. Each continuous culture flow system contained a flow cell, a medium reservoir, silicon tubing, Masterflex pumps, and a 400 mL continuous flow stirred flask bio-reactor to supply growing bacteria and medium to the surface of the crystals and was assembled as shown in Figure 1. The dilution rate in the reactor was controlled by a pump A (Figure 1). Pump B (Figure 1) controlled the flow rates in the ATR-FT/IR and QCM flow cells. The flow rates were 0.5 mL/min and 0.7 mL/min for the ATR-FT/IR and QCM experiments respectively. The flow cells were sterilized with ethylene oxide and the medium was filter sterilized. The tubing, inlet and outlet reservoirs were steam sterilized (121°C for 25 minutes).

ATR-FT/IR Analysis. ATR-FT/IR analyzes were performed in a flow cell manufactured by Harrick Scientific Corp. (Ossining, NY). A germanium crystal (50 X 10 X 2 mm) with the entrance face cut at a 45° angle was used. HPLC grade water (Burdick and Jackson, Muskegon, MI), medium or growing cells plus medium were pumped into the flow cell by pump B (Figure 1). All data were collected as single-sided interferograms (time domain spectra) at a resolution of 4 cm^{-1} using a Nicolet 60SX (Madison, WI) FT/IR spectrometer equipped with a KBR beamsplitter, Michelson interferometer, and a mercury-cadmium-telluride (MCT) detector. Data files were zero-filled and multiplied by a Haap-Genzel apodization function prior to Fourier transformation. A stable air spectrum served as an instrument background to assure proper mirror and flow cell alignment. The reference water spectrum was obtained by initially pumping HPLC grade water through the ATR flow cell. Once stable conditions were achieved, the bio-reactor solution was pumped into the flow cell. A ratio of all the solution data files to air background data files produced the transmission spectra. All transmission spectra were converted to adsorption spectra. The reference water spectrum was subtracted from each biofilm spectrum to obtain the spectra in their final form[14].

The poly-ß-hydroxybutyrate (PHB) standard was obtained from Sigma Chemical (St. Louis, MO). Spectra were obtained by drying 0.1 mg onto a germanium crystal. The PHB was isolated from Alcaligenes sp.

QCM Analysis. One inch diameter AT-cut 5 MHz quartz crystals (Valpey-Fischer, Hopkinton, MA) were suspended between two o-rings and secured into a Delrin flow cell. Two flow cells and two bio-reactors were utilized. Medium was pumped though one flow cell and medium plus growing cells were pumped into the other flow cell. The flow cells were placed in a Faraday cage (to reduce environmental noise) contained within a constant temperature box that maintained the flow cells at 25.0°C. Homemade oscillators provided the alternating voltage to the crystals. The frequency of the crystals were monitored by Hewlett Packard (Palo Alto, CA) 5385A frequency counters. Data were collected through a general purpose interface bus by an AT computer. The controlling software was written in ASYST (Rochester, NY) language.

Acridine Orange Direct Counting Microscopy (AODC). The direct count of microorganisms was determined by AODC epi-fluorescence microscopy. Two methods of preparation were used for attached organisms. The first method involved fixing the bacteria in 3% formaldehyde solution, staining and counting directly on the crystal. The second method involved fixing the attached cells with 5 mL of the 3% formaldehyde solution, recovering the bacteria by sonication, transferring them to a Nuclepore (Pleasanton, CA) filter (0.2 μm pore size), staining, and counting. The bulk phase organisms were sonicated, transferred to a filter, stained, and counted.

RESULTS

In the ATR-FT/IR experiments, balanced medium (carbon limiting) and C. crescentus cells (less than 10^6 cells/mL) were pumped across the germanium crystal for the initial 3.5 days. During balanced growth, the infrared absorption bands at 1650, 1543 and 1084 cm^{-1} increased in intensity (Figure 2) and have been identified as amide I, amide II, and C-O stretch, respectively[16]. With biofilm studies, the amide I and II bands are associated with proteins and the C-O stretch with carbohydrates[16]. The amide II band reached approximately 0.013 absorbance units and the C-O stretch band attained a value of 0.027 absorbance units. After 78 hours, the ammonium ion concentration was decreased from 5 $mg \cdot L^{-1}$ to 2 $mg \cdot L^{-1}$ by pumping the "low $[NH_4^+]$" medium into the bio-reactor. Within 10 hours, signs of unbalanced growth emerged in the biofilm. An infrared absorption band at 1730 cm^{-1} appeared (Figure 2). This band was identified as a carbonyl stretch (C=O), and is associated with poly-ß-hydroxybutyrate (PHB) production. In addition, the C-O stretch band increased in intensity from 0.027 to 0.050 absorption units. The amide I and II bands stayed constant for the remainder of the experiment. After 5.5 days, the C. crescentus biofilm was removed from the crystal and stained with acridine orange and sudan black. The cell density was 1.1 X 10^7 cells/cm^2. The staining procedures also confirmed the existence of intracellular PHB. In addition, acridine orange staining revealed a gelatinous matrix surrounding the microorganisms.

