Analysis of Biocide / Biofilm Interactions by Attenuated Total Reflection Fourier Transform Infrared Spectrometry

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FINAL REPORT

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ABSTRACT

Biological fouling of reverse osmosis membranes is of great concern as the attachment, growth and multiplication of microorganisms at the aqueous/polymer interface eventually lead to the formation of an incessant fouling layer. The presence of this biofilm ultimately leads to the degradation of membrane transport properties thereby reducing efficiency and increasing operating cost. The sustained application of chemical biocides to feedwater is effective at controlling biofilm growth; however, complete disinfection of the membrane surface is rarely achieved. A greater understanding of biocide/biofilm interactions is desired so that more cost effective methods for controlling membrane biofilm can be developed. The feasibility of using internal reflection spectrometry to study *in situ* adsorption phenomena at an aqueous/polymer interface was investigated using Maxwell and Fresnel equations. Thin films (80 - 200 nm) of cellulose acetate (43% acetyl) were cast onto a germanium internal reflection elements. An unidentified organic macromolecule(s) readily accumulated at the polymer surface forming a 'conditioning film'. Deterioration of the cellulose acetate film occurred at an accelerated rate in the presence of a membrane fouling bacterium tentatively identified as mycobacterium. Penetration of Proxel GXL (active ingredient 1,2-benziosthiazolin-3-one) into a mycobacterium biofilm was investigated. A greater quantity of chemical components associated with Proxel GXL accumulated within the biofilm than on bare Ge, and a slower rate of biocide desorption from the biofilm was observed as compared to desorption from a Ge substratum. Biofilm growth was inhibited by biocide addition; however, complete disinfection was not achieved as microbial growth resumed after application of the biocide was terminated.

INTRODUCTION

Bacteria readily attach to the surface of reverse osmosis (RO) membranes where they grow and multiply at the expense of nutrients dissolved in the feedwater (1,2,3). This sequence of events: attachment, growth and multiplication eventually lead to the formation of a biofilm. As biofilm growth progresses, membrane transport properties deteriorate (*i.e.* loss in water flux and salt rejection) and transmembrane operating pressure (Δp) increases (4). Treatment with chemical antimicrobial agent have only limited success. Cells present in the biofilms are typically 10-100 times more resistant to chemical biocides and antibiotic substances than planktonic microbes (5,6). The increased resistance to biocides is primarily a function of biofilm structure and composition, but may also be influenced by the physiologic state of bacteria within the biofilm. To effectively disinfect an RO biofilm, a biocide must completely penetrate the biofilm to the underlying substratum. The presence of extracellular polymeric substances (EPS) synthesized by the attached bacteria inhibit convective mixing processes, thereby retarding biocide transport into the biofilm. Our understanding of biocide penetration and transport has been hampered to date by lack of suitable analytical techniques for tracking biocide migration into biofilms. Recently, attenuated total reflection Fourier transform infrared spectrometry has been successfully employed to explore biofilm formation (7) and biocide transport at solid-liquid interfaces (8).

In the current study by ATR-FTIR, thin films (80-200 nm) of cellulose acetate were cast onto internal reflection elements (*i.e.* germanium). These polymer-coated IREs were incorporated into a flow cell. Infrared radiation was passed through the IRE to achieve multiple internal reflections. Chemical substances (*e.g.*, bacterial cells, their products, biocides, *etc.*) sorbed onto the polymer surface absorb a portion of the evanescent radiation at each internal reflection giving rise to a unique IR absorption spectrum. Thus, it was possible to observe not only the type but the amount of EPS produced by the bacteria within the biofilm. This method was also used to measure the kinetics of biocide penetration into a biofilm. The advantage of this analytical technique was that the analysis was nondestructively in real time on a native biofilm growing on polymer-coated IREs within a flow cell.

The following research issues were addressed by this project: (1) Theoretical and experimental investigation of internal reflection of aqueous solutions in contact with a cellulose acetate-coated germanium internal reflection element, (2) Stability of thin cellulose acetate (CA) films exposed to aqueous solutions, (3) Biocide sorption on a CA film, (4) Biofilm formation on a Ge IRE, (5) Biocide penetration into a biofilm formed on a Ge IRE and (6) Biofilm formation on a thin film of cellulose acetate.

MATERIALS AND METHODS

Deposition of Cellulose Acetate on Ge Internal Reflection Elements. Thin films (80-200 nm) of cellulose acetate (43% acetyl) were case onto germanium internal reflection elements. Cellulose acetate was dissolved in methylene chloride and the solution allowed to flow over the surface of the IRE. The solvent evaporated leaving the polymer on the surface of the Ge IRE. The polymer casting was done by Separation Systems Technology, San Diego, CA.

Theoretical Calculations of Internal Reflection. A computer program written in FORTRAN 77 was used to calculate reflectance from parallel-planed stratified media. The program was written and published by Richard Dluhy (University of Georgia) (9). Maxwell and Fresnel equations were applied to calculate the theoretical reflectance for systems of parallel-boundary stratified media. Optical constants (refractive index, n and absorption index, k) were obtained from the literature. A three-layer system of germanium/polymer/ water was characterized in terms of its reflectance and mean square electric field intensity (MSEFI).

Organism and Culture Medium. A bacterium was isolated from a reverse osmosis membrane (polyamide thin film composite) operated in Water Factory 21, a 5 mgd waste-water treatment facility located at Orange County Water District. Feedwater to the RO elements consists of secondary municipal wastewater that has undergone lime clarification, multimedia sand filtration and chlorination (3-4 ppm, monochloramine). Despite low level chlorination viable microorganisms are still present and thus the membrane are subject to biological fouling. The dominate genus of bacteria within these biofilms is *Mycobacterium*. The isolate has tentatively identified as mycobacterium and has been designated as FT-8.

The bacterium was grown on a defined minimal salts medium (MSM) consisting of 2.0 g *D*-mannitol, 0.017 g $CaCl_2$, 0.24 g NH_4Cl, 0.051 g $MgSO_4$, 1.0 g K_2HPO_4 (anhydrous) and 0.6 mL Wolfe's mineral salts solution per liter of E-pure® (18 Mohm) deionized water (Barnstead/ Thermolyne). The pH of the medium was adjusted to 7 with HCl or NaOH. Concentrated solutions of mannitol, $CaCl_2$ and Wolfe's mineral salts were filter sterilized (0.2 μm pore size) and added to the balance of the medium that was sterilized by autoclaving. The composition of Wolfe's mineral salts was 1.5 g nitrilotriacetic acid, 3.0 g $MgSO_4 \cdot 7H_2O$, 0.5 g $MnSO_4 \cdot H_2O$, 1.0 g NaCl, 0.1 g $FeSO_4 \cdot 7H_2O$, 0.1 g $CoCl_2 \cdot 6H_2O$, 0.1 g $CaCl_2$, 0.1 g $ZnSO_4 \cdot 7H_2O$, 0.01 g $CuSO_4$, $AlK(SO_4)_2 \cdot 7H_2O$, 0.01 g H_3BO_3 and 0.01 g $NaMoO_4 \cdot 2H_2O$ per liter of deionized water. The bacterial isolate FT-8 was maintained on R2A agar medium (Difco) and stored at 4°C.

Chemical Biocide. Proxel GXL (Zeneca/ICI Americas) with an active ingredient of 17% 1,2-benzisothiazolin-3-one (BIT) was selected for its microbicide activity, membrane compatibility and unique infrared spectrum. All biocide solutions were made with MSM and filter sterilized at 0.2 μm pore size. The pH was adjusted to 7 with HCl or NaOH prior to filtration. The ability of Proxel GXL to inhibit

growth of a biofilm formed on a reverse osmosis membrane was determined by 'biocide-swatch test'. Stripes of membrane (3 x 1 cm) were cut from a fouled RO element that was operated at Water Factory 21. The RO elements were operated at 220 psi for approximately 2 months before being remove for use in the biocide-swatch test. The fouled swatches were placed in 20% R2A broth containing serial dilutions of Proxel GXL. Test tubes were incubated for 14 days. The highest dilution showing no growth in the test tube was reported as the minimal inhibitory concentration (MIC). All swatches that tested negative for growth after 14 days were aseptically transferred to sterile R2A broth and incubated for 14 more days. The highest serial dilution showing no growth was reported as the minimum bactericidal concentration. The MIC of Proxel GXL based on its active ingredient, BIT was determined to be 80 μg/mL. The minimum microbicidal concentration was approximately 150 μg/mL.

FT-IR Spectrometry / Data Acquisition. Germanium internal reflection elements (50 x 10 x 2 mm, Harrick Scientific) were polished successively with 6 μm and 1 μm diamond paste (METADI® II, Buehler), washed with MICRO detergent (Baxter), rinsed with tap water, rinsed with deionized water and air dried. The IRE was sterilized with ultraviolet light (15 min) within a laminar flow biological hood. The stainless steel flow cell, Buna o-rings and silicon tubing (1/16" i.d.) were sterilized by autoclaving. The flow cell with internal reflection element and tubing were assembled in the laminar flow hood and then installed on the optical accessory within the sample compartment of a Nicolet Magna 550 Fourier transform infrared spectrometer equipped with a (medium range) mercury-cadmium-telluride detector. The flow cell was filled with deionized water (pH 7). A water reference spectrum, consisting of 128 scans, was collected at 4 cm^{-1} resolution and stored for later use in spectral processing. A macro data acquisition program (Nicolet OMNIC, version 1.2a) was written such that single-beam spectra were collected and stored at set intervals of time. Sample spectra were ratioed against a bare Ge background spectrum and converted to absorbance. A scaled water reference spectrum was digitally subtracted from each sample spectrum such that the region between 2000 and 1800 cm^{-1} was flat. Deconvolution and curve fitting were performed with Grams/386™ software (Galactic Industries Corp.). These techniques were used to enhance spectral resolution in the region between 1844 and 1462 cm^{-1}. All the major absorption bands associated with cellulose acetate, the biofilm and the biocide were observed in this region of the spectrum.

Effect of Chemical Biocide on Biofilm Growth. A 50 mL volume of MSM was inoculated with FT-8 taken from the refrigerated stock culture. The culture was incubated at room temperature (22-23 °C) and stirred with a Teflon® stir bar. When the culture became turbid (approximately 48 hr), 0.2 mL was aseptically transferred to 200 mL of MSM. After 48 hr incubation at room temperature, the FT-8 culture was pumped through a flow cell with a peristaltic pump at ~15 mL/hr. The flow cell volume was approximately 600 μL. After 2 hr the flow of bacterial culture was shut off. A biofilm was allowed to form at the surface of the Ge IRE as a continuous culture with constant flow of MSM. After 10 days a 0.2% BIT (Proxel GXL) was

substituted for MSM. The biofilm was exposed to the biocide for 24 hr and then MSM flow was turned back on for approximately 6 more days. An analogous experiment was run in the absence of a biofilm. An aqueous solution of 0.2% BIT was pumped through a flow cell containing a bare Ge IRE.

Fluorescence Microscopy. Application of a 1 μg/mL solution of a DNA-binding fluorochrome 4',6-diamidino-2-phenylindole (DAPI) allowed for the observation of total (viable and nonviable) cell within the biofilm. DAPI was applied directly to the biofilm on the IRE. The cells were examined with the 100X oil immersion fluorescence objective of an Olympus Vanox model AHBT3 microscope equipped with a 200-W mercury burner.

RESULTS AND DISCUSSION

Theoretical and Experimental Investigation of Internal Reflection at a Germanium/Polymer/Water Interface

Theoretical. The feasibility of studying adsorption phenomena at an aqueous/polymer interface by internal reflection was investigated. A three-layer system of germanium/ polymer/water was characterized in terms of its reflectance and mean square electric field intensity (MSEFI) as a function of polymer thickness. Calculations of MSEFI and the 1640 cm^{-1} band intensity of water allow an estimate of the feasibility of detecting the adsorption of microbial cells at the cellulose acetate/water interface to be made.

Attenuated total reflection spectrometry makes use of the fact that infrared radiation striking the interface between the optically dense medium (*i.e.* the IRE) and the optically rare medium (*i.e.* polymer, water, *etc.*) is totally internal reflected. Light striking the interface actually penetrates a short distance (~ 1 μm) into the adjoining medium before reflecting back. If a very thin film (less than the wavelength of IR radiation) of polymer material is deposited on the IRE, the radiation will pass through the film and into the adjoining phase (*i.e.* water). Therefore, the polymer thickness is of critical importance to the success of making IR measurements at the polymer/water interface. Of greatest significance is the effect the polymer film has on the MSEFI, E_o^2, at the polymer/water interface and the effective thickness, d_e, which represents the actual thickness of a film (*i.e.* sample) required to obtain the same absorption in a transmission measurement as that obtained in the reflection measurement using a semiinfinite bulk sample. The relationship of E_o^2 to d_e is represented by the equation below,

$$d_e = n_2 / n_1 E_o^2 \frac{d_p}{2\cos\theta}$$

where n_1 and n_2 is the refractive index of the internal reflection element and polymer film respectively. Theta, θ, is the angle of incidence, and d_p is the depth of penetration. Depth of penetration is defined as that distance at which the MSEFI drops to $1/e$ of its value at the interface. The magnitude of the MSEFI, E_z^2, at the polymer/water interface is plotted as a function of polymer film thickness in Figure 1. The z-direction is defined as being parallel to the plane of incidence and normal to the substrate. The MSEFI drops exponentially with increasing polymer film thickness; therefore, the effective thickness, d_e, decreases with increasing film thickness. This means that the intensity of absorption bands of any material adjacent to the polymer (*i.e.* in 'layer' 3) will decrease with increasing polymer film thickness. For example,

the MSEFI at the polymer/water interface for a Ge IRE coated with a 80 nm thick film drops by 27% relative to its intensity at a bare Ge/water interface. If the polymer thickness is increased to 200 nm, a 56% drop in the MSEFI occurs. In effect, the polymer film attenuates the electric field. Therefore, there is a finite range of CA thickness that can be deposited on IREs for use in ATR-FTIR experiments. If too thick of a polymer layer is cast onto the IRE, a sample spectrum with insufficient signal-to-noise ratio will be obtained. To compensate for the reduction in d_e, a crystal with multiple internal reflections is used. However, there is a limit to the number of internal reflections that can be used as energy is lost at each reflection. If too many internal reflections are used, insufficient energy will fall onto the infrared detector. Therefore, the polymer thickness and dimensions of the IRE must be carefully selected.

The depth of penetration at 1640 cm^{-1} at a Ge/water interface is 400 nm. In the presence of 80 nm thick polymer film, the MSEFI drops by 27%; therefore, the effective thickness, drops to 283 nm. As a result, the water band intensity will drop (and continue to drop with increasing polymer thickness). The theoretical 1640 cm^{-1} band intensity is plotted as a function of polymer film thickness for the three-phase system of Ge/polymer/water and 20, 22 and 24 internal reflections (see Figure 2). The intensity of the water band does not drop monotonically with increasing polymer thickness which is contrary to what calculations of electric field intensity predict. A small enhancement of the water band occurs at film thicknesses less than 100 nm. The MSEFI represents the absolute energy intensity at the aqueous/polymer interface whereas the 1640 cm^{-1} band intensity displayed in Figure 2 is represented by the logarithm of the reflection ratio ($R = R_{ge/polymer/water}/R_{ge/polymer/air}$) which also takes into account the polarization of the radiation. In short, optical phenomena occur at polymer thicknesses below 100 nm that lead to an enhancement of the 1640 cm^{-1} band intensity. This effect actually increases the sensitivity of measurements made near the polymer interface. At polymer thicknesses greater than 100 nm, the water band intensity does drop exponentially as predicted.

A typical bacterial cell is about a 0.5 μm (500 nm) in width. If we assume, d_e is about 283 nm then it should be possible to monitor the sorption of a monolayer of bacterial cells at the aqueous/polymer interface by ATR/FTIR spectrometry. Since the evanescent wave only penetrates a short distance from the surface of the IRE, the initial events of biofouling can be observed. Studies of surface treatment with chemical surfactants and biocides as well as biocide penetration into a mature biofilm may also be investigated.