In the QCM experiment presented in Figure 3, two bio-reactors and two QCM flow cells were utilized. After a 12 hour equilibrium period, one bio-reactor was inoculated with C. crescentus while the other remained sterile. The bacteria attached to the crystal for approximately 16 hours. A temperature perturbation can be seen at 6 hours. The response of each microbalance to this temperature change is different. At the end of the experiment, the bacteria on the crystal were stained with acridine orange and counted. The number of bacteria on the surface were 26 ($\pm$ 12) X 10^4 cells/cm^2. A calibration curve for C. crescentus attachment onto gold is presented in Figure 4. The frequency response produced a linear curve ($r = 0.981$) with cell densities ranging from 10^4 to 10^6 cells/cm^2.

DISCUSSION

A continuous flow stirred flask bio-reactor (Figure 1) was operated under "washout" conditions so that dilute medium and growing C. crescentus cells were supplied simultaneously to the crystals mounted in the flow cells. Flowing systems were used because they could provide better control over experimental parameters than batch systems. In batch systems, bulk phase parameters such as bacterial, nutrient and waste product concentrations are always changing, while in flowing systems, they can reach a steady state. The "washout" conditions were achieved by operating Pump A so that the bulk phase dilution rate was greater than the maximum growth rate of the microorganisms. These "washout" conditions were used to minimize the number of cells in the bulk phase so that nutrients could be supplied to the crystal's surface. Dilute medium was used to more accurately reflect oligotrophic conditions (common in many MIC environments) and to reduce the amount of adsorption of medium components onto the crystals. An ATR-FT/IR medium control experiment established that the adsorption of these dilute medium components was negligible.

The results from the ATR-FT/IR experiment demonstrate that the ATR-FT/IR technique can be used to nondestructively monitor the attachment of bacteria such as C. crescentus. The number of cells on the surface can be estimated by the intensity of the amide II (1543 cm^{-1}) band [17]. This absorption band results from the amide linkages found in proteins. Protein assays are well established estimates of microbial biomass. During balanced growth, the

amide II data indicated that the surface density of bacteria increased from zero under sterile conditions to 1 X 10^7 cells/cm^2.

The ATR-FT/IR technique can also be utilized to monitor unbalanced growth in a microbial biofilm. When the concentration of ammonium ion was decreased at 78 hours, nitrogen instead of carbon became the limiting growth substrate. Under these nitrogen-poor conditions, three significant physiological effects were monitored by the ATR-FT/IR technique. First, the carbonyl stretch band increased in intensity indicating that the bacteria were producing PHB, a carbon storage product. PHB production is a signal for unbalanced growth and has been used as a marker for nutritional status[15,18]. The existence of PHB was verified by microscopic methods. Secondly, the amide I and II bands remained constant, suggesting a that the amount of protein present remained constant. The third response was an increased intensity in the C-O stretch region (1084 cm^{-1}) of the spectra. Some of this increase can be attributed to the C-O bonds that exists in the ester linkage of the PHB polymer. The strongest band in the PHB absorption spectrum is the carbonyl stretch at 1730 cm^{-1} (Figure 2, insert). In the final biofilm spectra, the intensity of the carbonyl band only increased 0.004 absorption units. Therefore, the production of PHB could account for only a fraction of the 0.022 absorption units increase that occurred in this C-O stretch region in the final biofilm spectrum. The most likely explanation for the increase in the intensity of the C-O band is that it is due to an accumulation of carbohydrate material, which is most likely composed of extracellular polysaccharide secreted by the bacteria[16]. Extracellular polymers, which form the gel-like matrix which fills the space between bacteria in a biofilm, are known to accumulate in some bacteria during nutritional stress[19]. A gel-like matrix was also observed during AODC analysis.

The results of the QCM experiments demonstrate that the QCM is sensitive enough to detect initial microbial attachment and that the microbalance can be used to predict the number of cells on the gold surface for cell densities ranging between 10^4 and 10^6 cells·cm^{-2}. Furthermore, the mass of the biofilm can be determined accurately by Z-match theory if the shear modulus and solution density are known. Sauerbrey utilized a simpler theory[8], which assumes that the shear modulus of the attached film equals the shear modulus of quartz (rigid films). Using the Sauerbrey equations (equation 1) and the slope of the calibration curve (Figure 4), it is possible to estimate the average mass of an attached cell. For C. crescentus the calculation yielded a mass of 1.8 x 10^{-12} g per cell. This value is similar to the average wet mass of a smaller Escherichia coli cell, which was estimated to be 0.95 x 10^{-12}g [20]. Therefore, the Sauerbrey equation can provide a reasonable estimate of biomass during initial biofilm formation, even for nonrigid biofilms. Thicker and older biofilms that contain more extracellular polymers may require Z-matching theory for accurate frequency to mass conversions.

Although AT-cut quartz crystals have a temperature coefficient of zero at approximately 25°C, temperature can interfere with the frequency measurement. For this study, the quartz crystals were placed in a constant temperature environment to reduce the temperature fluctuations. The temperature at the start and the end of the experiments was maintained within 0.1 degrees. Even with these precautions, during these experiments the room temperature fluctuations (± 1°C) were correlated with frequency fluctuations (Figure 3). At first it was believed that the sterile medium signal could be subtracted from the biofilm signal to compensate for frequency shifts due to temperature fluctuations. Due to slight differences between AT-cut quartz crystals (angle of the cut, impurities in the crystal lattice), a temperature change produced a different frequency response for

each crystal (Figure 3). Methods designed to compensate for temperature fluctuations are presently being investigated.

CONCLUSIONS

1. The ATR-FT/IR technique can be used for nondestructive and on-line monitoring of bacterial attachment, the nutritional status of bacteria within a biofilm, and the accumulation of carbohydrate material, which is probably extracellular polysaccharide.

2. These results demonstrate that the QCM can also be used nondestructively to monitor biofilm formation and provide an estimate of biomass.

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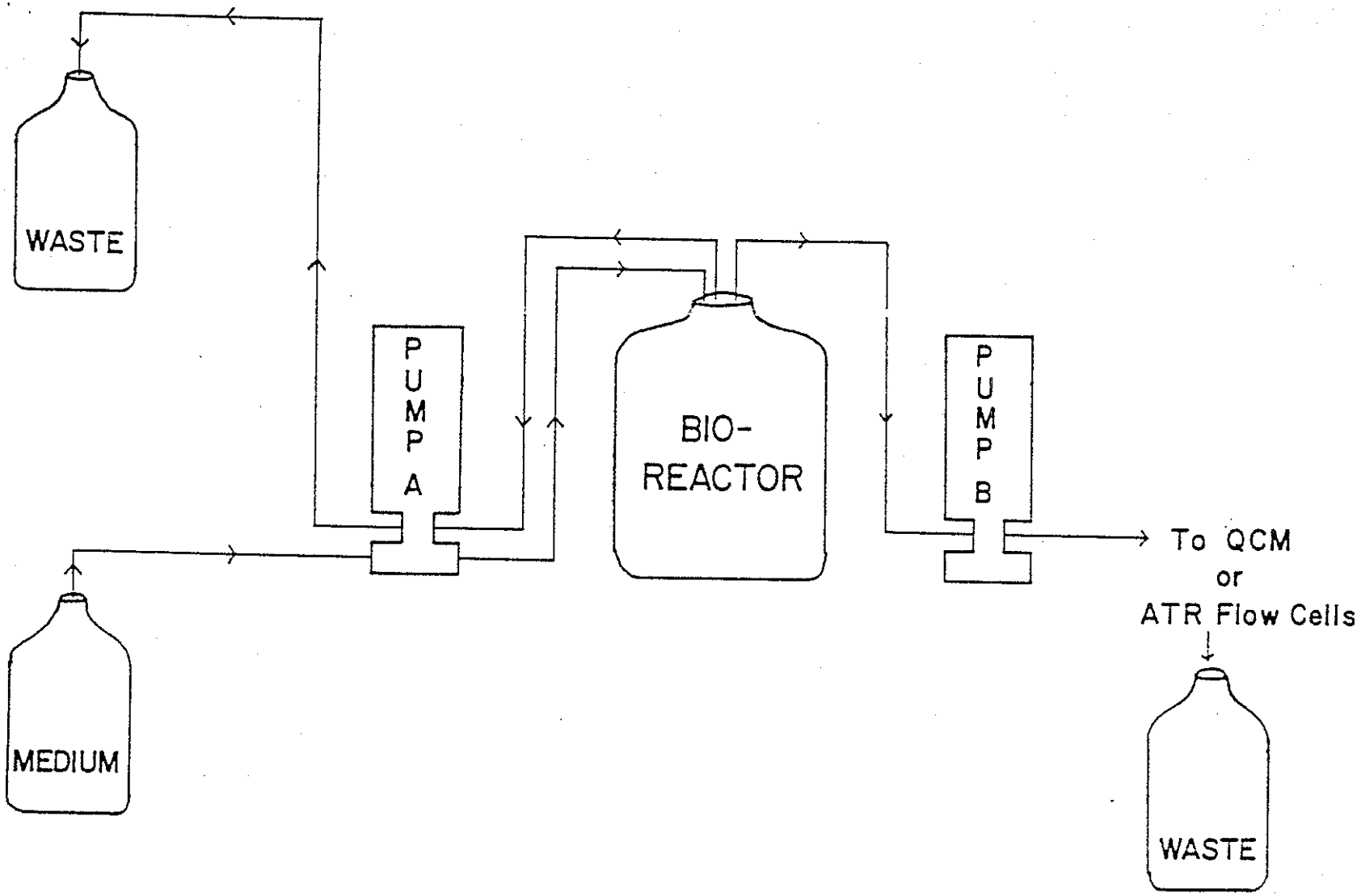