Experimental. A 1640 cm^{-1} band intensity of 0.662 absorbance units (A.U.) was measured with a 50 x 10 x 2 mm parallelepiped (45°) Ge IRE (see Figure 2). This measured intensity corresponds to approximately 21 internal reflections at the Ge/water interface. Note that the entire surface of the IRE is not in contact with water as the o-rings leave a 'footprint' approximately 45 x 8 mm in dimensions. The water band intensity dropped to 0.647 A.U. when measured with the Ge IRE coated with a CA film estimated to be 80 nm thick. The theoretical water band intensity for 20 internal reflections is 0.660 A.U. When the film thickness was increased to

approximately 200 nm, the measured 1640 cm^{-1} band intensity dropped to 0.355 A.U. The theoretical water band intensity is 0.416 A.U. for this same (200 nm) polymer thickness. The theoretical equations used in the program threat the three-phase system (Ge/polymer/water) as three distinct layers. However, in practice the polymer layer is actually permeable to water. Therefore, the experimentally measured water band intensities should be greater than the theoretical values. Despite this fact, our initial results indicate that the 1640 cm^{-1} water band intensity can be used to estimate the thickness of the cellulose acetate film.

Stability of a 200 nm Thick Cellulose Acetate Film Cast on a Ge IRE

Thin films of cellulose acetate (43% acetyl) were deposited on Ge internal reflection elements by first dissolving the polymer in a suitable solvent (methylene chloride). The polymer solution was then poured over the IRE and the solvent allowed to evaporate leaving the polymer on the Ge surface. Estimates of the polymer thickness were made based on the polymer-solvent composition (10)

Deionized Water Control. A thin film of CA approximately 200 nm thick was deposited on a Ge IRE and placed into a flow cell within the FT-IR spectrometer. A mid-infrared spectrum of the CA film in the region between 4000 and 950 cm^{-1} is displayed in Figure 3. An expanded region of the spectrum between 2000 cm^{-1} and 850 cm^{-1} is shown in Figure 4. Major absorption bands of cellulose acetate appear near 1753, 1368, 1230 and 1050 cm^{-1} and represent carbonyl(C=O), methyl($-CH_3$), ester(C-O-C) and sugar backbone (C-O-C) vibrations respectively. The chemical structure and band assignments for CA are shown in Figure 5.

Deionized water (pH 7) was pumped through the flow cell containing the CA-coated IRE. All the major cellulose acetate bands dropped between 21 and 24% during the first 5 min (see Figure 6). After approximately 1 hr of flow, all the major CA band intensities stabilized. Approximately 65 - 70% of the polymer film is exposed to the aqueous phase; therefore, a 70% drop in band intensities is the most that could occur if all the CA in contact with the aqueous phase were removed. Thus, a significant amount of the film is still in contact with the IRE after 1 hr exposure.

Between T = 24 hr and T = 48 hr, all band intensities dropped and then gradually increased in intensity over the following 110 hr (see Figure 6). The origin of this effect is not know but may be associated with detector nonlinearities as the MCT detector warmed to room temperature before being recooled between T = 24 hr and T = 48 hr. At the end of 157 hr, the following changes in CA band intensities had occurred.

Absorption Band	Percent Change
1750 cm^{-1}	-36.7%
1370 cm^{-1}	-38.9%
1050 cm^{-1}	-47.2%
1232 cm^{-1}	-31.6%

Therefore, approximately 50% of the original CA film remained in contact with the Ge IRE.

Benzalkonium Chloride Adsorption on a Thin Film of Cellulose Acetate

Benzalkonium chloride has proven to be a very effective biocide against membrane fouling bacteria. However, this chemical biocide is known to cause severe membrane flux decline at concentrations as low as 100 μg/mL (11). The cause of the flux decline is presumably a result of membrane binding and plugging of the pores within the polymer film. In an effort to gain a better understanding of the biocide-polymer interactions, benzalkonium chloride was exposed to the thin film of cellulose acetate.

Following 157 hr exposure to deionized water, a 0.5% (wt/vol) benzalkonium chloride solution was pumped through the flow cell at ~7.5 mL/hr. No changes in the major absorption bands of CA were observed over a 12 hr period (see Figure 7). An ATR spectrum of a 1% (wt/vol) solution of benzalkonium chloride measured with a ZnSe IRE is shown in Figure 8. Major absorption bands associated with this compound are located near 1484, 1468 and 1467 cm^{-1}. These bands are assigned to the methyl($-CH_3$) and methylene ($-CH_2-$) deformation of the n-alkyl 'tail' of the molecule. The ATR spectrum of benzalkonium chloride is overlaid with a spectrum of CA that was in contact with 1% benzalkonium chloride (see Figure 9). No absorption bands associated with benzalkonium chloride are visible in the biocide-treated CA spectrum. These results may be interpreted in number of ways: (1) Benzalkonium chloride is somehow excluded from making contact with the cellulose acetate film, (2) the polymer film is so thick that very little IR radiation penetrated into the bulk aqueous phase or (3) the biocide is not present in sufficient quantity to detect. The latter case is probably true. In theory, band intensities of a 0.5% benzalkonium chloride solution measured with the Ge IRE would be approximately 6-fold less at ~0.01 A.U. Since no biocide bands were detected, the Ca film must have been too thick, that is, insufficient energy was transmitted through the film and into the bulk aqueous phase to obtain an IR spectrum. If all the CA in contact with the aqueous phase had been removed from the Ge surface, then certainly absorption bands associated with benzalkonium chloride would have been detected.

After 12 hr the flow cell was rinsed with deionized water (pH 7). Virtually no

change in the CA band intensities occurred during the 12 hr rinse period indicating that the CA film was still stable (Figure 7). Approximately 50% of the polymer was still left on the at the end the 181 hr experiment.

Stability of a 80 nm Thick Film of Cellulose Acetate Cast on Ge IRE

Deionized Water Control. The 200 nm film appeared to be too thick for use in studying macromolecular adsorption by internal reflection spectrometry; therefore, the CA film thickness was reduced to 80 - 100 nm in an effort to increase the sensitivity of the infrared sampling technique. By reducing the film thickness over 2-fold, the MSEF1 was increased by approximately 65%. The stability of the CA film was investigated by pumping deionized water (pH 7) through the flow cell containing the polymer-coated IRE. Infrared spectra were collected periodically over a 171 hr period and are displayed in Figure 10. (The 'sharp' bands in the spectra between 1700 and 1400 cm^{-1} are due to water vapor.) The major CA band intensities are plotted as a function of time of flow (see Figure 11). The 1053 cm^{-1} C-O and the 1750 cm^{-1} carbonyl band intensities initially dropped at the same rate. The 1053 cm^{-1} band intensity leveled off at approximately T = 100 hr as did the 1238 cm^{-1} carbonyl ester band intensity. This effect was presumably due to the adsorption of an organic macromolecule(s). From the start of the experiment an organic chemical species slowly adsorbed onto the surface of the CA-coated IRE. Major absorption bands near 1374 cm^{-1} and 1493 cm^{-1} were observed (Figure 10). The spectrum of this 'contaminant' was obtained by taking the difference between the spectrum collected at T = 169.5 hr and T = 0 hr (see Figure 12). Other absorption bands are visible in the contaminant spectrum near 1421, 1264, 1244, 1202, 1140 and 970 cm^{-1}. This chemical species was not observed in the previous experiment where 200 nm thick film was exposed to deionized water. The identity of this contaminate has not been determined but can be viewed as a 'conditioning film'.

By the end of 170 hr, the carbonyl band intensity dropped to 0.0772 indicating a 30% loss in the carbonyl moiety due to either deacetylation or physical separation of the polymer from the surface of the IRE. If a portion of the polymer film were to swell or pull away from the surface of the IRE then all absorption bands associated with cellulose acetate would drop at the same rate and to the same extent. This effect was observed when the IRE coated with 200 nm of CA was exposed to deionized water (see Figure 6).

Hydrolysis of CA Film. Bacteria typically produce acidic metabolic waste products that reduce the pH within and around the bacterial colony (12,13). Local areas of high acidity *i.e.* under a microcolony or biofilm may eventually lead to hydrolysis of the polymer membrane. The effect of low pH on a thin film of cellulose acetate was investigated by pumping 0.01 M HCl through the flow cell

containing the polymer-coated IRE that had previously been exposed (170 hr) to deionized water at pH 7. Significant changes in the IR spectra occurred with time of flow. The 1238 cm^{-1} carbonyl ester, 1371 cm^{-1} methyl and 1493 cm^{-1} bands associated with the organic contaminant all dropped rapidly at the start of HCl flow. Each of these bands dropped approximately 2 mAU after approximately 15 min then remained constant for the remaining 161 hr. The contaminant bands did not completely disappear indicating that some of this material was still firmly bound to the surface polymer film.

The 1053 cm^{-1} C-O stretching band of CA increased in intensity from 0.063 A.U. to 0.081 A.U. after 12 hr exposure to the dilute HCl. The difference spectrum of the CA film before and after exposure to HCl is displayed in Figure 13. The 1050 cm^{-1} C-O stretching band increased throughout the 161 hr due in part to a broad series of absorption bands that appeared in the region between 1250 and 970 cm^{-1}. The 1750 cm^{-1} carbonyl intensity continued to drop at the same rate. These results suggest that polymer deacetylation and hydrolysis had occurred. When cellulose acetate undergoes deacetylation, carbonyl (C=O), carbonyl ester (C-O-C) and methyl (CH_3) bonds are lost and are replaced by carbon-hydroxyl (C-O-H) bonds. Therefore, the band intensities near 1750, 1370 and 1230 cm^{-1} should decrease in intensity and absorption bands associated with C-O-H group should increase (*i.e.* in the infrared region between 1200 cm^{-1} and 1000 cm^{-1}). When hydrolysis occurs the backbone (C-O-C) ester linkage between glucose subunits are broken forming more C-O-H bonds; therefore, significant changes in the region between 1200 and 1000 cm^{-1} should occur. When and if CA undergoes hydrolysis, the lose or cleavage of the C-O-C backbone linkages will more than likely cause the adjacent C-O-C vibrational bands to shift in frequency due to the proximity of these two bonds to each other. The C-O-H vibrational bands are also likely to shift in frequency. Thus, it is very difficult to make specific assignments to the bands associated with polymer hydrolysis, especially as they relate to the C-O-C and C-O-H absorption bands.

Because all the major cellulose acetate bands are affected by both hydrolysis and deacetylation it is difficult distinguish between separation of the polymer film from the Ge IRE and polymer degradation in the form of both hydrolysis and deacetylation. Presumably the organic contaminant is bound to the CA and not to the Ge surface; therefore, the fact that the 1493, 1371 and 1238 cm^{-1} bands do not drop during this time indicates that the film is still intact. If all the CA in contact with the aqueous phase is removed from the surface of the IRE, the carbonyl band intensity would drop to a value of 0.03 A.U. (*i.e.* a 70% drop in band intensity). Therefore, a combination of events appear to occur at the aqueous/polymer/IRE interface during exposure to dilute HCl, (1) deacetylation of CA as evidenced by the drop in carbonyl band intensity, and (2) hydrolysis of CA as evidenced by the increase in 1053 cm^{-1} band intensity and the appearance of C-O-H band in the region between 1250 and 970 cm^{-1}.

Effect of Proxel GXL on Mycobacterium Biofilm Formed on a Ge IRE

A 48 hr old culture of isolate FT-8 was pumped through a flow cell containing a bare Ge IRE. Infrared spectra were collected at predefined intervals such that biofilm formation could be monitored as a function of time of flow. After 2 hr, MSM was substituted for the bacterial culture. MSM was pumped through the flow cell for 10 days and then an aqueous solution of 0.2% BIT (Proxel GXL) was substituted for the culture medium. After 24 hr, the flow of MSM was reinitiated and allowed to continue for 6 days after which the experiment was terminated. A series of infrared spectra collected throughout the experiment (T = 0 - 828 hr) is shown in Figure 14.

Bacterial Attachment and Biofilm Growth. Initially, bands near 1546 cm^{-1} and 1080 cm^{-1} rose rapidly (Figure 15). The 1546 cm^{-1}, amide II band (and 1650 cm^{-1}, amide I band, not plotted) are attributed to protein from the bacterial cells. The 1080 cm^{-1}, C-O stretching band, is indicative of carbohydrate material and is a combination of contributions from the mannitol in the culture medium and from bacterial cells that had adsorbed to the surface of the IRE (see discussion below). The 1546 cm^{-1} and 1080 cm^{-1} band intensities leveled off shortly after the flow of bacterial culture was shut off indicating that bacterial adhesion had stopped and that the cells were firmly attached to the membrane. After a 24 hr 'lag period', the amide II band intensity began to increase at a constant rate, rising from 11 mAU to 69 mAU by the end of a 267 hr (11 day) period. The 1546 cm^{-1} band never reached a plateau indicating biofilm growth was within the depth of penetration of the evanescent wave (400 nm at 1550 cm^{-1}). The 1080 cm^{-1} band intensity only increased 2-fold from 11 mAU to 23 mAU over the same period time seeming to indicate that carbohydrate was less of a factor than protein at the surface of the IRE (Figure 15).

An estimate of the biofilm thickness in terms of protein and carbohydrate (or polysaccharide) can be made based on the amide II and C-O stretch band intensities (14). If we assume that the absorptivity (*i.e.* 1450 cm^{-1})[1] of the biofilm protein(s) is similar to proteins such as myoglobin or β-lactoglobulin (15) and that 21 internal reflections occur at the Ge/biofilm interface then a protein layer approximate 15-20 nm thick exits on the surface of the IRE. If similar assumptions are made with the carbohydrate component of the biofilm (*i.e.* absorptivity of 2700 cm^{-1} similar to the polysaccharide, dextran and 21 internal reflections), the polysaccharide contribution to the biofilm is approximately 2-3 nm. These calculations assume that the protein and polysaccharide material is spread as a uniform layer across the surface of the IRE.

Biocide Penetration into a Biofilm. After 11 days from the start of the experiment, an aqueous solution of 0.2% BIT (Proxel GXL) was pumped through the flow cell. The goal at this point was to monitor biocide penetration into the biofilm and to determine what effect BIT had on the biofilm. The original intent was to use

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[1] In this case, cm^{-1} does not represent frequency in wavenumbers.

the 1602 cm^{-1} secondary amine of BIT to monitor biocide penetration into the biofilm (see Figure 16). However, it was not possible to do this as BIT was never present in high enough concentration at the Ge/biofilm interface to be detected. Instead, the effect of BIT was assessed indirectly by monitoring the 1381 cm^{-1} methyl deformation band of propylene glycol the chemical agent BIT is dissolved in. While chemical components in MSM contributes to this region of the IR spectrum, it was still possible to attribute changes in the 1381 cm^{-1} band intensity to the presence of propylene glycol.

A series of IR spectra of the biofilm in the presence of 0.2% BIT are displayed in Figure 14. The 1381 cm^{-1} band rose rapidly upon the addition of Proxel GXL to the flow cell. This initial rapid rise was due to a combination of bulk and adsorbed propylene glycol. The rapid rise in 1381 cm^{-1} intensity was followed by a slower constant rate of increase (see Figure 17). The 1080 cm^{-1} dropped immediately after Proxel GXL was pumped into the flow cell. The biocide solution was mistakenly made with deionized water instead of MSM (0.2% mannitol); therefore, the rapid drop in the 1080 cm^{-1} band was attributed to the loss of the bulk mannitol contribution to the infrared spectrum. After 7 hr the error was rectified, and a new solution of 0.2% BIT in MSM was substituted for the solution made with deionized water. At this point, the 1080 cm^{-1} band intensity rapidly increased to a level of 19.6 mAU slightly below its initial level of 22.5 mAU.

After 12 hr the rate at which the 1381 cm^{-1} band increased dropped indicating a slower rate of propylene glycol accumulation at the Ge/biofilm interface. The 1546 cm^{-1} band intensity dropped throughout the entire 24 hr period indicating that biofilm growth was inhibited by the presence of the chemical biocide. The slow drop of the amide II band intensity may be indicative of the sloughing of dead and/or loosely bound cells from the surface of the biofilm. However, closer inspection of the IR spectra revealed that the drop in 1546 cm^{-1} band intensity was due to the displacement of MSM with chemical components of Proxel GXL. Despite the complications associated with overlapping absorption bands, Proxel GXL did appear to inhibit growth of bacteria within the biofilm.