FIG. 1. Continuous culture flow system for ATR-FT/IR and QCM experiments. This system is used to supply (pump B) the flow cells with medium and growing cells. The number of cells in the bulk phase of the bio- reactor can be controlled with pump A.

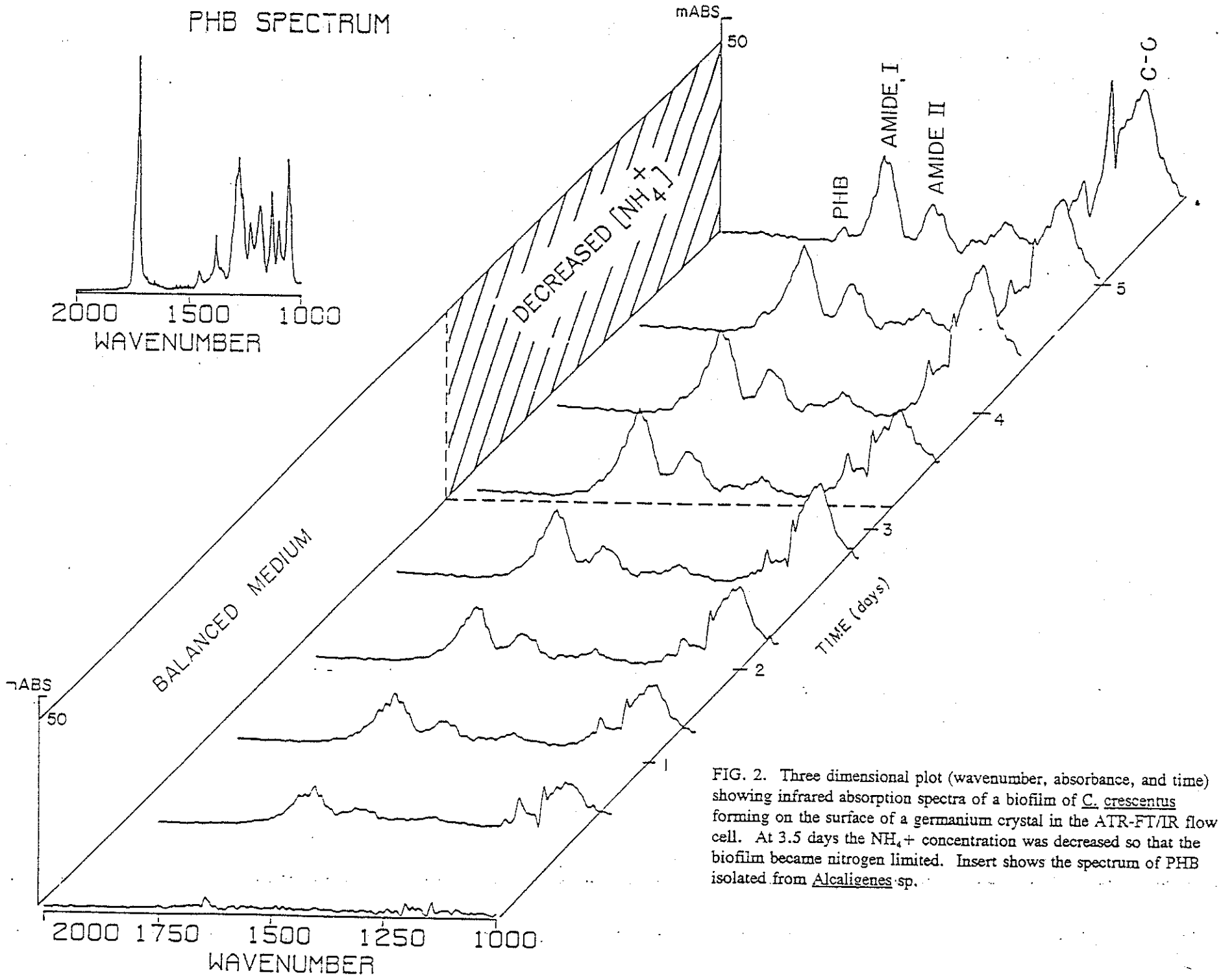
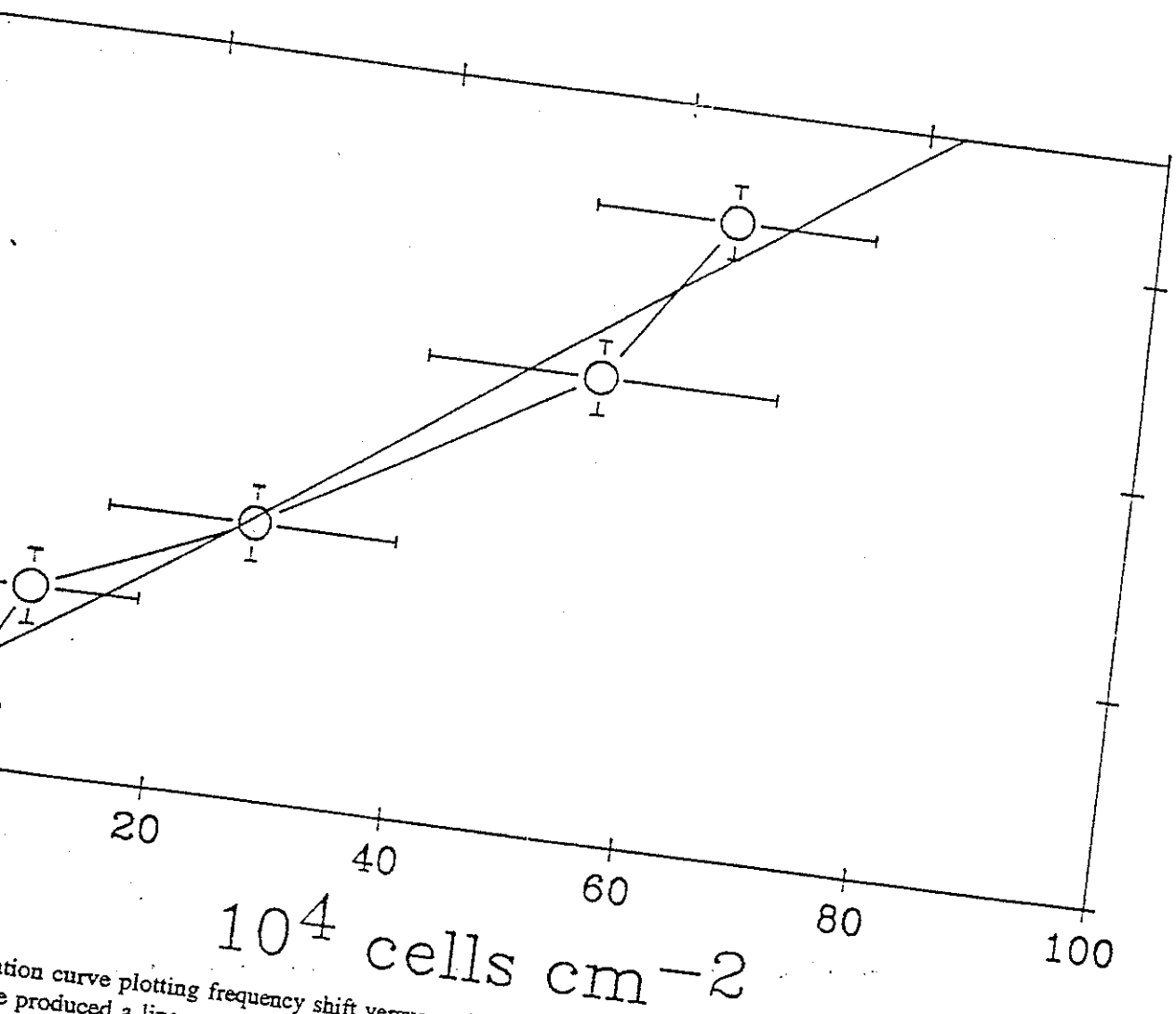