Biofilm Regrowth and Biocide Desorption. After 24 hr the biocide flow was stopped, and the flow of MSM restarted. Much of the propylene glycol was retained at the Ge/biofilm interface as the 1382 cm^{-1} band dropped at a slow constant rate over the remaining 6 days. When MSM was reintroduced to the flow cell, a 2 - 2.5 mAU increase in the 1546 cm^{-1} band intensity occurred within the few hours. The increase in the 1546 cm^{-1} band intensity is believed to be associated with the displacement of Proxel GXL at the interface by MSM and not to the rapid production of biomass (in the form of protein) at the interface. The rate of increase in 1550 cm^{-1} band intensity was followed by an approximate 20 - 24 hr lag period where little change in the amide II band intensity was observed. As the bulk and adsorbed propylene glycol concentration around the biofilm was reduced bacterial cells within the biofilm began to grow and multiple again. The gradual increase in the 1546 cm^{-1} was not due to the accumulation of chemical components of MSM at the interface as both the amide I and amide II bands increased concurrently. The protein band rose at a steady rate for

the remaining 6 days indicating complete disinfection of the attached biofilm was never achieved.

At the conclusion of the experiment, the Ge IRE was removed from the flow cell and visually inspected. Most of the biofilm had formed around the edges of the flow cell where the o-rings made contact with the IRE. The biofilm was stained with DAPI and observed by fluorescence microscopy (Figure 18).

Proxel GXL Adsorption on and Desorption from a Ge IRE

Adsorption. The adsorption of Proxel GXL on a bare Ge IRE was investigated. The Ge IRE was preconditioned with MSM for 24 hr before 0.2% BIT was pumped through the flow cell. After 24 hr, MSM was substituted for the biocide solution and pumped through for 24 hr. The 1381, 1240 and 1078 cm^{-1} band intensities associated with Proxel GXL are plotted as function of time in Figure 19. The 1381 cm^{-1} CH_3 deformation and C-O stretching bands of propylene glycol increased rapidly. However, the magnitude of the 1381 cm^{-1} band increase was 2-fold less as compared to sorption into the mycobacterium biofilm. These results indicates that propylene glycol was concentrated on or within the biofilm.

Desorption. A much slower rate of desorption was observed in the presence of the biofilm as compared to desorption of Proxel GXL from the bare Ge IRE. When MSM was substituted for the biocide solution, the 1381 cm^{-1} band intensity dropped at two different rates. Initially the rate of loss was rapid (see Figure 20). This rapid drop in 1381 cm^{-1} band intensity was associated with the removal of the bulk (nonadsorbed) biocide from the flow cell. A second slower rate of decrease followed which was attributed to the desorption of propylene glycol that was reversibly adsorbed to the surface of the Ge IRE. During the MSM rinse period, the 1381 cm^{-1} band intensity dropped to a level of 17.2 mAU and then gradually increased to 20.3 mAU, near the level (21.1 mAU) prior to Proxel GXL addition. This effect is believed to occur as propylene glycol desorbed from the Ge/water interface and was slowly replaced by the sorption of chemical components in the MSM.

Mycobacterium Biofilm Formation on a Thin Film of Cellulose Acetate

Attachment and Growth. Microbial attachment and growth on cellulose acetate was investigated by pumping a 48 hr old culture of FT-8 through a flow cell

containing a Ge IRE coated with a thin film (80 - 100 nm) of CA. A series of IR spectra collected periodically over 11 days are displayed in Figure 21. The four major bands of CA all *increased* steadily from the start of flow (see insert Figure 22). In previous experiments, the intensities of absorption bands dropped when the thin film was initially exposed to aqueous solutions. This effect was attributed to swelling of the polymer so that less material was retained within the depth of penetration of the evanescent wave. At this time, we are unable to explain why the CA band intensities increased when the bacterial culture was pumped into the flow cell. After 50 min all four CA band intensities dropped. An almost periodic trend of fluctuations occurred in the CA band intensities during the first 72 hr. However, careful scrutiny of variables such as the water subtraction factor and the time at which the detector was refilled with liquid nitrogen did not reveal a specific cause for the observed fluctuations in band intensities. The general trend of rapid CA removal from the Ge surface occurred over a 72 hr period, *i.e.* rapid removal relative to the 'control' experiment where a similar CA thin film was only exposed to sterile deionized water. The carbonyl band intensity dropped from 0.151 A.U. to 0.067 A.U. in 72 hr. By the end of 11 days, the 1753 cm^{-1} band intensity was 0.062 A.U. This change corresponds to a 58% drop in intensity and means that virtually all of the CA in contact with aqueous phase had been removed after 72 hr of exposure to mycobacterium isolate FT-8.

The 1653 cm^{-1} amide I and 1547 cm^{-1} amide II band intensities are plotted as a function of time in Figure 23. The amide II band overlapped with the 1495 cm^{-1} band associated with the organic contaminant. Therefore, to verify the that the amide II band did increase with time and that the increase in peak height was not due to contributions from the adsorbed contaminant, the overlapping bands were resolved by deconvolution and curve fitting. The integrated intensities of the amide I and II bands are plotted as a function of time in the bottom trace of Figure 23. Similar changes in band intensity were observed for plots of area and peak height. There are minor contributions from MSM to the region of the spectrum around 1550 cm^{-1}; however, the component that gives rise to this band did not accumulate at the surface of IRE (data not shown). The organic contaminant does not contribute to the spectrum between the 2000 cm^{-1} band and 1500 cm^{-1}. Therefore, these changes in the amide I and II bands were attributed to protein from the bacterial cells attached to the surface of the IRE. The amide I and II band intensities appeared to reach a plateau level near T = 200 hr. An IR spectrum of the biofilm that had existed at the polymer/Ge interface at T = 262 hr is displayed in Figure 24. Protein and the organic contaminant are the most dominate components of the biofilm. The most intense bands of the organic contaminant are visible near 1500, 1376, 1242 and 1142 cm^{-1}.

Deterioration of Thin Film of Cellulose Acetate. Initial results indicate that deterioration of the CA film is enhanced in the presence of a monoculture of mycobacterium. Major CA band intensities at 1753 cm^{-1}, 1236 cm^{-1} and 1051 cm^{-1} dropped approximately 60% after 84 hr exposure to mycobacterium indicating that very little (5 - 10%) polymer remained on the surface of the IRE exposed to the aqueous phase. The methyl deformation band near 1369 cm^{-1} only dropped by 20%

in the same period of time; however, this was due in part to the adsorption of the organic contaminant that contributes to this region of the spectrum. A similar (80 - 100 nm) CA film that was exposed to deionized water demonstrated much greater stability. In that experiment, the carbonyl band intensity only dropped 16% after 85 hr exposure to deionized water at pH 7, and after 170 hr 40 - 45% of the film still remained in contact with the aqueous phase (see Figure 11).

CONCLUSIONS

The feasibility of using attenuated total reflection Fourier transform infrared spectrometry to study biofilm/biocide interactions at an aqueous/polymer interface were investigated. Theoretical calculations of internal reflection at a Ge/polymer/water interface indicate that a monolayer of bacterial cells can be detected at the surface of a thin film (~80 - 100 nm) of cellulose acetate cast onto a Ge internal reflection element.

The physical and chemical stability of cellulose acetate (43% acetyl) films cast onto a Ge IRE were investigated by exposing the polymer to deionized water. Significant changes occur in the IR spectrum when the CA film is initially exposed to water. These changes were attributed to polymer swelling and/or physical separation of the polymer from the surface of the IRE. A 200 nm thick film deposited on the 45°, 50 x 10 x 2 mm, parallelepipped Ge IRE was too thick to allow for measurements of adsorption phenomena at the aqueous/polymer interface. Typically, a 40 - 50% drop in CA band intensities were observed after 160 - 170 hr exposure to deionized water. The optimum film thickness appears to be between 100 - 150 nm.

An unidentified organic contaminant readily adsorbed to the surface of 80 - 100 nm thick CA films (and the bare Ge IRE). The exact source of the contaminant has yet to be determined. The adsorption of this macromolecule(s) to the IRE and CA film was viewed as a 'conditioning film'. The organic species was not displaced by the chemical biocide or the mycobacterium biofilm.

A significantly greater rate of deterioration was observed when the CA film was exposed to a monoculture of an RO membrane fouling bacterium (*Mycobacteria sp.*). This result suggests that the presence of a biofilm on an RO membrane may contribute to the degradation of transport properties apart from the physical fouling or blockage that occurs. The presence of a biofilm retarded penetration of a chemical biocide to the aqueous/solid interface. However, approximately 2-fold more chemical components from the biocide, Proxel GXL, were concentrated near the surface of the IRE in the presence of the mycobacterium biofilm as compared to sorption on bare Ge. The presence of this chemical biocide inhibited biofilm growth. Chemical components of the biocide were retained in the biofilm to a greater extent as compared to the bare Ge surface. However, once the flow of the biocide to the flow cell was stopped, biofilm growth continued after a brief lag period, indicating that the substratum was not completely disinfected.

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Figure 1.

Effect of Polymer Thickness on Mean Square Electric Field Intensity at the Aqueous/Polymer Interface

Germanium / Polymer / Water

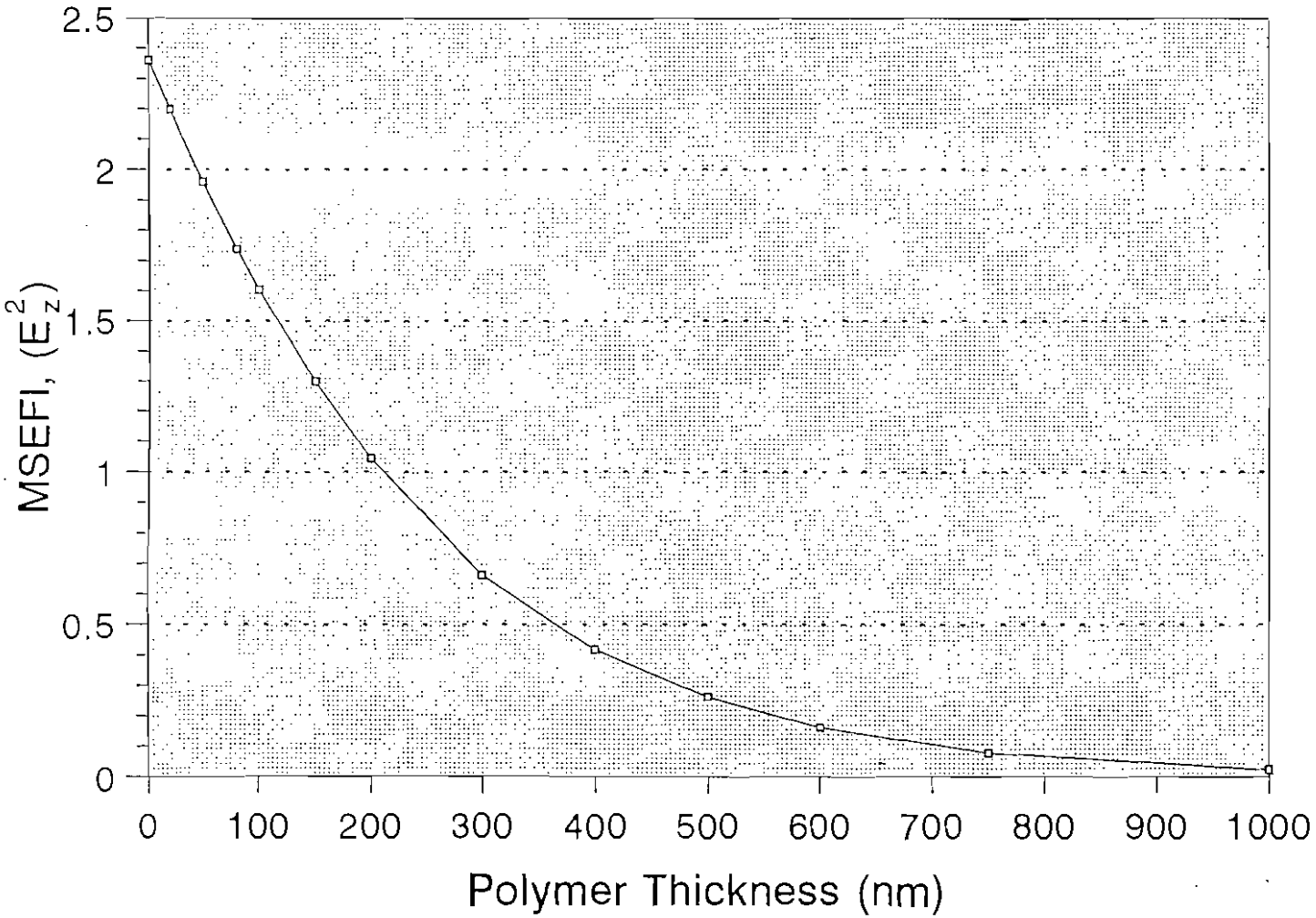


Figure 2.

Effect of Polymer Film Thickness on Water Band Intensity

Three-Phase System: Germanium / Polymer / Water

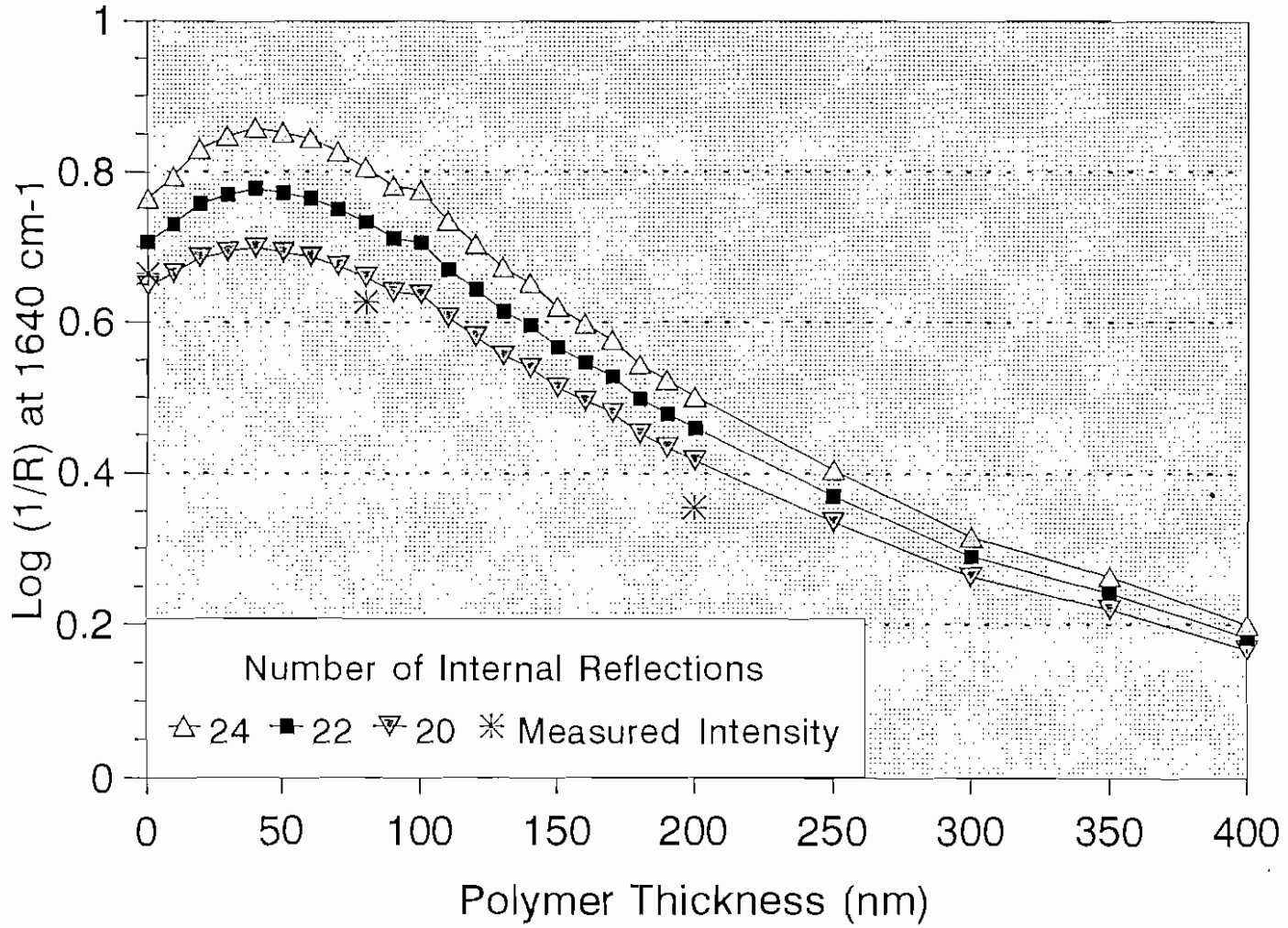


Figure 3.

Cellulose Acetate Film (43% Acetyl) 200 nm Thick

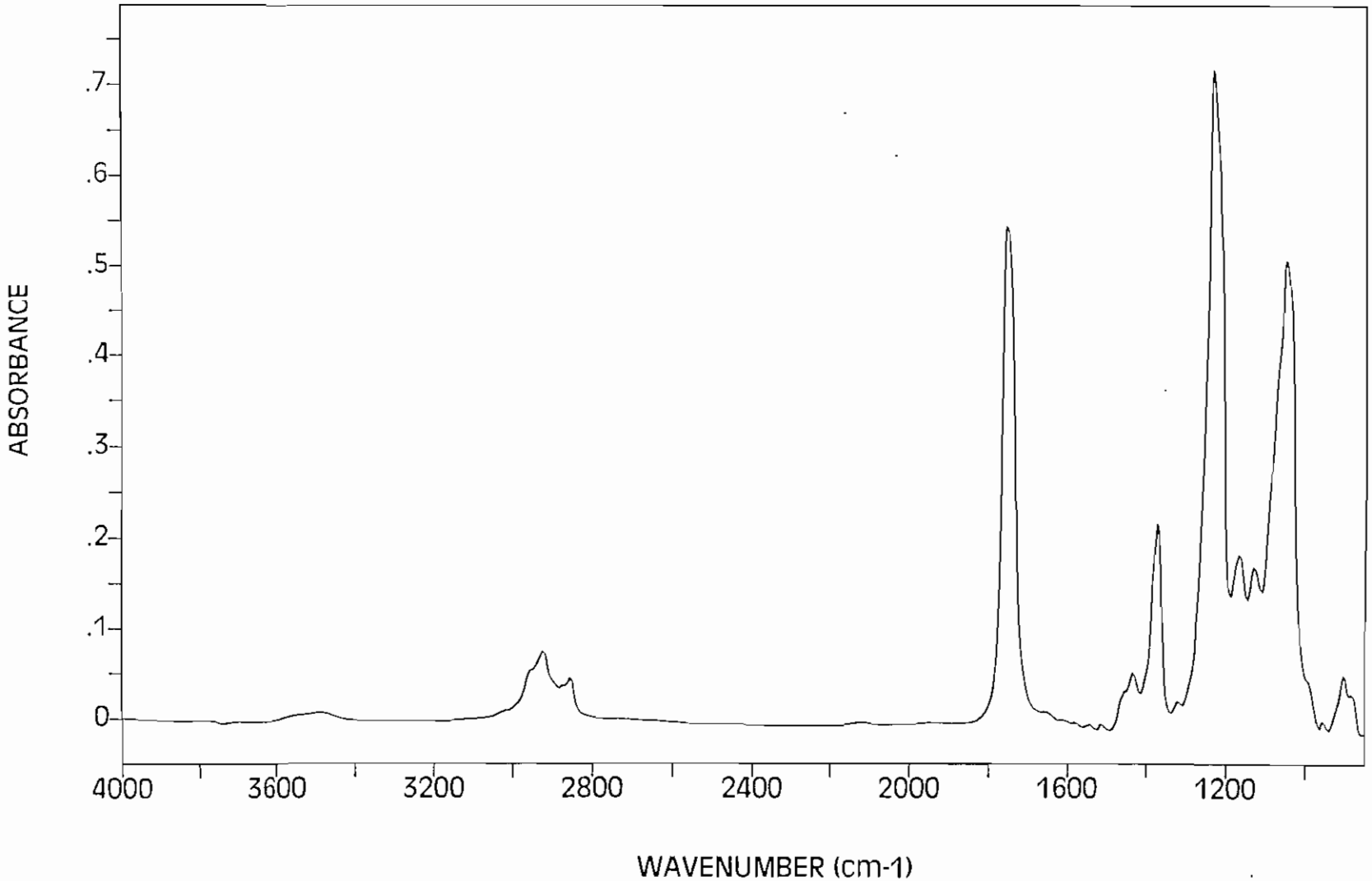


Figure 4.

Cellulose Acetate Film (43% Acetyl) 200 nm Thick

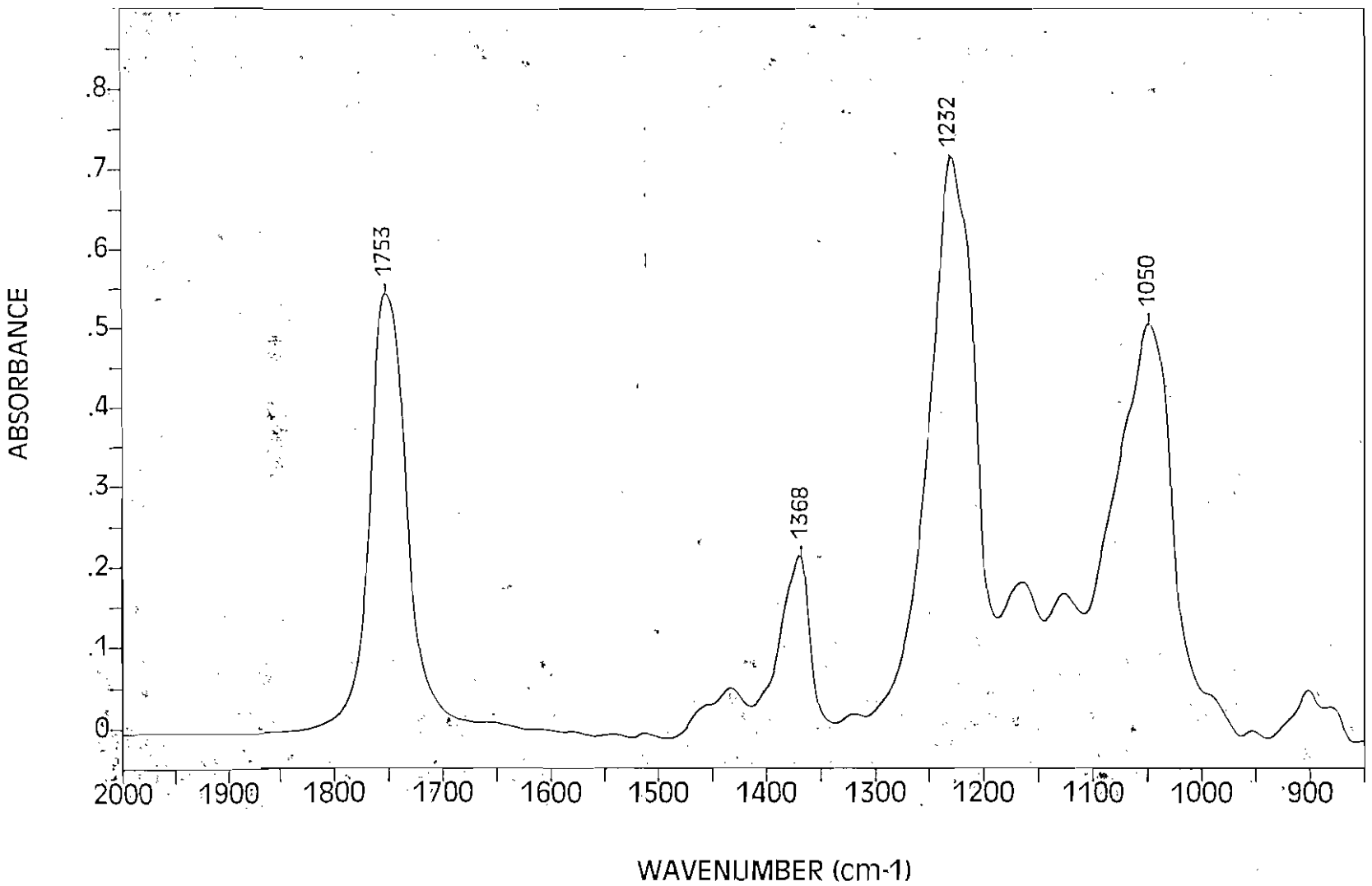


Figure 5.

Cellulose Acetate (triacetate and diacetate)

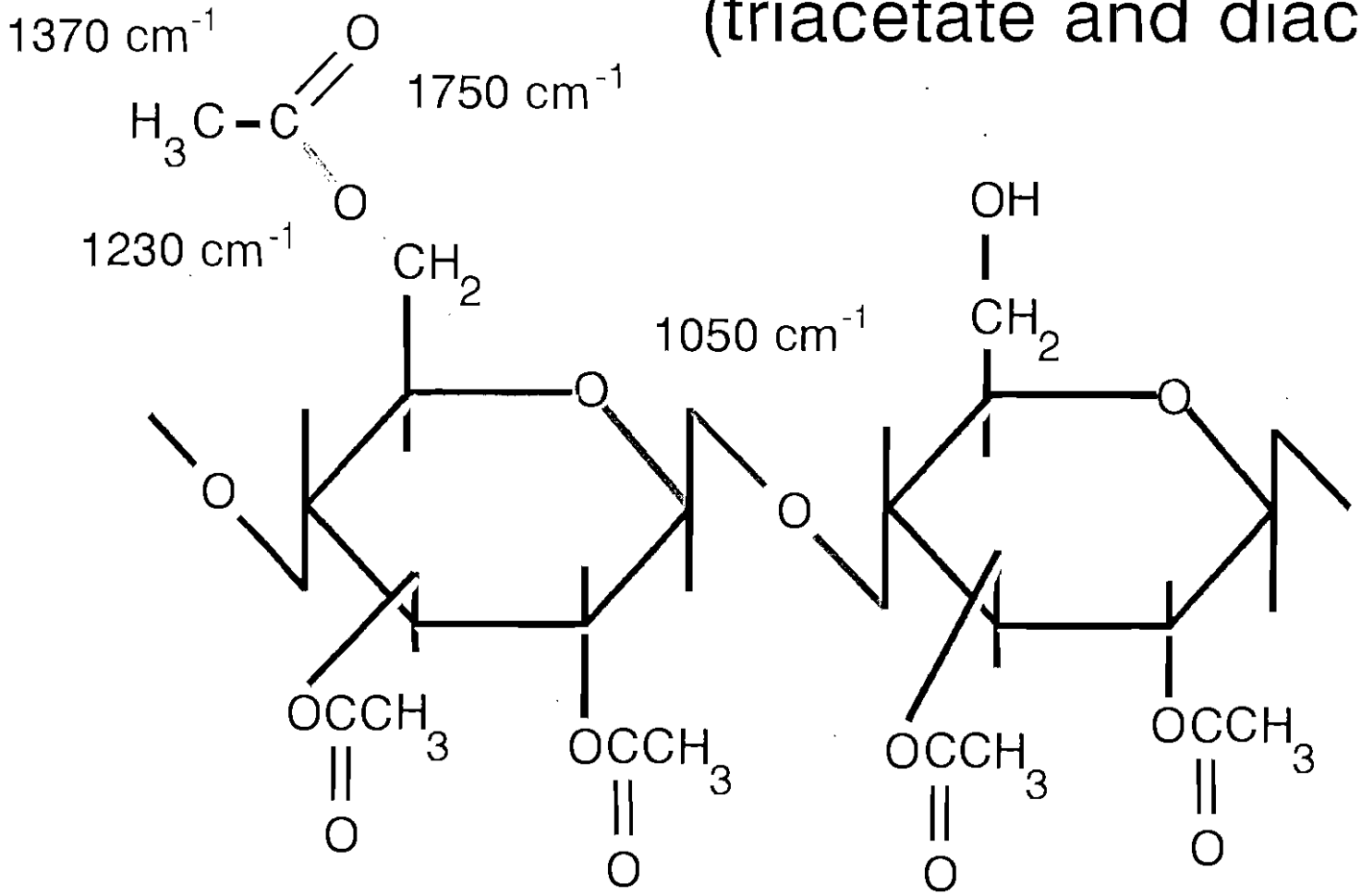


Figure 6.

Cellulose Acetate Film

Ultrapure Water Control pH 7

200 nm Thick Film (43% Acetyl) on Ge IRE

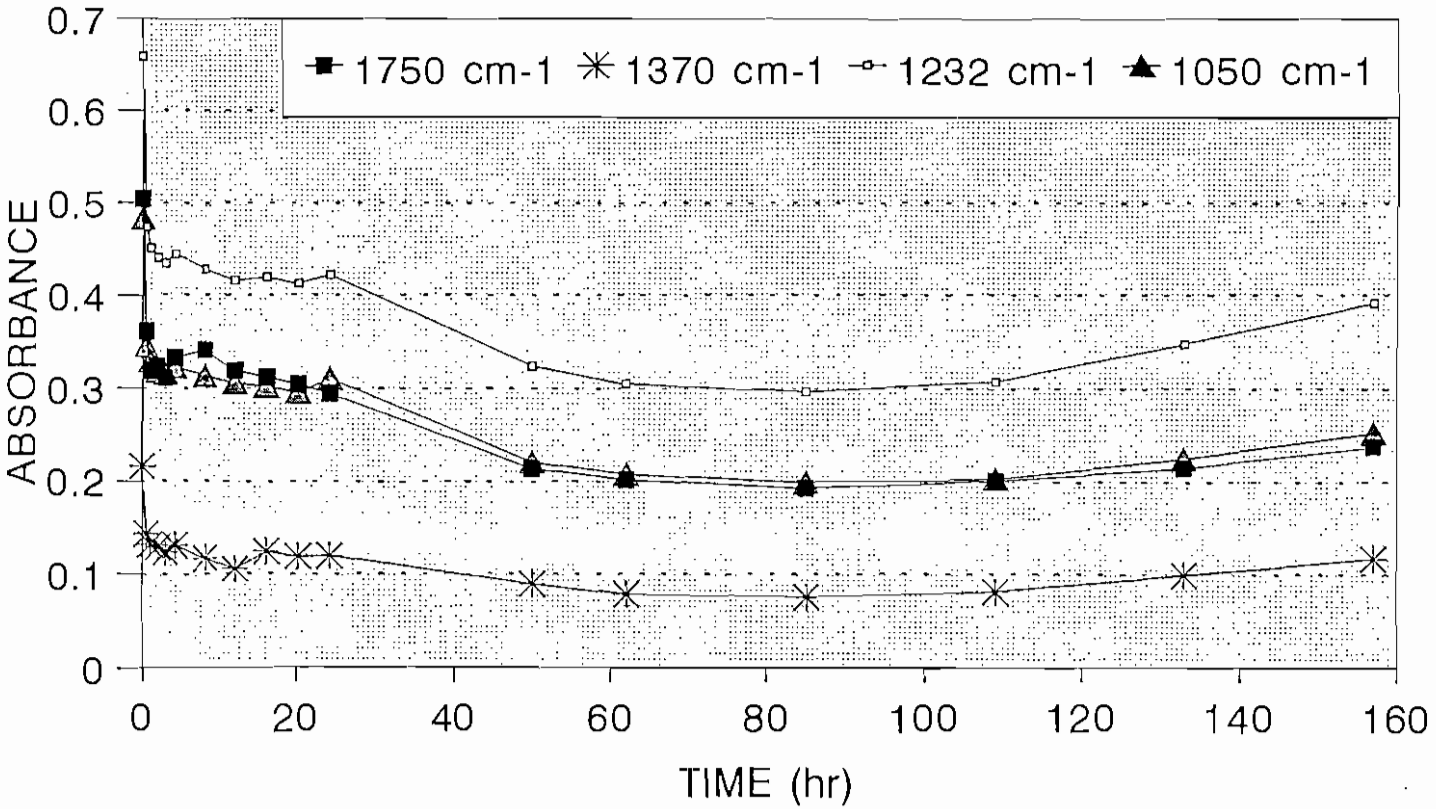
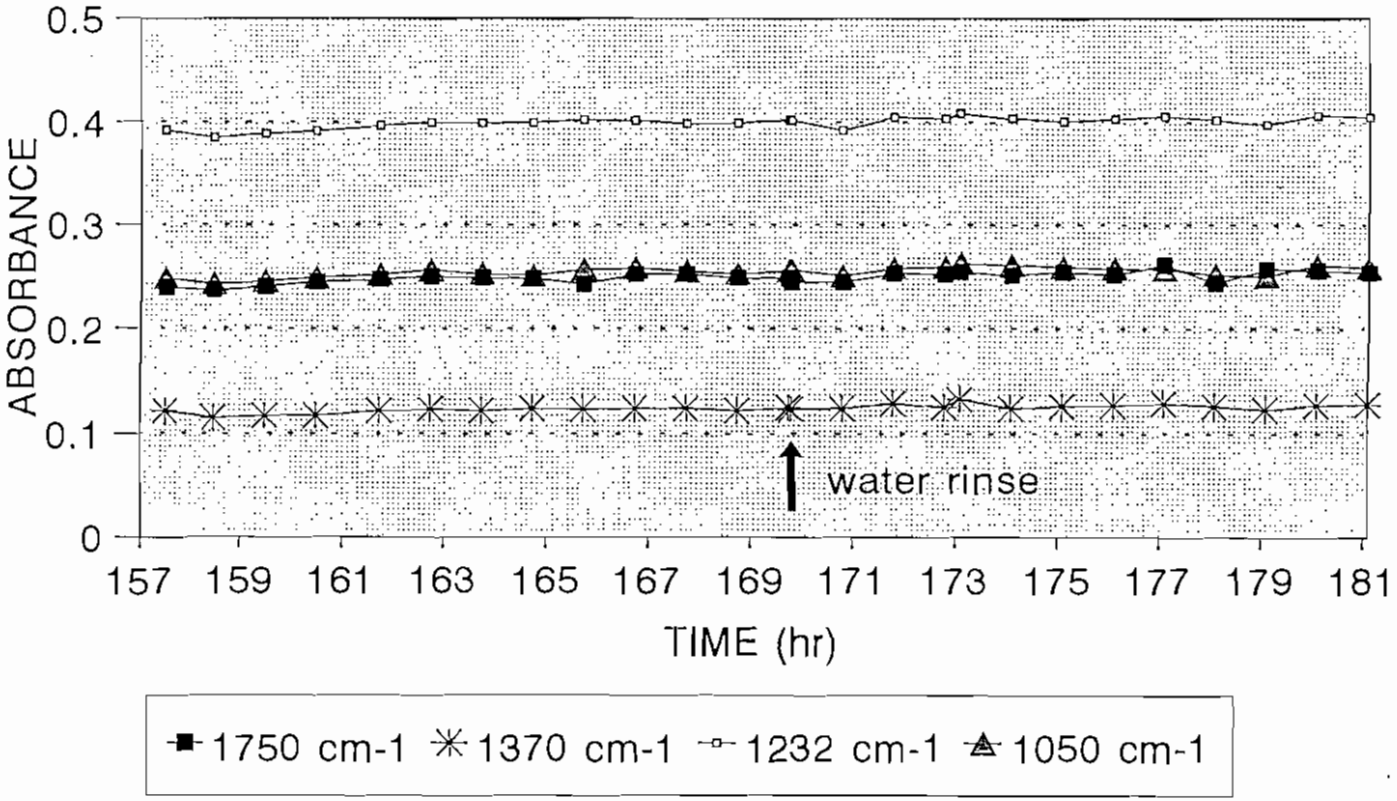