FIG. 2. Three dimensional plot (wavenumber, absorbance, and time) showing infrared absorption spectra of a biofilm of C. crescentus forming on the surface of a germanium crystal in the ATR-FT/IR flow cell. At 3.5 days the NH_4+ concentration was decreased so that the biofilm became nitrogen limited. Insert shows the spectrum of PHB isolated from Alcaligenes sp.



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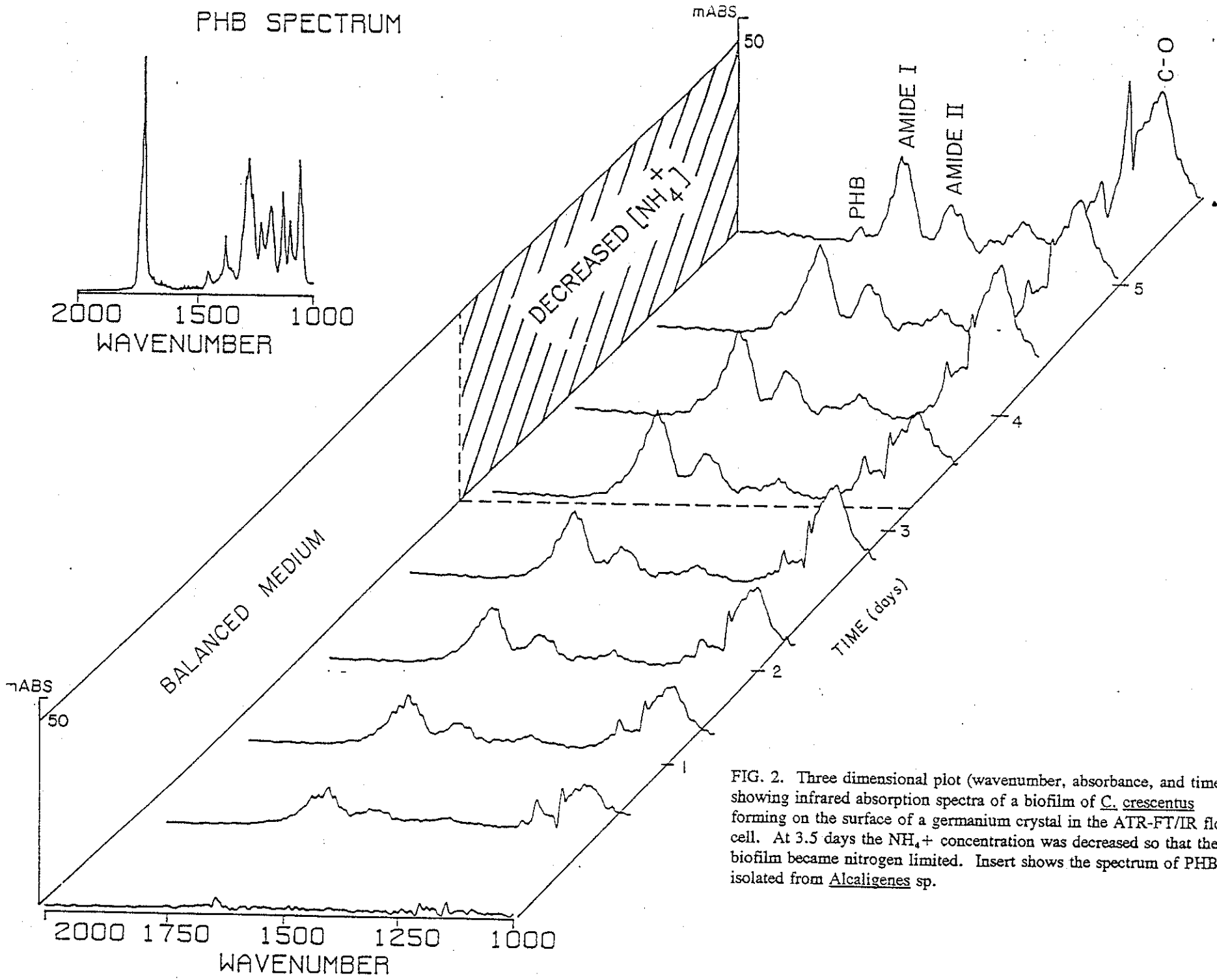