Figure 7.

Cellulose Acetate Film

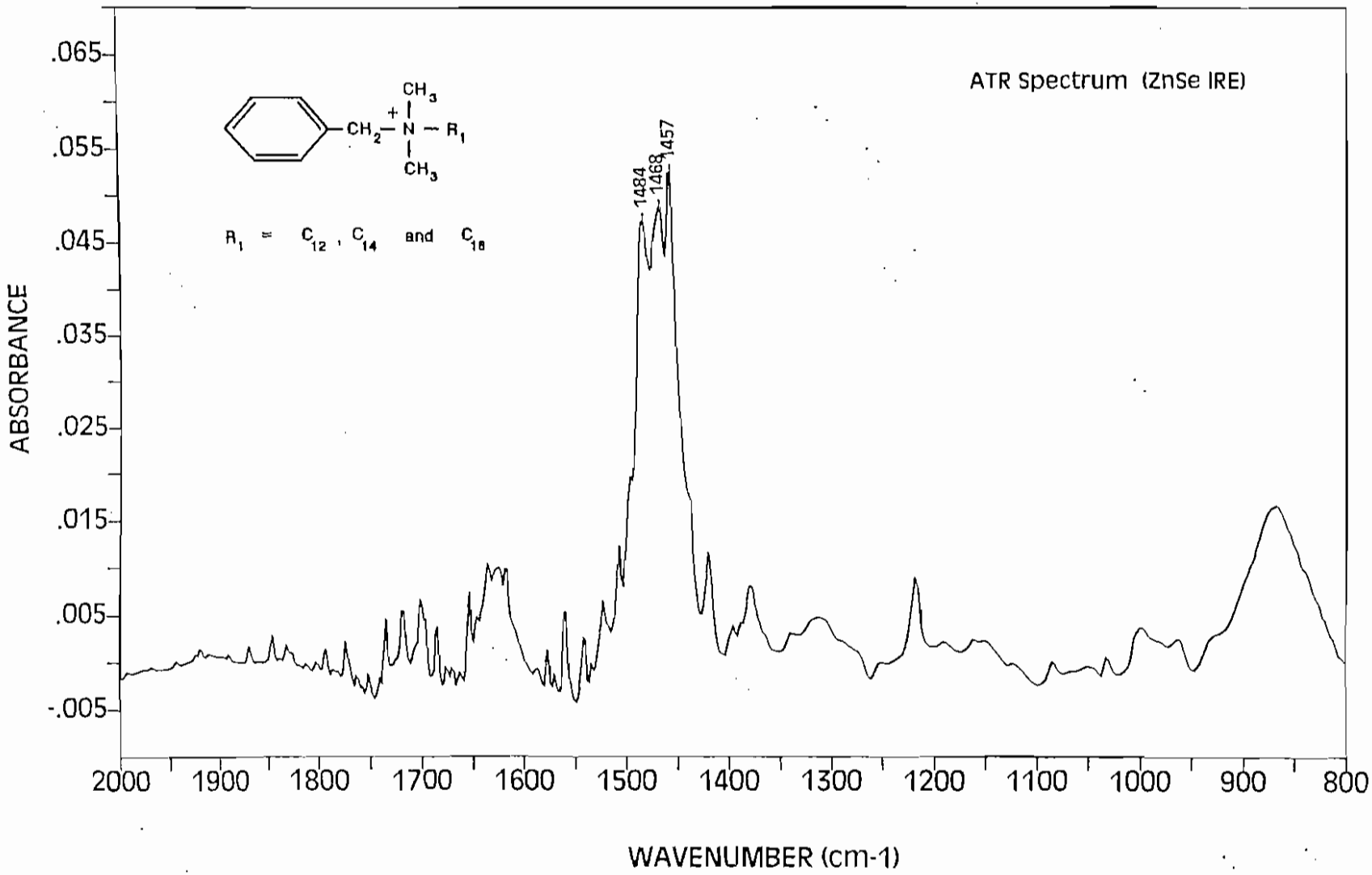
0.5% Benzalkonium Chloride Addition pH 7

200 nm Thick Film (43% Acetyl) on Ge IRE



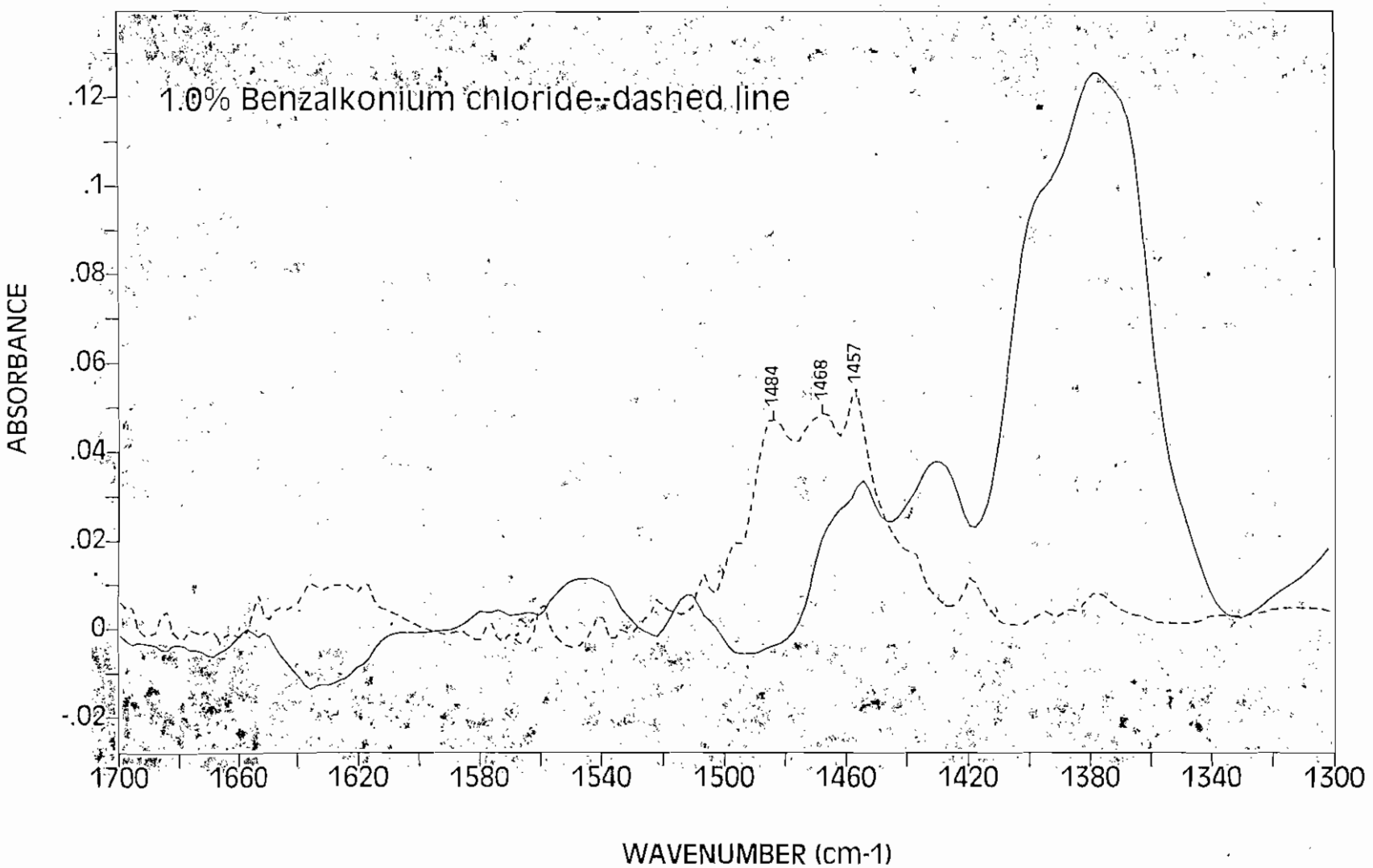
1.0% Benzalkonium chloride

Figure 8.



Cellulose Acetate Film on Ge IRE (200 nm) T = 170 hr

Figure 9.



Cellulose Acetate Film 80 - 100 nm Thick

Figure 10.

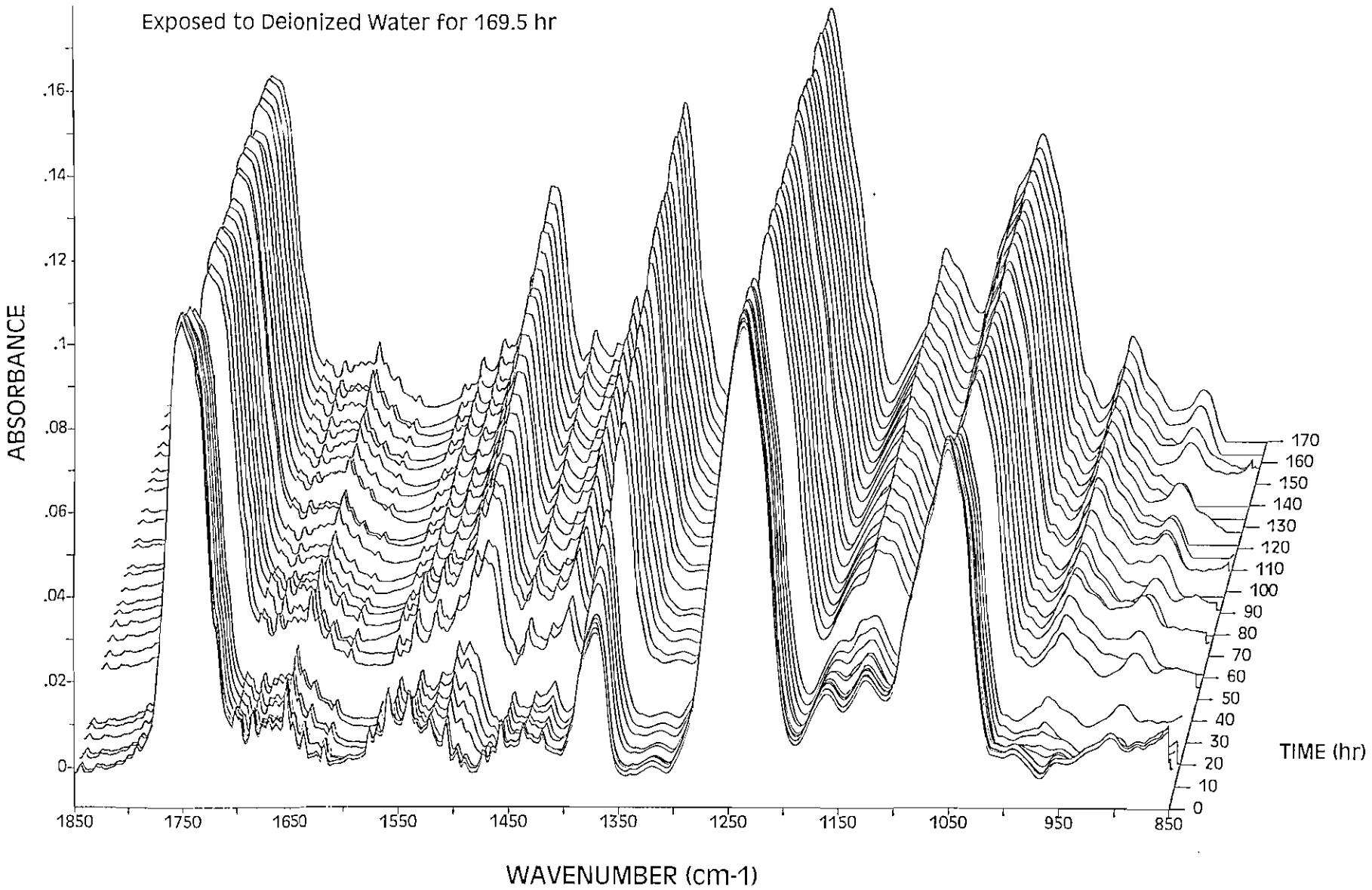
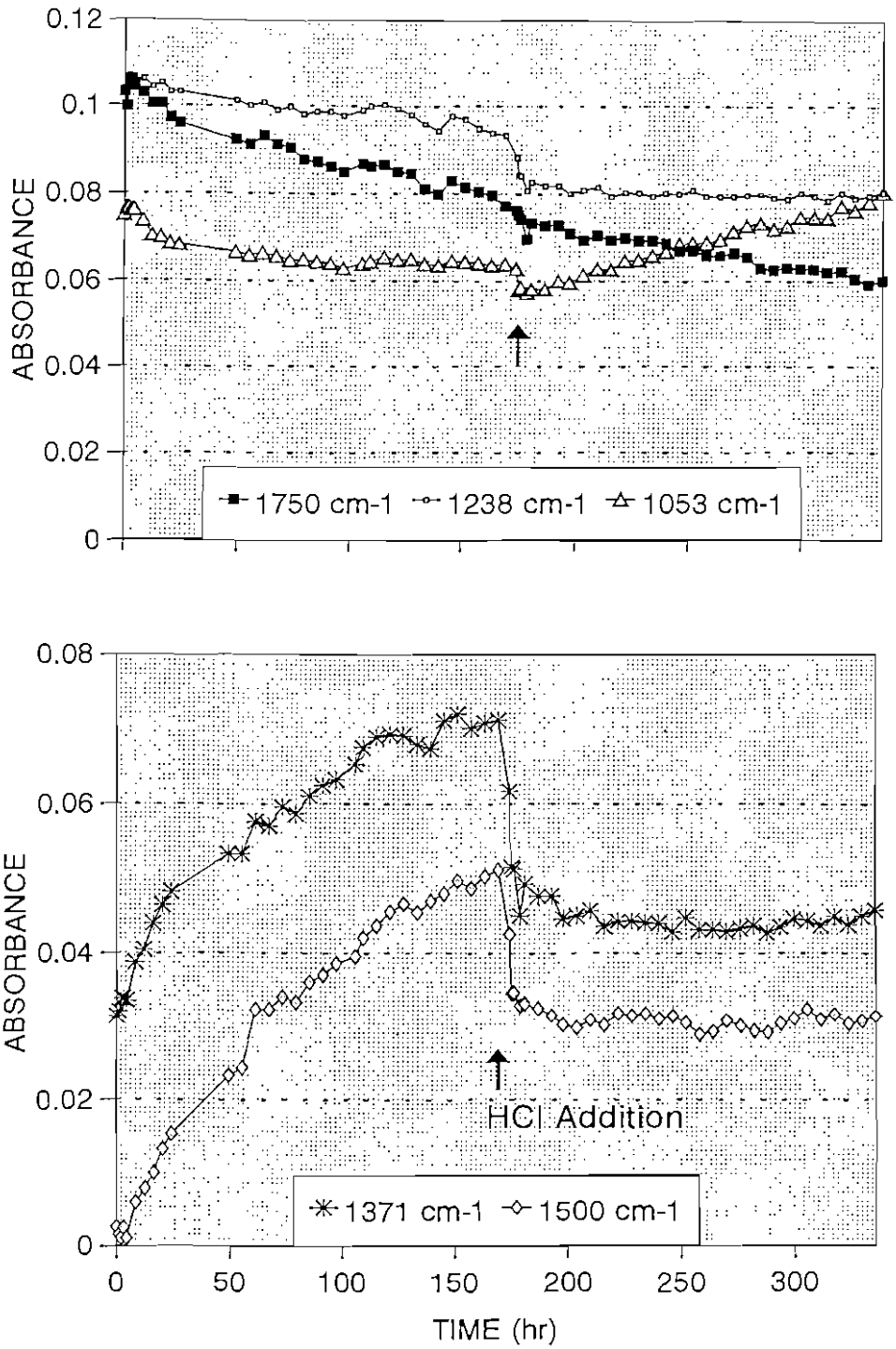


Figure 11.

Cellulose Acetate Film

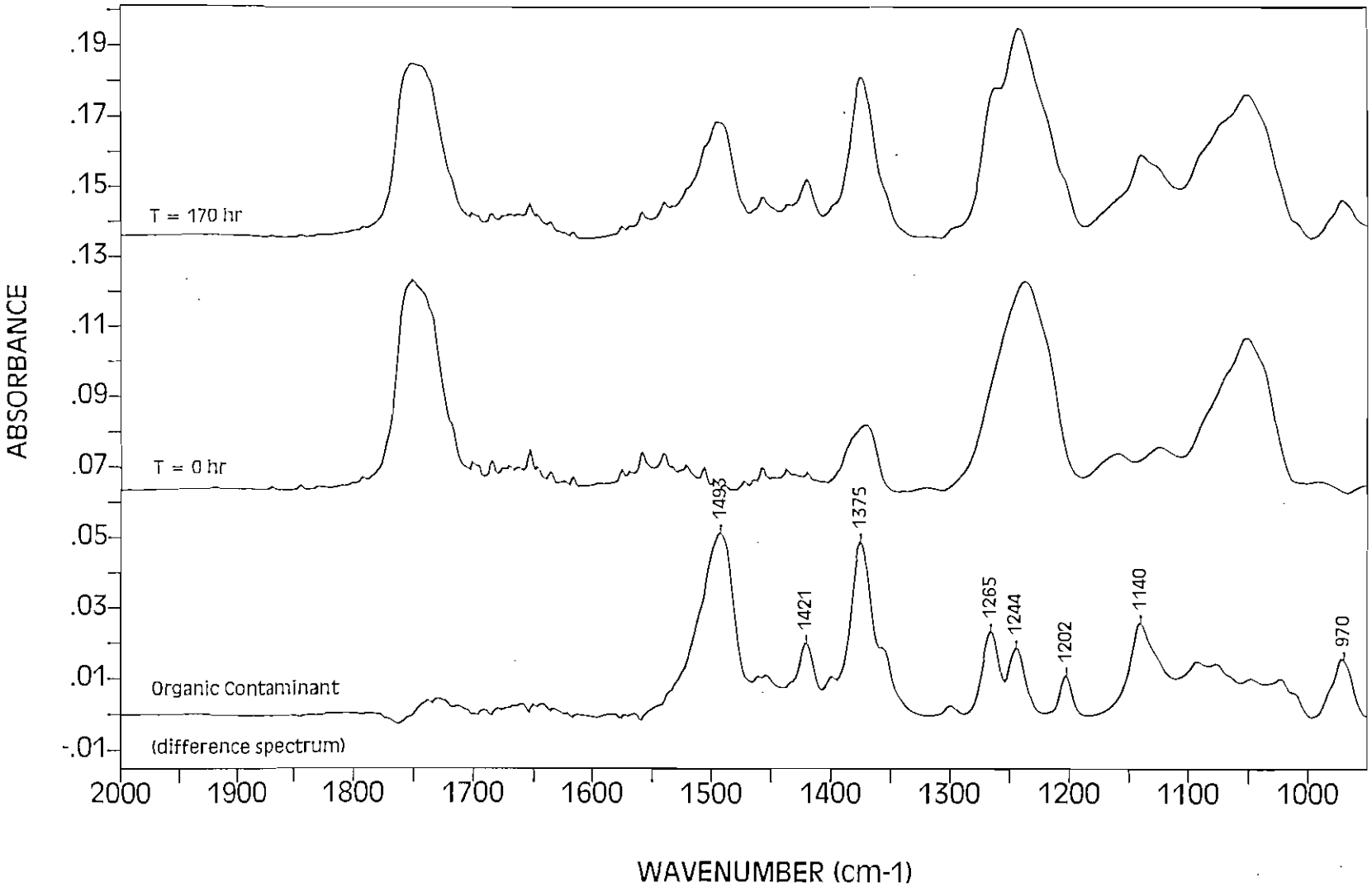
HCl Hydrolysis pH 2

80 nm Thick Film (43% Acetyl) on Ge IRE



Cellulose Acetate Film (43% Acetyl) 80-100 nm Thick

Figure 12.



Cellulose Acetate Film (43% Acetyl) 80-100 nm Thick

Figure 13.

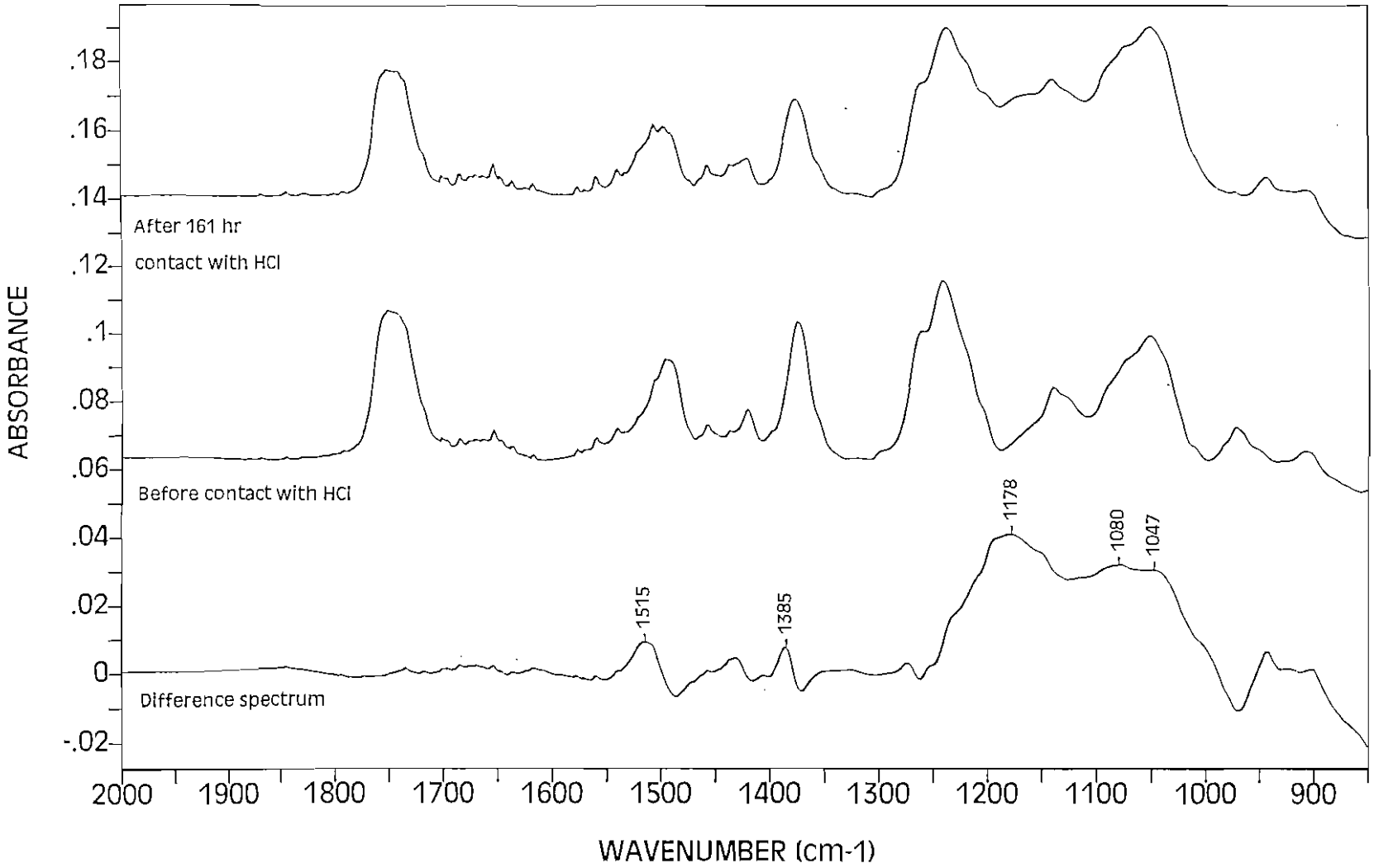
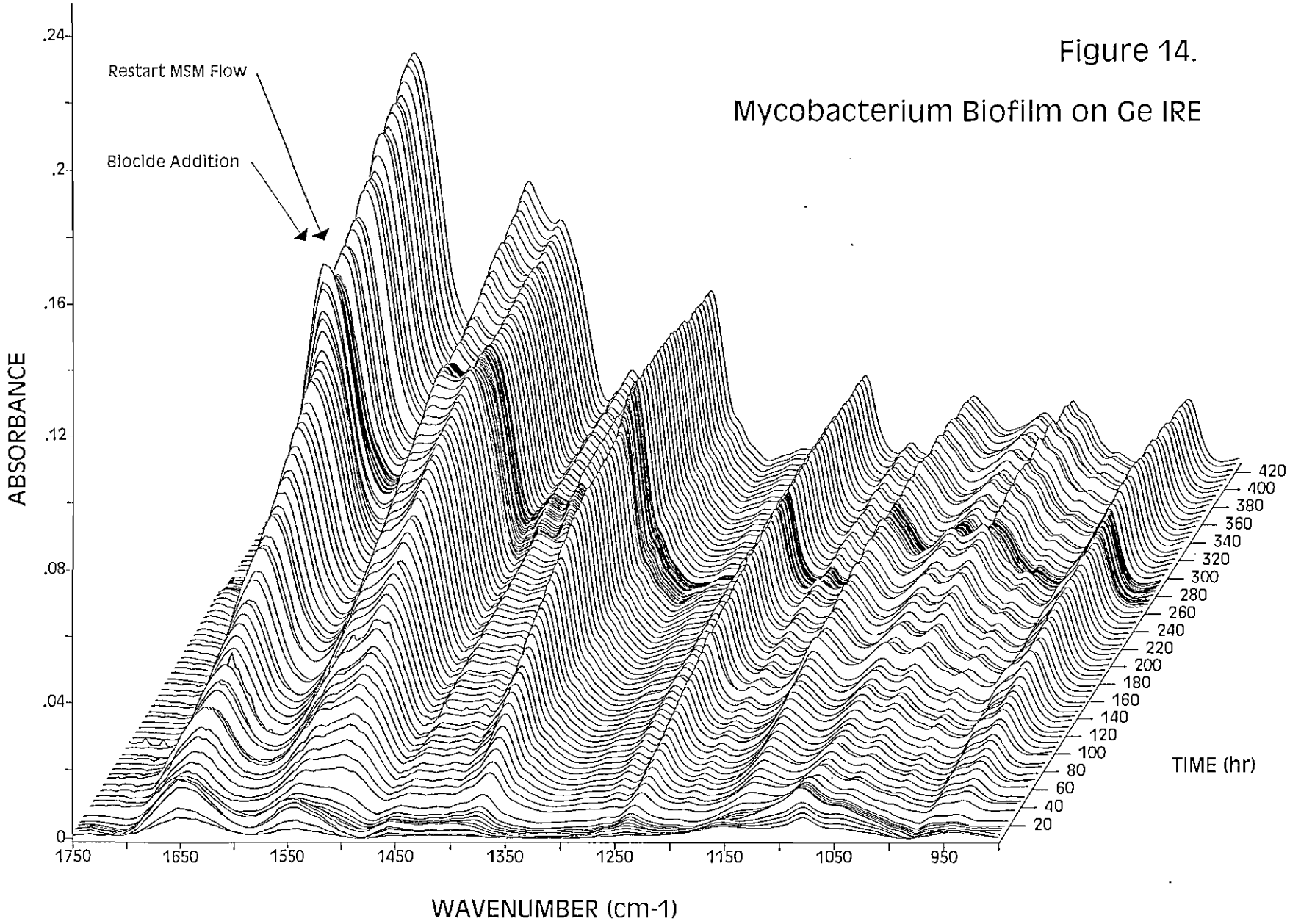
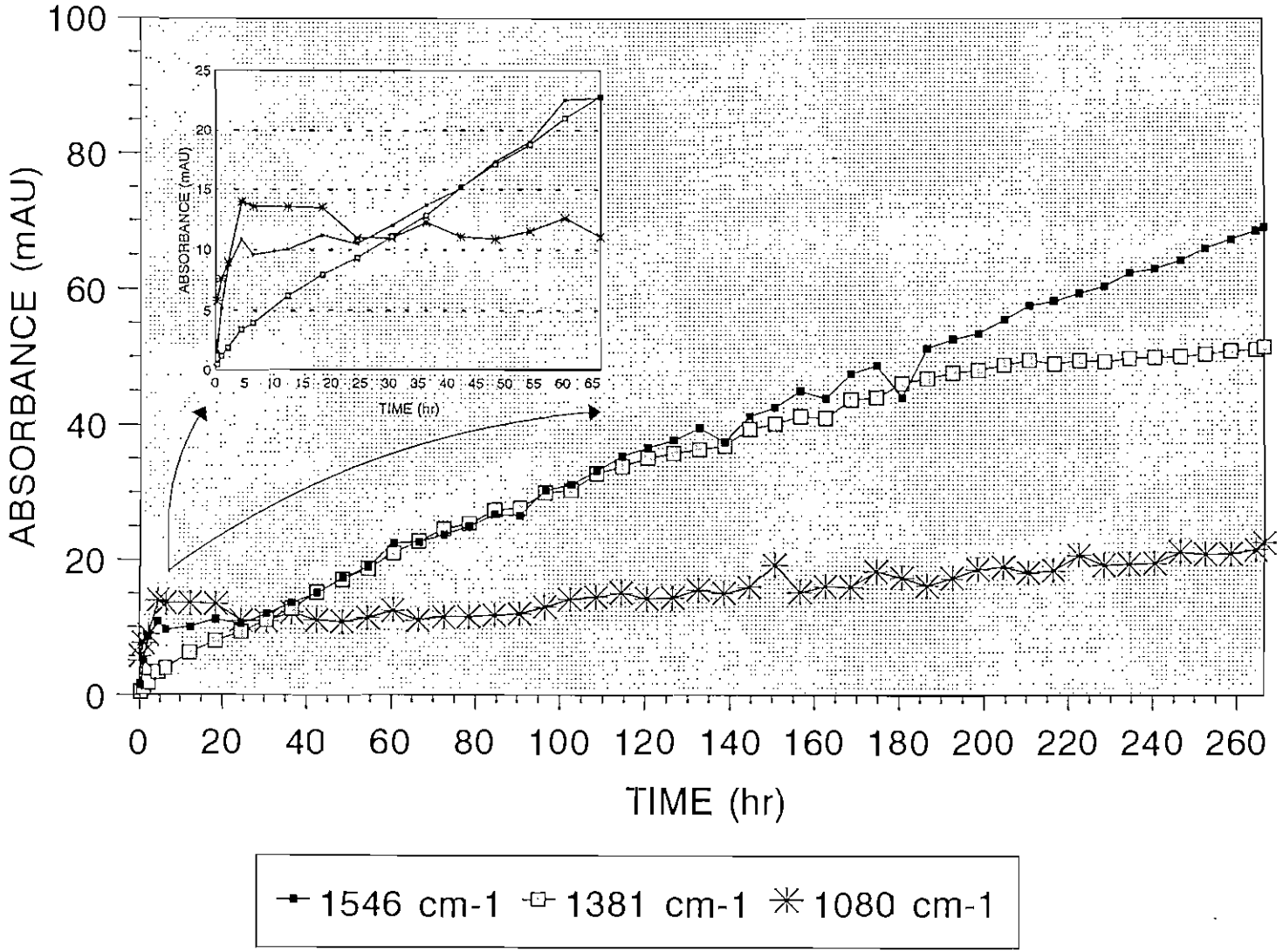


Figure 14.