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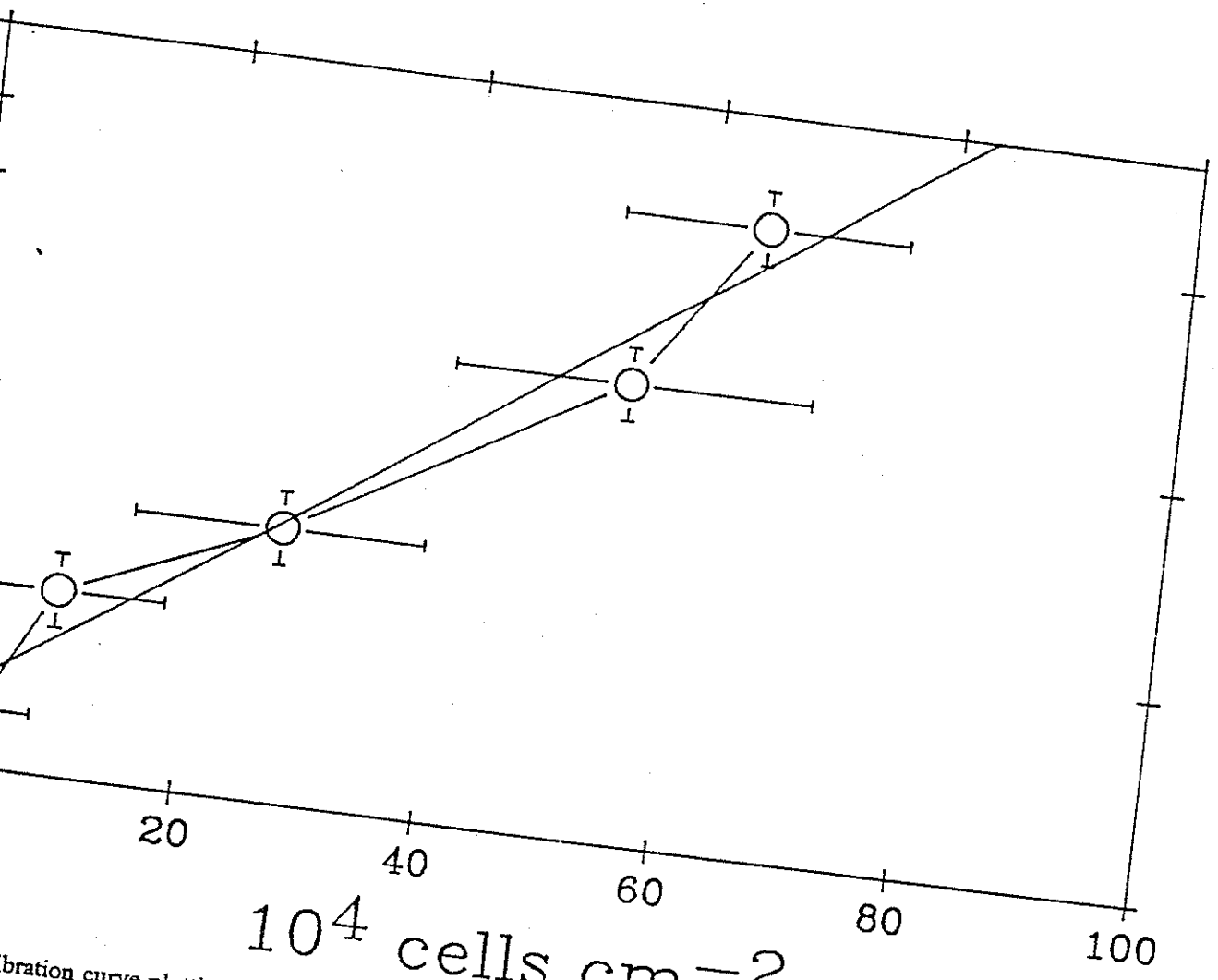