Mycobacterium Biofilm on Ge IRE



Biofilm Formation on Ge IRE Figure 15.



0.1% BIT (Proxel GXL) pH 7

Figure 16.

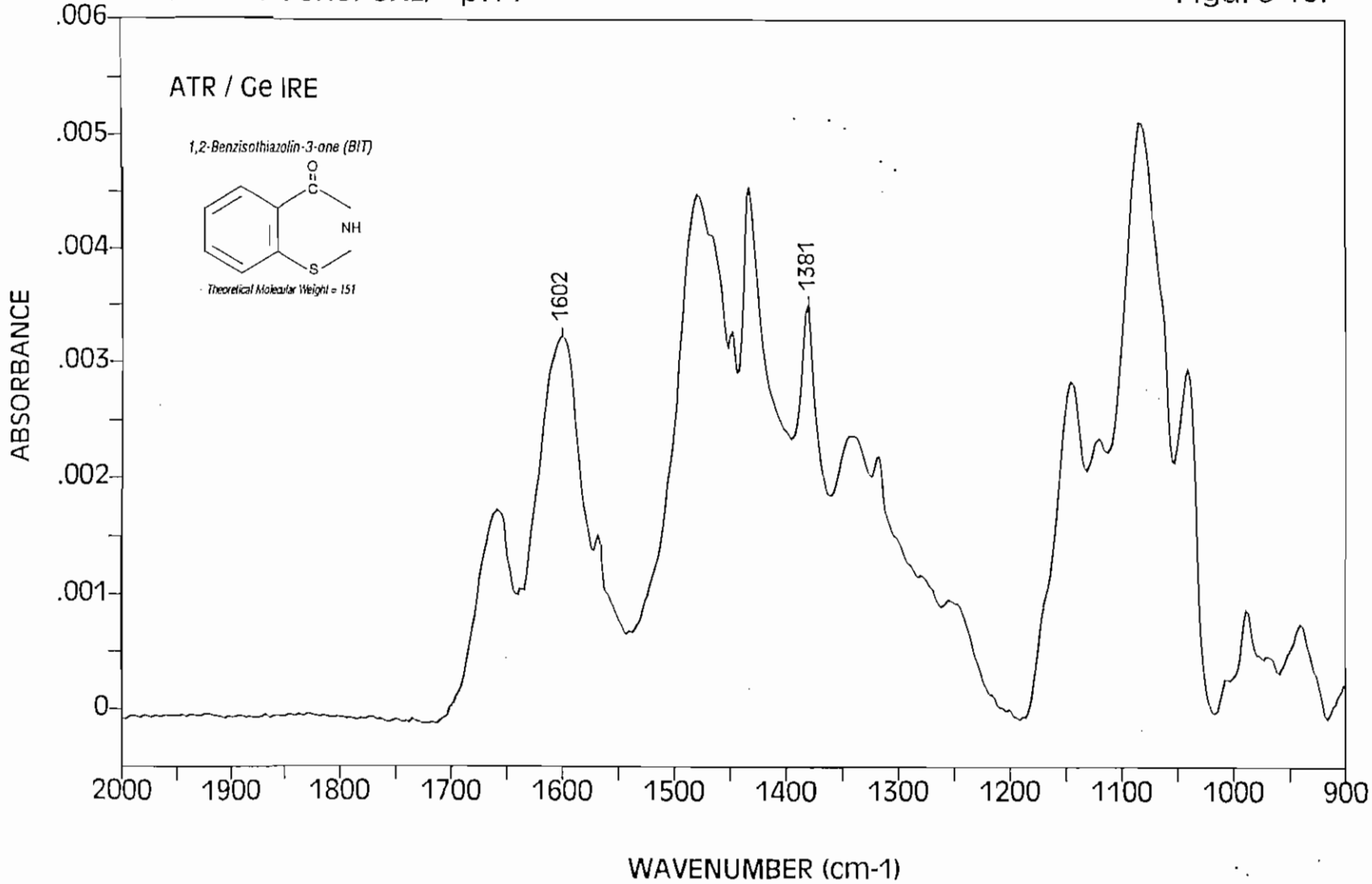


Figure 17.
Biocide Penetration into Biofilm

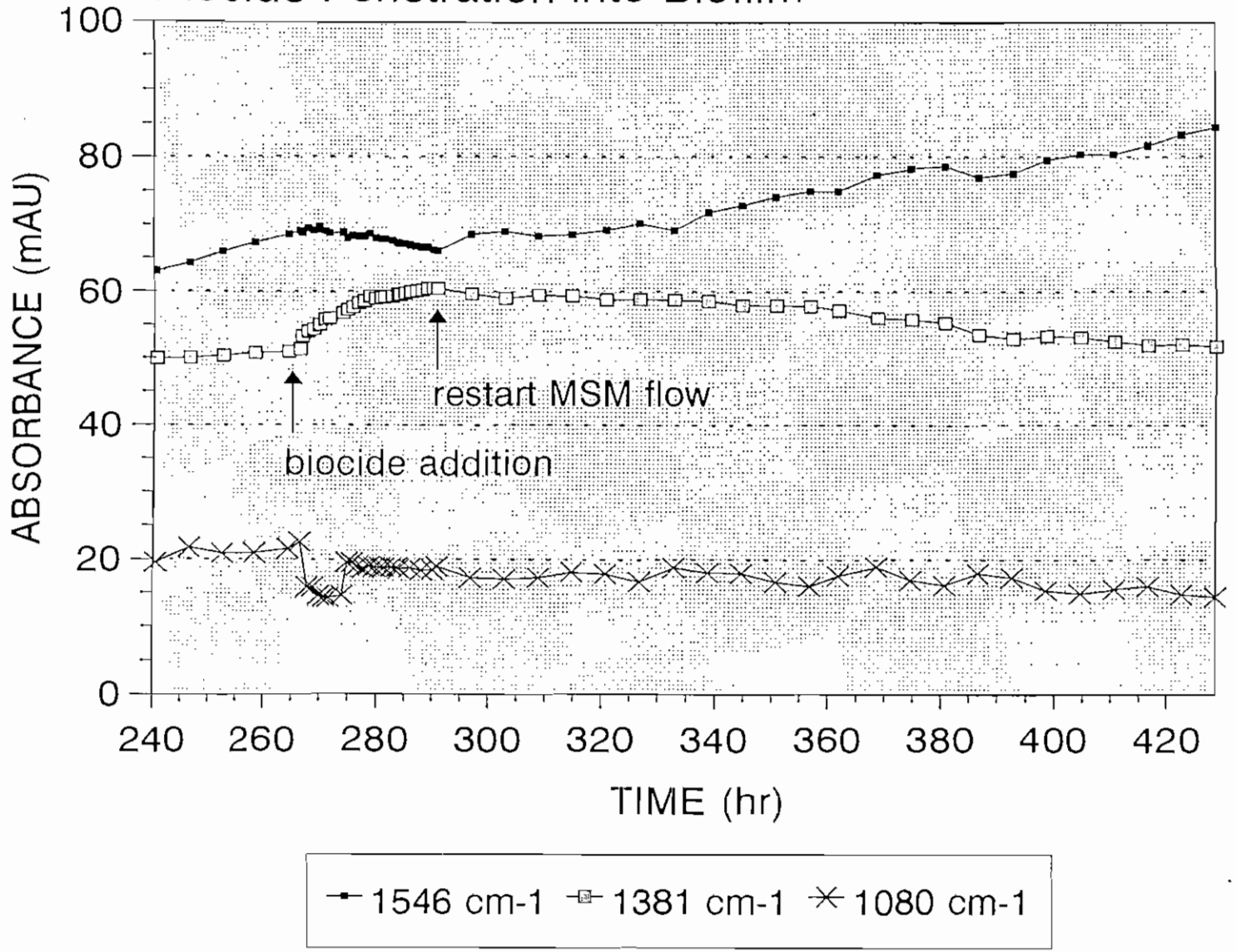


Figure 18.

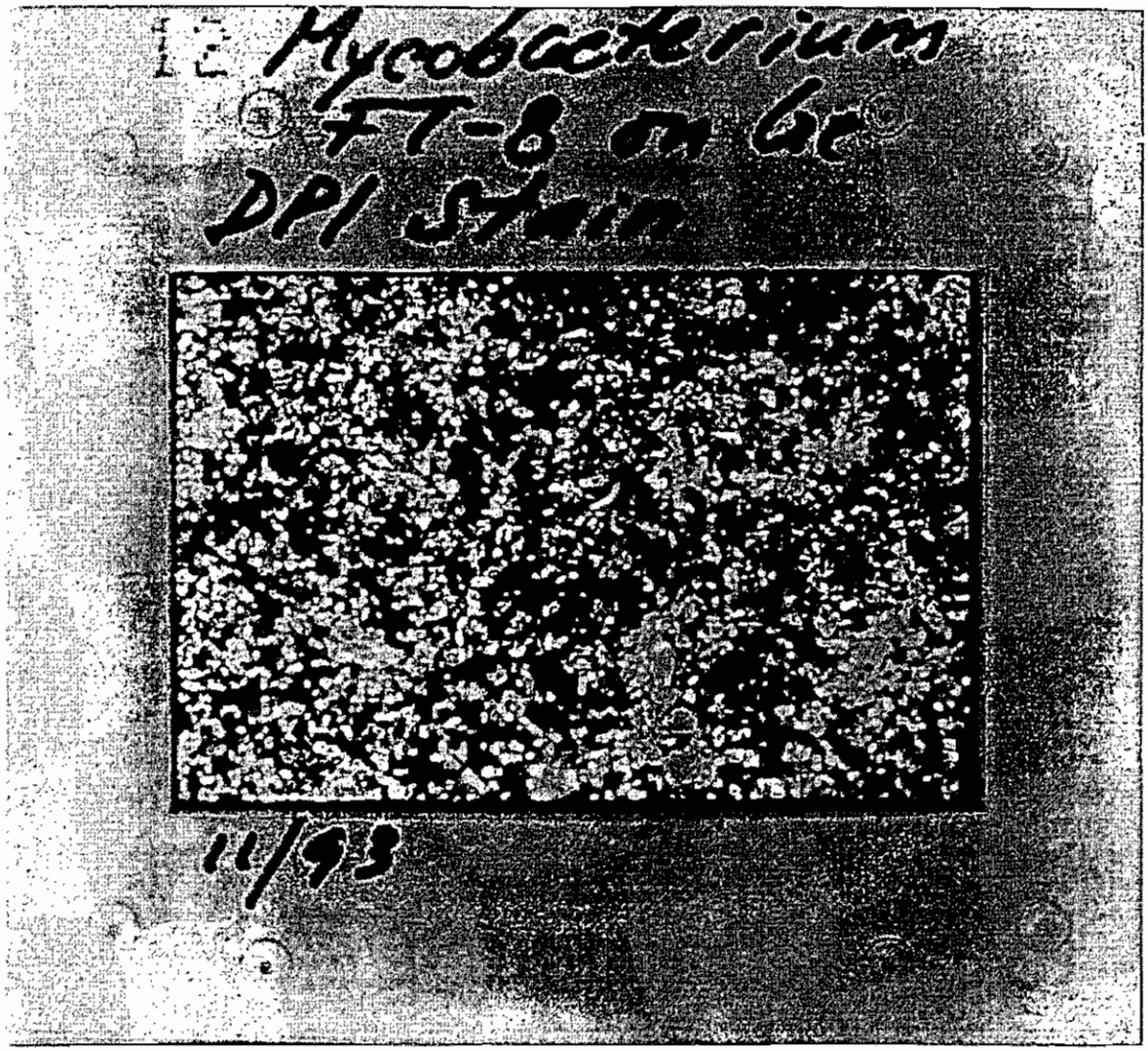


Figure 19.
Biocide Adsorption on Ge IRE

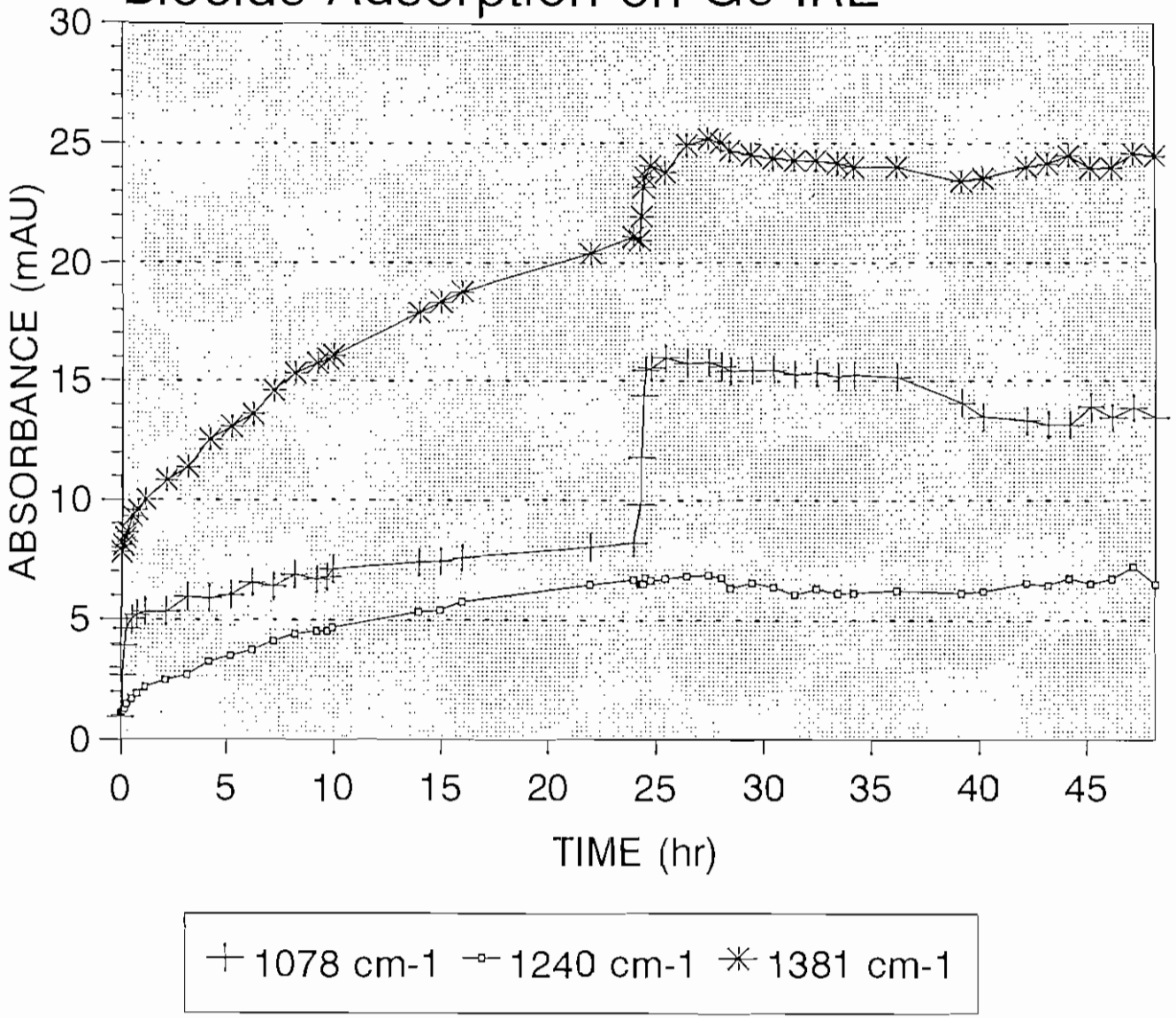
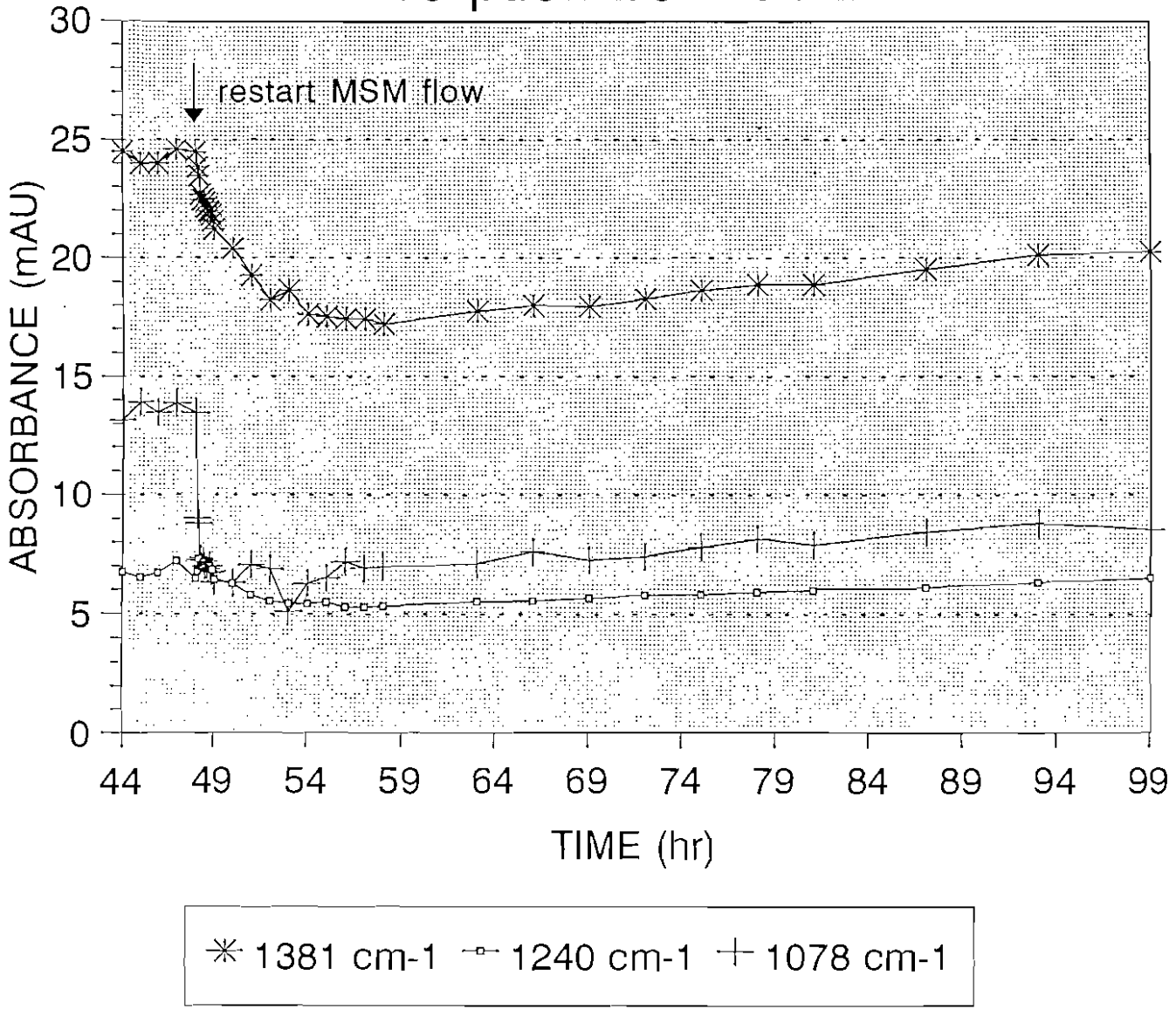
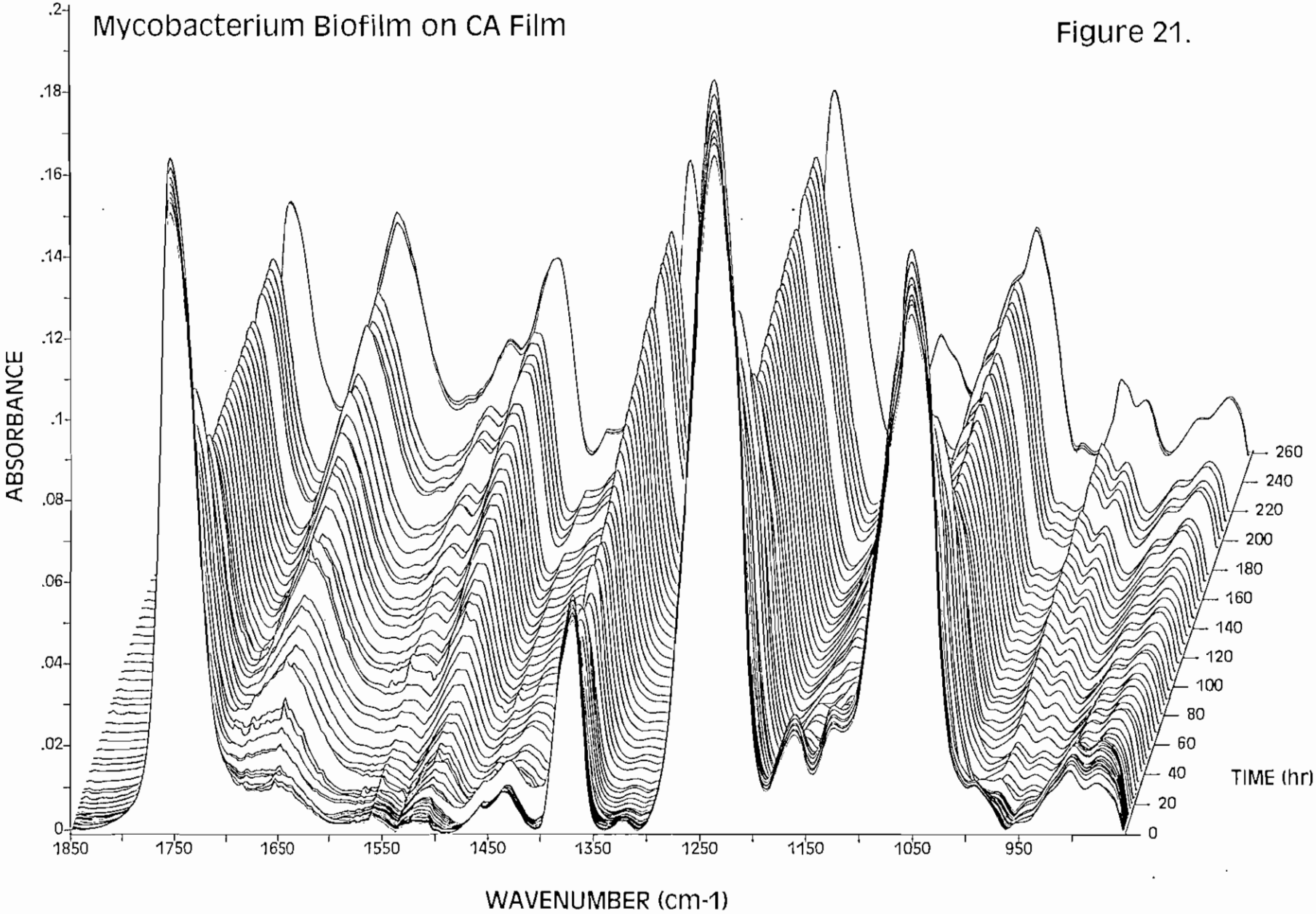


Figure 20.
Biocide Desorption from Ge IRE



Mycobacterium Biofilm on CA Film

Figure 21.



Mycobacterium Biofilm
on Cellulose Acetate Thin Film Figure 22.

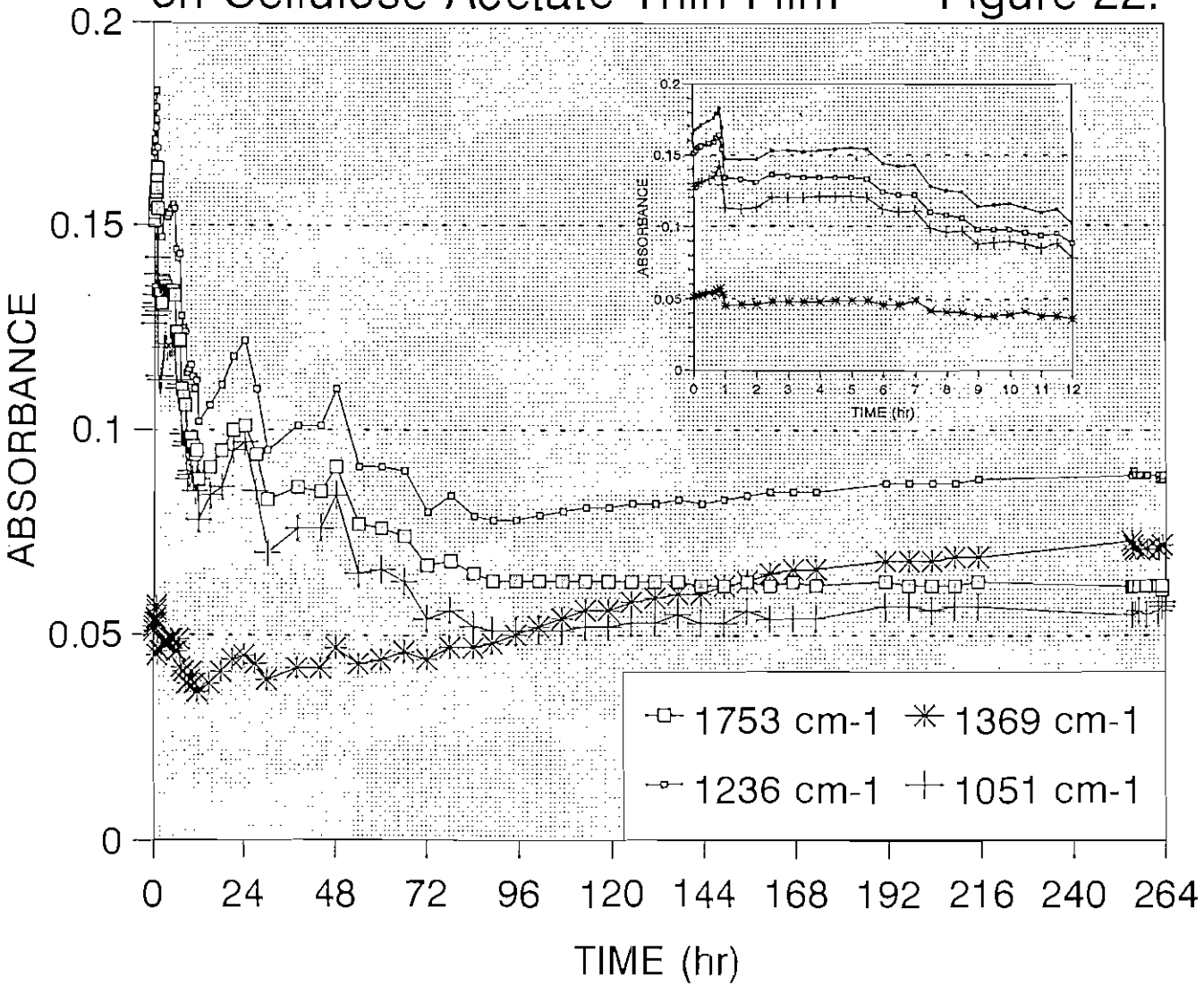
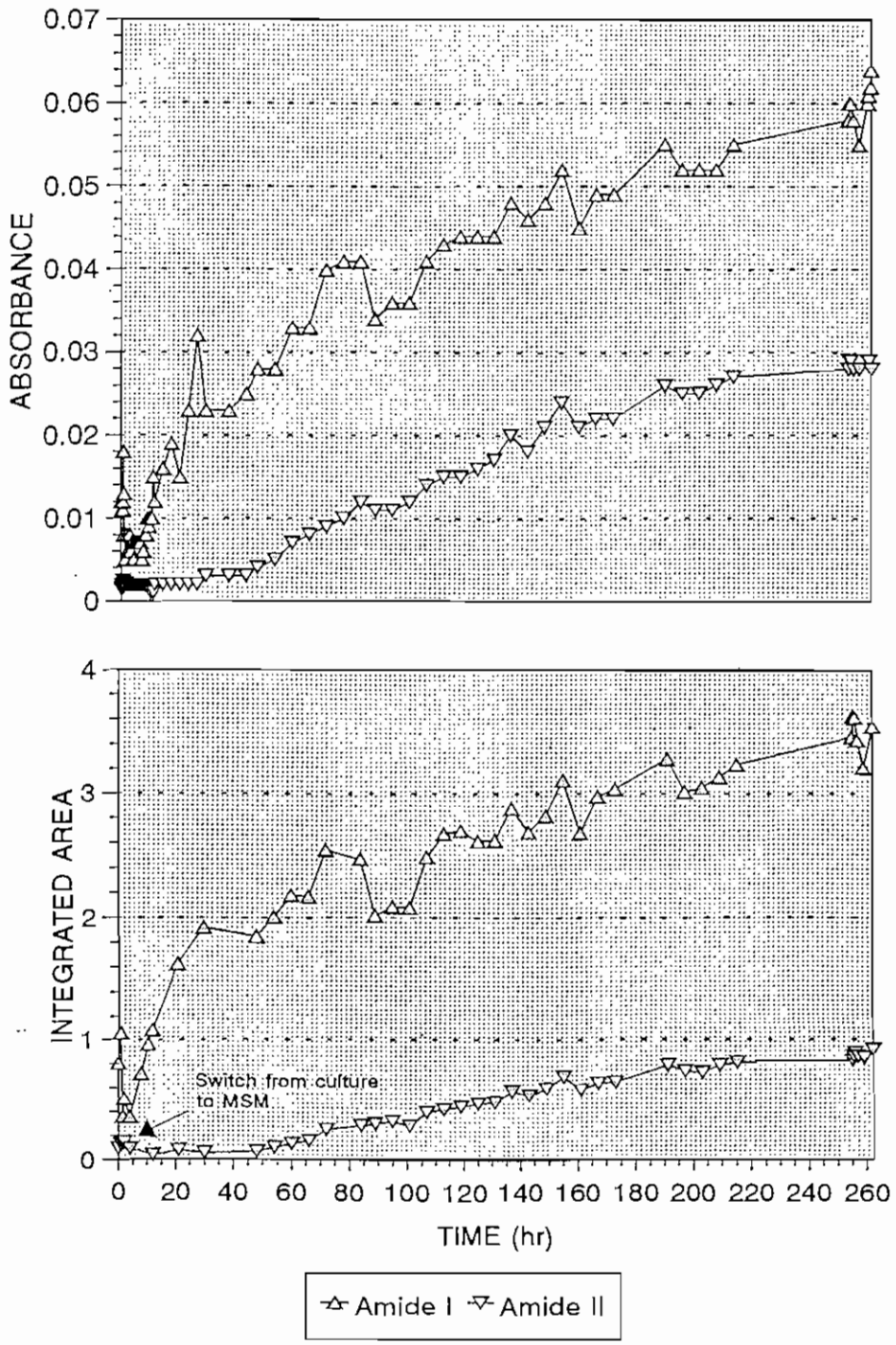


Figure 23.

Mycobacterium Biofilm on Cellulose Acetate

80 - 100 nm Thick Film on Ge IRE



Mycobacterium Biofilm
on Cellulose Acetate Cast on a Ge IRE

Figure 24.