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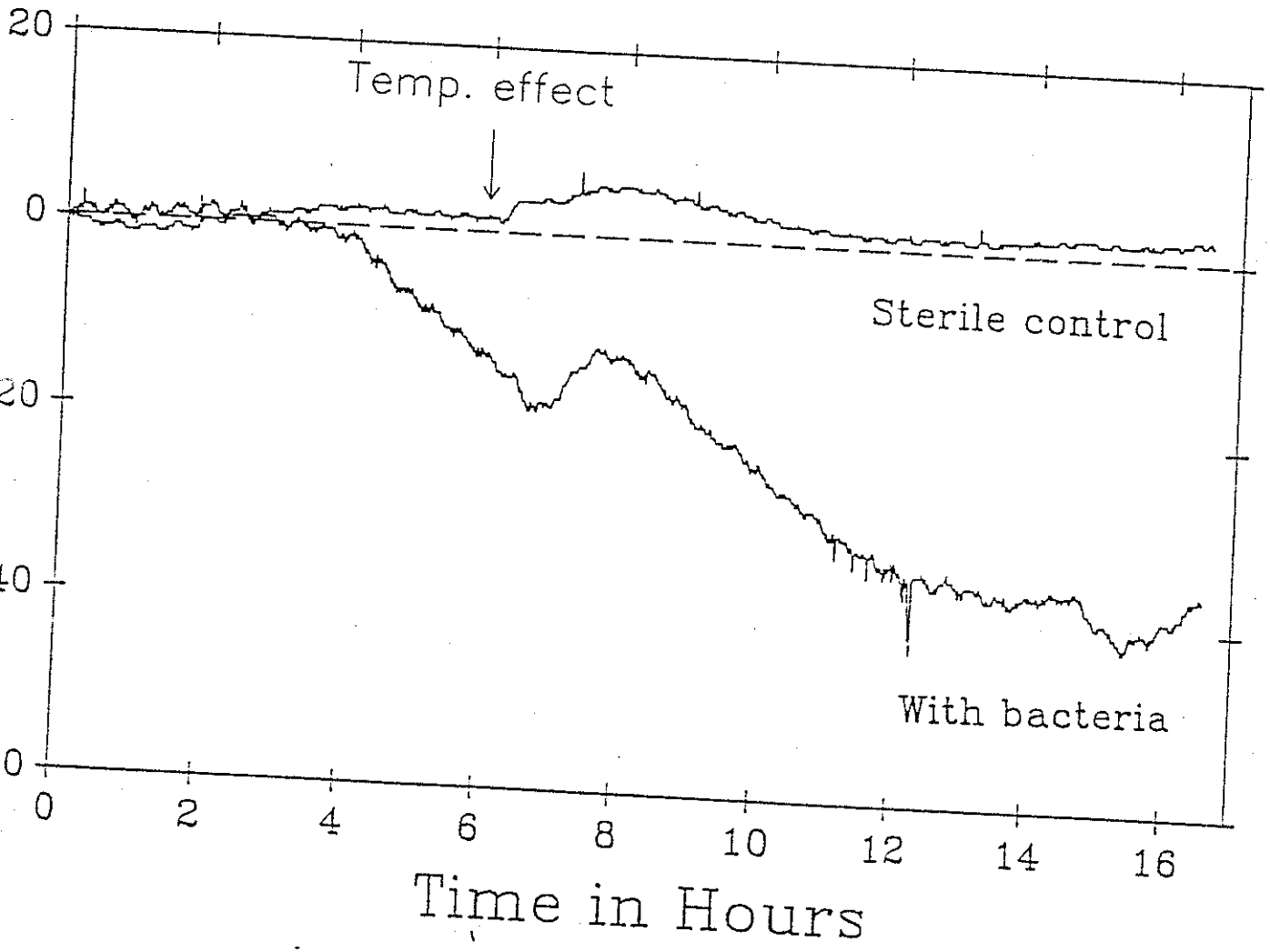

FIG. 3. Response of QCM to bacterial colonization by C. crescentus (lower trace) compared with sterile control (upper trace). The arrow indicates a temperature effect.